University of Alberta

Virus Induced Asthma Exacerbations: Immunologic Mechanisms and Metabolomic Biomarkers

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DEDICATION

This thesis is dedicated to my brother, Jordan Skappak, who passed away suddenly in the spring of 2013. Jordan was the type of person who would do what ever he could to help those around him. I miss him everyday.

I also would like to dedicate this to my parents, Dwaine and Nola Skappak. The two of you have been an inspiration throughout my entire life and the best role models I could ever hope for. I will continue to strive to follow the ideals and values you taught me.

ABSTRACT

Asthma is a complex multifaceted chronic inflammatory disease that is characterized clinically by reversible airflow obstruction, shortness of breath, coughing, and wheezing. Asthma is the most common chronic disease in children, and is differentiated into many phenotypes with multiple triggers, the most common being respiratory virus infection. Allergic asthma, the most abundant phenotype, is defined by the airway inflammation made up of IgE, mast cells, CD4+ T helper cells, and eosinophils.

My research attempted to explain the mechanism behind why individuals with allergic asthma have longer and more severe responses to repeat respiratory virus infections. I hypothesized that the inflammatory cells in the asthmatic airway, particularly eosinophils, are activated by re-exposure to viral antigens due to immunological memory, and subsequently cause increased airway reactivity. My model demonstrated that the atopic status of an animal with virus specific immune memory altered its response to inert viral antigens. Both atopic (n=5) and non-atopic (n=5) animals with immune memory demonstrated increased airway reactivity in response to re-exposure to a live virus. Non-atopic animals with immune memory did not demonstrate increased airway reactivity when re-exposed to inert viral antigens (n=5), while atopic animals did demonstrate increased airway reactivity when re-exposed to inert viral antigens (n=8). These results suggest a new mechanism for the triggering of asthma exacerbations via virus specific immune memory..

Diagnosing and prognosticating asthma in children is a field lacking precise and non-invasive techniques. The other aspect of my research was to identify urinary biomarkers of asthma and acute hypoxia. Using metabolomics, the study of breakdown products of physiological and pathological processes; I hypothesized that I could identify phenotypes of asthma and acute hypoxia in animal models (n=7). The metabolomic analysis of acute neonatal hypoxic injury identified 14 metabolites, 7 of which have not been reported elsewhere. Analysis of urinary metabolites of my animal models of respiratory virus exposure resulted in the identification of 13 metabolites (n=6). These experiments have identified metabolites that can be used to determine asthma phenotypes and mild to moderate acute hypoxic episodes.

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LIST OF SYMBOLS, NOMENCLATURE AND ABBREVIATIONS:

ACh – Acetylcholine

AHR – Airway Hyperreactivity

AP-1 – Activator Protein 1

ANOVA –Analysis of Variance

APC – Antigen Presenting Cell

BAL – Bronchoalveolar Lavage

BPM – Breathes per Minute

BrdU – 5-bromo-29-deoxyuridine

cAMP – Cyclic Adenosine Monophosphate

CC10 – Clara Cell Protein 10 kDa

CCL(X) – Chemokine (C-C motif) (number X)

CD(X) – Cluster of Differentiation (number X)

CDHR(X) – Cadherin-Related Family Member (number X)

CO – Cardiac Output

$CRTh_2$ – Chemoattractant Receptor-homologous Molecule Expressed on Th2

$CysLT_1R$ – Cysteinyl Leukotriene Receptor 1

cGMP – Cyclic Guanosine Monophosphate

D_2O – Deuterium Oxide

dsRNA – Double Stranded RNA

DSS – disodium-2,2–dimethyl-2-silapentane-5-sulphonate

EEG – Electroencephalography

eNO – Exhaled Nitric Oxide

EPR – Early Phase Reaction

FiO_2 – Fraction of Inspired Oxygen

F Protein – Parainfluenza Fusion Protein

GR – Glucocorticoid Receptor

GSDMB – Gasdermin B

GM-CSF – Granulocyte Monocyte Colony Stimulating Factor

GP – Guinea Pig

GWAS – Genome-Wide Association Study

HCO^-_3 – Hydrogen Carbonate

HN – Hemagglutinin-Neuraminidase

^{1}H-NMR – Proton Nuclear Magnetic Resonance

H–R – Hypoxia-Reoxygenation

H_1R – Histamine Receptor Type 1

ICAM-1– Inter-Cellular Adhesion Molecule 1

IFN – Interferon

Ig(X) – Immunoglobulin (type X)

Ik β – Inhibitor of Kappa β

IL – Interleukin

I.P. – Intraperitoneal

IRF(x) – Interferon Regulatory Factor (number X)

I.S. – Induced Sputum

I.V. – Intravenous

LCF – Lymphocyte Chemoattractant Factor

LC-MS – Liquid Chromatography and Mass Spectroscopy

L Protein – Parainfluenza Large Protein

LPR – Late Phase Reaction

$M_{(X)}R$ – Muscarinic Receptor (Type X)

MAB – Monoclonal antibody

MAP – Mean Arterial Pressure

MBP – Major Basic Protein

Mcl-1L – Myeloid Leukemia Cell Differentiation Protein 1

MCP-1 – Monocyte Chemoattractant Protein 1

MDI – Metered Dose Inhaler

MHC – Major histocompatibility complex

MIP-1 α – Macrophage Inflammatory Protein -1 α

M Protein – Parainfluenza Matrix Protein

MRI – Magnetic Resonance Imaging

mRNA – Messenger Ribonucleic Acid

ms – millisecond

NANC – Non-adrenergic, Non-cholinergic

NEP – Neutral Endopeptidase

NF-AT – Nuclear Factor of Activated T cells

NF-k β – Nuclear Factor Kappa β

NK cells: Natural Killer cells

NMR – Nuclear Magnetic Resonance

NP – Parainfluenza nucleocapsid protein

PaO_2 – Arterial Partial Pressure of Oxygen

$PaCO_2$ – Arterial Partial Pressure of Carbon Dioxide

PBS – Phosphate Buffered Saline

PCLS – Precision Cut Lung Slices

pH – Power of Hydrogen

PIP – Pulmonary Inflation Pressure

PIV – Parainfluenza Virus

PKA – Protein Kinase A

PLS-DA – Partial Least Squares Discriminant Analysis

ppm – Parts Per Million

P Protein – Parainfluenza Phosphoprotein

PRAM – Pediatric Respiratory Assessment Measure

PSGL-1– P-selectin Glycoprotein 1

RAD50 – Radiation protein 50

RANTES – Regulated upon Activation, Normal T-cell Expressed, and Secreted

RPMI – Roswell Park Memorial Institute medium

RSV – Respiratory Syncytial Virus

RV – Rhinovirus

SABA – Short Acting β_2 Agonist

SeV – Sendai Virus

SLP-1 – Secretory Leukocyte Protease Inhibitor 1

SNP – Single Nucleotide Polymorphisms

ST2L – IL-33 cell-associated receptor

TCR – T Cell Receptor

TLR: Toll-like receptor

Th – T helper cell

TNF – Tumor Necrosis Factor

TP – Thromboxane A2 Receptor

T_{regs} – T Regulatory Cells

TSLP – Thymic Stromal Lymphopoietin

UV – Ultraviolet light

VCAM-1– Vascular Cell Adhesion Molecule 1

VLA-4 – Very Late Antigen 4

CHAPTER 1: *The Tip of the Iceberg: An Examination of the Multifaceted Disease that Is Asthma Exacerbation and How We Can Study It*

1.1 The Societal Impacts of Asthma

Asthma is a heterogeneous chronic inflammatory disease of the airways that is clinically manifested by reversible airflow obstruction, shortness of breath, coughing, and wheezing. Asthma is the most common chronic illness in children, and one of the leading health expenses around the world. In 2004 it was estimated that over 300 million people around the world have asthma (1). In 2007 it was estimated that 6.7 million children in the United States have asthma, and in 2001 it was estimated that over 0.58 million children in Canada have the disease (2, 3). In 2012 it was estimated that 8.5% of Canadians over the age of 12 had been diagnosed with asthma (4).

Airway obstruction from asthma can lead to severe shortness of breath, which can be life threatening. As such people may present to the emergency department and require hospitalization. The need for such care makes asthma a leading medical expense. In the province of Alberta, Canada, during the period from 1999 to 2005, over 105,000 adults and over 94,000 children presented to emergency departments because of their asthma (5, 6). These hospital visits have high direct and indirect costs associated with them. In 2004 it was estimated that the direct cost of urgent health care system access due to asthma across Canada was over $161,000,000 (7). The overall direct healthcare costs of asthma in one Canadian province, British Columbia was estimated to be $54,700,000 in

2007 alone (8). Over 47% of all asthmatic children missed 1 to 3 days of school due to asthma exacerbations per year, with over 5% missing 7 or more days of school per year (9). In 2005 in Alberta, Canada, asthma related work absences both direct and indirect cost the economy between $70,000,000 and 84,000,000 over one year (10). The total costs of asthma will vary between regions and country based on the definition and method for diagnosing asthma, but regardless of the potential variation, this disease has tremendous societal and economic impact. In addition, over 86,000 patients in Canada under the age of 20 were admitted to hospital with a first diagnosis of asthma (11). Of these patients over 20,000 (23.3%) had to be readmitted to the hospital because of their asthma (11). These high numbers of admitted and readmitted patients also highlight the need for more accurate diagnostics and prognostics to improve asthma treatment.

1.2 Understanding the Development of the Asthmatic Airway

The development of an asthmatic airway with subsequent asthma exacerbation requires pathologic immunologic processes to occur. For the most common form of asthma, allergic asthma, the immunologic basis of this disease is the Th_2 inflammatory phenotype. This type of inflammation is characterized by unique effector cells and cytokines (Interlukin-4, IL-5, IL-6, IL-9, IL-13, lL-33, and thymic stromal lymphoprotein [TSLP]), which are different than that of a Th_1 response. This is discussed in detail in the sections below.

This allergic phenotype is derived from the polarized differentiation of naive Cluster of Differentiation (CD) 4+ lymphocytes (Th_0 cells) into CD4+ lymphocytes with a Th_2 rather than Th_1 response (12). Th_0 cells are antigen/allergen naïve lymphocytes derived

from the bone marrow and matured in the thymus, that have successfully passed positive and negative selection to self-antigens *in utero* and postnatally. The purpose of a portion of these cells is to become memory T cells with the ability to respond to future harmful effectors in the body (i.e. bacteria, virus, cancer, parasites, etc.). Once these T cells move into circulation and cycle through the various lymphatic organs, they wait for the chance encounter of recognizable antigens being presented on major histocompatibility complex (MHC) class-II cell surface proteins by primary antigen presenting cells (APCs), such as macrophages and dendritic cells.

Differentiation of Th_0 cells into Th_1 or Th_2 is complex and incompletely understood. The basic mechanism occurs during antigen presentation with the effects of contact dependent factors, various cytokines released into the Th_0's surrounding microenvironment, including those produced by other T cells such as T_{regs} and Th_{17} cells (13). The contact factors include the duration and binding strength between the T-cell Receptor (TCR) recognizing the APC's antigen-MHC class-II complex and the co-stimulatory molecules. Studies have demonstrated that strong binding of the antigen-MHC class-II and TCR skews differentiation towards development of memory lymphocytes with a Th_1 response. In contrast to the strength of recognition of antigen presenting and co-stimulatory molecules, having high concentrations of antigen present has been reported to skew T cell differentiation towards Th_2 (14, 15). Another study by Kuchroo et al. demonstrated how Th_2 differentiation could by prevented by blocking the B7-1/CD28 co-stimulatory molecules from recognizing each other during antigen presentation (16). The other well-defined factor affecting Th_1/Th_2 differentiation is the presence of cytokines produced by

Th_1 and Th_2 cells in the microenvironment. The presence of IL-4 during TCR/antigen-MHC class-II recognition is known to skew Th_0 differentiation towards Th2 cells, while the presence of interferon (IFN)-γ, IL-12 and IL-18 has been shown to skew the differentiation towards Th_1 cells (17-19). Once differentiated Th_1 cells, but not Th_2 cells, produce IL-2, lymphotoxin-α, and IFN-γ, while Th_2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 (12, 20, 21). Th1 cytokines are known to promote activation of macrophages via IFN-γ, T-cell survival via IL-2, and production of IgG_2a by B cells (22-24). Th_2 cytokines are involved in B cell maturation and immunoglobin (Ig)E class switching via IL-4, IL-6, IL-9, and IL-13, eosinophil hematopoiesis and survival via IL-5, co-stimulatory factors for Th_0 differentiation via IL-4 and IL-6, and eosinophil and mast cell activation via IL-9 (25-30).

It is the downstream consequences of Th_2 cells and their cytokines that creates the allergic phenotype, which includes effector cells such as mast cells, basophils, eosinophils, and plasma cells releasing allergen specific IgE. A study by Akdis et al. reported this process in cells *in vitro* isolated from individuals with severe allergies (31). They found that when APCs recognized a common allergen such as phospholipase A_2 from bee venom they could activate Th_2 T cells. The Th_2 cells then released high levels of IL-4, IL-5, and IL-13 and induced IgE production by B cells. In studies of animal models of allergic asthma, Hofstra et al. reported that by inhibiting the development of allergen specific Th_2 CD4+ T cells in the lung, they decreased serum IgE production, prevented antigen induced airway hyperreactivity (AHR), and inhibited migration of eosinophils and neutrophils into the lung tissues (32).

There are other factors that contribute to the generation of allergic airway inflammation, and one such factor is the cytokine, TSLP. TSLP is constitutively expressed in human airway smooth muscle, and can be induced in human airway epithelial cells by inflammatory mediators and respiratory viral infections (33-35). TSLP is known to activate mast cells and dendritic cells, induce eosinophil chemoattraction, and promote Th2 CD4+ T cell differentiation (36-39). Increased TSLP expression in bronchial mucosa is associated with severe asthma, and appears to be involved in the propagation of Th_2 inflammation (40).

1.3 The Genetics of Asthma

With an understanding of the pathology of asthma, researchers have begun to study its genetic elements. By 2006 over 500 gene association studies of asthma and atopy had already been published (41). From these 500 published studies researchers identified 25 different genes associated with asthma and atopy. For example a international multi-ethnic genome-wide association study (GWAS) of 5415 individuals with asthma of European, Spanish, and African American descent identified SNPs that were associated with disease in all three ethnicities near the 17q21 locus (Gasdermin B (GSDMB) – nearest gene) and the IL-1RL1 (ST2L), TSLP, and IL-33 genes (42). Another GWAS study investigating children with severe asthma found several similar single nucleotide polymorphisms (SNPs were associated with this disease (TSLP, IL-33, IL-1RL1), in addition to two newly identified gene, cadherin-related family member (CDHR) 3 and radiation protein 50 (RAD50) (43). Clearly these common genetic differences cannot

explain the complexity of the disease. Environmental factors and epigenetic regulation must play a critical role in the generation of the disease.

1.4 The Allergic Mechanism of Airway Obstruction

One mechanism described in an allergen induced asthma exacerbation is called the early phase reaction (EPR). The EPR can be mediated by allergens binding cell surface bound IgE on mast cells and airway macrophages (44, 45). Once the IgE is crosslinked by allergen molecules on mast cells, it sends a signal to the cells to degranulate releasing products like histamine and to produce eicosanoids such as prostaglandins, leukotrienes, and thromboxane A_2 (46, 47). These products reach their respective receptors (histamine receptor 1 (H_1R), chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTh2), cysteinyl leukotriene receptor 1 ($CysLT_1R$), and thromboxane A2 receptor (TP)) on various cells within the airways such as goblet cells, smooth muscle cells, leukocytes and vascular endothelial cells (48-50). For example, stimulation of airway smooth muscle through H_1R, $CysLT_1R$, and TP cause bronchoconstriction. Airway parasympathetic nerves also have H_1R and $CysLT_1R$, which induce smooth muscle contraction, but also mucus gland stimulation. (51-53). Stimulated H_1R, $CysLT_1R$, and TP on vascular endothelial cells increase vascular permeability causing movement of proteins and fluid to the extracellular space, with subsequent airway narrowing (53, 54). Histamine has also been demonstrated to induce airway mucus sections in humans via the H_2 receptor (H_2R) (55). When this occurs in the vasculature of the small airways it can lead to tissue edema, and serum protein build up in the airways causing decreased ciliary beating frequency and decreased airway clearance (56, 57). The initial affects of allergen

activation of mast cells and macrophages can have immediate and potentially fatal consequences.

A second mechanism causing bronchoconstriction is known as the later phase reaction (LPR) (58). Six to 9 hours after the effects of EPR through IgE-bound mast cells or macrophages, another inflammatory cell influx occurs into the airways through the presence of various cytokines and chemokines. The inflammatory cell influx involves several cell types including eosinophils, basophils, and more CD4+ Th_2 T cells (59-63). Chemoattractant agents like chemokine (C-C) motif (CCL) 5 (RANTES), CCL3 (macrophage inflammatory protein (MIP)-1 α), CCL2 (macrophage chemoattractant protein (MCP)-1), IL-16 (lymphocyte chemoattractant factor (LCF)), CCL11 (Eotaxin) have all been found in samples taken from the lungs of humans with atopic asthma (64-66). Th_2 cytokines include, IL-4, IL-5, IL-6, and granulocyte/monocyte colony stimulating factor (GM-CSF) are also released (67, 68). In order for these inflammatory cells to enter the airways, cells adhesion molecules must be present to allow the cells to enter the lungs from the vasculature. Humans with asthma express abnormal increased amounts of several cell adhesion molecules such as vascular cellular adhesion molecule (VCAM)-1, very late antigen (VLA)-4, intercellular adhesion molecule (ICAM)-1, ICAM-2, P-selectin, P-selectin glycoprotein (PSGL)-1, an L-selectin in bronchial tissues (69, 70). Due to the influx of these various inflammatory cells and their subsequent activation causes damage and long-term changes to the structure and function of the airways. Once drawn into the lungs, these inflammatory cells are able to sustain themselves by producing their own cytokines (71).

Another key cytokine released from the plethora of cells plays an important role in the generation and maintenance of the allergic phenotype. IL-33 acts as a chemoattractant and/or enhances chemoattraction for inflammatory cells such as Th_2 cells and neutrophils (72, 73). Additionally IL-33 aids maturation and survival of inflammatory cells like mast cells and eosinophils (74, 75). In animal models of Influenza A virus infections, it was found that IL-33 messenger ribonucleic acid (mRNA) and IL-33 protein had increased expression in in the lungs of infected animals (76). In studies of bronchial biopsies from individuals with asthma and healthy controls, individuals with asthma express higher levels of IL-33 from bronchial epithelial, smooth muscle, and endothelial cells (77, 78). In studies of inflammatory markers in sputum from children with asthma, it was found that IL-33 correlated with other inflammatory markers such as lymphotoxin-α (79). It is hypothesized that IL-33 plays a key role in the continuation of chronic inflammation in asthma.

In addition, obstruction can occur through damage and remodeling of the airway. Damage to the airways and the epithelium in particular is readily apparent in both fatal asthma and stable chronic asthma in humans (80). Lung biopsies demonstrated large amounts of epithelial cell shedding and destruction of ciliated cells, while basal cells appear to remain attached to the basal lamina (80-82). Current literature demonstrates that various cytotoxic proteins, reactive oxygen species, and proteolytic enzymes released from inflammatory cells cause the damage to the epithelium (83-87). Other changes to the epithelium include a thickened basement membrane, and in severe cases of asthma a

large increase in the number of goblet cells (88, 89). These changes decrease the protection offered by the epithelium to the underlying structures, and make the airway more sensitive to allergens, irritants, and pathogens.

1.5 Asthma In Children: the clinical presentation

Diagnosing a child with asthma involves an assessment of clinical history and physical exam. The history taking should be focused on evaluating the patient for past wheeze, chronic cough especially at night with a paroxysmal nature, prolonged coughing/wheeze after common colds (2-3 weeks), other triggers of paroxysmal cough/wheeze (i.e. exercise, aero-allergens), family history of asthma, and other allergic co-morbid diseases (i.e. rhinitis, atopic dermatitis, food allergies, anaphylaxis) (90). Physical signs to evaluate include: shortness of breath, use of accessory respiratory muscles, tripod breathing position, tachypnea, tachycardia, pulsus paradoxus, decreased air entry in lungs, cough, and wheeze (90).

The clinical severity in an emergency room setting can be established using a scoring system such as the PRAM score, which has been validated for children between the ages of 2 and 17 (91, 92). PRAM establishes a severity score out of 10 based on O_2 saturation and 4 physical exam findings: wheezing, changes in air entry, and accessory respiratory muscle involvement (demonstrated by suprasternal in-drawing and scalene retractions).

In addition to history and physical examination, other tests are possible. Spirometry has been used to assess the severity of a patient's asthma, and also determine whether there is

a reversible airway obstruction in response to B2 agonists. Unfortunately the utility of spirometry is limited to children older than 5 years of age because of the required effort needed for accurate test results. In addition, most children are unable to perform a lung function test during an exacerbation. It is interesting that many studies have shown that spirometry does not correlate well with the clinical severity of the asthma exacerbations (93, 94). While induced sputum is a valid method of measuring airway inflammation (95, 96), there are significant barriers to its use including limited availability outside a tertiary care setting and the inability of children (and many adults) to expectorate sufficient or valid samples. While eNO values correlate with asthma inflammation and are commercially available, the test is invalid in children less than 4 years, and it appears to lack the sensitivity and specificity of induced sputum for improving patient outcomes (97).

Given the impact of asthma, the goal has been to prevent these spells of shortness of breath and hypoxia by decreasing airway obstruction. To do this has required decades of research to improve our understanding of the airway obstruction, but also to improve our ability to diagnose and monitor the obstruction. The general goal of this thesis was to study asthma exacerbation both in terms of understanding mechanisms of obstruction but also to improve diagnosis, especially in children.

