Role of Caveolin-1 in Airway Hyper-responsiveness and Inflammation in Response to House Dust Mite Challenge

by

Tyler Hynes

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Figure 4 – Razani B. et al. "Caveolin-1 null mice are viable but show evidence of hyperproliferative and vascular abnormalities". J Biol Chem. 2001 Oct 12;276(41):38121-38. Figure 9

List of Abbreviations

AHR – Airway hyperresponsiveness
APC – Antigen presenting cell
ASM – Airway Smooth Muscle
BAL(F) – Bronchoalveolar lavage (fluid)
C – Compliance
Ca - Calcium
Cav - Caveolin
CFTR – Cystic fibrosis transmembrane conductance regulator
COPD – chronic obstructive pulmonary disorder
CSD – Caveolin scaffold domain
EC20 – 20% effect concentration
ELISA - enzyme linked immunosorbant assay
ERK – Extracellular signal-regulated kinases
FEV_1 – Forced expiratory volume in 1 second
FOT – forced oscillation technique
FRC – Functional residual capacity
FVC – forced vital capacity
G – Resistance (of peripheral airways)
H – Elastance (of peripheral airways)
chloride channel
hCLCA1 - human calcium activated
HDM – house dust mite
IL – Interleukin
LOX - Lipoxgenase
MAP – Mitogen activated protein
MBP – Major basic protein
MCh – Methacholine
MMP – Matrix metalloproteinase
MUC – Mucin
NOS – nitric oxide synthase
OVA - ovalbumin
P – pressure
PAH – pulmonary arterial hypertension
Rt-PCR – real time polymerase chain reaction
PEEP – Positive end expiratory pressure
Penh – Enhance pause
Raw – Resistance (of central airway)
SEM – standard error of the mean
TGF – Transforming growth factor
TLC – Total lung capacity
TNF – Tumor necrosis factor
VEGF – Vascular endothelial growth factor
V – volume
Zin – Impedence (of the entire system)

Abstract

Allergic asthma is the name given to a syndrome that produces respiratory distress in response to environmental allergic or non-allergic triggers. The source of this distress in response to an allergen is an over reaction of the immune system causing an influx of inflammatory cells into the airway and concomitant airway smooth muscle constriction. As the incidence of asthma continues to rise throughout the developed world, a more complete understanding of the immunology, pathology and physiology of this disorder is needed. In this regard, the goals of the experiments in this project were two-fold: to create a relevant mouse model of human asthma and to better understand cellular and molecular mechanisms critical in the processes that propagate the pathophysiology of allergic asthma. The overall hypothesis of this project is: Caveolin-1 confers protection against airway inflammation and hyperresponsiveness in mice exposed to the common aeroallergen, house dust mite.

In the first set of experiments, designed to establish optimal protocols for generating murine models of allergic airway inflammation, we demonstrate that using whole house dust mite (HDM) extract as a sensitizing inhaled allergen produces an equivalent or more robust inflammatory reaction than can be achieved with ovalbumin (OVA) sensitization / challenge despite the fact that the latter protocol is more widely used in the field. Testing different exposure timelines and allergen exposure doses; we found that repeated inhaled HDM challenge for 10 days induced a significant increase (391.66±4.41%, $P<0.001$) in total inflammatory cells in bronchoalveolar lavage fluid (BALF). Eosinophils and neutrophils comprised 44.8±1.42% and 21.4±1.56% of inflammatory cells, respectively, a distribution that mimics profiles seen in mild-to-moderate human asthmatics. In contrast to the predominantly eosinophil inflammation observed in HDM challenged mice, only 25.6±1.27% BALF cells from OVA treated animals were eosinophils. Furthermore, using a small animal ventilator that

imposed low frequency forced oscillation maneuvers, 10 days of HDM challenge also induced a significant 146.86±45.6% increase in inhaled methacholine (MCh)-induced airway resistance (R_{aw}), as well as a 274.49±22.4% increase in tissue resistance (G) and a 169.06±37.79% increase in tissue elastance (H) compared to allergen-naïve control animals. Airway mechanics measured in OVA sensitized/challenged mice were shown to be similar to that of the HDM challenged mice.

To address our second objective, we attempted to elucidate the role of caveolae in the allergic asthmatic response by studying the response to inhaled HDM challenge in caveolin-1 (Cav-1) knock out (KO) mice. Caveolin-1 is the main structural component of caveolae within the pulmonary tissue, and the protein acts as a scaffold for multiple receptor-mediated signaling proteins that are thought to be involved with allergic inflammation and lung function. We subjected Cav-1 null mice to the HDM challenge protocol developed in concert with our first objective and found that lack of Cav-1 was associated with ~88% greater maximum central airways resistance (R_{aw}; $p<0.01$), ~159% higher peripheral tissue resistance (G: $p< 0.001$) and ~131% higher lung elastance (H: $p<0.001$) after HDM treatment compared with wild type mice. Cav-1 KO was also associated with increased airway sensitivity to inhaled MCh ($EC20_{control}$ =2.90 μg/ml versus $EC20_{Cav\text{-}1}$ = 1.76 μg/ml: $p<0.01$).

Additionally, we examined HDM exposure-induced inflammation and mucous production in Cav-1 KO and wild type mice. Assessment of airway inflammation revealed no difference in BALF inflammatory cell differential between mouse strains, but a greater total number of eosinophils was apparent in Cav-1 null mice, accounting for a ~71% augmentation in total inflammatory cells compared with HDM-challenged wild type animals (B6129SF2/J) animals ($p<0.001$). Inflammatory cytokines were also assessed using enzyme linked

immunosorbant assay (ELISA) in which allergen-naïve Cav-1 KO animals exhibited baseline increase in some cytokines compared to wild type animals: significant elevation seen for TNF-α (50.50 Cav-KO vs 11.17 WT $p<0.01$), TGF-β (57.01 Cav-1 KO vs 15.19 WT $p<0.01$) and IL-5 (74.19 Cav-1 KO vs 5.27 WT $p<0.01$). However after animals were challenged with HDM the only difference between strains was that of significantly higher levels of IL-5 (267.79 Cav-1 KO vs 121.73 WT $p<0.001$) in the BALF. Lastly, we investigated goblet cell number and mucus production in the four groups using Alcian blue staining. This demonstrated that the HDM exposed control animals had a greater staining area (normalized to basement membrane length) than Cav-1 KO counterparts (0.22 vs 0.03 pixels/um $p>0.001$). The distribution of staining differed greatly between strains also, with the majority of staining being found within the airway lumens of Cav-1 KO mice (73%) whereas the majority of staining was contained within epithelial cells in WT animals (71%). Using rt-PCR we further investigated mucus production, and observed that Cav-1 KO animals had 6-fold higher levels of MUC5a/c mRNA compared to the control groups ($p<0.01$)

Our findings demonstrate that repeated inhaled HDM challenge offers an outstanding platform for studying the acute episode of airway inflammation on lung physiology and its underlying biological mechanisms. Furthermore, our data suggest an important role for Cav-1 in down regulating allergic airways inflammation, leading to reduced airways hyperresponsiveness and mucus overproduction. Future studies targeting Cav-1 are needed to fully understand its role in modulating the onset and progression of allergic lung diseases, and in particular the pathophysiology of asthma.