1.6 The Diversity of Asthma: presentations and disease phenotypes

Every patient does not have the same amount, type, or severity of airway dysfunction at the same time. As such, asthma is a heterogeneous syndrome that can be categorized in

many different ways. These include separating the syndrome into phenotypes based on the inflammatory cells in the lungs (i.e. eosinophilic versus neutrophilic), by the triggers that induce symptoms (i.e. allergic versus stress), and by clinical groupings seen by physicians (i.e. steroid sensitive versus insensitive). Part of this diversity may be due to the complexity and overlapping nature of genetic variations and epigenetic variability of asthma (98).

Classifying asthma based on the inflammatory cells in the lung is important with respect to response to treatment. Eosinophilic asthma has been identified as one of two major inflammatory subtype of asthma. In a study by Ater et al. 50% of preschool children with wheeze presenting to the emergency department were found to have eosinophilia in induced sputum (I.S.) samples (99). Schleich et al found similar results in adults with asthma, that 46% of subjects had eosinophilia in their I.S. samples (100). Despite the eosinophil's susceptibility to corticosteroids, Wenzel et al. found that 58% of severe asthmatics with a mean inhaled corticosteroid dose of 29 mg/day had eosinophils in their lung biopsy samples (101). Neutrophilic asthma is another important phenotype as 18% of adults have only neutrophilic inflammation in their lungs, additionally neutrophilia is often found in the I.S. and bronchial biopsies from severe asthmatics alongside eosinophils (100-102). Opposite of eosinophils, neutrophils in the lungs become activated and release inflammatory products even in the presence of corticosteroid treatment (103). This ability to resist apoptosis and continue propagating inflammation may be the underlying cause of treatment resistant asthma. The final inflammatory phenotype of asthma is found when both eosinophilia and neutrophilia are absent, and is

called paucigranulocytic asthma. This phenotype makes up a large portion of asthmatics with 40% of adults having paucigranulocytic asthma (100).

Asthma associated with obesity is an increasingly seen phenotype. It is typically associated with two different presentations. The first is a early onset atopic asthma phenotype, and the second is a late onset non-atopic phenotype with primarily neutrophilic inflammation found in women (104). Obesity is known to act synergistically with asthma through mechanical airway obstruction due to increased adipose tissue on the chest wall, through local and systemic inflammatory pathways meditated through adipokines such as leptin, and by influencing environmental exposure. The next method for classifying asthma phenotypes is by identifying patients based on their clinical presentation.

The use of clinical presentations to classify asthma is especially useful for guiding the treatment of children based on disease severity. PRAM scores offer a quick validated method for establishing the clinical severity of a phenotype of asthma and a treatment pathway (91, 92). Severe asthma that is prone to exacerbation is a diverse phenotype which can have multiple underlying causes such as: underlying medical conditions, drug resistance/insensitivity, and airway abnormalities (105).

The age at which asthma symptoms present is another grouping by clinical presentation of the disease. Asthma with loss of lung function and chronic airflow obstruction is typically found in a small number of males, who had hyperreactive airways and low lung

function in childhood (106) Patients with asthma that develops before the age of 12 are typically noted to have more allergies and airway inflammation compared to individuals who develop asthma later in life (107). Defining asthma phenotypes by clinical presentation offers multiple ways to subcategorize this highly heterogeneous disease, however the clinical presentation may not always reflect the underlying pathophysiology.

1.7 Asthma Treatment: acute and chronic therapy

Regardless of the phenotype of asthma, treatment for acute asthma exacerbation is aimed at maintaining the child's ability to oxygenate, and to reverse the airflow obstruction. The aggressiveness of the therapy is completely dependent on the degree of respiratory distress. For children with mild to moderate respiratory distress (PRAM scores 0 to 4/10), the initial step of treatment is to begin oxygen therapy to keep arterial oxygen saturations greater than 95% (108, 109). Once oxygen therapy has begun the patients SaO_2 needs to be monitored to assess further response to treatment. Bronchodilation through activation of lung β_2 receptors is key to the successful treatment of an acute exacerbation. β_2 receptors are located throughout the airways on bronchial smooth muscle, and induce bronchodilation and smooth muscle cell relaxation via the generation of cAMP, the activation of PKA, and sequestering of intracellular calcium (110-113). The child is treated with a SABA given either by a nebulizer or a MDI with a chamber for up to three doses in the first hour (90). Adrenergic therapy for asthma was proposed after blockade of β receptors in asthmatics induced bronchoconstriction, and the most commonly used preparation, salbutamol/ventolin, has been used since 1968 (114, 115).

Oral corticosteroids are incredibly valuable for reducing inflammation in most types of asthma. The use of oral corticosteroids in children with mild asthma exacerbations is recommended in cases where the child failed to respond to SABA therapy after 6 to 8 hours or has recently received systemic corticosteroids (108, 130). Glucocorticoids are steroid based molecules that bind intracellular GR, which when activated translocate to the nucleus of the cell where they directly and indirectly inhibit gene transcription (116). Glucocorticoid treatment enhances expression of multiple different anti-inflammatory proteins and adrenoreceptors in the lung, such as lipocortin-1 which blocks eicosanoid production in mast cells, SLP-1 which degrades proteases in the airways, IL-1 receptor antagonist which blocks the pro-inflammatory effects of IL-1 β, cell surface NEP which breaks down circulating substance P and other tachkinins, CC10, increased production of Ik β which inhibits the NF-κB inflammatory pathway, β_2 receptor expression on smooth muscle, and IL-10 production in macrophages which in turn causes a down regulation of TNF-α, IL-1 β, IL-6, IL-8, MIP-1 α, and GM-CSF, (117-124). In addition to the increase in anti-inflammatory protein production, glucocorticoid therapy also down regulates the production of numerous cytokines and chemokines such as IL-1 β, IL-2, Il-3, IL-4, IL-5, IL-6, IL-11, TNF-α, GM-CSF, RANTES (125). The mechanism for the down regulation of all these inflammatory mediators is not completely known for each, but for certain cytokines such as IL-2, glucocorticoids are able to inhibit transcription factors such as NF-AT and AP-1 (126). RANTES is another cytokine/chemokine that glucocorticoids prevent being produced by inhibiting the transcription factors NF-κB and AP-1 (127). Glucocorticoids also have a direct effect

on the apoptosis of human eosinophils and neutrophils. The apoptosis of eosinophils is greatly accelerated by glucocorticoids by affecting the mitochondrial apoptotic pathway, particularly by causing localization of the pro-apoptotic protein, Bax, and increasing degradation of the anti-apoptotic protein, Mcl-1L (128). In neutrophils, the affect is the opposite with Mcl-1L degradation decreased through the production of antisense Mcl-1L siRNA. Another way glucocorticoids are able to exert an anti-inflammatory effect is by blocking cell migration into tissues. Van de Stolpe et al. demonstrated that treatment with glucocorticoids decreased the expression of ICAM-1 on the surface of macrophage/bronchial epithelial cells (129). Without the expression of proper cellular adhesion molecules, inflammatory cells are less able to enter sites of inflammation.

For children who have moderate to severe asthma, more steps must be taken to ensure that their respiratory distress does not progress to the point of becoming life threatening. In moderate asthma (PRAM scores 5 to 8/10), the same treatment pathway is followed as for mild asthma with the addition of 3 doses of ipratropium bromide to the SABA in the first hour (109). Ipratropium bromide is a non-specific cholinergic receptor antagonist created in 1973, that prevents parasympathetic induced contraction of bronchial smooth muscle by inhibiting the binding of ACh to receptors and the down stream production of cGMP (131). After the combined therapy of inhaled SABA and ipratropium bromide, and less then 4 hours after oral corticosteroid the child should be reassessed, and if they haven't improved, continuous inhaled SABA should be given for 30 min to 1 hour (109). If the child's PRAM score still remains greater then 3, then the child should be admitted to hospital.

In severe asthma exacerbations (PRAM score 8 to 10/10), the same therapy as moderate exacerbations should be initiated with vital signs being monitored every 20 minutes, administration of 100% oxygen, and treatment with oral corticosteroids after the first inhalation of SABA/ipratropium bromide (109). If the child is not responding to treatment continuous inhaled SABA should be given in addition to the insertion of a peripheral intravenous (I.V.) line and the administration of magnesium sulfate (109). Magnesium sulfate has been demonstrated to have a bronchodilatory and vasodilatory effect in children with asthma, which is believed to be mediated by correcting a Mg^{2+} deficiency in asthmatic patients which causes smooth muscle excitability and bronchoconstriction (132-134). If the child doesn't respond after the magnesium therapy (PRAM score of 9 to 10/10), they should continue to receive continuous inhaled SABA and be admitted to a pediatric intensive care unit (109). In cases when a child is at immediate risk of complete respiratory failure the following procedures should be taken, administration of 100% oxygen at a rate of 8 to 10 liters/minute, continuous inhaled SABA/ipratropium bromide, continuous cardiopulmonary monitoring, insertion of two peripheral I.V. lines (or intraosseous lines), administration of injectable corticosteroids, rule out pneumothorax, determine appropriateness of I.V. magnesium sulfate, administration of I.V. SABA (109). If there is no improvement following these therapies, clinicians should consider intubating the child. The treatment of acute asthma exacerbations requires good clinical acumen and a detailed knowledge of the pharmacology behind therapies.

1.8 The Causes of Asthma Exacerbations

The most common trigger for asthma exacerbations is respiratory viral infections. In a study by Johnston et al. investigating children with wheeze and/or cough ages 9 to 11, it was reported that between 80 and 85% of all asthma exacerbations were associated with respiratory viral infections (135). Of these virus triggered asthma exacerbations, the most commonly detected viruses were rhinovirus, coronavirus, influenza virus, and parainfluenza virus (135). A similar study investigating asthma exacerbations in adult men and women ages 19 to 46 reported that 50-60% of asthma exacerbations were associated with colds (136).

Allergic asthma, as previously discussed, is one of the largest phenotypes of asthma, and as such allergies play a prominent role in triggering asthma exacerbations. In the United States in 2006 over 13% of all individuals with IgE allergen positive atopy were also diagnosed with asthma (137). A 1996 survey of 28,000 children aged 5 to 9 across Canada, reported that 13% of children had asthma, and up to 58% of these children had aeroallergen associated asthma (138). Other triggers for asthma include exercise and cold air, exposure to aspirin, menstruation, exposure to occupational irritants, and air pollution (139-143).

1.9 Virus Induced Asthma Exacerbations: the controversy

As stated previously respiratory virus infections have been described as the primary cause of asthma exacerbations in both adults and children. In addition to being the most

common trigger of asthma, asthmatics have more frequent symptoms from respiratory viruses that are longer and display more severe lower respiratory tract symptoms then healthy individuals (144). The rational behind why this is a debated issue among researchers. One theory is based on the difference in immune response to respiratory virus infections between asthmatics and healthy individuals. It has been hypothesized that individuals with asthma have an impaired immune response to respiratory virus infections. To test this hypothesis individuals with asthma to determine if they have elevated lung viral load during respiratory infections compared to healthy individuals. A study by Denlinger et al. compared the effects of viral load in natural RV infections in non-asthmatic individuals, individuals with asthma, and asthmatics undergoing an acute exacerbation and found that each group had similar levels of lung viral burden (145). Differences in inflammation were observed in asthmatic individuals and asthmatics experiencing an acute exacerbation compared to non-asthmatic individuals. Asthmatics and those experiencing an acute exacerbation had sputum neutrophilia. In another study by Bardin et al. individuals with atopy had more severe upper respiratory tract inflammation in response to experimental RV infection compared to non-atopic individuals, but there were no differences in viral shedding between atopic and non-atopic individuals (146). In contrast to studies that found no difference in viral burden, Message et al. found that in experimental RV infections asthmatic individuals had more severe lower respiratory tract symptoms, higher levels of airway inflammation, and increased viral burden compared to non-asthmatics (147). They also found that serum Th_1 cytokines such as IFN-γ and IL-10 were decreased and BAL Th_2 cytokines such as IL-5, IL-6, and IL-13 were increased in asthmatic individuals compared to non-

asthmatics. They concluded that increased viral burden, impaired Th_1 function, and enhanced Th_2 function were associations with more severe symptoms in asthmatics.

Investigations into the impairment of the immune response of asthmatics to respiratory virus infections have branched into studies of the innate immune response. The expression of interferons by bronchial epithelial cells in responses to respiratory virus infections has been a subject of much scrutiny. An *in-vitro* study of RV infection of primary cultures of bronchial epithelial cells from asthmatics by Wark et al. showed that epithelial cells from asthmatics produced less IFN-β mRNA in response to RV infection than cells from non-asthmatics (148). When they added exogenous IFN-β, the asthmatic cells responded to the infection in a manner similar to cells from non-asthmatics. Another study by Contoli et al. investigated the *in-vitro* and BAL derived role of type III interferons, IFN-λ_1 and -$\lambda_2/_3$ in response to respiratory virus infections (149). Primary cultures of asthmatic bronchial epithelial cells and alveolar macrophages had decreased expression of IFN-λ_1 and -$\lambda_2/_3$ mRNA relative to higher viral loads compared to cells from non-asthmatic individuals during infection, and that overall IFN-λ was decreased in cells from asthmatics. They also examined IFN-λ production in BAL cells from the same asthmatics and non-asthmatics and non-asthmatics that donated cells to the *in-vitro* arm that had been given experimental RV infections. IFN-λ production was decreased in asthmatic samples compared to non-asthmatic samples. In contrast to the *in-vitro* work by Wark et al. and Contoli et al., Lopez-Souza et al. demonstrated that primary culture of bronchial epithelial cells from asthmatics infected with RV produced significantly more IFN-β then non-asthmatic cells (150). They hypothesized that differing methods of

primary cell culture we responsible for producing artifacts in the *in-vitro* data of Wark et al. and Contoli et al. (151). Earlier, Lopez-Souza et al. reported that the differentiation of the cultured epithelial cells affected viral replication and the degree of innate immune responses by the cells (151). Cells cultured under methods known to produce low levels of cellular differentiation had higher viral loads and a larger degree of impairment of their innate immune response compared to cells grown under conditions that produced high levels of differentiation. Poor differentiation of bronchial epithelial cell cultures maybe the reason behind differing levels of interferon production in some of the *in-vitro* work, but it still does not explain the *ex-vivo* assessment of IFN generation in BAL samples.

Another hypothesis behind the more severe and frequent lower respiratory tract symptoms associated with respiratory viral infections involves the presence and increased activity of allergic inflammation, including eosinophils, in the lungs of individuals with atopy and asthma. As previously stated, airway eosinophilia is a common aspect of asthma, with almost 50% of adults and children reporting this in I.S. samples (99, 100). The presence of the eosinophils is important not only because their degranulation products to cause airway damage, but for the effect they have on airway smooth muscle regulation (152, 153). Children with more severe respiratory symptoms associated with viral infections of the lower respiratory tract were found to have higher levels of eosinophilic cationic protein, a degranulation product (154). How eosinophils are involved in worsening respiratory symptoms has been well studied over the past 2 decades.

1.10 Airway Hyperreactivity: viruses, eosinophils, and nerve dysfunction

The vagus nerve and its cholinergic branches provide the majority of airway innervation (155). This is achieved by signaling through acetylcholine and muscarinic receptors throughout the airways (156). M_1Rs are found on airway parasympathetic ganglions and when inhibited by a specific M_1R antagonist, pirenzepine, there is increased expiratory flow at low lung volumes (157). M_2Rs have been identified on parasympathetic postganglionic presynaptic nerves, and act as inhibitory autoreceptors suppressing ACh release (158). Activation of airway M_2Rs by a selective agonist, pilocarpine, inhibited cholinergic induced bronchoconstriction in healthy individuals (159). M_3Rs are located in abundance on airway smooth muscle and submucosal glands throughout the human airway (160). These receptors once activated by ACh are able to stimulate the contraction of smooth muscle through the release of intracellular calcium stores via phospholipase C (161). As the primary source for smooth muscle innervation, dysfunction of parasympathetic nerves in the lung is a serious clinical concern.

There have been several studies investigating airway parasympathetic nerve dysfunction associated with the effects of respiratory viruses, cytokines, and eosinophil degranulation products. Jacoby et al. reported that when cultured airway parasympathetic neurons were either infected with parainfluenza virus or exposed to interferon-γ *in-vitro* they released more ACh in response to electrical stimulation (162). In control cultures when M_2Rs were either activated or inhibited with methacholine and atropine respectively they could decrease or increase the amount of ACh released in response to electrical stimulation. When the nerves were cultured with virus or IFN-γ, ACh release was increased, and

adding methacholine or atropine to the cultures did not change the release of ACh. Jacoby et al. also demonstrated that M_2R expression decreased by a log with viral infection or exposure to IFN-γ. They concluded from these experiments that the virus could be triggering IFN-γ release from T-cells or that the virus infected the neurons themselves and caused decreased receptor expression. Bowerfind et al. was able to demonstrate in an *in-vivo* model that inducing IFNs with double stranded RNA caused M_2R dysfunction not only in the parasympathetic airway nerves, but also in the cardiac parasympathetic nerves (163). Matsuda et al. also reported that respiratory viruses could directly infect the vagus nerve (164).

In addition to IFNs and viral infections, eosinophil degranulation products cause M_2R dysfunction in the lungs. In 1973 Gleich et al. reported that eosinophils contained an arginine rich basic protein, major basic protein (MBP), in their cytoplasmic granules which was released upon degranulation (165). Costello et al. reported that eosinophils were found in close association with airway parasympathetic nerves in allergen challenged models of asthma (166). Additionally in lung samples from patients with fatal asthma, they reported 30% of eosinophils identified in biopsies were associated with airway nerves. This close association could lead to release of MBP directly on M_2Rs of airway parasympathetic nerves. MBP and another eosinophil product, eosinophil peroxidase, were reported by Jacoby et al. to be allosteric antagonists of M_2Rs in humans (167).

Two studies investigated the outcome of blocking MBP with specific antibodies in animal models of allergic asthma. Inhibition of MBP prevented antigen induced AHR, Evans et al. reported that the anti-MBP antibodies specifically prevented M_2Rs dysfunction and AHR (168, 169). Further studies investigating the role of eosinophils in airway nerve and smooth muscle dysfunction, found that eliminating an essential cytokine, IL-5, prevented M_2R dysfunction and AHR in response to antigen challenge in an animal model (170). Thus, the close association of eosinophils to airway nerves, and the ability of MBP to antagonize the effects of M_2Rs, establishes another plausible mechanism for airway nerve and smooth muscle dysfunction.

With the knowledge of the eosinophil's role in M_2R related airway nerve and smooth muscle dysfunction, additional investigations were conducted into eosinophils and virus induced asthma exacerbations. The first study by Adamko et al. reported that by blocking MBP with specific antibodies, or depleting eosinophils via an anti-IL-5 antibody prevented AHR in animal models of allergic asthma that were given acute parainfluenza virus infections (171). In a second study Adamko et al. prevented M_2R dysfunction and AHR in response to respiratory virus infections in animal models of allergic asthma by depleting the T cells that were activating and sustaining the eosinophils located near airway nerves (172). Gelfand et al. also reported the importance of having CD8+ T cells and eosinophils in RSV infections of mice in order to develop AHR (173, 174). Not only did the elimination of eosinophils and their degranulation products appear to inhibit AHR in response to antigen challenge and viral infection, but eliminating cells that activated the eosinophils also prevented the airway dysfunction.

1.11 T cells: immune memory and eosinophil degranulation

Development of immunological memory is one of the hallmarks of the adaptive immune system. T cell memory plays a key role in eliciting the appropriate immune response over a faster time frame then compared to an original reaction to a particular pathogen. The presence of activated memory CD4+ and CD8+ T cells has been confirmed in the BAL fluid from humans and animals with atopic asthma (175, 176). Not only can these T cells contribute a direct response to an antigenic threat, they can influence the responses of other cells, including T cells. Coyle et al. demonstrated how cytokines produced by allergen specific CD4+ Th_2 cells could induce virus specific CD8+ T cells to produce Th_2 cytokines such as IL-5 in response to activation (177).

As stated previously Adamko et al. reported that depletion of these CD8+ T cells prevented eosinophil degranulation and airway dysfunction (172). Interestingly, Davoine et al. demonstrated that CD4+ memory T cells from humans with allergies could induce eosinophil degranulation in the presence of viral antigens and antigen presenting cells (178). These results suggest a mechanism for eosinophil degranulation on re-exposure to any commonly encountered airway antigen for which immune memory exists.

1.12 Guinea Pigs: an animal model of human allergic asthma

Guinea pigs have been used as animal models for experimentation in allergies and anaphylactic reactions for over 100 years (179, 180), primarily due to their ease in care

and handling, but more importantly because of the anatomical and physiological similarities between human and guinea pig airways and immune systems. Structurally the two species share many anatomic similarities. The structure of the airway generations in humans and guinea pigs is quite similar, with the primary difference being that humans have between 25 to 32 generations to the respiratory bronchioles and guinea pigs have between 15 to 17 generations (181, 182). Ultrastructural analysis of guinea pigs large airways revealed a pseudostratified columnar epithelium populated by ciliated cells, goblet cells, basal cells, mucous glands, as well as intraepithelial granulocytes and lymphocytes (183). The distal bronchial epithelium is populated by cuboidal ciliated cells and Clara cells (184). In the respiratory bronchioles the epithelium transitioned into non-ciliated cuboidal cells, which eventually transition into alveolar ducts lined by type I and II pneumocytes. Humans have a similar epithelium, with their large airways covered by ciliated pseudostratified columnar epithelium interspersed by goblet cells and mucus glands supported by a lamina propria containing a high amount of elastin (185). In the distal bronchioles the epithelium transitions into a ciliated simple columnar cell, and then into a ciliated simple cuboidal cell in the terminal bronchioles, which transition into the alveolar ducts and alveoli that are lined by type I and II pneumocytes(186). Additionally both humans and guinea pigs have a robust subepithelial vascular and lymphatic network located in the lamina propria throughout the airways (88, 187, 188). The cholinergic innervation of both humans and guinea pigs is localized to the smooth muscle throughout the lungs as well as in the lamina propria of the trachea and large bronchi (189-191). Sensory (afferent vagal) nerve endings including C-fibers and stretch receptors in human bronchi are found throughout the lamina propria and between epithelial cells, and in large

airways these nerves can be located close to the lumen (192, 193). Guinea pigs share an equivalent distribution pattern of sensory nerve endings throughout the lung (194, 195). Guinea pigs have a large network of non-adrenergic, non-cholinergic (NANC) nerve fibers throughout the epithelium of the trachea and large bronchi, as well as the lamina propria and smooth muscle of the bronchi, and in the alveolar walls and around pulmonary vessels (196). These nerves contain neuropeptides such as substance P, vasoactive intestinal peptide (VIP), calcitonin gene related peptide, and neuropeptide Y (196, 197). Humans also have a diverse NANC nerve fiber network in the trachea, the smooth muscle of the bronchi, and around bronchial glands (198, 199). With comparable structural, vascular, and neuronal components of the lung, guinea pigs offer a logical basis for modeling human airway disease.

Physiologically the respiratory and the mechanical aspects of respiration in humans and guinea pigs are also comparable. Humans and guinea pigs share similar levels of lung elasticity when the airway compliance is adjusted for the size of the lung, with humans having a compliance to weight ratio of 0.139 ($^{L/cm\ of\ H_2 0}/_{kg}$) compared to the guinea pigs 0.137 ($^{L/cm\ of\ H_2 0}/_{kg}$) (200). However, guinea pigs have a higher resting respiratory rate (100 – 110 BPM) than humans (60 – 100 BPM) (201). Guinea pigs and humans have similar acid-base profiles in their arterial blood samples at rest, with pH = 7.41(humans) vs. 7.44 (guinea pig), $PaCO_2$ = 38.9 mmHg (humans) vs. 38.9 mmHg (GP), HCO^-_3 = 24.4 $^{mM}/_{L}$ (humans) vs. 24.4 $^{mM}/_{L}$ (guinea pig), and PaO_2 = 91.0 mmHg (humans) vs. 91.9 mmHg (guinea pig) (202, 203). *In-vitro* testing of PCLS confirmed that guinea pigs and humans responded in a similar fashion to the following bronchoconstrictive agents,

leukotriene D_4, histamine, and methacholine (204). Additionally antigen induced bronchoconstriction in passively sensitized PCLS of human and guinea pigs could be mitigated by treatment with thromboxane-, histamine-, and leukotriene-receptor antagonists (204). Ressmeyer et al. established that treating Guinea pig PCLS with salbutamol also reversed methacholine-induced bronchoconstriction in a dose dependent manner similar to the effect seen in humans. Having such comparable physiological aspects establishes the guinea pig as a suitable model for human lung function experiments.

The generation of an allergic phenotype and an asthmatic immune response in guinea pigs is the primary reason for the continued use of this animal model in asthma experimentation. Guinea pigs develop an allergic response following inhalation or injection of antigens (205, 206). The allergen sensitization is necessary to generate the 4 responses (early bronchoconstrictor response, late bronchoconstrictor response, airway hyperreactivity, and cellular influx to the airways) typically seen when individuals with allergies and asthma encounter an allergen (207, 208). Smith et al. achieved all four responses in guinea pigs through an optimized sensitization and allergen challenge protocol using a well studied allergen, ovalbumin (209, 210). The cellular influx into the airways is also similar between asthmatics and guinea pigs, with the influx of eosinophils having particular significance to asthma pathology (211, 212). The ability of guinea pigs to replicate the hallmark responses seen in an allergen induced asthma exacerbation in addition to replicating the inflammatory cell influx into the airways confirms that guinea pigs are an appropriate model of human allergic asthma.

1.13 Parainfluenza Virus: a viral pathogen in guinea pigs

Parainfluenza virus is a common respiratory pathogen in humans, and it is also virulent in guinea pigs. In Alberta over 12% of samples from individuals with suspected respiratory virus infection were positive for PIV over a 3-year period from 2009 to 2012 (213). Initial PIV inoculation occurs through the nasal mucosa, and produces symptoms of a URTI after a 2 to 4 day incubation period following infection of epithelial cells (214). The 4 different strains of the virus (PIV1 – PIV4) and the murine analogue SeV also infect the lower respiratory tract, and can cause severe infections in children (214, 215). The PIV virion consists of a filamentous nucleocapsid core containing an RNA genome, all within a lipid envelope covered in virus glycoproteins. The PIV genome is a linear negative sense single stranded RNA genome with an average length of 15,000 nucleotides and encodes eight genes in the following order 3'-NP-P/C/V-M-F-HN-L-5' (216). The nucleocapsid is made up of the nucleocapsid protein (NP), phosphoprotein (P), and large (L) protein, with three proteins combined producing the RNA dependent RNA polymerase activity (217). The C protein is a non-structural protein found only within the cytoplasm of the infected cell, and has been demonstrated to limit the formation of double stranded (ds)RNA preventing the activation of an innate immune response (218). The V protein has been reported to inhibit IFN production in infected cells by interfering with the activation of IRF3 and IRF7 (219, 220). The matrix (M) protein, is involved in organizing virion assembly through the binding of NP and its interaction with membrane glycoproteins (221). The fusion (F) protein is involved in accumulating structural components at assembly sites inside the cell, and an activated

trimer of the F protein is used to insert into the membrane of a target cell to initiate viral fusion (222, 223). The hemagglutinin-neuramadase (HN) protein has a combined activity and cleaves cell surface sialic acid as well as bind sialic residues on cell membranes; additionally it can cleave F protein trimers exposing their fusion domain and activating them (224). The well-documented biology and the ability of PIV to cause infections in both humans and animals make it an ideal pathogen for studying the effects of respiratory viral infection *in-vivo* (225).

1.14 Metabolomics: biomarkers of asthma and hypoxia

Metabolomics is the study of the biological products of normal physiological processes, disease pathophysiology, and gene function (226). An initial idea was to use comprehensive metabolite profiling to provide a rapid personalized health assessment (227). Researchers are using metabolomics to diagnose and prognosticate various diseases. There are two primary technologies that are used for identifying metabolites in biological samples, mass spectroscopy and nuclear magnetic spectroscopy (NMR) spectroscopy (228, 229). Both of these technologies have strengths and weaknesses. Mass spectroscopy allows for identification of compounds in nano-molar concentrations, requires small volumes, and is ideal for identifying lipid based compounds. However, using mass spectroscopy results in the destruction of whatever sample is introduced into the instrument, and is unable to differentiate between isomers of a specific compound. NMR spectroscopy is advantageous because it does not destroy or change the sample, and allows for qualitative and quantitative analysis of compounds based on their resonant

frequencies and their signal intensities. Unfortunately NMR is limited to detect only micro-molar concentrations, typically requires 60x larger volumes of sample compared to mass spectroscopy, and has poor ability to differentiate lipid molecules. Despite these limitations many researchers use NMR spectroscopy, while others combine it with mass spectroscopy.

The most commonly used form of NMR spectroscopy in metabolomic studies is 1D ^{1}H-NMR. ^{1}H-NMR involves aligning protons of a molecule with or against a strong external magnetic field. Once the protons are aligned they can be elevated to a higher energy state through bombardment with energy from radio waves. Eventually the energized proton will emit the absorbed radio wave energy, and the frequency of this emission can be detected. The proton's ability to absorb energy is dependent on the electron environment of the proton. If the proton is bonded to molecules with a strong local electro-magnetic field density this additional field will block the proton and deflect the radio wave energy resulting in more energy being required to excite the proton, this effect is called shielding. The differing electro-magnetic environment of the protons allows for compounds to be identified based on their differing resonance frequencies. To account for the variation of magnetic fields between magnets, unknown compounds are compared to standard compounds with known resonance frequencies. To provide a universal location unit for a compound, the compound resonance frequency in Hz is divided by the frequency of the magnet in MHz, to give a standard unit in parts per million (ppm). To quantify the amount of a particular compound present in a sample, the signal intensity of a proton's resonance frequency must be examined. This is due to the signal intensity of a

proton resonance peak being proportional to the concentration of a compound. In high concentrations a larger number of environmentally identical protons will resonate at the same radio frequency providing a stronger emission. Although this is a summary of how ^{1}H-NMR functions, it provides the core concepts needed to understand NMR spectroscopy compound identification and quantification.

Different biofluids and tissues have been used for metabolomic analysis. A biofluid preferred by most researchers for NMR metabolomics is urine due to its ease of collection, large sample volume, and physiologic filtering through the kidneys. Sample collection must be standardized to minimize potential changes to the urinary metabolites; these changes can be caused by bacterial degradation of the metabolites, enzymatic activity in the urine, and structural changes in temperature sensitive compounds (230). Ensuring the study has the appropriate control samples is essential as individual metabolite concentrations can vary considerably (231). Optimization of the ^{1}H-NMR analysis is just as important as standardizing sample collection and handling. Saude et al. reported standardized methods for performing ^{1}H-NMR based metabolomic analysis for analyzing biofluids such as urine (232).

Metabolomics analysis of asthma is becoming a popular research field due to the lack of non-invasive diagnostic and prognostic techniques for asthma in children. Current targets include urinary-based metabolites, serum metabolites, and metabolites from exhaled breath condensates (233-235). My research has investigated urinary-based metabolite markers of asthma in children, animal models of asthma, and animal models of acute

hypoxia, which represents the most severe stage of an asthma exacerbation and bronchoconstriction (232, 236, 237). Urine samples were collected from study subjects and their respective healthy controls. The 1D ^{1}H-NMR spectra collected from each of these samples were analyzed using targeted metabolite profiling and quantification software (Chenomx, Edmonton, AB) (238), as outlined in Chapter 2. The targeted analysis allowed for the identification of known compounds in the urine.

1.15 Thesis Rational

The pathophysiology and the immunological pathways behind asthma and exacerbations are complex. The high number of individuals afflicted with this condition and the gap in our knowledge necessitate the continued research in the mechanisms behind this disease's development, triggers, and management. Understanding the role of viruses and resident cells like eosinophils and T cells may lead to future research that uncovers novel targets or even changes clinical practices or patient behavior. Additionally by using metabolomics to discover new biomarkers of asthma for diagnostic and prognostic applications helps fill a serious gap in the evaluation and treatment of the disease. With more accurate assessment of stable disease, acute exacerbations, and severe airway hypoxia we will be able to provide a real time assessment of the patients condition. This thesis attempts to provide a clearer understanding of how virus–induced asthma exacerbations can occur in-vivo, and how NMR based metabolomics can identify markers of acute hypoxia, an aspect of the most severe asthma exacerbations (Figure 1.1).

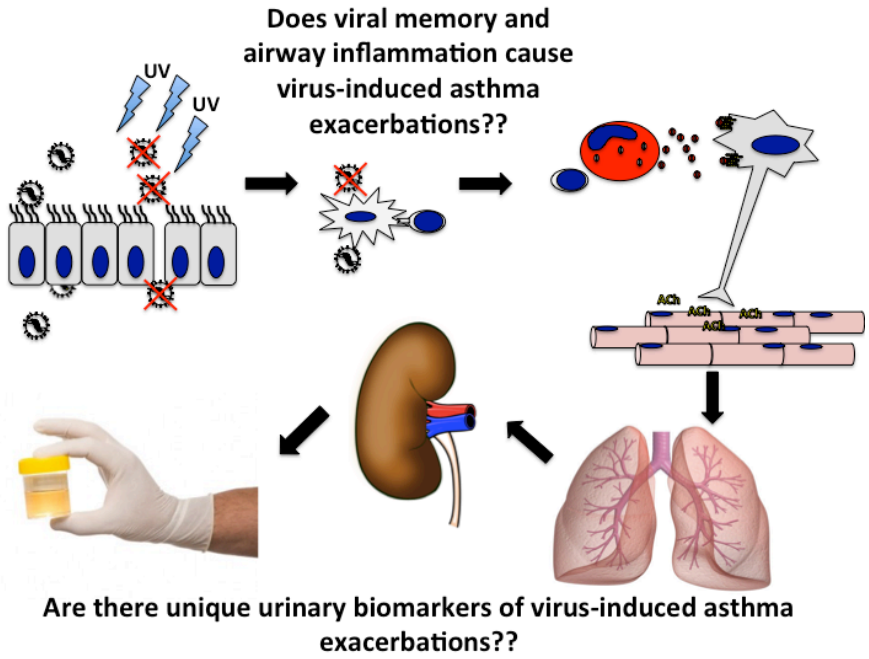

Figure 1.1 The Defining Questions Behind This Thesis

The goal of this thesis is to answer two questions. 1. Does viral memory and airway inflammation cause virus-induced asthma exacerbations? 2. Are there unique urinary biomarkers of hypoxia and virus induced asthma?

Specific Aim #1 – To evaluate eosinophil induced airway hyperreactivity in the context of activation by immunological memory to respiratory viral antigens in an allergen-sensitized model of asthma. I hypothesize that sensitized animals with viral immunological memory will have eosinophil degranulation and airway hyperreactivity in response to re-exposure to viral antigens.

Chapter 3 – Infection with a respiratory virus is the most common trigger for an asthma exacerbation. In everyday life humans encounter respiratory viruses to which they already have developed immunity and immunological memory. *This chapter reviews*

experiments evaluating the role of eosinophils in mediating AHR in an animal model of allergic asthma re-exposed to viral antigens. Comparisons are made between the physiological and immunological responses of atopic and non-atopic animals in response to re-exposure to live virus and inactivated virus. Investigations were also made into the ability of specific and non-specific therapies to reverse the AHR through depletion of eosinophils and suppression of inflammation.

Specific Aim #2– To identify metabolites of hypoxia in a newborn piglet model using urinary NMR metabolomic profiling. I hypothesize that newborn piglets that undergo an episode of hypoxia will have an altered urinary metabolite profile compared to animals that have not experienced a hypoxic episode.

Chapter 4 – In the creation of a metabolomic based prognostic for asthma, all stages of the disease must be examined, from stable quiescent asthma to exacerbations inducing severe bronchoconstriction and acute hypoxia. Acute hypoxia can be the result of severe bronchoconstriction, and it can potentially be fatal if prolonged long enough. Currently there are few techniques able to determine the extent of hypoxic injury. Additionally developing a urinary prognostic for hypoxic injury is particularly valuable to other fields such as neonatology for the evaluation of hypoxic injuries of birth. *This chapter reviews experiments evaluating metabolomic urinary biomarkers of acute hypoxic injury.* Comparisons are made between accuracy of physiological data and metabolomic based data in the diagnosis of hypoxic animals.

Specific Aim # 3 – To identify metabolites of virus-induced asthma exacerbation using urinary NMR metabolomic profiling in an animal model. I hypothesize that animals that have been exposed to a live respiratory virus will have a different urinary metabolite profile compared to animals that were not exposed to a respiratory virus.

Chapter 5 – Asthma has many different phenotypes with each having their own underlying pathophysiology. By identifying the biomarkers associated with multiple different asthma phenotypes we can better understand the pathophysiology and create better diagnostics. Virus infection is a leading cause of asthma exacerbation. *This chapter discusses ongoing experiments investigating urinary biomarkers in an animal model of viral induced asthma exacerbation.* Urine samples have been collected and targeted metabolite identification of the 1H-NMR spectra has been performed. The number of animals studied was small, which limited the strength of the data. Despite this, statistical analysis was performed to assess the possibility of using a metabolomic approach to the differentiate between virus infected and non-infected animals, and whether allergic inflammation created an additional unique metabolome.

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CHAPTER 2: *Methods*

Introduction

This chapter describes the methods used for the experimental analyses. While I completed the majority of this work, some of the methods were performed by collaborators and this is identified.

2.1 Guinea Pig Model Development

2.1.1 Guinea pig allergen sensitization

Specific pathogen-free female Hartley guinea pigs (GPs) (180-200 g; Charles River Laboratories, Saint-Constant, Canada) were obtained and allowed to adjust to their new environment for 14 days. After 14 days the animals were sensitized to an inert antigen, ovalbumin via intraperitoneal (i.p.) injections of 10 mg/kg of ultra pure ovalbumin dissolved in NaCl 0.9% (Sigma-Aldrich, St Louis, MO, USA). A second i.p. injection of the same dose was given 2 days later. Sensitization was confirmed after airway hyperreactivity measurements, with intravenous (I.V.) injection of 10mg/kg of ovalbumin dissolved in NaCl 9% at day 75 (1). This injection induced bronchoconstriction in sensitized GPs, but not in non-sensitized GPs.

2.1.2 Parainfluenza virus culture

Parainfluenza type 1 (Sendai virus, VR-105; American Type Culture Collection) was grown in cynomoglus monkey kidney cell monolayer (ViroMed Laboratories, California, USA) in LHC-8 medium for 1 week at 34°C 5% CO2. Cells and medium were frozen and

thawed, cleared by low-speed centrifugation (1000 x g 10min at 4°C), the resulting PIV titer was determined by the hemoadsorption test 1x07 serodilution, and the virus was stored in aliquots at −70°C

2.1.3 Guinea pig virus and sham virus inoculation

GPs were anesthetized intramuscularly with ketamine (30 mg/kg) and xylazine (5 mg/kg), and were inoculated intranasally with 0.5 mL of virus stock diluted in HBSS (Sigma-Aldrich) to produce a solution containing a 10^5/mL tissue culture infectious dose (TCID50; 10^5 times the concentration required to produce infection in 50% of rhesus monkey kidney monolayers) (1). Infected and non-infected animals were housed in individually sealed cages with their own laminar airflow in a level 2 bio-containment facility.

2.1.4 Aerosolized antigen challenge

GPs were placed individually into a sealed chamber with one access port. The animals were exposed to an aerosolized solution of 0.5% ovalbumin dissolved in PBS (Sigma-Aldrich, St Louis, MO, USA) for 20 seconds. Ovalbumin sensitization is confirmed with this treatment because ovalbumin sensitized GPs developed visible respiratory distress after aerosolized antigen challenge.

2.1.5 Dexamethasone treatment

GPs were given i.p. injections of 0.6 mg/kg dexamethasone dissolved in NaCl 0.9% (Sigma-Aldrich, St Louis, MO, USA) for 4 consecutive days before being re-exposed to

live PIV or UV-inactivated PIV. This dose was selected due to its use to treat moderate to severe Croup in children infected with PIV (2).

2.1.6 Anti-IL5 treatment

GPs were given i.p. injections of 240 μ g/kg anti-IL5 monoclonal antibodies (mepolizumab, Galaxo-Smith Kline, Mississauga, Ontario, Canada) once 5 days before virus re-exposure and again 3 days before virus re-exposure. This dose was selected due to its ability to decrease eosinophil numbers in humans with allergic asthma (3).

2.2 Guinea Pig Outcome Measurement

2.2.1 Assessment of bronchoconstriction and airway hyperreactivity

GPs underwent airway assessment 5 days after re-exposure to either live PIV or UV-inactivated PIV using a modified technique proposed by Dixon et al (4). GP were anaesthetized with i.p. injections of 1.5 g/kg of urethane (Sigma-Aldrich). Making a vertical incision above the sternum exposed the trachea following dissection below the superficial fascia and sternohyoid muscles. By extending the initial incision laterally, either the left or right external jugular vein can be accessed after dissecting through fascia and the deep pectoral muscle. The external jugular vein was catheterized with a dual lumen 4 Fr i.v. catheter (Cook Medical, Indiana, USA). GPs were tracheostomized, paralyzed with succinylcholine (10 g/kg/min. i.v., Sigma-Aldrich), and ventilated (tidal volume of 1 mL/100 g body weight; 100 BPM; Harvard Apparatus, South Natick, MA). PIP was recorded as a measure of bronchoconstriction at the tracheal cannula using a

DTX pressure transducer and Powerlab software (AdInstruments, Colorado Springs, Co, USA). GP were given doubling doses of histamine i.v. in 5-minute intervals (0.1-2.0 μg/kg, Sigma-Aldrichand the resulting change in pulmonary pressure was recorded (5). Sensitization was confirmed after airway hyperreactivity measurements, with the I.V. injection of 10mg/kg of ovalbumin dissolved in NaCl 9% (Sigma-Aldrich). The animals were then euthanized with an overdose of urethane (3 g/kg i.v.).

2.2.2 Bronchoalveolar lavage and cell staining

BAL was performed immediately after euthanization using 4-5 10 ml aliquots of 0.9% NaCl until 40 mL of lavage fluid was collected on ice. The BAL was centrifuged at 300 x g for 10 minutes at 4°C. The pellet was resuspended in 1 mL of erythrocyte lysis solution (R&D Systems Inc, Minneapolis, MN, USA), and diluted with 9 mL of 0.9% NaCl. The cells were then centrifuged again at 300 g for 10 minutes, and a cell pellet was collected. The pellet was then resuspended in 10 mL of 0.9% NaCl. Total cell numbers was determined from a 10 μL sample pipetted onto a hemocytometer. 100,000 cells from lavage fluid were cytospun on to glass slides. The slides were then stained using a commercial staining solution (Diff-Quik, Baxter Healthcare Corp, Mississauga, Ontario, Canada). The slides were allowed to dry, then put into the fixative solution (containing fast green in methanol) for 30 seconds, and then the solution was allowed to drain off the slide. The slide was next put into solution 1 (containing Eosin G in phosphate buffer) for 30 seconds. After 30 seconds the solution was allowed to drain off the slide. The slide was then put into solution 2 (thiazine dye in phosphate buffer) for 30 seconds, and the seconds had passed the slide was rinsed with distilled water and allowed

to air dry. A slide coverslip was then glued over top of the slide using (Permount, Fisher Scientific, Ottawa, Ontario, Canada), and the cell differential determined using light microscopy.