Literature Review

Asthma

Significant effort and resources have been allocated towards researching the asthmatic condition with significant knowledge and results being achieved, but it remains difficult to solidify a unanimous definition. There are however hallmark characteristics that have become widely accepted in the medical and scientific community: 1) intermittent and reversible periods of difficult breathing due to a decrease in the sufferer's airway lumen (first observed by Dr. Henry Hyde Slater in the mid-19th century); and 2) structural changes in the airways that are known collectively as airway remodeling (described in detail in a latter section) [1, 2]. The most common type of asthma is associated with atopy [2], where individuals become sensitized or over sensitized to a particular antigen(s), which leads to an inflammatory response that compromises airflow during breathing. Over time these episodes, which trigger repeated inflammation and wound healing, lead to permanent changes within the pulmonary tissue that are associated with chronic respiratory symptoms.

Epidemiology

Worldwide, 300 million people suffer from asthma and the number will continue to rise of the next generation with another 100 million expected to be diagnosed by 2025. This places projected prevalence rates between 45-60% in urbanized areas. Although asthma is frequently mentioned as a disease of the developed world, majority of the deaths occurring due to asthma occur in low to low-middle income countries [3].

In Canada approximately 3 million people are living with asthma, accounting for approximately 9% of the total population over the age of 12 [4]. Canadian prevalence rates are much like the rest of the western world, which has seen a steady increase of the past 40 years.

A study released in 2008 by Statistics Canada showed that 13% of children between 0-11 had been diagnosed with asthma [5]. Although the incidence and prevalence of the disease continue to rise within the adolescent population, the rate of those experiencing regular exacerbation or uncontrolled symptoms is on the decline with rates falling from 41-36% and 51-39% respectively between 1994 and 2001 [5]. Although this may indicate improving treatment of asthmatics, a large population asthmatic suffers do not have control of their symptoms putting them at a higher risk for severe airway hyperresponsive events which can lead to death [4]. In 2003 asthma proved fatal to 287 people in Canada. Although mortality rates have been on a steady decline since 1991 [5] this number remains too high for a disease that; when treated, can be very well controlled, indicating that progress is still needed to fully understand and better control the condition.

As one would expect with a high prevalence rate, the economic burden of asthma is large. In fact, asthma is one of the most draining chronic health concerns to the worldwide economy. Figures show the direct costs due to asthma, such as medication and hospitalization, is approximately $400 annually per person in Canada, while the indirect annual cost of asthma (eg. missed days of work and decreased productivity) is approximately $250 [6]; these numbers stem from an estimated total $2 billion spent annually due to the costs of asthma.

Etiology

The etiology of asthma has proven to be just as complicated as defining the syndrome itself. Three main causal factors for atopic asthma have emerged: 1) a genetic component 2) viral infections and 3) environmental factors [7, 8]. It appears likely that each factor contributes in combination to the development of asthma in individuals.

Genetics play a large role in determining the asthma phenotype and severity of disease [9]. An area that has received a lot of attention is focused on polymorphisms of genes responsible for the expression of cytokines. *IL-13* genes play a role in the development of a wheeze and susceptibility to infection that induce asthma symptom exacerbations [10]. Another cytokine gene, *IL-4* and its variants have been shown to increase one's risk of developing asthma, particularly atopic asthma [11] due to its role in T-cell propagation. Genes responsible for the expression for IgE [12] and its receptors [13] have also been implicated in the development of atopic asthma through their role in the propagation of the hypersensitivity reaction via activation of mast cell degranulation. A unique gene that has garnered significant interest is *ADAM33,* which encodes a metalloprotease that may play a role in fetal lung development, thereby predisposing individuals carrying specific single nucleotide poloymnorphisms (SNPs) to higher risk for developing asthma [14]. This is a superficial overview of asthma genetics, but the topic has been reviewed in detail elsewhere [15, 16].

Genetically susceptible individuals with infection of respiratory syncytial virus is typically thought to trigger an immune hypersensitive state, thus increasing one's risk of atopic development [17]. However, viral infection can have the opposite effect, and has been shown to be protective against atopy development, by inducing immune system towards a Th-1 'pre-set' [18]. Clearly, understanding etiological factors linked to viral infection remains a topic of significant interest.

As stated above, allergic asthma is a multifactorial disease; another major factor believed to play a role in its etiology is the environment in which an individual is born and raised. There are two aspects to the role of environmental etiology of atopic asthma. First, is the concept called known as the 'hygiene hypothesis' [19]. The basis of the hypothesis is that the decrease

in immune system stimulation by infectious agents and antigens daily (due to increased personal and social hygiene) has spawned increased susceptibility to atopic disease. This premise appears to be supported by the dramatic increase in prevalence of allergic disease concurrent with an opposite reflection of infectious diseases prevalence [19, 20]. Historically, exposure to infections and other antigens stimulates the naïve immune system of a child, causing it to be pushed towards a Th-1 dominant mature system. If such exposures are absent, the immune system develops a Th-2 dominant phenotype, thus predisposing for development of hypersensitivity disorders. The Hygiene Hypothesis is not without its shortcomings, for example, there is no evidence that vaccination either improves or worsens the likelihood of developing atopy [21]. Furthermore a study performed in the UK by Bremner and colleagues examined prevalence and incidence of two birth cohorts for which exposure to 'clinically important infection' was known and found there was no direct impact on propensity to develop allergy [22].

Furthermore with increased industrialization of the world, rising levels of air pollution have been suggested as a cause for increasing incidence of asthma. Hulin et al. [23] demonstrated increased incidence of childhood asthma correlates with increased home air pollution levels. An environmental toxin that has garnered much attention as a possible risk factor for asthma is cigarette smoke. Exposure to the toxins in cigarette smoke is proven to increase the risk of developing asthma [24], severity of symptoms, rate of lung function decline, and negatively affects the efficacy of treatment [25]. The mechanism behind the negative effects of pollutant inhalation on the development and severity of asthma may be through induction of an inflammatory reaction within the airways. Smokers exhibit increased T-cell, macrophage and neutrophil number in sputum samples compared with non-smokers

[26]. Furthermore, it has been shown that babies born to parents who smoke are at increased risk of developing asthma both in childhood and later in life [27]. Together this shows that the environment has a large impact on the likelihood of development of the asthma syndrome and the severity of its symptoms.