2.2.3 Lung removal, fixation, and sectioning

Lung removal from the GPs occured immediately after BAL collection. An incision was made laterally across the GP's abdominal cavity below the lower border of the ribs. The abdominal cavity was opened and the diaphragm is exposed. A lateral incision across the diaphragm was made, and surgical scissor were used to make a vertical incision to level of the tracheotomy tube taking care not to cut the trachea. Following the vertical incision the thoracic cavity was manually exposed by bilateral breakage of the ribs. The tracheal cannula was removed, and the trachea ligated as proximally to the skull as possible. The aorta, superior and inferior vena cava, and supporting ligaments were ligated and cut allowing for the heart, lungs, and trachea to be removed together. The tracheobronchial lymph nodes were dissected from the hilum of the lung and from the connective tissue surrounding the trachea, and stored in 5 mL of complete RPMI (RMPI 1610 with HEPES, 10% FBS, 2mmol/L glutamine, and non-essential amino acids, Invitrogen, Carlsbad, Calif, USA) on ice. A section of lung tissue was also removed and stored in an -80°C freezer for viral titer assessment. The lung was then inflated and fixed in 3.7% formaldehyde in 0.1% phosphate buffer for 24 hours. After 24 hours the lungs were cut into 1.5 cm^2 pieces placed in tissue cassettes and stored in 70% ethanol at 4°C until fixed in paraffin and sectioned (6.0 μ m). The resulting slides were saved for future analysis of eosinophil degranulation.

2.2.4 Lymphocyte enrichment, culture, and BrdU incorporation assay

The tracheobronchial lymph nodes were manually broken down under sterile conditions by using abrasive rubbing between glass slides in 10 mL of complete RPMI solution at 4°C. The lymph node homogenate solution was then pipetted up and down 10 times to remove adhered cells and disrupt clumps. The remaining tissue was removed by passing the homogenized sample through a 40 μ m cell strainer (BD Biosciences, Canada). The resulting cell solution was centrifuged at 300 x g for 10 minutes at 4°C, and the resulting cell pellet was resuspended in 10 mL of complete RPMI. The cell solution was then incubated in a 6-well plate for 2 hours at 37°C to remove adherent cells. The non-adherent cells were collected and the plates were lightly washed with room temperature RPMI to remove any remaining loosely adherent cells. The collected cells were then centrifuged in 10 mL of Ficoll (Pharmacia Biotech, Uppsala, Sweden) at room temperature. Lymphocyte bands were collected and resuspended in 1.5 mL of complete RPMI solution, and centrifuged at 300 x g for 10 minutes at 4°C. The cells were resuspended in 10 mL of complete RPMI and were incubated for 45 minutes in a nylon wool column (Polyscience Inc, Warrington, PA, USA) to enrich for T cells (6). The enriched T cell solution was collected and cell viability was determined using trypan blue exclusion test (7). Cells were then cultured in a 96 well plate with 100,000 cells in 200 µL volume per well. Viral memory responses were measured via a cell proliferation assay using BrdU incorporation (BrdU Cell Proliferation ELISA Chemiluminescent Kit; Roche, Mannheim, Germany) with the FLx800 luminometer (Biotek, Winooski, Vt, USA) and determined as relative light units (8). Cells were cultured in complete RMPI solution

with either BrdU alone (background luminescence), BrdU + phytohemagglutinin (Life Technologies Inc, Burlington, Ontario, Canada) (positive proliferation control), BrdU + $^{1}/_{1000}$ dilution of Rhino Virus (RV type 16) (virus specific proliferation control), or BrdU + $^{1}/_{1000}$ dilution of PIV stock solution (viral memory).

2.2.5 Hemadsorption assay and determination of UV-inactivation of virus

Confirmation of UV-virus inactivation was determined through analysis of lung samples from all animals re-exposed to UV-inactivated virus. Before GP lungs were fixed samples were taken and stored at −80°C and sent to the laboratory of Dr. David B Jacoby for analysis under the following protocol (1). Frozen samples were thawed, weighed, and homogenized in 2 mL PBS (Polytron™; Brinkmann). Virus was eluted from the tissue homogenate by incubating at 34°C for 1 hour. The suspensions were centrifuged at 450 x g for 30 minutes, and the supernatants were inoculated in serial 10-fold dilutions into fresh rhesus monkey kidney cell monolayers. After 1 week of incubation at 34°C, the monolayers were washed and the medium replaced with a 0.5% suspension of guinea pig erythrocytes in Hanks' PBS. After 1 hour, the erythrocytes were washed off and the monolayers were examined under an inverted phase–contrast microscope (Olympus Corp.) for evidence of hemadsorption (sticking of erythrocytes to the surface of cells because of expression of viral hemagglutinin on these surfaces). Viral content was determined as the amount of lung homogenate required to produce infection in 50% of rhesus monkey kidney monolayers (the TCID50), and is expressed as TCID50/g lung wet weight. Only data from virus-exposed GPs with confirmed PIV are reported.

2.2.6 Statistical analysis

T-cell proliferation data were analyzed using ANOVA with Dunnett's multiple comparison test to identify statistical significance between the means. All other data were analyzed using Two-Way ANOVA with the Bonferroni multiple comparison test to identify statistical significance between the means. All data analyses were performed on GraphPad Prism version 6.0 software for Macintosh (GraphPad Software, La Jolla, CA, USA).

2.3 Neonatal Piglet Hypoxia Evaluation

2.3.1 Surgical preparation of animals

All neonatal piglet experiments were carried out at the laboratory of Dr. Po-Yin Cheung (9). Male newborn Yorkshire-Landrace piglets 1-3 day of age weighing 1.6 to 2.5 kg were obtained from the University of Alberta Swine Research Centre. Animals were anesthetized initially maintained with inhaled isoflurane (2–3%) (Abbott Lab. North Chicago, IL, USA), and then switched to fentanyl (0.005– 0.05 mg/kg/h), midazolam (0.1–0.2 mg/kg/h) and pancuronium (0.05–0.1 mg/kg/h) (Sandoz Canada, Quebec City, Quebec, Canada) once mechanical ventilation was commenced. Oxygen saturation was continuously monitored with a pulse oximeter (Nellcor, Hayward, CA,USA), and heart rate and blood pressure were measured with a 78833B monitor (Hewlett Packard Co., Palo Alto, CA, USA). Fractional inspired oxygen concentration (FiO_2) was measured by an oxygen monitor (Ohmeda Medical, Laurel, MD) and maintained at 0.21–0.24 for oxygen saturation between 90 and 97%. Argyle catheters (5F; Sherwood Medical Co., St.

Louis, MO, USA) were inserted via the right femoral artery and vein for continuous measurement of MAP and central venous pressure, respectively. All medications and fluids were administered via the femoral venous catheter. Via a tracheotomy, pressure-controlled assisted ventilation was commenced (Model IV-100, Sechrist Industries Inc., Anaheim, CA, USA) with pressure of 20/4 cm H_2O at a rate of 18–20 breaths/min. A left anterior thoracotomy was performed to expose the main pulmonary artery. A 6-mm transit time ultrasound flow probe (6SB, Transonic Systems Inc., Ithaca, NY, USA) was placed around the main pulmonary artery to measure the blood flow as a surrogate of CO. The ductus arteriosus was ligated. A 20G Insyte-W angiocatheter was inserted into bladder transcutaneously to drain the urine.

Maintenance fluids during experimentation consisted of 5% dextrose at 10 mL/kg/h and 0.9% normal saline solution at 2 mL/kg/h. The dosages of fentanyl, midazolam and pancuronium were adjusted to maintain minimum body movements throughout the experimental period. Propofol (0.1–0.2 mg/kg/h) (AstraZeneca Canada, Quebec City, Quebec, Canada) was given as needed to maintain anesthesia. The body temperature was maintained at 38.5–39.5°C using an overhead warmer and a heating pad.

2.3.2 Hypoxia experimental protocol

After surgery, animals were stabilized for at least 60 min. Piglets were block-randomized into a sham-operated group (ventilation with FiO_2 of 0.21 without hypoxia for 6 h, n= 15) or a H-R group (ventilation for 2 h with an FiO_2 of 0.10–0.13 using nitrogen and oxygen gas mixture achieving a PaO_2 20–40 mmHg, n= 17). After hypoxia, the HR piglets were

resuscitated with a FiO_2 of 1.0 for 0.5 h, followed by 0.21 for the last 3.5 h of the experimental period. Blood gases were studied every 30-60 min throughout the experiment. Peak inspiratory pressure (18–25 cm H_2O) and respiratory rate (12–20 breaths/min) were adjusted in all animals in an attempt to maintain normocapnia during experimentation. At the end of the experiment, each piglet was euthanized with an overdose of pentobarbital (100 mg/kg, i.v.).

2.3.3 Physiological recordings and calculations

Hemodynamic parameters (heart rate, MAP and pulmonary artery flow) were recorded at specific predetermined time points at baseline and throughout hypoxia and reoxygenation.

2.3.4 Statistical analysis of physiological data

All results were expressed as median and inter-quartile range. A one-way ANOVA test with Bonferroni correction was used to study the differences among groups. Statistical analyses were performed using Graphpad Prism version 6.0 software, (San Diego, CA) Significance was set at $p<0.05$.

2.4 Metabolomic Analysis

2.4.1 Urine sample preparation

Urine samples were collected either by direct cystocentesis in GPs, or through a bladder catheter in newborn piglets. GP urine collection occurred immediately after the last PIP value was collected before euthanasia of the animal. Neonatal piglet urines were collected at 3 time points, the first was taken right after experimental surgeries were completed and ventilation was begun, the second was taken during controlled hypoxia episodes, and the third was collected at the end of re-oxygenation following hypoxia. Urine samples were collected in a sterile eppendorff tube, and 25 μL of sodium azide was added as a bacteriostatic agent. The sample was then stored in a −80°C until the day of NMR analysis. On the day of analysis the samples were thawed and the pH adjusted to between 6.60 and 6.70 through the addition of NaOH and/or HCL. Following the pH adjustment 630 μL of the sample was then added to 70 μL of an NMR standard solution (containing 4.9 mM of DSS and 100 mM of imidazole in D_2O). Then 600 μL of the sample was then transferred into a standard 5-mm NMR glass tube (Wilmad LabGlass, Wilmad, NJ) and the sample stored at 4°C until analyzed (10).

2.4.2 ^{1}H-NMR spectral acquisition

All ^{1}H-NMR spectra were acquired on a 600-MHz VNMRS spectrometer (Varian Inc., Palo Alto, Calf) equipped with a 5-mm inverse-proton (HX) probe with z-axis gradient coil (11). One-dimensional ^{1}H-NMR spectra were collected at 258C by using the first increment of a 2-dimensional-^{1}H, ^{1}H-NOESY (1-dimensional, 3-pulse NOESY, with a transmitter presaturation delay of 900 ms for water suppression during the preacquisition delay and 100 ms mixing time), and a spectral width of 7200 Hz (phase cycle available

on request). The time-domain data points were 64 kilo complex points, acquisition time was 4 seconds, the 908 pulse was 6.8 microseconds, repetition time was 5 seconds, there were 4 steady-state scans, and the number of acquired scans was 32 per free induction decay. The data were apodized with an exponential window function corresponding to a line broadening of 0.5 Hz, 0-filled to 128 k complex points, and Fourier transformed (12).

2.4.3 Targeted NMR spectral analysis

Spectral identification and quantification of metabolites was performed using the Chenomx NMR Suite Professional software package Version 7.1 on NMR spectra obtained with the aforementioned methods (Chenomx Inc., AB, Canada). The software contains a database of known metabolites with their referenced ^{1}H-NMR spectral resonant frequencies. These known resonant frequencies were matched to the observed resonant frequencies of the collected spectra, enabling the qualitative and quantitative analysis of metabolites in urine. To account for potential differences in hydration, each metabolite value was standardized to the sample's individual measurement of urine creatinine (12).

2.4.3 PLS-DA analysis of urinary metabolite concentrations

PLS-DA was performed (SIMCA-P 11, Umetrics, USA), which determines the relationship between the response vector Y (i.e. sham control group versus hypoxemia) and the matrix X (concentration of each metabolite) by simultaneous projections of both Y and X spaces to a plane. Seven-fold internal cross validation was performed. PLS-DA generates a prediction score (0-1) for each animal based on the value of the metabolites

(i.e., scores <0.5 would be predicted to be sham control animals versus hypoxic animal >0.5). This process identifies the metabolites whose concentrations differed significantly between groups of animals. The significance of a metabolite depends on the level of concentration variation between treatment groups. Metabolites of low significance are detrimental to model accuracy and should be removed (12).

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CHAPTER 3: *Eosinophils induce enhanced airway reactivity via activation by immunological memory to respiratory viral antigens in an allergen-challenged model of asthma*

Acknowledgements

Animal experiments described in this chapter were performed by Mr. Christopher Skappak and Dr. Darryl Adamko. All cellular experimental procedures described in this chapter were performed by Mr. Christopher Skappak. Statistical Analysis was performed by Mr. Christopher Skappak.

3.1 Introduction

Asthma is a heterogeneous chronic inflammatory disease of the airways that is clinically manifested by reversible airflow obstruction, shortness of breath, coughing, and wheezing. There are multiple phenotypes of asthma with the most abundant being allergic (atopic) asthma. The most common trigger for asthma exacerbations in adults and children, are infections with common respiratory viruses. Individuals are likely to encounter the same respiratory virus multiple times during interactions in their home, work, or school environments.

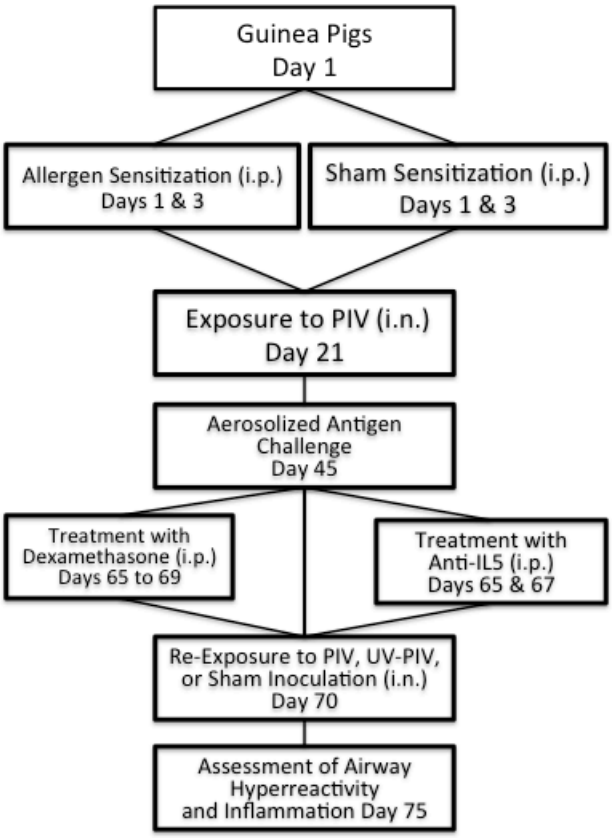
For an unknown reason, individuals with allergies and asthma develop more severe clinical symptoms that last longer when infected with a common respiratory virus then healthy individuals. There are multiple hypotheses as to why this happens with one of

them focusing on the characteristics of inflammation in the lungs of asthmatics. The airway inflammation in allergic asthma is defined by Th_2 inflammation and the presence of effector cells like eosinophils. The eosinophils have been reported to induce airway hyperresponsiveness in the context of virus infection. Our previous studies have demonstrated a mechanism where eosinophils undergo degranulation in response to activation from memory CD4+ T cells when cultured with respiratory virus antigens, *in vitro*. We propose that this same mechanism can happen *in vivo* in the natural contact with respiratory antigens in humans. The goal of this chapter is to determine the role of immunological memory in viral induced exacerbation in an *in vivo* model of allergic asthma.

3.2 Methods

For the detailed methods see **CHAPTER 2** sections 2.1 and 2.2. Figure 3.1 outlines the different treatment groups used for this experiment and the relative timelines of treatment.

Figure 3.1: **Experimental timeline flowchart**. Total duration of the experiment was 75 days for all treatment groups.



3.2.1 Animal ethics statement

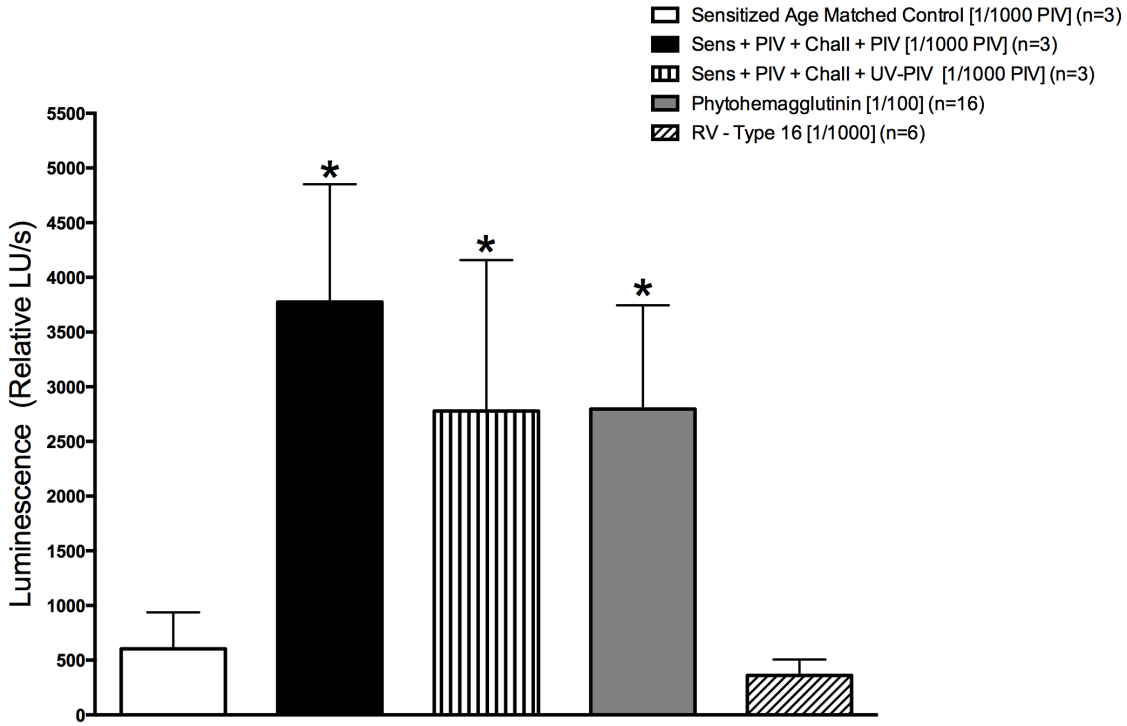
All animals were used in accordance with the standards and protocols of the University of Alberta's Animal Care and Use Committee for the Health Sciences (ACUC-HS, protocol number 061/03/06/D) and the guidelines of the Canada Council on Animal Care.

3.3 Results

3.3.1 Lymphocyte proliferation in-vitro confirms T cells with immune memory to parainfluenza virus

A T cell enriched population of lymphocytes was created from the tracheobronchial lymph nodes of each animal group. Lymphocytes from GPs expected to have virus-specific immune memory (Sens+PIV+Chall+PIV and Sens+PIV+Chall+UV-PIV) showed significant proliferation when cultured with a $^{1}/_{1000}$ dilution of para-influenza virus ($p<0.001$ and $P<0.01$, Figure 3.2) compared to cells from GPs without immune memory (Sens Age Matched Control). In addition, specific memory to PIV was confirmed, as lymphocytes from these animal groups did not show proliferation in response to another respiratory virus, Rhinovirus-16.

Figure 3.2: **Primary T cell cultures demonstrated virus specific proliferation when immune memory was present.** Animals re-exposed to live (black bar) (n=3) and UV-inactivated virus (vertical striped bar) (n=3) demonstrated clonal expansion and BrdU incorporation when exposed to PIV *in-vitro*. [Error bars represent SEM]



3.3.2 Re-exposure to live parainfluenza virus enhances airway hyperreactivity to histamine and increases airway inflammation in both non-sensitized and ovalbumin sensitized guinea pigs

As might be expected, both non-sensitized and ovalbumin sensitized GPs re-exposed to live PIV demonstrated a significant increase in airway reactivity compared to respective age matched and sham controls (Figure 3.3, $p<0.0001$ each). The degree of increased bronchoconstriction was higher in the sensitized GP compared to non-sensitized GP ($p<0.05$). Both non-sensitized and sensitized animals re-exposed to live PIV showed significantly increased total cell numbers in the BAL compared to their respective controls (Figure 3.4, $p<0.001$ each). In sensitized animals, this cell count was made up specifically of a significant increase in the number of macrophages and eosinophils relative to both controls ($p<0.0001$ and $p<0.001$). In non-sensitized animals this increase in cell count was made up primarily of eosinophils and macrophages.

Figure 3.3: **Repeat exposure to live virus increased histamine induced dependent airway reactivity in non-sensitized and sensitized animals at Day 75. (A)** Non-sensitized animals re-exposed to live virus (star) (n=5) have significantly higher airway reactivity compared to sham controls (closed square) (n=3) and age-matched controls (closed triangle) (n=5). **(B)** Sensitized animals re-exposed to live virus (star) (n=5) have significantly higher airway reactivity at 1.0 and 2.0 μg/kg of histamine compared to sham controls (open square) (n=4) and age-matched controls (open triangle) (n=3). [Error bars represent SEM]

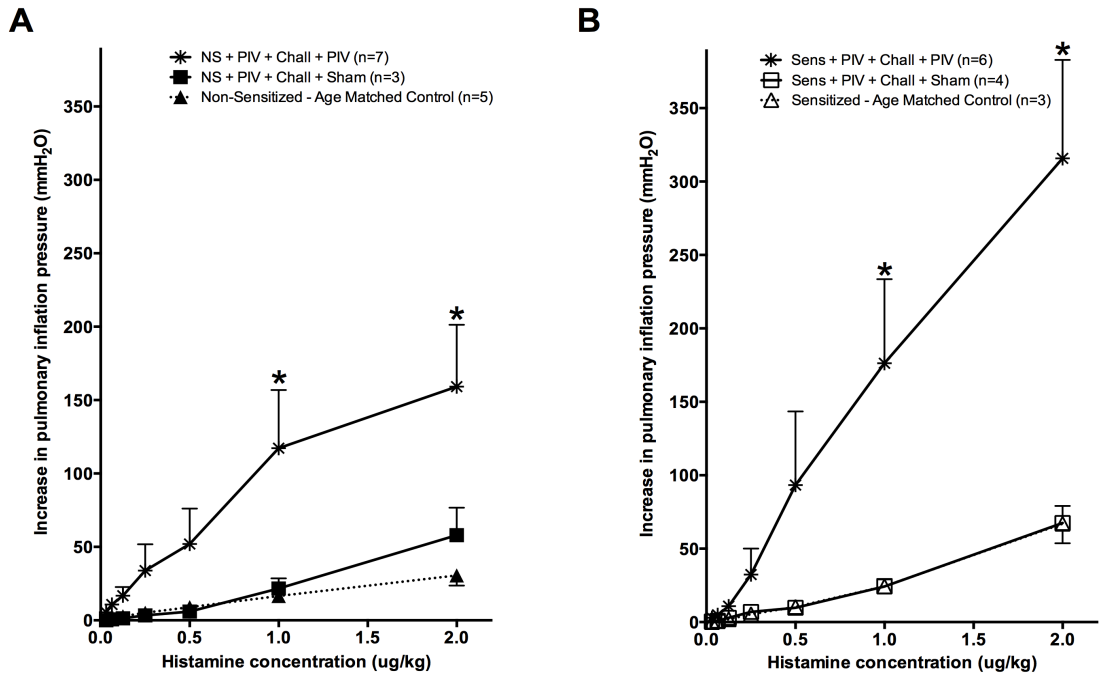
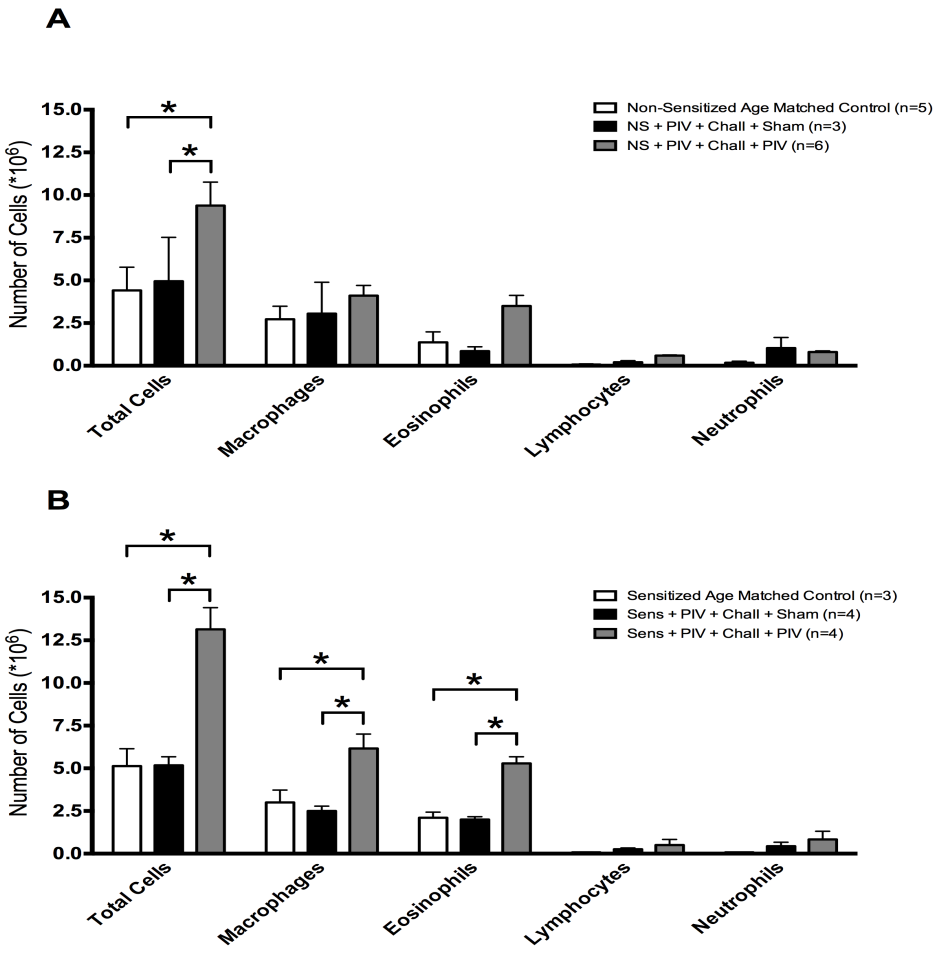


Figure 3.4: **Sensitized animals had significantly higher numbers of eosinophils and macrophages in the airways following re-exposure to live virus as seen in BAL at Day 75. (A)** Non-sensitized animals re-exposed to live virus (grey bar) (n=6) have significantly higher increases in total cell counts compared to age-matched controls (white bar) (n=5) and sham controls (black bar) (n=3). **(B)** Sensitized animals re-exposed to live virus (grey bar) (n=4) have significantly higher increases in total cell counts, macrophage numbers, and eosinophil numbers compared to age-matched controls (white bar) (n=3) and sham controls (black bar) (n=4). [Error bars represent SEM]



3.3.3 Re-exposure to UV-inactivated parainfluenza virus enhanced airway reactivity to histamine in only the sensitized guinea pigs

Non-sensitized animals exposed to UV-inactivated PIV did not develop significantly increased reactivity to histamine compared to animals that were re-exposed to live PIV or uninfected controls (Figure 3.5). In contrast sensitized GPs re-exposed to a UV-inactivated PIV demonstrated a significant increase in airway reactivity compared to sham controls at histamine doses of 1.0 and 2.0 μ g/kg (Figure 4, $p=0.05$ and $p<0.0001$). Both non-sensitized and sensitized animals had significantly increased total cell numbers in the BAL compared to their respective sham controls (Figure 3.6, $p=0.05$ and $p<0.0001$). In regard to individual cell types, this increase was only significant in the sensitized animals with an increase in macrophages and eosinophils relative to sham controls ($p<0.01$ and $p<0.01$).

Figure 3.5: **Exposure to UV inactivated virus induced airway reactivity to histamine in sensitized animals, but not in non-sensitized animals at Day 75. (A)** Non-sensitized animals re-exposed to UV- inactivated virus (star) (n=5), did not have significantly higher levels of airway hyperreactivity at 1.0 and 2.0 μ g/kg of histamine compared to sham controls (closed triangle) (n=3). **(B)** Sensitized animals exposed to UV-inactivated virus (star) (n=8) had significantly higher airway hyperreactivity at 1.0 and 2.0 μ g/kg of histamine compared to sham controls (open triangle) (n=4). [Error bars represent SEM]

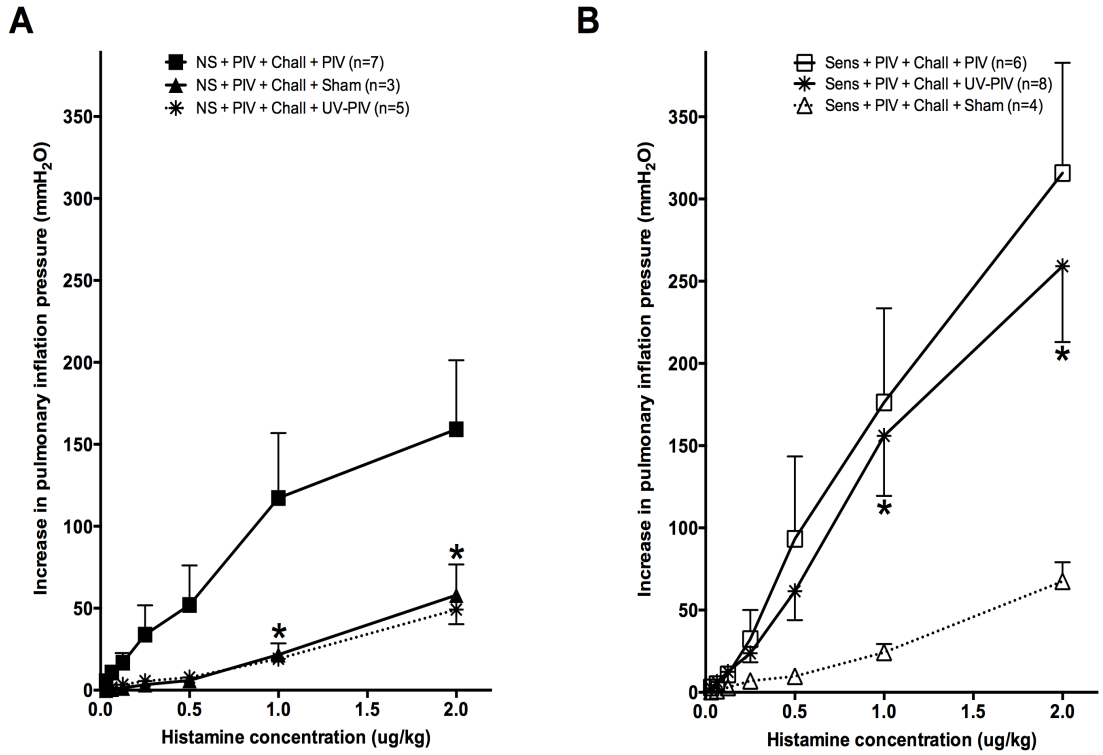
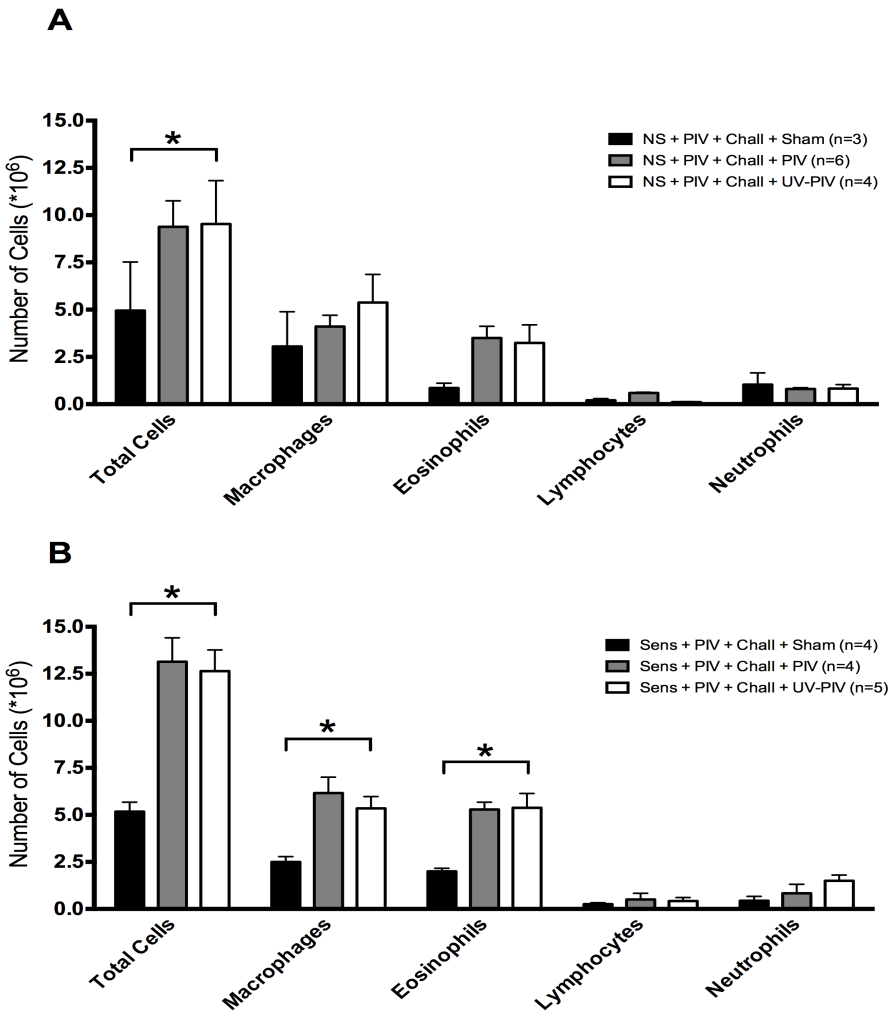


Figure 3.6: **Sensitized animals had significantly higher numbers of eosinophils and macrophages in the airways following re-exposure to UV inactivated virus as seen in the BAL at Day 75. (A)** Non-sensitized animals exposed to UV-inactivated virus (white bar) (n=5) had a significant increase in total cell numbers compared to sham controls (black bar) (n=3). **(B)** Sensitized animals exposed to UV-inactivated virus (white bar) (n=5) have significantly higher increases in total cell numbers, which was made of a significant increase in macrophages, and eosinophils compared to sham controls (black bar) (n=4). [Error bars represent SEM]



3.3.4 Treatment with dexamethasone prior to re-exposure with live PIV decreases airway reactivity to histamine in only sensitized animals

Non-sensitized animals treated with DEX and re-exposed to live PIV continued to have significantly increased airway reactivity compared to sham treated animals (Figure 3.7, $p=0.05$). In contrast, sensitized animals treated with DEX and re-exposed to live PIV demonstrated a significant decrease in airway reactivity compared to animals exposed to live virus alone (Figure 3.7, $p=0.05$ and $p<0.0001$). While, both non-sensitized and sensitized animals treated with DEX before live PIV showed significantly decreased total cell numbers in the BAL compared to animals re-exposed to live PIV alone (Figure 3.8, $p=0.05$ and $p=0.05$), no statistical significance was seen in the individual cell counts.

Figure 3.7: **Treatment with dexamethasone prevented histamine dependent airway reactivity in sensitized animals re-exposed to live virus, but not in non-sensitized animals at Day 75. (A)** Non-sensitized animals treated with dexamethasone (DEX) and re-exposed to live virus (star) (n=6) have significantly increased airway reactivity at 2.0 μ g/kg of histamine compared to sham controls (closed triangle) (n=3). This level was similar to untreated animals re-exposed to live virus alone (closed square) (n=7). **(B)** Sensitized animals treated with DEX and re-exposed to live virus (star) (n=4) had significantly lower airway reactivity at 1.0 and 2.0 μ g/kg of histamine compared to animals re-exposed to live virus alone (open square) (n=6) (n=4). [Error bars represent SEM]

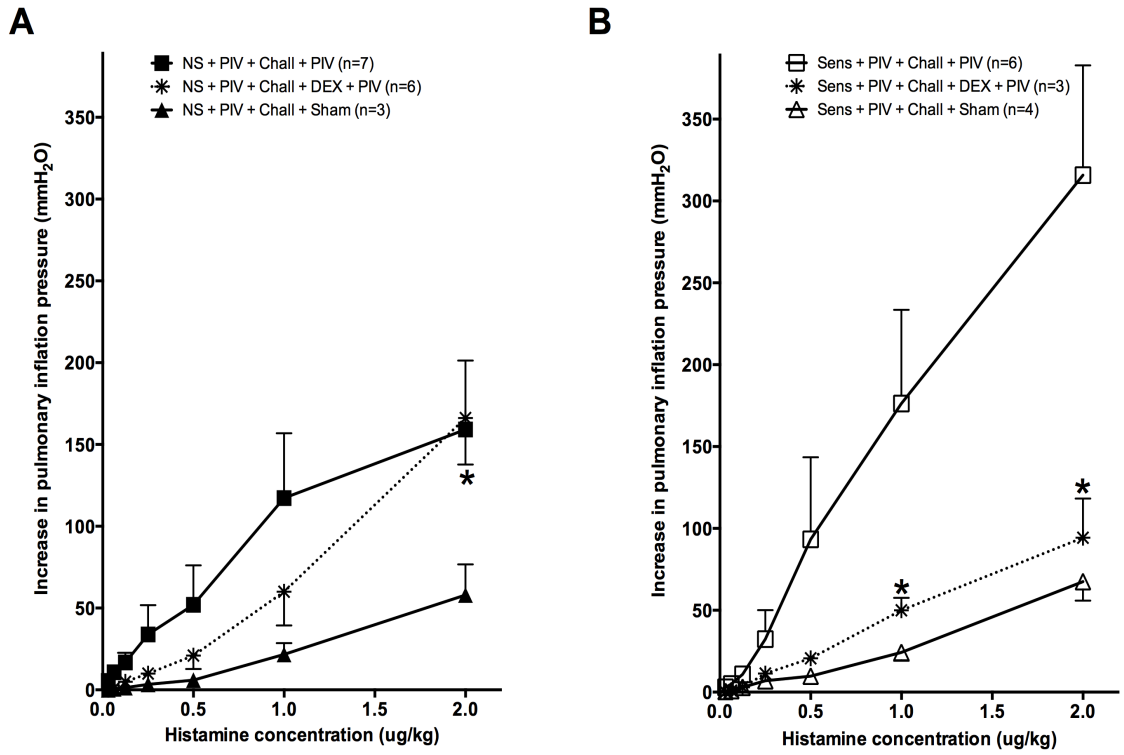
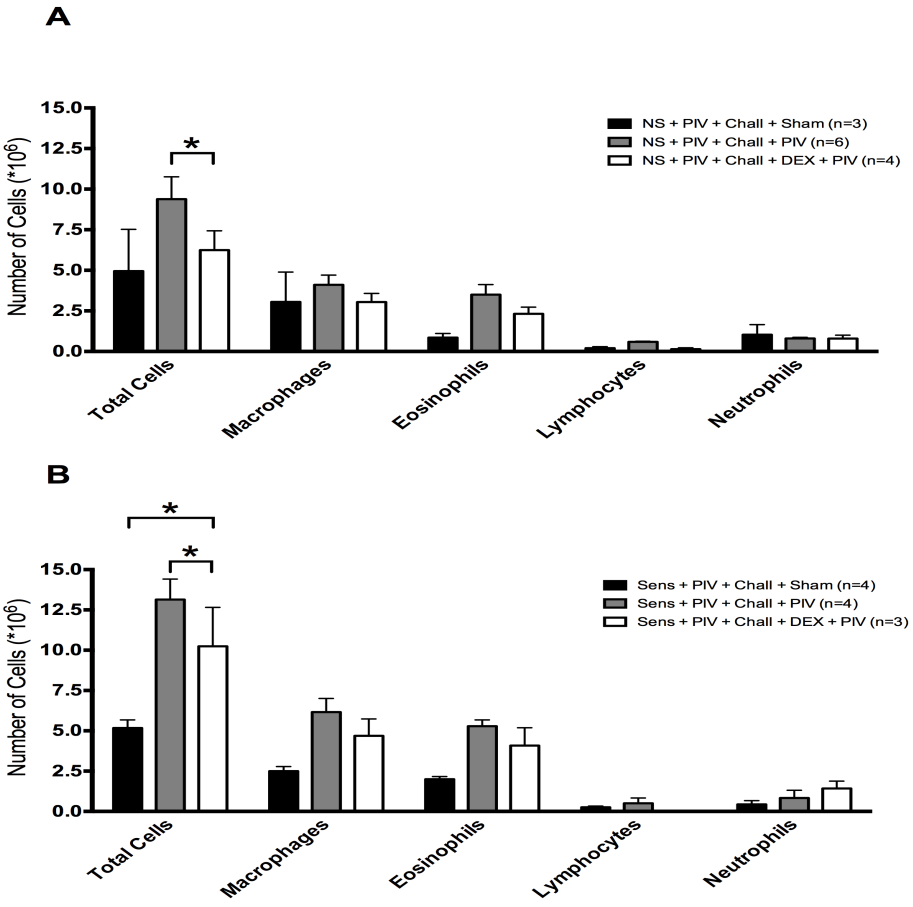


Figure 3.8: **Treatment with dexamethasone decreased total cell numbers in the airways of both non-sensitized and sensitized animals re-exposed to live virus at Day 75. (A)** Non-sensitized animals treated with DEX and re-exposed to live virus (white bar) (n=4) had significantly decreased total cell numbers compared to animals re-exposed to live virus alone (grey bar) (n=6). **(B)** Sensitized animals treated with DEX and re-exposed to live virus (white bar) (n=3) had significantly decreased total cell numbers compared to animals re-exposed to live virus alone (grey bar) (n=4), but levels remained higher compared to sham controls (black bar) (n=4). [Error bars represent SEM]



3.3.5 Treatment with dexamethasone prior to exposure to UV-inactivated PIV decreases airway hyperreactivity and airway inflammation in only the sensitized animals

Non-sensitized animals treated with DEX and exposed to UV-inactivated PIV demonstrated significantly increased airway reactivity compared to animals not given DEX that were exposed to UV-inactivated PIV alone and sham animals (Figure 3.9, $p<0.0001$). In contrast, sensitized GPs treated with DEX and exposed to UV-inactivated PIV demonstrated a significant decrease in airway reactivity ($p<0.0001$ and $p<0.0001$) compared to animals not given DEX but exposed to UV-inactivated PIV alone and sham controls. While total cell numbers and individual cell counts did not change significantly in non-sensitized animals treated with DEX before exposure to UV-inactivated PIV, in sensitized animals, treatment with DEX did induce a significant decrease in total cell number (Figure 3.10, $p=0.05$ and $p<0.0001$). The counts for individual cell types did not reach significance.