Pathogenesis

Asthma is characterized by difficulty breathing due to airway obstruction. Both acute and chronic symptoms and mechanisms are thought to contribute to asthma pathophysiology. Acute exacerbations of the disease, known more generally as 'asthma attacks', are characterized by airway obstruction induced by airway inflammation. These processes include mediators that can lead to reversible airway obstruction from acute bronchospasm and airway

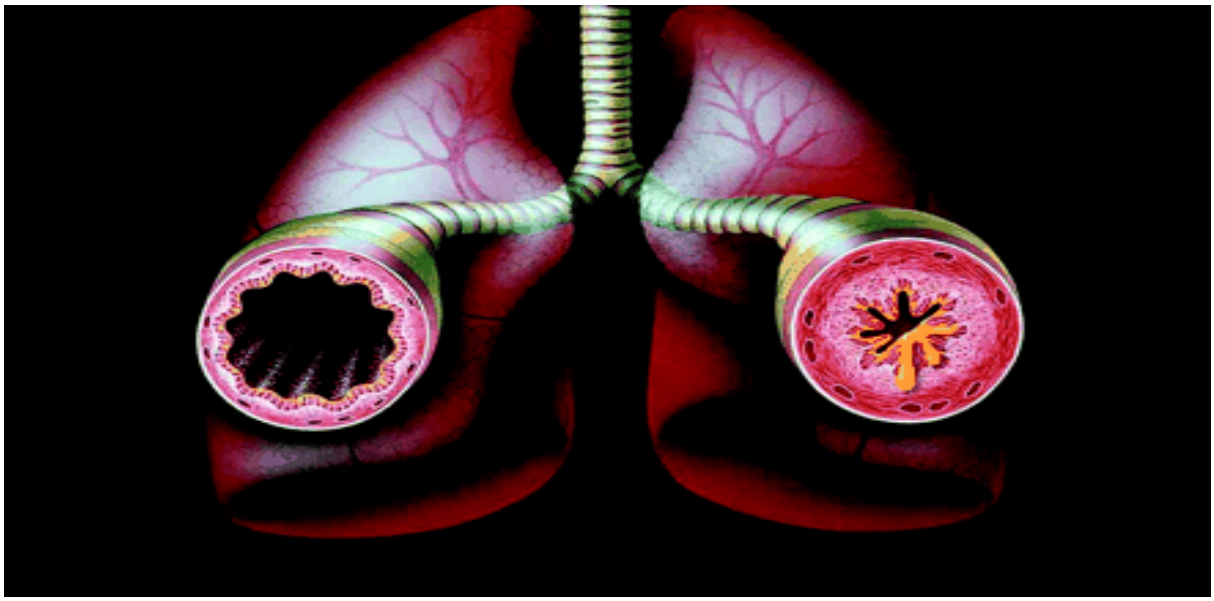

Figure 1 –. Representation of airways in the normal lung (*left*) and in asthma (*right*), demonstrating the thickening of the airway wall in asthma due to, increased ASM contraction, injury, chronic inflammation, and remodeling of epithelium and subepithelial tissue, leading to lumen narrowing. Also depicted is increased mucus secretion which occludes the airway further. The result is encroachment of the airway lumen and a marked increase in resistance to airflow. *Reproduce with permission from HighWire Publishing. Peter K. Jeffrey; "Remodeling in Asthma and Chronic Obstructive Lung Disease" .Volume 164, S28-S38, 2001; American Journal of Respiratory and Critical Care Medicine. Figure 3*.

hyperresponsivenss (AHR) along with increased secretion of airway mucus (Figure 1).

Atopic asthma is characterized as a type I hypersensitivity reaction: meaning that the assaulting antigen causes cross linking of IgE antibodies on the surface of mast cells, leading to a mass release of inflammatory mediators. Classical mediators released from this degranulation include histamines, prostaglandins, and leukotrienes. These mast cell mediators lead to vasodilation and permeability that potentiate an inflammatory process that induces airway tissue infiltration by eosinophils and neutrophils that migrate from the circulation through trophic effects of chemokines [28]. Antigen is also taken up by antigen presenting cells (APCs) which process the protein and define whether subsequent IgE or IgG-based antibodies are produced by B-cells, and stimulate T-cell activation and proliferation [29]. Interleukin (IL)-5 released from neutrophils and mast cells directs the T-cell response towards a Th-2 type immune response featuring eosinophil infiltration and the activation of B-cells to predominantly produce IgE [30]. In the case of atopic asthmatics, this establishes immune sensitization towards the assaulting antigen to trigger harmful Th-2 inflammation in the airways upon subsequent exposures. A Th-2 dominated inflammatory reaction is characterized by a distinct cytokine profile, as measured in airway lavage samples from asthmatics: these characteristic Th2 cytokines include IL-4, -5, and -13 [31]. The overall effect of this inflammatory reaction is many-fold: 1) it causes epithelial damage, possibly mediated by IL-13 [32], leading to easier antigen penetration on subsequent attacks [33]; 2) the release of cytokines can also promote airway smooth muscle contraction, migration and proliferation [34], thereby contributing to airway narrowing and airway remodeling; and, 3) these

inflammatory events induce injury and repair cycles that over time can lead to fibrosis and thickening of within the airway wall [35].

IL-5 and leukotrienes also promote the development of eosinophilia inflammation, another classical feature of the Th-2 inflammation observed in asthma. Notably, the administration of anit-IL-5 antibodies significantly diminishes eosinophilia, however, though initial clinical trials with anti IL-5 therapy did ablate eosinophila in asthmatics it was not associated with decreased AHR [36], the symptom that is most troublesome to asthmatics. Similarly, in mice with disabled major basic protein (MBP), the major secretory product of eosinophils, there was no impact on allergen exposure-induced AHR [37]. However, in recent clinical trials it has been shown that there can be significant clinical symptom improvement if IL-5 therapy is used in a select group of asthmatics that exhibit markedly elevated sputum eosinophil number [38].

Airway hyperresponsiveness (AHR) is an exaggeration of sensitivity and reactivity of normal constriction of the airway in response to bronchospastic agents, such as methacholine, leading to swifter and exaggerated development of airway resistance. The narrowing of the airway occurs due to bronchospasm underpinned by airway smooth muscle contraction, making expiration, in particular, extremely difficult. In asthma this can result in air trapping, which manifests as symptomatic chest tightness and difficulty in breathing experienced by the patient. Gas trapping leads an increase in the work of breathing due to areas of higher elastance being used for gas diffusion. These areas increase the alveolar dead space, equating to decreased alveolar ventilation, leading to hypercapnia as well as hypoxia. AHR is identified using spirometry to measure the amount of a provocative agent, such as methacholine or histamine, needed to decrease forced expiratory volume in one second (FEV_1) by 20%[39].

AHR is correlated with the extent of a patient's symptoms and degree of disease control [40]. Mechanistically, the degree of AHR correlates with airway inflammation, in particular the extent of eosinophilia and mast cell infiltration [41, 42].

Columnar epithelial cells line the respiratory tree lumen; interspersed within these cells are specialized cells known as goblet cells. These cells produce and secrete glycoproteinaceous mucus, which consists of peptide backbones with hundreds of O-linked carbohydrate side chains. Glycoproteins are encoded by a specific group of mucin (*MUC*) genes, with MUC5A being principally generated by goblet cells, whereas MUC5B is the primary product synthesized in submucosal glands [43, 44]. Acute inflammatory cytokines or possibly antigen itself may stimulate excess release of mucus, which occludes the airway, sometimes even forming 'mucus plugs' that reduce or prevent airflow. Mucous plugs are common findings in patients who die in *status asthmaticus* [45]. IL-13 provokes MUC5A secretion via mechanisms that are dependent on the presence of IL-4 and transforming growth factor (TGF)-β and their requisite receptors [46, 47].

Acutely, inflammatory mediators can drive (over) excitation of airway smooth muscle (ASM), and chronic inflammation can induce accumulation of ASM, increasing contractile capacity [48]. Repeated episodes of acute atopic asthma exacerbation are thought to drive structural changes in the airway wall that contribute to fixed airway obstruction (reviewed in [49, 50]. Airway wall remodeling includes accumulation of extracellular matrix, basement membrane thickening, mucus-gland and goblet-cell hyperplasia, and increased smooth muscle mass [51]. TGF-β, IL-4, IL-6, IL-9, IL-13, IL-17, and vascular endothelial growth factor (VEGF) have all been implicated in process of airway remodeling [52, 53]. TGF-β is of major interest because it affects multiple structural cell types in the airway. For example, promoting

fibroblast proliferation, differentiation & increased collagen deposition [54], ASM proliferation and migration [55] and, goblet cell hyperplasia and mucus secretion [47]. Over time these changes accumulate lead decreased baseline lung function and increase the extent of AHR.

Animal Models of Asthma

Due to the ethical and practical limitations of using human asthmatic patients to study mechanisms for disease development, animal models have become a very effective and beneficial tool to investigate and understand the pathophysiology, leading to advancement of clinical treatment [56, 57]. A particularly good example is work that was done to dissect the role of leukotriene receptors in inflammation in a sensitized and aerosol allergen-challenged guinea pig model, which lead to the development of leukotriene receptor antagonist and 5-lipoxygenase (LOX) inhibitors [58].

A large number of animal models have been and are currently being used in the study of allergic asthma. Mouse models have become particularly useful as they are relatively easy to alter genetically, and have been shown to mimic airway inflammation, hyperresponsiveness, and remodeling [59]. Logistically mice are of relatively low cost, their genetics are very well understood, and the use of transgenic technology has become routine leading to strains being widely available commercially. Overall murine models of have been shown to be a very good model for studying the sequealae of acute inflammation [59], thus proving very valuable in the evaluation of the specific goals of this project.

Although mice provide an excellent research platform because of genetic engineering potential, there are drawbacks for the study of atopic asthma. First, there are a number of differences between the mature murine and human respiratory system: 1) mice cannot breath

through their mouths, 2) murine airways have fewer cilia, 3) the human bronchial tree contains twenty three airway generations while the mouse only has six, and 4) mice lack sub-mucosal mucous glands. There are also differences between murine and human immune systems, such as higher numbers of alveolar macrophages, and lack of a late asthmatic response in murine asthma models. Lastly and possibly most importantly, mice do not spontaneously develop asthma, hence experimental protocols can only model specific aspects of the human disease [59, 60]. Murine models of allergic airway inflammation, hyperresponsiveness and remodeling have typically used ovalbumin (OVA) as a sensitizing agent and aero-challenge allergen [57]. However, concerns have arisen about the appropriateness of using OVA as it is not considered a relevant inhaled antigen in the context of human asthma. As a protein OVA is relatively large (45 kDa), compared with most clinically relevant proteinaceous allergens [61]. Additionally, OVA interacts with bound IgE in monomeric form, which is unlike most relevant human allergens that interact as a dimeric structure with membrane bound Ig E [62]. The use of an adjuvant to intensify the immune response to OVA is necessary, and this has also raised questions of relevance to human pathology where no adjuvant is involved. Furthermore, the route of OVA/adjuvant sensitization in mouse models is most often intra-peritoneal, which is not consistent the inhaled route of allergic sensitization in most humans [63]. In contrast to OVA, use of whole lysates of house dust mite (HDM) such as *Dermatophagoides farinae* as an allergen is more relevant to the human condition because HDM is something all humans come in contact with daily, and exposure can cause acute asthmatic exacerbations [63, 64]. Also, HDM can induce a Th2-polarized immune response in mice that is characterized by eosinophilic inflammation and accompanying airway hyperreactivity and remodeling (with chronic HDM exposure) [65].

Airway Mechanics

Asthma features a spectrum of clinical symptoms and the clinical portrait is characterized by reversible airway obstruction. To assess this hallmark symptom and aid in making a diagnosis of asthma, respiratory mechanics are typically measured, with particular attention paid to functional evidence for airway patency. Further testing can also include measurement of indices of the sensitivity and reactivity of the airways to inhaled non-allergic constrictors (eg. methacholine) or dilators (eg. β2-adrenerigic agonists).

As asthma is characterized by excess airway narrowing the most relevant physiologic measure is airway resistance, which is the opposition to flow as air moves through the pulmonary tree. Within the healthy lung 50% of this resistance is created by the large or central airways, only 10% resides within the small or peripheral airways, and the remaining resistance derives from the contribution of the mechanical properties of the lung and connective tissue [66]. Spirometry is the most widely used technique for assessing airway resistance, which is interpolated from direct measurement of forced flow and volume of expired air. The outputs obtained from this relatively simple test are forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC). The ratio of these measurements has proven very useful in diagnosing asthma, as by definition an obstructive respiratory disease such as asthma manifests as an FEV_1/FVC ratio below 70%. Use of this cutoff has very good specificity when applied to a patient with a clinical picture suggesting asthma.

It has become increasingly evident that peripheral airway pathology contributes significantly to asthma symptoms [67], as asthmatics represent a heterogeneous population in terms of where in the bronchial tree their respiratory symptoms arise [68, 69]. Quantifying peripheral airway properties is best achieved by considering both lung compliance (or its

inverse, elastance) and resistance. Compliance (C) is the extent to which a hollow organ resists recoil force towards its original form or the change in volume of the organ divided the increase in pressure that causes the change. This can be represented mathematically by:

$$C = \Delta V / \Delta P$$

When discussing the airways ΔV is the change in cylindrical volume of the bronchial tree and ΔP is the increase in air pressure needed to induce this change.