Figure 3.9: **Treatment with dexamethasone prevents enhanced histamine dependent airway reactivity in sensitized animals exposed to UV-inactivated virus at Day 75. (A)** Non-sensitized animals treated with dexamethasone and exposed to UV-inactivated virus (star) (n=5) developed significantly increased airway reactivity at 2.0 μg/kg of histamine compared to sham controls (closed triangle) (n=3), and to untreated animals exposed to UV-inactivated virus (closed square) (n=5). **(B)** Sensitized animals treated with dexamethasone and exposed to UV-inactivated virus (star) (n=5) had significantly lower airway reactivity at 1.0 and 2.0 μg/kg of histamine similar to sham controls (open triangle) (n=4) and when compared to untreated animals exposed to UV-inactivated virus (open square) (n=8). [Error bars represent SEM]

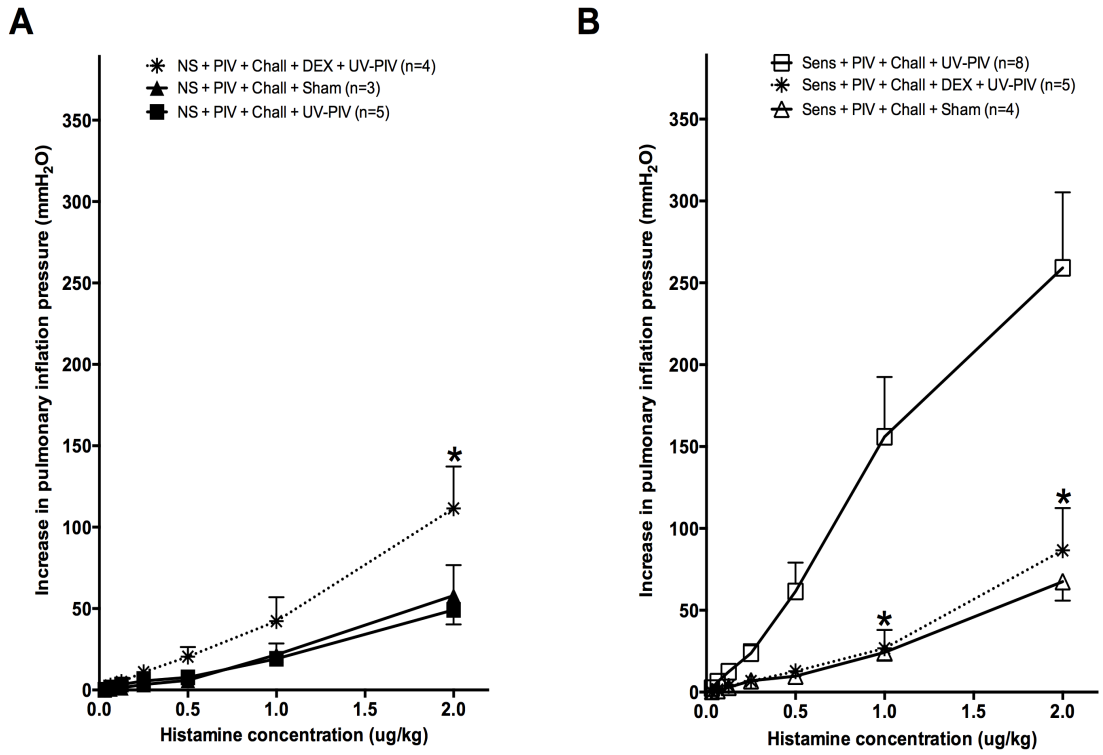
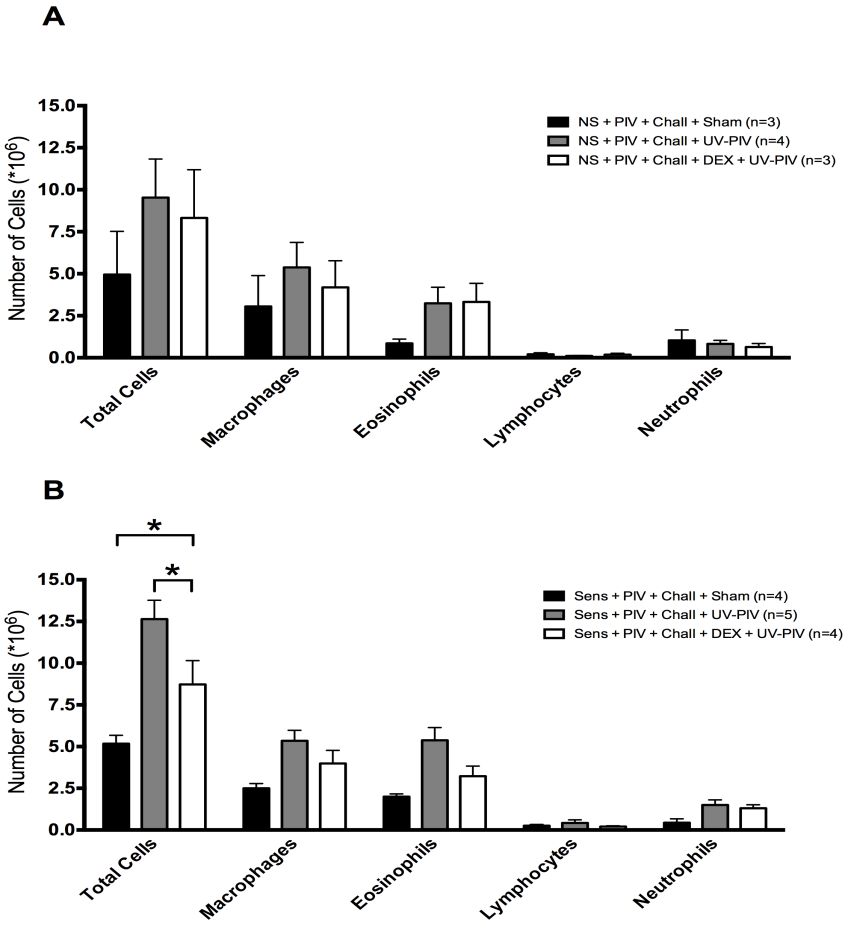


Figure 3.10: **Treatment with dexamethasone decreased total cell numbers in the airways of sensitized animals exposed to UV-inactivated virus at Day 75. (A)** Non-sensitized animals treated with dexamethasone and exposed to UV-inactivated virus (white bar) (n=3) did not have any significant changes in cell numbers compared to sham controls (black bar) (n=3) or untreated animals exposed to UV-inactivated virus (grey bar) (n=4). **(B)** Sensitized animals treated with dexamethasone and exposed to UV-inactivated virus (white bar) (n=4) had significantly decreased total cell numbers compared to untreated animals exposed to UV-inactivated virus (grey bar) (n=5). Levels were similar compared to sham controls (black bar) (n=4). [Error bars represent SEM]



3.3.6 Treatment with anti-IL5 monoclonal antibody (mepolizumab) prior to re-exposure to live PIV decreased airway reactivity in only sensitized animals: Sensitized animals treated with aIL-5 and re-exposed to live PIV demonstrated a significant decrease in airway reactivity compared to animals exposed to live virus alone (Figure 3.11, $p=0.05$). Non-sensitized animals demonstrated no changes in airway reactivity when treated with anti-IL-5 (data not shown). Both non-sensitized and sensitized animals had significantly increased total cell numbers when treated with anti-IL-5 and re-exposed to live PIV in the BAL compared to animals re-exposed to live PIV alone (Figure 3.12, $p=0.05$ and $p<0.0001$). Sensitized animals also saw a significant increase in the number of macrophages ($P<0.01$) compared to animals re-exposed to live PIV alone (figure 3.12), but no significant decrease in eosinophil numbers. Unfortunately experiments with an isotype control were not able to be preformed.

Figure 3.11: **Treatment with a monoclonal anti-IL5 antibody (mepolizumab) did not prevent histamine dependent bronchoconstriction in non-sensitized animals re-exposed to live virus at Day 75. (A)** Non-sensitized animals treated with anti-IL5 and re-exposed to live virus (star) (n=3) did not have significant decreases in airway reactivity compared to animals re-exposed to live virus alone (closed square) (n=7), and sham controls (closed triangle) (n=3). **(B)** Sensitized animals treated with anti-IL5 and re-exposed to live virus (star) (n=3) had a significant decrease in airway reactivity at 2.0 μ g/kg of histamine compared to animals re-exposed to live virus alone (open triangle) (n=5), and sham controls (open square) (n=4). [Error bars represent SEM]

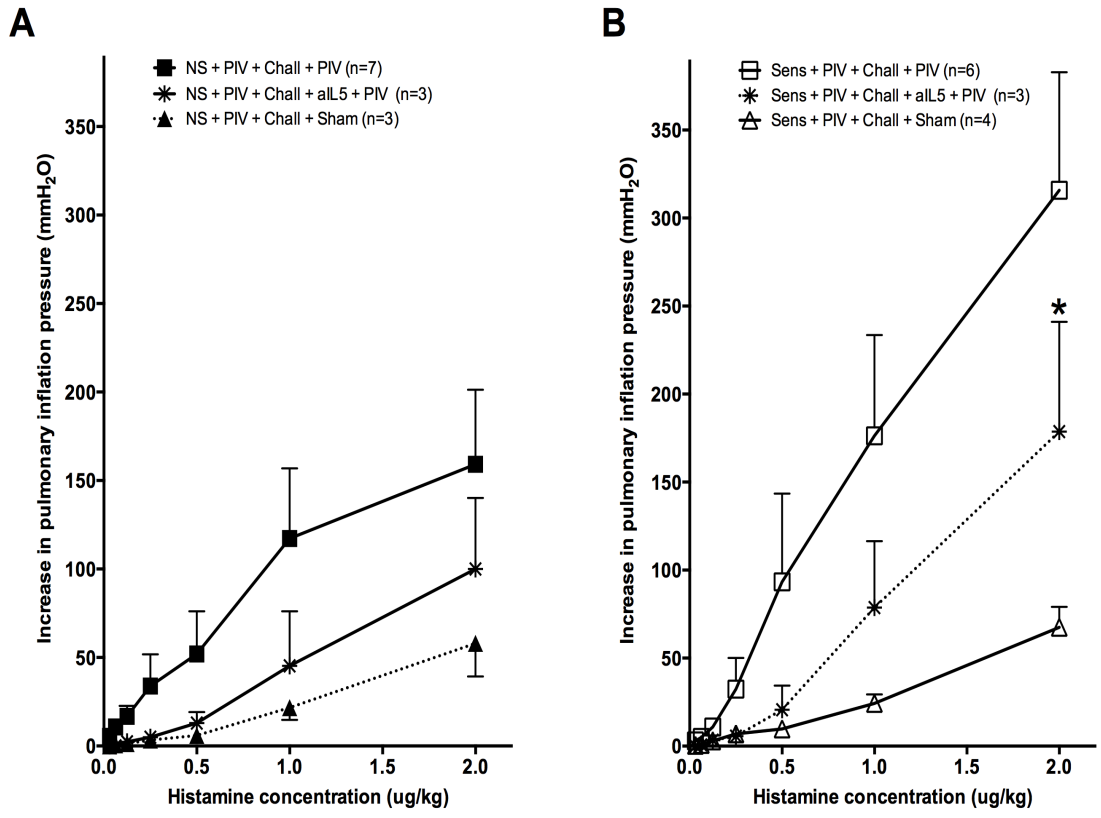
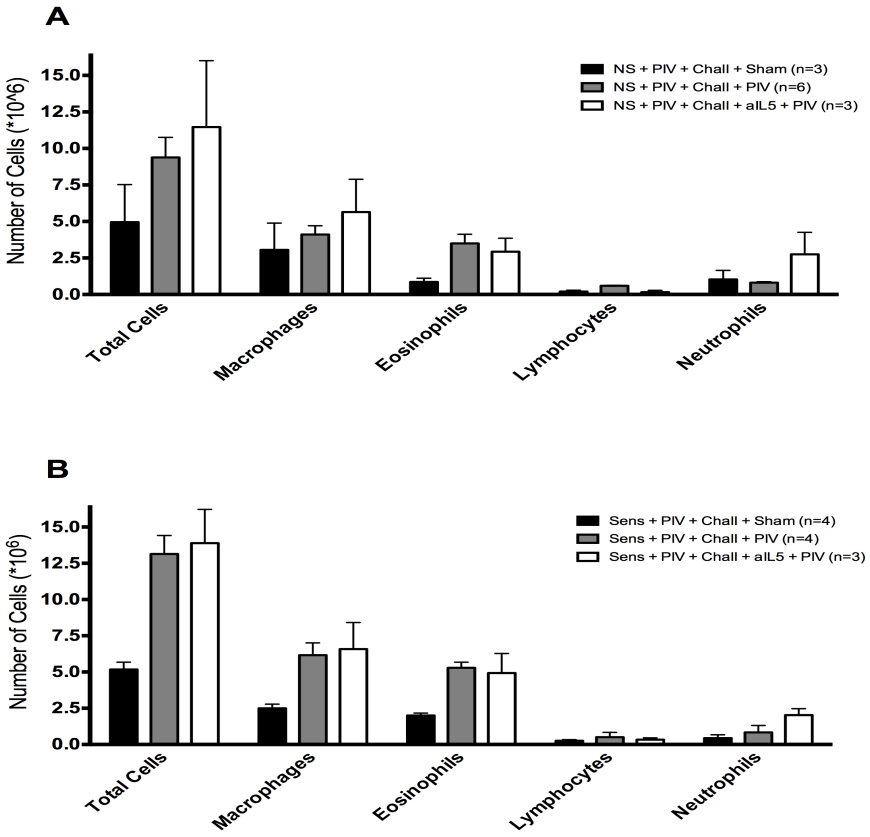


Figure 3.12: **Treatment with a monoclonal anti-IL5 antibody increased cell numbers in the airways of both non-sensitized and sensitized animals re-exposed to live virus**

at Day 75. (A) Non-sensitized animals treated with anti-IL5 and re-exposed to live virus (white bar) (n=3) had significantly increased total cell numbers compared to sham controls (black bar) (n=3), which were similar to untreated animals re-exposed to live virus (grey bar) (n=6). **(B)** Sensitized animals treated with anti-IL5 and re-exposed to live virus (white bar) (n=3) had significantly increased total cell numbers including macrophages compared to sham controls (black bar) (n=4), which was similar to untreated animals re-exposed to live virus (grey bar) (n=4). [Error bars represent SEM]



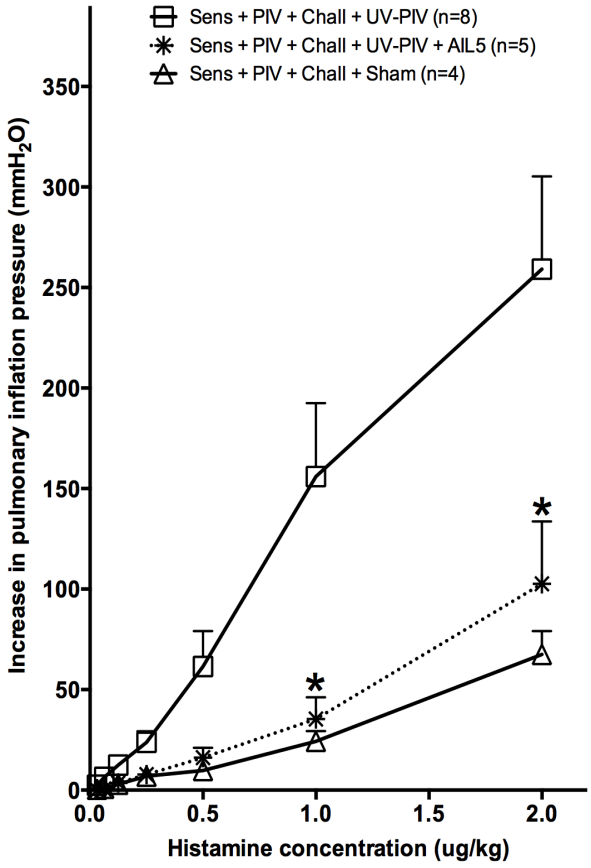
3.3.7 Treatment with anti-IL5 monoclonal antibody (mepolizumab) prior to exposure to UV-inactivated PIV decreased airway hyperreactivity: Sensitized GPs treated with anti-IL-5 before exposure to UV-inactivated PIV demonstrated a significant decrease in airway reactivity compared to untreated animals exposed to UV-inactivated PIV (Figure

3.13A, $p<0.0001$ and $p<0.001$). Non-sensitized animals treated with anti-IL-5 and exposed to UV-inactivated PIV did not demonstrate any AHR (data not shown). Sensitized animals treated with anti-IL5 before UV-inactivated PIV exposure demonstrated significant decreases in BAL total cell number ($p<0.0001$), which was made up of a significant decrease in the number of macrophages ($P<0.01$), and the number of eosinophils ($p<0.0001$) compared to untreated animals exposed to UV-inactivated PIV (figure 3.13B). Non-sensitized animals demonstrated no changes when treated with anti-IL-5 prior to exposure to UV-inactivated PIV (data not shown).

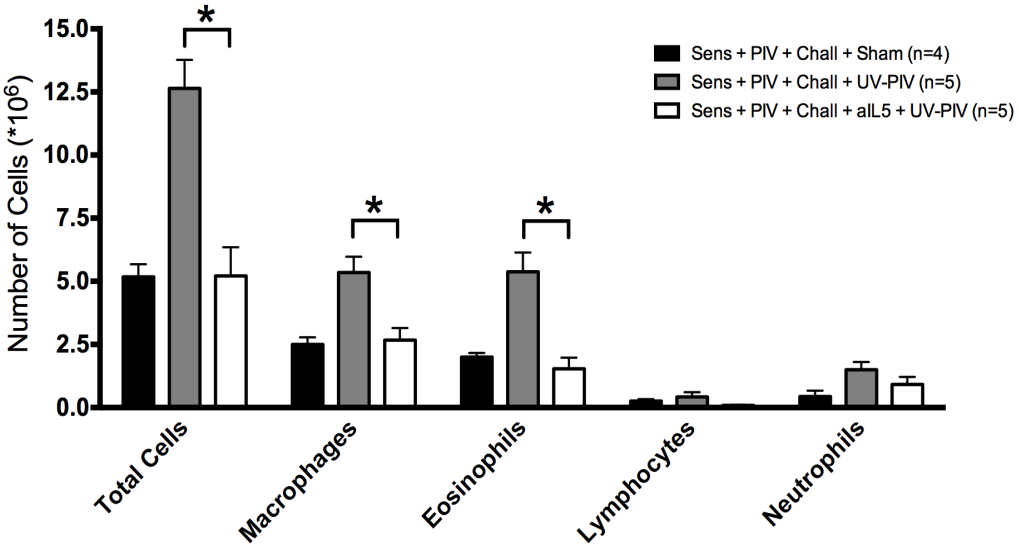
Figure 3.13A: **Treatment with a monoclonal anti-IL5 antibody (mepolizumab) prevented histamine dependent airway hyperreactivity in sensitized animals exposed to UV-inactivated virus at Day 75.** Sensitized animals treated with anti-IL5 and exposed to UV-inactivated virus (star) (n=5) had significantly lower airway reactivity compared to exposed to untreated UV-inactivated virus animals (open square) (n=8), similar to sham controls (open triangle) (n=4). [Error bars represent SEM]

Figure 3.13B: **Treatment with a monoclonal anti-IL5 antibody (mepolizumab) decreased cell numbers in the airways of sensitized animals exposed UV-inactivated virus.** Sensitized animals treated with anti-IL5 and exposed to UV-inactivated virus (white bar) (n=5) had significantly decreased total cell numbers, macrophages, and eosinophils compared to animals exposed to UV-inactivated virus alone (grey bar) (n=5), which were similar to sham controls (black bar) (n=4). [Error bars represent SEM]

A


Sens + PIV + Chall + UV-PIV (n=8)
Sens + PIV + Chall + UV-PIV + AIL5 (n=5)
Sens + PIV + Chall + Sham (n=4)
Increase in pulmonary inflation pressure (mmH2O)
350
300
250
200
150
100
50
0
0.0
0.5
1.0
1.5
2.0
Histamine concentration (ug/kg)
*
*

B


Sens + PIV + Chall + Sham (n=4)
Sens + PIV + Chall + UV-PIV (n=5)
Sens + PIV + Chall + aIL5 + UV-PIV (n=5)
Number of Cells (*10^6)
15.0
12.5
10.0
7.5
5.0
2.5
0.0
*
*
*
Total Cells
Macrophages
Eosinophils
Lymphocytes
Neutrophils

3.4 Discussion

These results demonstrate that allergic inflammation is critical to the development of airway reactivity in animals re-exposed to a viral antigen for which they have previously developed immune memory. We developed this model in an effort to mimic natural responses, as asthmatics in real life will be exposed multiple times to the same viral antigens after developing immune memory to a specific respiratory virus. Over the last decade two theories have been proposed as to why asthmatics have increased and more severe lower respiratory tract symptoms in response to respiratory viral infections than non-asthmatics (1).

Increased viral burden and immune system dysfunction being the underlying factor behind the severity of respiratory virus induced AHR in humans with asthma is one school of thought that have been studied. It is hypothesized that individuals with asthma will have more severe viral infections and higher viral loads in their lower airways than normal humans. Bardin et al. found that atopic patients given experimental RV infections had increased albumin levels in nasal washes, which corresponded to a higher level of upper respiratory tract inflammation compared to normal humans with the same viral infection (2). However, they found no difference in the level of viral shedding or viral loads between atopic and non-atopic individuals (2). Another study by Message et al. found the opposite with atopic asthmatics having increased nasal virus shedding in response to experimental RV infection, while also demonstrating increased airway reactivity in response to histamine challenge (3). A later study by Denlinger et al.

evaluated the viral load in natural RV infections in asthmatics, asthmatics undergoing an acute exacerbation, and non-asthmatic individuals (4). They found that non-atopic and atopic asthmatic individuals had similar viral burden and neutrophila in their sputum. Differences were only observed in the asthmatic individuals experiencing an acute exacerbation, where viral burden was the same, but there was increased neutrophilia in sputum (4). These three studies demonstrate the conflicting results in testing this hypothesis, while suggesting that inflammatory cells in the lung are associated with differences in acute viral induced airway reactivity in patients with asthma.