Accurate measurement of flow velocity and pressure is complex. Some of the methods used to assess lung function include plethysmography, single breath nitrogen washout, changes in pressure volume curves, and the forced oscillation technique (FOT) [70, 71].

As mice have become a primary model for asthma research, the need to measure lung function in the species has grown. Unfortunately this has proved difficult because scaling down techniques that are used in humans has not always proven possible or reliable. This difficulty is mainly rooted in the small volumes and rates of airflow, and a lack of laminar airflow in mice [72]. In addition, there are significant differences in anatomy, for instance, mice have a larger relative airway lumen than that seen in the human lung. Moreover, the chest wall in the mouse is extremely compliant [72]. As methods to measure murine respiratory mechanics have evolved, it has been necessary to balance accuracy with maintaining natural breathing conditions. The first and simplest popular method of measurement is unrestricted plethysmography, in which a conscious, unrestrained, breathing mouse is placed in a small-sealed box and pressure changes in the box during breathing are recorded. In this modality, temporal changes in pressure during inspiration and expiration can be used to derive the term, ‘enhanced pause’ (Penh), which may be linked to airway resistance. However, Penh is flawed

in that it has no direct association with airway mechanical properties such as resistance or elastance and has been shown to be less then ideally correlated with lung function [73, 74].

In contrast to unrestrained whole body plethysmography, an alternative approach is to employ a small animal ventilator with an anesthetized (and usually paralyzed) mouse that is subjected to maneuvers employed during the forced oscillation technique (FOT). The approach provides external pressure to the respiratory system of a trachestomized mouse via a computer controlled piston in order to enable measurement of respiratory system impedance (*Zin*), which is dependent on oscillation frequency [75]. Being a complex function, *Zin* consists of both a tangible component (airflow resistance) and a virtual component, reactance (a derivative of peripheral elastance). Measuring *Zin* in a mouse is technically challenging because of the animal's small size, but it is now routinely achieved using commercially available devices, such as the *flexi*Vent (Scireq Products, Montreal) small animal ventilator device, used for experiments in the present work. The system is based around a computer-controlled piston oscillator, allowing for precise control of frequency, pattern and magnitude of ventilation. A computer uses a basic model of the lung and its mechanical properties, in conjunction with the so-called constant phase model [74] and the equation of motion (see below) to calculate central resistance (Rn or Raw) and peripheral resistance (G) and tissue elastance (H) [72, 74, 76]:

$$Zin(f) = R_N + i2\pi f I + \frac{G - iH}{(2\pi f)^{\alpha}}$$

FOT has shown to have excellent correlation with lung function [77], though some criticize the technique due to the lack of spontaneous respiration. Nonetheless, at present it clearly represents the most accurate and reliable measurement approach of murine lung function.

Caveolae

Caveolae, so named for their cave like appearance are 50-100 nm invaginations of the plasma membrane on a variety of cells that were first described in ultrastructural studies in the 1950's [78]. These membrane structures represent specialized membrane lipid rafts, which are distinguished as highly organization plasma membrane microdomains characterized by a high concentration of sphingolipids and interacting cholesterol moieties [79, 80]. These membrane structures have important and numerous important roles in daily cell functioning [81-83]. The factor that distinguishes caveolae from other lipid raft domains is the presence of critical structural proteins called caveolins. There are three caveolin isoforms, caveolin (Cav)-1, -2 and -3. Cav-1 associates with caveolae in most cell types, including, but not limited to, smooth muscle, fibroblasts, epithelium, endothelium, and adipocytes. Cav-3 is chiefly expressed as a skeletal and cardiac muscle protein. Expression of Cav-1 or -3 drives formation of cell membrane caveolae, whereas Cav-2 is not required for caveolae formation but does form heterocomplexes with Cav-1 or -3, an interaction that stabilizes Cav-2 and its biological half-life [84-86].

Caveolin proteins form hairpin like structures that have a hydrophobic portion with two hydrophilic ends. Each protein includes a 33 amino acid hydrophobic portion that inserts into the membrane bi-layers, while N- and C-termini are hydrophilic and found on the cytosolic side of membranes (Figure 2). The interaction of multiple caveolin proteins forms a network that supports formation and stability of caveolae. Inter-cavolin protein interaction involves the N-termini, which form oligomers of caveolins that also interact with membrane cholesterol. The C-terminal domain is highly conserved among the caveolins and is responsible for anchoring the protein to the membrane [78]. Within the N-terminus there is a 20 amino acid

domain, known as the caveolin scaffold domain (CSD), which accommodates binding of many signaling proteins that harbor a complementary caveolin binding domain, thus giving caveolae the ability to affect cell signaling [79, 87] (Figure 3).

Caveolin-1 is expressed by many cell types in pulmonary tissues, including airway smooth muscle cells, airway fibroblasts, epithelium, endothelium and type I pneumocytes [88]. As Cav-1 stabilizes caveolin-2 protein, the latter is found in similar cells and tissues as Cav-1 [85]. Interestingly Cav-1 has particularly high expression in airway and vascular smooth muscle, whereas there is almost no expression in surfactant secreting type II pneumocytes [89, 90]. These findings suggest an important role within many cells of the lung and its integral role in the health/disease balance [91, 92].

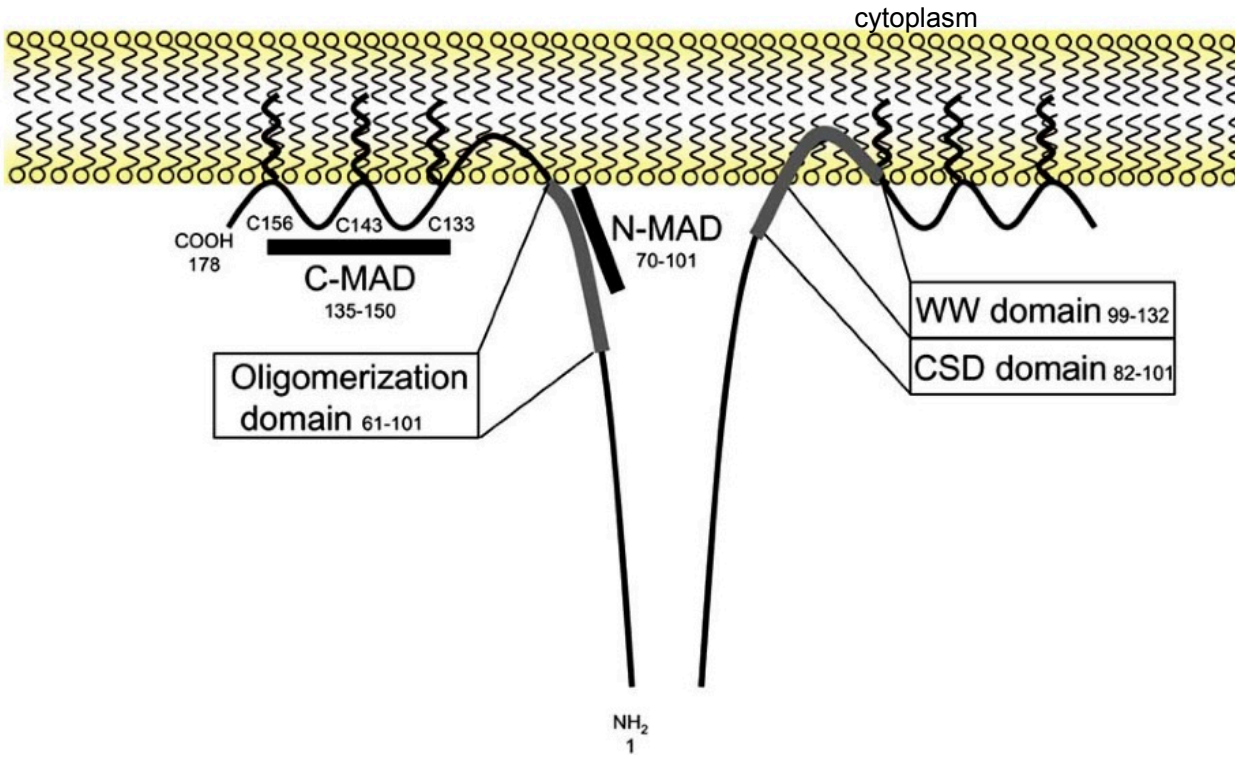

Figure 2 –*. Cavoelin and its structural and Functional domains. 33 AA hydrophobic domain within lipid bi-layer, WW domain interacts with cytoskeleton, CSD cell signal binding and transduction, N terminal forms caveolin oligomers. Reproduced with permission from Bentham Publishing.* Gosens, R. et al. "Caveolae and Caveolins in the Respiratory System" Current Molecular Medicine, Dec 2008, Vol. 8 Issue 8, p741-753. Figure 1.

Caveolins have been implicated in many cellular processes such as vascular transport, cholesterol homeostasis, cytoskeletal stabilization, ion channel and pump localization and signal transduction [79, 81]. It is believed that caveolae contribute to cell signaling by helping or hindering effector molecules being brought to close proximity to their receptors, thus giving caveolae micro-domains the ability to both promote or suppress different signal transduction cascades [93]. These effects are achieved through both upstream and downstream components such as G-protein coupled receptors, steroid hormone receptors, heterotrimeric G-proteins, and ion channels [94]. Although a review of all cellular pathways affected by caveolins is beyond the scope of this paper, several comprehensive reviews have been published, for example one by Patal et al. [94].

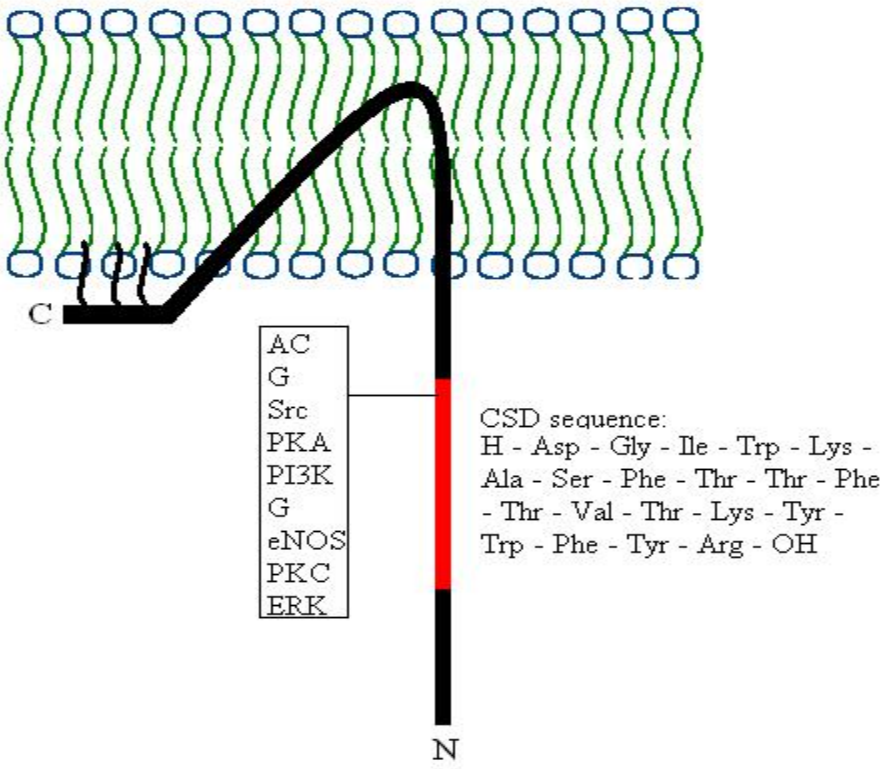

Figure 3 – Schematic depicting caveolae, resident structural proteins, caveolin (with its topology in the plasma membrane), and certain binding partners that interact with the caveolin scaffolding domain *(CSD red)* and its AA sequence

Caveolae, caveolins and obstructive airway disease

Caveolins can modulate both acute and chronic aspects of asthma pathology. First, as mentioned above, Cav-1 is abundant in ASM cells and appears, through impact of signaling related to regulation of contraction, to possess the capacity to regulate airway responsiveness. In primary culture it has been shown that as ASM cells switch from a synthetic to a contractile phenotype in mitogen-free conditions there is a corresponding, and necessary, increase in Cav-1 expression [95]. Furthermore, Cav-1 has a role as a cell signal modifier, thus affecting ASM function on numerous levels: (1) Ca++ mobilization: it is well accepted that elevation of free cytosolic Ca++ is the key step to induce smooth muscle contraction. It has been shown that L-type Ca++ channels, calsequestrin and calreticulin (calcium binding proteins) and the plasma membrane Ca++ pump are all present within caveolae. Further evidence shows that caveolae play a role in movement of calcium via its close proximity to intracellular calcium stores (sarcoplasmic reticulum and mitochondria) [96]. (2) K+ homeostasis: the balance between Na+ and K+ is key in the control of the creation of action potentials and it has been shown that Cav-1 can modulate Ca++ activated K+ channels [97] which in theory could affect the resting potential and thus the ease at which an action potential is created, and (3) adenylate cyclase and cyclic AMP have been shown to be negatively regulated by Cav-1 [98]. These molecules are part of the down stream pathways initiated by β2 adrenergic receptors that mediate pathways regulating airway lumen narrowing via ASM contraction.