The rationale behind this hypothesis is that the increased viral burden is due to deficient aspects of the innate immune response to viral infections, particularly an impaired synthesis of interferons. Wark et al. proposed this hypothesis after demonstrating that primary cultures of bronchial epithelial cells from asthmatics produced less interferon (IFN)-β mRNA in response to *in-vitro* RV infection compared to cells from non-asthmatics (5). They found that when exogenous IFN-β was added to these cultures the cells responded to the virus in the same manner as non-asthmatic cells, displaying decreased viral load and increased apoptosis of infected cells. Another study on RV infections in primary cultures of bronchial epithelial cells from asthmatics by Contoli et al. demonstrated that IFN-λ1 and -λ2/3 mRNA production was also decreased compared to the levels found in infected cells from non-asthmatic controls (6). However, these results are in direct contrast to the findings of Lopez-Souza et al. where they found expression of IFN-β and IFN-λ1 mRNA expression was increased in RV infection of bronchial epithelial cells from asthmatics (7). Another paper by Lopez-Souza et al.

recognized a potential cause of these differing results; they found that differentiated *in-vitro* cultures of asthmatic bronchial epithelial cells respond differently to RV infection than non-differentiated cultures (8). They discussed how previous studies such as that of Wark et al. cultured cells in a manner that led to poor differentiation *in-vitro*, and that by using methods that encouraged a more "natural" level of differentiation in the cultures resulted in a more robust immunological response to RV infection (5). These studies suggest the potential importance of interferons in the immune response to respiratory viruses, but the exact role they play in the lungs of patients with asthma is debated due to methodological issues.

Another hypothesis involves the role of eosinophils in AHR in respiratory virus infections in asthmatic patients. Studies on models of asthma have identified the role of eosinophils, and their importance to allergen induced asthma exacerbations. Lee et al. demonstrated that eosinophil deficient atopic animals that had undergone antigen challenge did not develop AHR in response to inhaled methacholine, compared to animals with eosinophils that did develop AHR (9). Previously we demonstrated that eosinophils and their degranulation products were necessary for vagal induced AHR in atopic animals with an acute respiratory virus infection (10). This helped establish a mechanism of eosinophil involvement in animal models of asthma with virus induced AHR, but did not addressing the natural occurrence of re-infection by the same virus.

The effect of re-infection with a respiratory virus on inflammation and AHR has only been explored few studies, and these studies primarily focused on early childhood viral

infections and long-term immunological changes (11), (12). Unfortunately these studies failed to address what would occur in an animal model that had developed a Th_2 phenotype prior to initial viral infection, nor did they investigate the cellular pathway behind virus induced AHR. However, the studies do corroborate our previous findings that a pre-existing Th_2 phenotype alters severity of AHR caused by respiratory viral infection.

Our previous work went established how Th_2 based inflammation is involved in eosinophil mediated airway smooth muscle dysfunction (10). In an allergen sensitized model without immune memory to acute PIV infection, Adamko et al. demonstrated that viral mediated airway hyperreactivity could be prevented by depleting the animal of eosinophils via administration of an anti-IL5 antibody (10). Additionally by removing a potential activator of eosinophils in an acute viral infection, CD8+ T-cells, Adamko et al. demonstrated the importance of T-cells in mediating eosinophil activation in an allergen-sensitized model of respiratory virus induced AHR (13). Additional human *in-vitro* work by Davoine et al. established that virus specific memory CD4+ CD25+ CD45RO+ T-cells were essential for inducing eosinophil activation and degranulation in the presence of antigen-presenting cells and the specific respiratory virus (14). These studies provided a mechanism behind the eosinophil hypothesis of viral induced AHR in asthmatics, and established a role for immune memory in triggering AHR.

Exposure to a live respiratory virus is known to induce AHR and increase the number of inflammatory cells recovered in the bronchoalveolar lavage of humans regardless of their

allergy or asthma status. As expected our animal model responded in the same manner when re-exposed to live PIV (15, 16). Both sensitized and non-sensitized animals demonstrate increased airway reactivity to histamine, and an increased number of inflammatory cells in the BAL. This is the expected response to an acute viral infection before the adaptive immune system is able to mount an effective response, although the types of inflammatory cells involved are dependent on the animals' allergic status.

The antigenic effects of UV-inactivated viruses has been known since the mid-twentieth century (17). It is the ability of viral antigens to trigger an adaptive immune response that led to their use in vaccine development. In our model the lack of airway reactivity to histamine by non-sensitized animals to the UV-inactivated PIV is more likely to be dependent on the inflammatory cells reacting in the airways, specifically the lack of significant numbers of eosinophils. It is this absence rather than the model's ability to generate immunological memory to respiratory virus infections that may the source of this difference with sensitized animals (18).

Corticosteroid therapy is the backbone of long-term asthma management due to the role of airway inflammation in the disease. Dexamethasone is known as a anti-inflammatory agent with broad effects that include actions on eosinophils and T cells. It is for these reasons this agent was used to reduce the effects of T cell and eosinophil activation in our experiments. Sensitized animals treated with DEX and re-exposed to live PIV had significantly decreased AHR and total inflammatory cell numbers in BAL. This result was not unexpected with the known effects of DEX on eosinophil apoptosis, and its

effects on AHR and inflammation in allergen challenged and virus infected animal models (19-21). DEX had an almost identical effect on sensitized animals that were re-exposed to UV-inactivated PIV.

IL-5 has long been known to be a major factor in human asthma and eosinophil hematopoiesis and regulation (22). Blocking the effects of IL-5 via an antibody has led to the inhibition of AHR and drop in the number of eosinophils in animal models of asthma and virus induced AHR (10, 23). In this study we used a humanized monoclonal antibody specific for IL-5 (Mepolizumab), which has been demonstrated to lower blood and sputum eosinophils in humans, while also decreasing the risk of an asthma exacerbation (24). Mepolizumab was able to significantly decrease AHR in sensitized animals that were re-exposed to live PIV, despite its lack of effect on numbers of eosinophils in BAL. However mepolizumab's largest effect was seen in sensitized animals that were exposed to UV-inactivated PIV. Depleting IL-5 caused a significant decrease in AHR and the numbers of cells in the BAL including eosinophils and macrophages. The effects on macrophages may be attributed to some partial binding of mepolizumab to the GM-CSF receptor, which shares a common subunit with the human IL-5 receptor (24).

These experiments have identified a mechanism where asthma exacerbation can be triggered in individuals with allergic asthma by respiratory virus without an active infection. This supports a more realistic model of repeated viral antigen exposure in an individual's environment inducing more frequent asthma exacerbations.

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Chapter 4: *Identifying hypoxia in a newborn piglet model using urinary NMR metabolomic profiling*

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4.1 Introduction

Every delivery of an infant is a physiologically stressful event and hypoxemic episodes occur. Current methods for evaluating the level of damage from a hypoxic episode are limited, and there is a clinical need for a precise assessment of a neonate's hypoxic state to improve patient care (1). Neuroprotective therapies used to treat hypoxic episodes are successful but must be applied within a relatively short time period (2). The clinical methods used to assess neonatal hypoxic episodes include Apgar scores, serum lactate levels, serum acid-base deficits, EEG recordings, and MRI. While each of these tests offer valuable information that can be clinically relevant in predicting some outcome of neonatal hypoxia, none of them have been proven to have the sensitivity needed to affect treatment. The lack of ability to identify acute hypoxic changes in the neonate leaves neonatologists without direction when it comes to implementing early therapies such as hypothermia treatment (3). Development of a diagnostic tool able to rapidly assess the

level of hypoxic damage experienced by a neonate would allow therapies to be initiated sooner, theoretically preventing long-term neurological deficits.

NMR based metabolomics is a powerful tool that offers the opportunity to investigate biochemical changes in response to a disease state and/or injury. The utility of this diagnostic technique is now being explored in the field of neonatal medicine (4). One critical area being investigated is the potential ability for metabolomics to identify and quantitate damage and the extent of recovery from periods of neonatal asphyxia (5-7). Currently animal models of neonatal hypoxia offer precise physiological measurements that can be directly compared to acute changes in the urine metabolome in order to establish a model of neonatal hypoxic injury (8, 9). In this study we used NMR metabolomics in conjunction with physiological measurements to establish a metabolomic profile of neonatal hypoxia-reoxygenation (H-R).

4.2 Methods

For the detailed methods please refer to CHAPTER 2 sections 2.3 and 2.4.

4.2.1 Ethics Statement

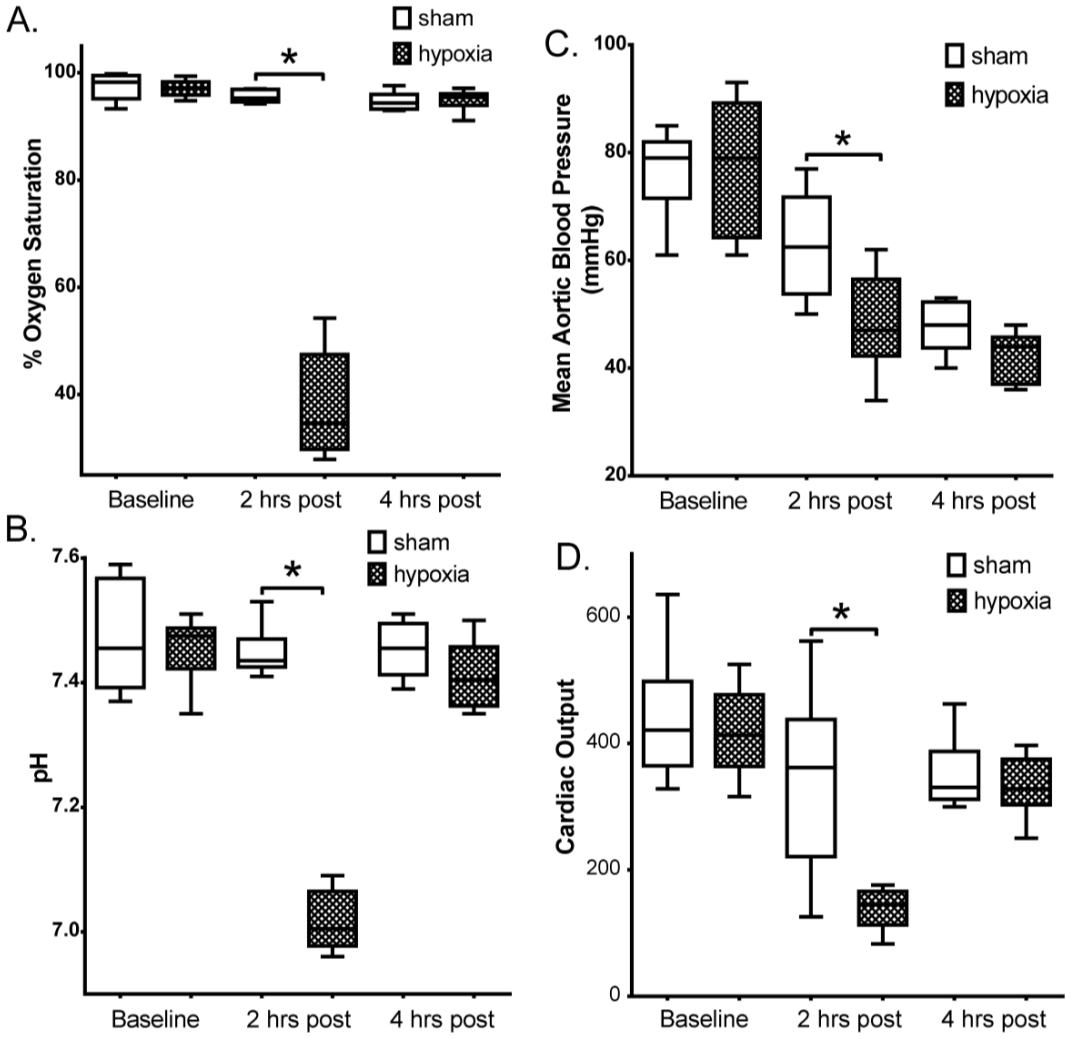
All experiments were conducted in accordance with the guidelines of Canadian Council of Animal Care (2001) and approved by the Animal Care and Use Committee: Health Sciences, University of Alberta (ACUC: HS Protocol #183/10/10B).

4.3 Results

4.3.1 Physiologic data alone cannot not accurately predict hypoxemia

Both groups of animals were subjected to the same invasive surgical preparation in order to acquire the required physiological measurements. As a result, all animals showed some physiologic stress and a decline in all parameters measured by the end of experiment (Figure 1, arterial oxygen saturation, pH, MAP, and CO). There were no statistically significant differences in these measurements between groups at baseline post-surgical set-up. As might be expected, after 2 h of hypoxia, the H-R animals had statistically lower values for all physiologic parameters measured. In contrast, after 4 hours of recovery at the 6 h period, the physiologic measurements in H-R animals returned to values similar to that of the non-hypoxia control animals.

Figure 4.1: **Physiological effects of hypoxia in newborn piglets**. Temporal changes in (A) oxygen saturation (% O_2), (B) blood pH, (C) mean arterial pressure (mmHg), and (D) cardiac output between hypoxia (n=7) and sham (n=6) treated animals. *P<0.05 Sham vs. Hypoxia for corresponding time point.



4.3.2 Despite normal physiology, the urine NMR profile of hypoxic challenged animals differs compared to non-hypoxic sham controls

While urine samples were collected from both groups at baseline, 2, and 6 hours, the 6-hour time-point was chosen to create the diagnostic metabolomic model. We thought this time period would best replicate the clinical scenario of an infant post-hypoxic insult. The concentrations of 50 metabolites were measured in the urine samples (Chenomx, Edmonton, AB) and standardized to their respective creatinine level. Based on these values for each group of animals, PLS-DA created a model of separation between hypoxemic (n=7) and sham treated animals (n=6). As would be expected, many of the metabolites excreted in the urine did not differ greatly between groups, and leaving metabolites of low importance rendered the metabolite model less accurate, including the possibility of false positive results. To remove these metabolites and improve accuracy, we used a test set of urine samples from sham treated animals not part of the model (n=9). We removed as many metabolites as possible while still maintaining the best possible correct classification score of blinded sham treated animals (PLS-DA prediction score < 0.5; 8 of 9 animals correct; figure 2). The final model for PLS-DA separation of non-hypoxic animals from hypoxic animals at 6-hours consisted of 13 metabolites. The model used one component giving an R2=0.911 and Q2=0.892. The differences in concentration of these metabolites between groups are shown as the Coefficient of Variation Plot (Figure 3), and the ranking of metabolite importance for separation is shown as the Variability of Importance Plot (Figure 4). The final metabolites chosen and their average concentrations for each animal group are shown in Table 1. To validate the proposed model as a diagnostic tool for hypoxic insult, we entered the concentrations of

metabolites from HR animals not originally part of the modeling exercise (n=10). The PLS-DA model correctly diagnosed the blinded hypoxic samples with 90% accuracy (9/10 samples).

Figure 4.2 **Differentiating hypoxic animals vs. sham treated animals.** The PLS-DA algorithm separates groups of data based on a score of 0-1; in this case a value closer to zero indicates no hypoxemia (sham, n=6) and above 0.5 indicates hypoxemia (n=7). Illustrated are the PLS-DA prediction scores for each animal including blinded test groups. Error bars represent medians and interquartile ranges.

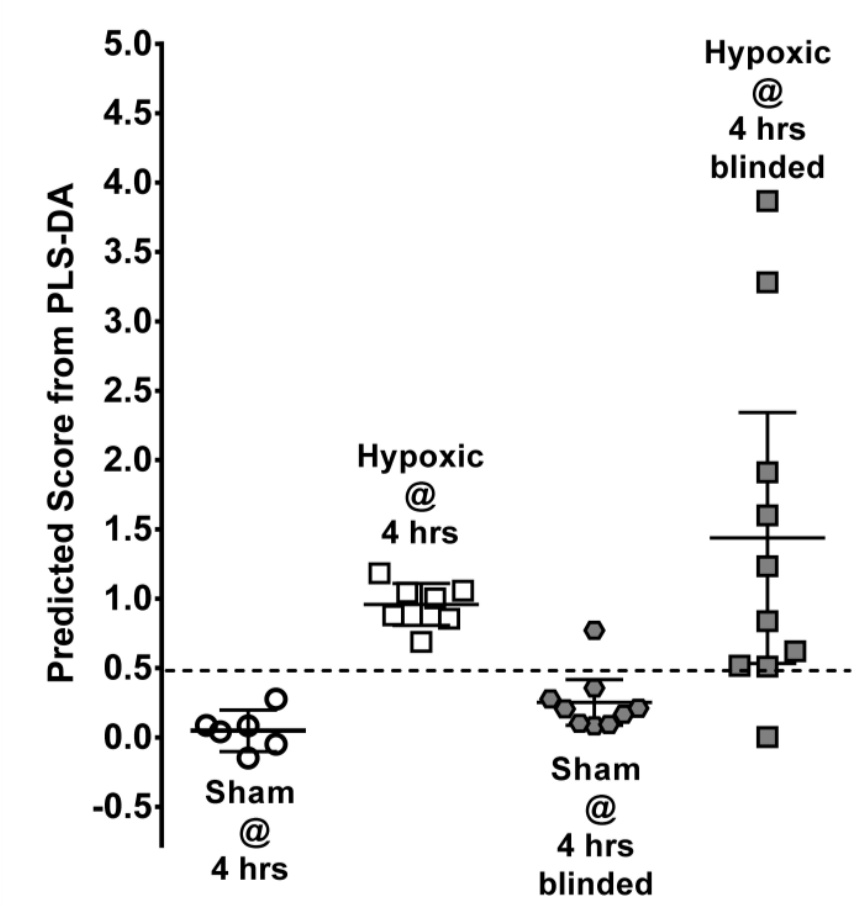


Figure 4.3: **The metabolomic model of hypoxic vs. sham treated animals**. PLS-DA analysis of urine from hypoxic versus sham treated animals was based on differences in metabolites between groups shown as the Coefficient of Variation (CoV) plot (A). The importance of each metabolite within the model is shown as the Variability of Importance (VIP) plot (B).

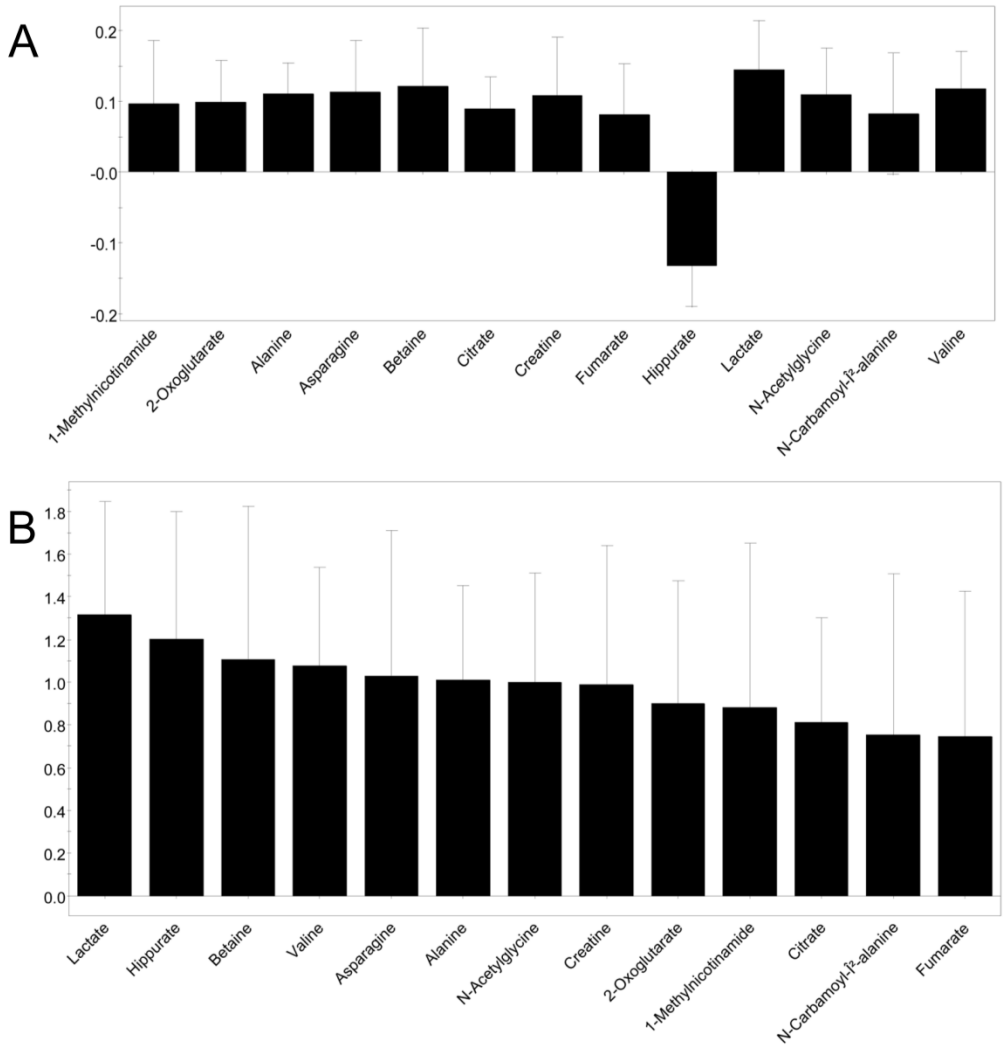


Table 4.1: **Metabolite Concentrations**

Average concentrations and inter-quartile ranges of urinary metabolites in hypoxia and sham treated newborn piglets used to generate the metabolomic model (in mmol of metabolite/mmol creatinine).