A key provocative component of acute asthmatic responses lies in the local inflammation process. Caveolin-1's role both acute responses and chronic pathogensis have been shown. Studies have confirmed the presence of Cav-1 in macrophages, dendritic cells, neutrophils and lymphocytes [99, 100] and it is believed that within these cells Cav-1 regulates multiple

responses, including cell migration. Caveolin-1 also modulates the expression of pro- and anti-inflammatory mediators through negative modulation of the MAP kinase pathway. Down regulation of Cav-1 results in increased production of IL-6 and TNFα, and decreased production of IL-10, pro- and anti-inflammatory cytokines, respectively by alveolar macrophages [101]. A peptide mimic of the CSD of Cav-1 is able to suppress inflammation by interacting with nitric oxide synthase (NOS), thus decreasing NO generation, which normally promotes inflammation via the NF-κB pathway [102]. Caveolin-1 can also influence the inflammatory response through inhibition of the SRC family kinases that phosphorylate cysteine residues on a number of targets, leading to up regulation of inflammatory gene expression [103]. Thus, Caveolin-1 has the capability to modulate pathways linked to inflammation during an acute allergic asthma 'attack', however the presence and extent of these effects have never been investigated fully.

In terms of chronic effects of reduced Cav-1 expression, more insight has emerged in relation to inflammation and lung modeling in the last five years, in particular with the generation of Cav-1 knock out mice [104]. Specifically, Cav-1 has been shown to have suppressive effect on TGFβ signaling [91]. This cytokine contributes significantly to development of fibrosis seen in the airways of long term asthma suffers. Furthermore Le Saux et al. [105] showed that IL-4 down regulates Cav-1 expression in the allergen challenged murine lung. Fibroblasts from lungs of Cav-1 knock out mice have increased activation of MEK and down stream MAP kinases, ERK 1 and ERK2, which supports increased collagen production and proliferation [91]. Much like asthma, COPD is also characterized by airway inflammation and AHR, thus the mechanisms discussed above could be relevant to the pathology of COPD. As COPD pathogenesis includes destruction of alveolar septae, mediated

chiefly by metalloproteases, it is notable that Cav-1 can modulate both the activity and expression of MMP-1, 2, 9 & 13 [106]. Collectively these findings support the hypothesis that Cav-1 could play an important role in both acute and chronic pathophysiology in asthma or COPD.

Caveolae, Caveolin and Pulmonary Hypertension (PAH)

It has been shown that as caveolin-1 knockout mice age they suffer from severe pulmonary hypertension [107]. Pathology of the pulmonary vasculature in these animals is not completely surprising as Cav-1 is typically abundant in vascular smooth muscle. Furthermore, Cav-1 deficient endothelial cells may also contribute to disease development; molecular processes for PAH may be linked with the fact that the CSD of Cav-1 has a down regulatory effect on tyrosine kinases, thus decreasing STAT3 activation and its down stream events [108]. Recent work, however, has presented evidence that although there is a decrease in overall expression of Cav-1 in the lungs of human suffers of PAH, there is actually an increased expression within the vascular smooth muscle and this is associated with increased DNA synthesis and intracellular Ca++ release, which contribute to vascular medial hypertrophy and increased vascular resistance, respectively [109].

Caveolin-1 knockout mouse

Cav-1 KO mice were generated simultaneously by two different groups in 2000 [104, 110]. When first attempting to construct this genotype, it was thought these animals may be non-viable due to the role caveolae play in the function of multiple cells types, however the mice were found to not only be viable but also fertile and able to reproduce [110]. Importantly,

as Cav-2 binding to Cav-1 is necessary for protein stability, Cav-1 KO mice present as dual null phenotype for both isoforms [111]. Indeed, when Cav-2 mice were generated, the phenotype, in particular associated with the lung, was very similar to that of Cav-1 KO animals, suggesting that both Cav-1 and Cav-2 contribute to the phenotype of Cav-1 KO mice [111]. The primary phenotypic features of Cav-1 KO mice appear in the lung, with changes in pulmonary phenotype including: hyper-cellularity of the peripheral lung parenchyma, thickening of alveolar walls, and vascular leakage leading to the presence of red blood cells in alveolae (Figure 4) [110]. In terms of overall health, Cav-1 KO mice develop pulmonary hypertension and have a higher incidence of lung and other cancers [104, 107]. Cav-1 null mice also exhibit vascular system dysfunction, including cardiac hypertrophy, retarded angiogenesis, and vascular smooth muscle hypertrophy and have also been shown to decrease rates of atherosclerosis development [88, 112].

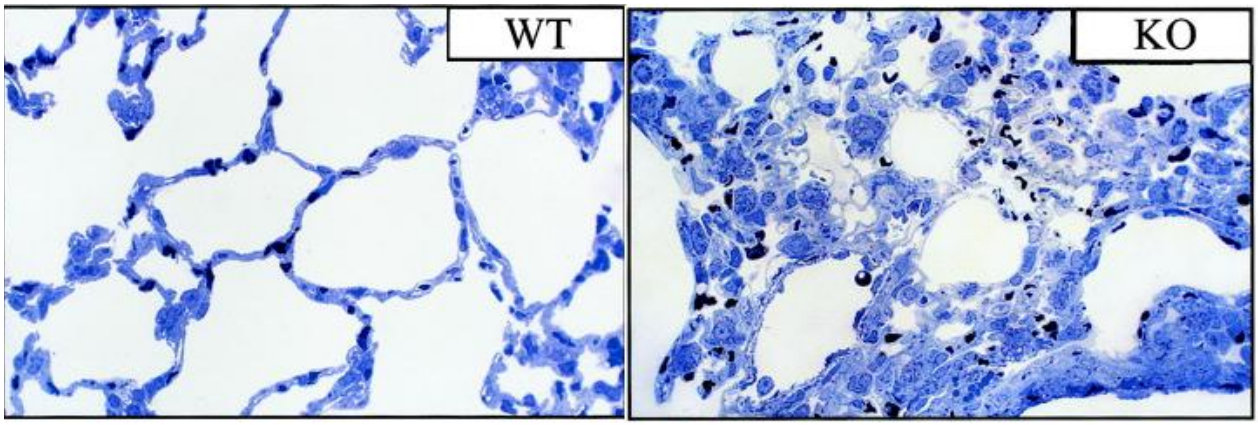

Figure 4 - Histological section of Cav-1 -/- mouse compared to it genetic control. Visualizing hyper-proliferative state and increased alveolar wall thickness phenotype. – *Reproduce with permission from American Society of Biochemistry and Molecular Biology.* Razani B. et al. "Caveolin-1 null mice are viable but show evidence of hyperproliferative and vascular abnormalities". J Biol Chem. 2001 Oct 12;276(41):38121-38. Figure 9

Hypothesis and Specific Aims

Asthma is a chronic respiratory condition that causes those affected to present with common symptoms that can include wheezing, chest tightness, excessive cough, and shortness of breath after minimal physical exertion [3]. Over the second half of the 20th century asthma has become one of the largest health concerns in developing countries, with prevalence rates reaching as high as 30% in Australian and British children. In North America, asthma complications are one of the three leading causes of emergency room visits and hospitalization among children [3, 5].