Metabolite	Sham (IQ range) n=6	Hypoxia (IQ range) n=7
Alanine	0.211 (0.137)	0.639 (0.364)
Asparagine	0.054 (0.030)	0.038 (0.007)
Betaine	0.116 (0.093)	0.705 (0.607)
Citrate	0.428 (0.239)	0.887 (1.412)
Creatine	0.030 (0.022)	0.166 (0.207)
Fumarate	0.062 (0.044)	0.127 (0.134)
Hippurate	1.375 (0.577)	0.685 (0.364)
Lactate	0.457 (0.359)	7.946 (5.630)
1-Methylnicotinamide	0.103 (0.030)	0.175 (0.178)
N-Acetylglycine	0.070 (0.081)	0.180 (0.157)
N-Carbamoyl-β-alanine	0.347 (0.282)	0.615 (0.489)
2-Oxoglutarate	0.265 (0.212)	0.465 (0.421)
Valine	0.007 (0.005)	0.038 (0.014)

IQ range = Q3-Q1

4.4 Discussion

Identifying neonatal hypoxemia and determining if and when to initiate potential neuroprotective therapy remains a challenging issue based on current diagnostic techniques. Apgar scores are affected by a poor method of assessing neonatal asphyxia (10). This is due to a number of variables such as maternal sedation and analgesia, and underlying cardiovascular or neurological conditions in the neonate (11). Measuring serum lactate levels and base deficits do show some positive predictability in identifying neonates that have experienced severe hypoxia, but unfortunately are not reliable when assessing mild to moderate hypoxia or in conditions like septicemia (1, 12, 13). Predicting the level of developmental delay is based around neurological examinations and the use of EEG recordings (14, 15). A recent examination of the use of EEG in staging neonatal hypoxic episodes and predicting severe outcomes found that EEG performed within 6 hours of a hypoxic birth did not predict long-term outcomes involving disability and death (16). The use of MRI to evaluate hypoxic brain injury in neonates has also been established, however MRI requires an anesthesia for the infant and is not able to identify and evaluate early hypoxic changes (17, 18). The limitations of the current diagnostic methods leave a large gap in the ability of physicians to accurately diagnose and assess the degree of hypoxia experienced by an infant. An ideal diagnostic to mitigate this deficiency would be one that is non-invasive, available at the bedside in the NICU, and would provide an accurate assessment of the patient's condition.

NMR metabolomic analysis of urine from hypoxemic neonates offers a non-invasive solution. It can be used to rapidly identify and quantify the compounds that could provide

the basis of a bedside urinary diagnostic test. Further, in contrast, to measuring just one variable like lactate, NMR metabolomics studies a large number of variables covering multiple metabolic pathways. Our report identified a combination of 13 metabolites that could be used to identify a hypoxemic episode in the animal model. Similar to other studies using NMR and mass spectrometry analysis of urine in fetal piglet models of hypoxia, we confirmed the presence of 6 compounds including, alanine, citrate, creatine, fumarate, lactate, succinate, and valine (5, 6). In addition, we found the following compounds were uniquely identified in our NMR analysis: 1-methylnicotinamide, 2-oxoglutarate, asparagine, betaine, hippurate, N-acetylglycine, and N-carbamoyl-β-alanine.

Many of the metabolites identified were directly related to cellular energy levels and metabolism. Not surprisingly, lactate, which was a key metabolite, is a product of anaerobic respiration and is known as an early marker of neonatal hypoxia in humans (19). Citrate, an important intermediate in energy metabolism particularly in the citric acid cycle, was increased. This is also reported in the CSF of hypoxic fetal sheep (20). Mitochondria appear to have up to a 40% decrease in the ability to use citrate and malate under hypoxic conditions (21). Creatine and one of its products phosphocreatine are also important components of energy metabolism in muscle. Rises in creatine in the absence of phosphocreatine may be indicative of an impaired metabolism due to an energy-depleted system (22). Creatine has also been found to have a neuro-protectant role as an anti-oxidant in hypoxic chick spinal cord neuron cultures (23). Fumarate and 2-oxogluterate are components of the citric acid cycle important for energy metabolism

(24). N-acetylglycine is an N-acytylated derivative of glycine that is normally broken down by an ATP dependent pathway; high levels of this molecule may indicate an energy-deprived state (25). Together these metabolites reflect the major impact mild to moderate hypoxia can have on aerobic respiration and cellular metabolism.

The remaining metabolites identified were likely the result of hypoxia's effects on inflammation and dysregulation of amino acids. Many of these metabolites relate to the body's ability to self-limit inflammation and cellular dysfunction that accompany ischemic injury. Alanine levels are known to increase in the cerebral spinal fluid (CSF) of fetal sheep during a hypoxic episode, and they remain high for 2 hours following the episode before gradually decreasing (20). The initial increase then gradual decrease may be due to the consumption of alanine to make the excitatory amino acid aspartate, which can also have damaging effects on the brain (26). Betaine, a trimethylglycine is known to act as a protective osmolyte and act as a methyl donor (27). Betaine and methyl donor insufficiency has been associated with metabolic disorders and impaired fetal development in humans (27). High levels of betaine are reported to decrease the inflammatory response of adipocytes stressed under hypoxic conditions (28). Hippurate an excretion product of benzoate and glycine was found to decrease in hypoxia (24). Hippurate was also shown to be decreased in the urine of human stroke patients with-in 72 hours of an ischemic event, this is believed to be related to folic acid deficiency and hyperhomocysteinemia (29, 30). N-carbomyl-β-alanine has been found to play a role in the protection of the liver against hypoxic injury in a rat model via aiding in ion homeostasis (31). 1-Methylnicotinamide (1MNT), a metabolite of nicotinamide was

found to have anti-inflammatory properties (32). High dose administration of 1MNT has been shown to inhibit a pro-inflammatory enzyme (matrix metalloproteinase 9) in the brains of neonatal rats during the acute phase of a hypoxic episode. High levels of this metabolite may be an endogenous anti-inflammatory agent of the brain (33). Valine, an amino acid, was found to be increased in the urine of our hypoxic animals, this corresponds with previous studies that demonstrated increased release of valine from dog brain tissue in-vivo over a 30 minute episode of hypoxia (34).

It is important to note that no single metabolite could diagnose all animals correctly. For example while critical, lactate was also elevated in some non-hypoxic animals. Sham animals did undergo a similar invasive surgical protocol but without hypoxemia. For an animal to be diagnosed as hypoxic, it required a combination of metabolites. At the 6-hour period, the hypoxic animals had time to recover, and both groups had similar levels of oxygen saturation, pH, MAP and CI. This might mimic the real world experience of a birth. We suggest that to have accuracy at diagnosis a combination of variables will be needed. Metabolomics provides this option. Ideally in the future this profile will be confirmed in human neonates, and a non-NMR based point of care diagnostic could be developed from this urinary metabolomic profile. The noninvasive nature of urine makes this test applicable to non-tertiary care settings where deliveries most often occur. A first urine sample may become very insightful for obstetricians and family doctors performing deliveries. Such metabolomic-based data might warrant the care of a neonatologist.

While this chapter was written for publication to those interested in neonatal hypoxia, hypoxic stressors exist in people with asthma exacerbation. Even in outpatient settings, patients with asthma have some airway obstruction, which could cause a degree of hypoxemic stress on body tissues. As such, this chapter provides the data to support the use of metabolomics as a means to monitor patients with asthma and its severity. It would be important to know if a patient is having abnormal hypoxemic stress, as this would alert the physician (and patient) to alter therapy and prevent the exacerbation.

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Chapter 5: *Urine metabolomic analysis in an animal model of virus-induced asthma exacerbation: a pilot study*

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5.1 Introduction

Using objective measurements of airway inflammation to guide asthma therapy has been shown to produce superior therapeutic results compared to using traditional measures (e.g., symptoms and lung function) alone (1-4). However, for clinicians especially those caring for children, detecting airway inflammation in a typical clinical setting is quite difficult. Instead of assessing inflammation to inform therapeutic intervention, most physicians simply give trials of therapy. Often this approach does not work, which leads to hardship and morbidity for the patient, and also added health care expense. Overall, a simple, non-invasive test of inflammation for patients with airway obstruction is not widely available.

Metabolomics is the study of the biological products of normal physiological processes, disease pathophysiology, and gene function (5). An excellent use of metabolomics was for comprehensive metabolite profiling to provide a rapid personalized health assessment (6). It is in this spirit that researchers are using metabolomics to diagnose and prognosticate various diseases. Metabolomic analysis of asthma is a popular research field, and current targets include urinary-based metabolites, serum metabolites, and metabolites from exhaled breath condensates (7-9).

Nuclear magnetic resonance (NMR) spectroscopy is one technique used to measure metabolites in biofluids. NMR is advantageous as it does not destroy or change the sample during analysis, and it allows for qualitative and quantitative analysis of compounds based on their resonant frequencies and their signal intensities. The 1D ^{1}H-NMR spectra can be analyzed using targeted metabolite profiling and quantification software (Chenomx, Edmonton, AB) (10). Different biofluids and tissues have been used for metabolomic analysis. A biofluid preferred by manyresearchers for NMR metabolomics is urine due to its ease of collection, large sample volume, and physiologic filtering through the kidneys.

The experiments described in CHAPTER 3, created an allergic and non-allergic model of virus induced airway hyperresponsiveness. *I hypothesized that atopic and non-atopic animals infected with live parainfluenza virus would have different urinary metabolomic biomarkers, which would also differ from healthy control animals.* Unfortunately, the number of animals from which urine was collected is small in this pilot study, but the

data analysis is provided to assist others in future studies. The Results then, while interesting, should be viewed in this context of a pilot study.

5.2 Methods

For the detailed methods see CHAPTER 2 sections 2.1, 2.2, and 2.4.

5.3 Results

5.3.1 The urine NMR profile of virus infected versus non-infected animals:

Urine samples were collected from animals previously infected with PIV or sham. After measurements of airway reactivity were completed a urine sample was taken by bladder puncture. The concentrations of 70 metabolites were measured in the urine samples (Chenomx, Edmonton, AB) and standardized to their respective creatinine level. Based on these values for each group of animals, PLS-DA created a model of separation between virus infected (n=6) and non-infected sham treated animals (n=3). A model for PLS-DA separation of non-infected from infected animals was derived using two components (metabolite concentrations vs. infection status) giving an R2=0.90 and Q2=0.50 (Figure 5.1). While this sample size is too low to make any strong conclusions, we were interested in the potential accuracy of this model. We entered the concentrations of metabolites from animals not originally part of the modeling exercise (n=10, 6 uninfected and 4 PIV infected). The PLS-DA model correctly diagnosed the blinded virus infected or non-infected samples with surprising excellent accuracy (10/10 samples

correct, Figure 5.1). The metabolites used for the separation and identification of infection are described in Table 5.1.

Figure 5.1: **Differentiating virus infected animals vs. sham infected animals.** The PLS-DA algorithm separates groups of data based on a score of 0-1; in this case a value closer to zero indicates no viral infection (n=3) and above 0.5 indicates live PIV exposure (n=6). Illustrated are the PLS-DA prediction scores for each animal including blinded test groups. Error bars represent medians and interquartile ranges.

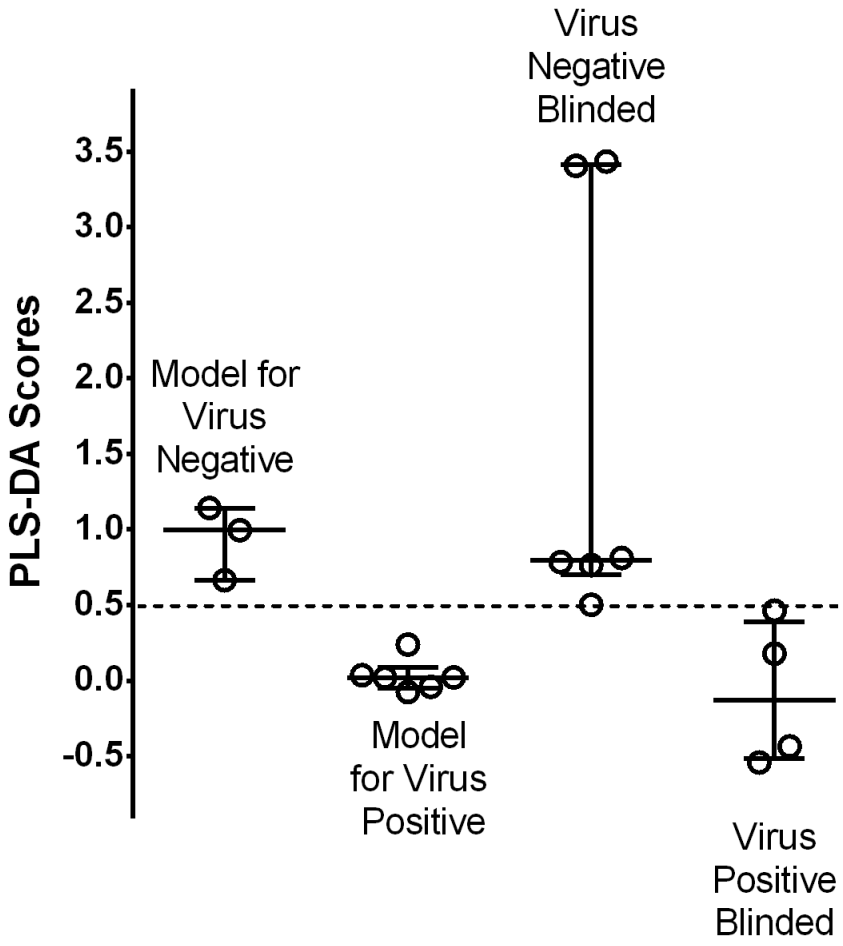

Table 5.1: **Urinary metabolite concentrations.** The mean concentrations (in mM) and the IQ range of the metabolites used to separate infected from non-infected animals as determined by PLS-DA analysis.

Metabolite	Animals With Virus Mean Metabolite Concentration mM (IQ range) n=6	Animals With No Virus Mean Metabolite Concentration mM (IQ range) n=4
Adipate	0.0213 (0.0069)	0.0299 (0.0027)
Succinate	0.0108 (0.0099)	0.0287 (0.0213)
4-Hydroxyphenylacetate	0.0586 (0.0339)	0.1179 (0.0190)
Hypoxanthine	0.0037 (0.0021)	0.0013 (0.0027)
N-Phenylacetylglycine	0.1174 (0.0987)	0.2344 (0.0803)
Glutamine	0.1772 (0.1129)	0.0819 (0.0312)
Trimethylamine	0.0055 (0.0072)	0.0163 (0.0152)
Hippurate	1.4701 (1.9067)	3.6900 (2.0993)
2-Hydroxyisobutyrate	0.0174 (0.0236)	0.0356 (0.0625)
Isovalerate	0.0046 (0.0049)	0.0093 (0.0035)
Fumarate	0.0004 (0.0006)	0.0068 (0.0115)
1,3-Dihydroxyacetone	0.0029 (0.0038)	0.0351 (0.0632)
Sarcosine	0.0521 (0.0491)	0.1009 (0.0451)

IQ range = Q3-Q1

5.3.2 The urine NMR profile of sensitized and PIV challenged animals compared to non-sensitized animals after virus infection

In a pilot study, we tested whether there was a difference in the metabolome of virus infected animals previously sensitized or not to ovalbumin. The urine samples from animals that had been previously sensitized, challenged with ovalbumin, and virus infected were compared to non-sensitized animals after virus infection. After measurements of airway hyperreactivity were completed a urine sample was taken. The concentrations of 70 metabolites standardized to creatinine were analyzed by PLS-DA. PLS-DA created a model of separation between the allergic virus infected (n=7) and non-allergic virus infected animals (n=6). A model for PLS-DA separation of non-infected from infected animals was derived using two components giving an R2=0.69 and Q2=0.25. Unfortunately we did not have any animals not part of the modeling exercise to use as a test set.

5.4 Discussion

The different responses in viral induced exacerbations in atopic versus non-atopic individuals with asthma are important to understand. Biomarkers identified in animal models offer a prediction of what to expect in human subjects (12). This work suggests that metabolomics could be a novel way to differentiate a cause of exacerbation in humans.

Our laboratory has investigated metabolomic-based biomarker profiles in asthma for diagnostic purposes has been an area my colleagues and I have pursued in the past. Our

animal work into a human study that identified urinary-based metabolites associated with healthy children and children with asthma (13). Interestingly, we are the only group that has studied children using true blinded analysis for accuracy, which makes our results compelling. These children studied included both the stable/controlled phenotype of asthma and those in an emergency room setting. This set of urinary biomarkers identified an atopic phenotype of childhood asthma. The experiments with PIV in this chapter provide the opportunity to investigate more selected phenotypes of asthma, namely vial induced exacerbation in an atopic or non-atopic population

While no other groups studying metabolomic biomarkers have attempted to differentiate virus infection in asthma; other researchers have attempted to identify biomarkers of other asthma phenotypes. Carraro et al. have used LC-MS analysis of EBC and measurement of eNO to distinguish between healthy children and those with severe and non-severe asthma (14). They reported that current diagnostic tools such as lung function and eNO were unable to differentiate the groups. The metabolomic analysis of EBC revealed that the concentrations of the following compounds helped separate the groups through computer modeling: retinoic acid, deoxyadenosine, ercalcitriol, thromboxane B2, 6-keto prostaglandin F1a, and 20-hydroxy-PGF2a. Interestingly some of these compounds are related to vitamin D and eicosanoid metabolism.

Another study by Ibrahim et al. examined EBC from healthy adults and individuals with asthma who were separated into the following groups asthmatics with sputum eosinophilia, asthmatics with sputum neutrophilia, well-controlled asthmatics, poorly

controlled asthmatics, and inhaled corticosteroid use (9). They analyzed these samples using NMR and a non-targeted approach known as spectral binning. By dividing up a 1D ^{1}H-NMR spectra into small sections, they can compare different regions of the spectra between patient groups to identify any variation. This method does not initially identify specific metabolites, but can direct researchers to potential metabolites based on their specific resonant frequency and chemical structure. Ibrahim et al. were able to separate healthy controls from asthmatics based on the variation in spectral regions determined from binning. However, they were unable to separate various asthma phenotypes from one another, which they attributed to low sample sizes.

Mattarucchi et al. reported findings using metabolomic urinary biomarkers to identify children with atopic asthma based on disease control and medication use (15). Through LC-MS analysis they separated healthy children from atopic children with well-controlled asthma, well-controlled atopic asthma using corticosteroids, and poorly controlled atopic asthma. They separated out children on corticosteroid therapy through the metabolites of corticosteroids (urocortisone and urocortisol), and also separate asthmatics from healthy controls using 3 metabolites (urocanic acid, Ile-Pro, methyl-imadazoleacetic acid).

My pilot work suggests that a metabolome for virus infection in asthma should exist, and that there likely is a difference in this metabolome between those with allergic airway inflammation or not. The results of this pilot study will offer insight into potential human biomarkers for the most common form of asthma exacerbation.

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Chapter 6: *Conclusions and Future Directions*

6.1 Introduction

Asthma is a diverse and complicated syndrome that impacts the lives of numerous people around the world. My research into the mechanism of viral induced asthma and the urinary biomarkers associated with hypoxia and viral induced exacerbations attempts to provide a better understanding of such a complex condition.

6.2 CHAPTER 3 Conclusions and future directions

The experiments in CHAPTER 3 demonstrated that allergic inflammation played an important role in an animal model of the airway's response to re-exposure with viral antigens. Re-exposure to a live virus in sensitized and non-sensitized animals induced an expected inflammatory response and enhanced airway reactivity to histamine. However, the fact that only sensitized animals responded with inflammation and AHR following re-exposure to UV-inactivated virus supports my hypothesis that the differing inflammatory make up of the airways is the responsible factor. The necessity of the cellular influx (primarily eosinophils and macrophages) response was supported with the inhibition of AHR with corticosteroid and anti-IL5 treatment respectively. These experiments supported previously proposed mechanisms for eosinophil mediated airway dysfunction, and offer a mechanism by which humans with asthma can have exacerbations based on interactions with re-exposure to viral antigens circulating in their environment. I suggest that this postulatedmechanism could be further tested with human testing by exposing

individuals with allergic asthma to non-infectious viral antigens to which they have immunity. No such work with inactivated virus in humans has been tried ina controlled experimental setting to our knowledge. Through the use of experimental rhinovirus infections in humans we could create a human model of immune memory in individuals with asthma. Human testing in such a model would potentially confirm this mechanism, and also may demonstrate the need for further research into improved controller therapies to prevent this specific immune response.

6.3 CHAPTER 4 Conclusions and future directions

The experiments in CHAPTER 4 demonstrated that one effect of asthma exacerbation, that being hypoxemia could be identified using urinary-based metabolites. Neonatal piglets with hypoxic episodes could be identified from other piglets that did not undergo hypoxia. The metabolomic approach was more sensitive than using physiologic parameters, which was surprising.

Currently are no rapid non-invasive test exists for identifying the severity of hypoxia in patients. These metabolite markers that we identified should be tested for their applicability to birth associated hypoxia. I would suggest that a clinical study be considered to investigate the correlation of the degree of hypoxic injury experienced by neonates following birth with their urine metabolomic profile. If physicians had such a test that was rapid, this could lead to earlier interventions to treat hypoxic brain injury in neonates. In addition, children with severe acute asthma exacerbations experience hypoxia to some degree. Future clinical studies based on these urinary biomarkers could

be done to identify if these markers are elevated in children with asthma. If true, then they could be used to adjust treatment accordingly and potentially decrease poor asthma control, emergency department visits, hospital admissions, and death.

6.4 CHAPTER 5 Conclusions and future directions

Experiments in CHAPTER 5 attempted to identify urinary biomarkers of the common phenotype of viral-induced exacerbation in an animal model of allergic asthma. Although our sample size was low, we provided evidence to support proof of concept that metabolomics has the ability to differentiate viral infections in the animal model. This data supports the need for additional studies with a larger sample size. If this project were successful, clinical studies of urinary biomarkers of acute viral induced asthma exacerbations in humans would be the next necessary step to further test the hypothesis.

6.5 Summary

Overall, my work has provided new information about viral-induced asthma exacerbations. I have provided evidence to support proof of concept data for the potential use of urine metabolomics in humans in hypoxemia and virus infection (Figure 6.1). I am hopeful that my research will eventually lead to changes in treatments for individuals suffering from recurrent viral induced asthma exacerbation, and better diagnosis and prognostication of an individual's asthma.

Figure 6.1: **The questions addressed by this thesis.** This thesis has provided information to help answer these questions. 1. Is immune memory to viral antigens and airway inflammation associated with virus-induced asthma exacerbations? 2. Are there unique urinary biomarkers of virus induced asthma exacerbations?