The pathophysiology of asthma is seen as a 'three headed monster' whose components all contribute to respiratory distress. The first prong of the syndrome includes episodes of bronchospasm that can typically be fully reversed using inhaled bronchodilators such as β2-adreneric receptor agonists. Asthma episodes are characterized by excessive narrowing of the airways leading to significant limitation of airflow, chiefly stemming from constriction of airway smooth muscle encircling the bronchi and bronchioles [42, 68]. Bronchospastic events are associated with episodes of airway hyperresponsiveness, thus the airway response to inhaled allergic and non-allergic stimuli is greatly increased. The second feature of asthma is airway inflammation, and it directly correlates with, and likely underpins, onset of airway hyperresponsiveness. Airway inflammation in asthma is generally of a Th-2 type phenotype [36, 113], and includes hallmark influx of eosinophils to the airways in most patients [28]. Bronchospasm and airway inflammation are both key components of the "asthma attack". The third hallmark of asthma pathophysiology, chiefly in severe asthmatics and patients who have had asthma for a long duration, is the presence of constant breathing difficulty that cannot be fully reversed with inhaled bronchodilators; this feature is often referred to as partially fixed airway obstruction. Airway remodeling refers to a number of anatomical changes that thicken

the airway wall, and specific features include increased thickness of airway epithelium goblet cell number, basal membrane thickness, extracellular matrix protein accumulation in the submucosa, and smooth muscle layers mass (due to cell hyperplasia and hypertrophy). These changes to the airways develop over time, likely driven by persistent episodes of airway inflammation, causing the airways to narrow excessively, decrease lung compliance, and increase airway resistance [51].

In trying to study a disease such as asthma it is not feasible to use human subjects to investigate and test basic mechanisms, therefore it is necessary to develop working animal models that mimic as closely as possible the hallmark symptoms of the human condition. This allows systematic investigation and clarifies factors that cause disruption of normal physiology, and to assess possible therapeutics for intervention. As no such model was established in our lab at the outset of my work, the first objective of the current project was to develop an effective and reliable working murine model of acute allergic airway inflammation and AHR using a relevant aerosol-sensitizing antigen; for this purpose we used house dust mite (HDM). Developing a model using HDM, rather than more commonly used ovalbumin (OVA) was important, as HDM is relevant to the human asthmatic population, being one of the leading triggers of allergic asthmatic exacerbations. Moreover, use of HDM does not require a sensitization exposure, thus changes are associated primarily with lung inflammation, and are not dependent on complex systemic immune and allergic responses. The advantages of using HDM over OVA have also been summarized in a number of published reviews [63, 65].

Caveolae are cellular membrane microdomains that can play a critical role in day-to-day cell functioning, including cellular signaling, cholesterol maintenance and calcium mobilization [93, 101, 105, 107]. Cav-1 is the key structural protein of caveolae, which can be

found throughout the pulmonary tissue in various cell types including smooth muscle cells, fibroblast, epithelium, endothelium and type I pneumocytes [89]. Cav-1 is a membrane-associated scaffolding protein that can sequester and modulate the activity of numerous intracellular signaling proteins associated with receptor-mediated cell responses. Caveolae and Cav-1 play a role in some disease pathology, for example it negatively regulates IL-4 release, a key pro-inflammatory cytokine, and it has also been implicated in modulation of fibrosis that occurs in lungs of patients with idiopathic pulmonary fibrosis [91]. The role of Cav-1 in response to allergen challenge is not clear, though it does appear that allergic inflammation marked by elevated levels of interleukin-4 (IL-4), can suppress Cav-1 expression in mice [105]. Work done previously in Dr. Halayko's lab indicates airway smooth muscle expresses abundant Cav-1, and Cav-1 facilitates Ca++ mobilization and contraction in response to several contractile agonists [95]. Despite these observations the precise role of Cav-1 in the context of the pathophysiology manifestations of asthma: airway inflammation, hyperresponsiveness and airway remodeling is not known.

The overall goal of my research project is to further the understanding of biological mechanisms that could be associated with asthma and its acute symptoms. In this regard, **experiments were designed to test the hypothesis that caveolin-1 protects against airway inflammation and hyperresponsiveness in mice exposed to inhaled house dust mite.**

To test this hypothesis I completed 2 objectives:

Objective 1: Develop a murine model of the acute atopic asthma event using a human relevant antigen, house dust mite (HDM) extract as a sensitizing agent.

Objective 2: Perform HDM exposure using genetically engineered caveolin-1 KO mice, and assess the impact on airway inflammation and AHR compared to Cav-1 expressing WT mice.

Asthma has complex origins and pathogenesis and sufferers represent a heterogeneous disease profile. Thus, a complete understanding of all factors that play role in pathogenesis is needed, as this knowledge provides a platform for future development of new treatments for asthmatic patients. The significance of the work conducted for my project is that it contributes to fundamental understanding of molecular mechanisms that can contribute to airway inflammation, remodeling and hyperresponsiveness. Thus the project fits with broader goals of better understanding of the pathogenesis of asthma-like disease.

Methods

Animals

In development of our HDM challenge protocols 8-10 week old female Balb/C mice (Charles River, Canada) were used for all procedures and measurements. For all studies, including those involving Cav-1 knock out mice, animals were housed in polycarbonate cages in a controlled environment within the University of Manitoba animal housing and care facility. The housing facility operates on a 12 hr. light/dark cycle and the animals have access to food and water *ad libitum*. Animals were given a one-week acclimatization period prior to any procedures being performed. All animal procedures were reviewed and approved by the Bannatyne Campus Protocol Management and Review Committee and are within the guidelines set forth by the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care (CCAC).

To examine the impact of constitutive suppression of Cav-1 expression, we completed studies using 8-10 week old female Cav-1$^{-/-}$ mice (Cav$^{tm1Mls/J}$, Jackson Laboratories, Bar Harbor, Maine) and compared that with data obtained from age- and gender-similar mice of the genetically matched control strain, B6129SF2/J (Jackson Laboratories, Bar Harbor, Maine).

Allergen Exposure

For the protocols described below, mice were either exposed to OVA (Grade V, Sigma-Aldrich, St Louis, MO, USA), whole aqueous lysates from lyophilized house dust mite (*Dermatophagoides pteronyssinus*) (Greer Laboratories, Lenoir N.C) or saline using an intranasal route. To facilitate delivery mice were anesthetized briefly using isoflourane (5%) to enable placement of aqueous drops (25μL) at the nasal opening. Drop placement stimulated the

animal to “sniff” and inhale the allergen, ensureing its delivery to the lung with minimal or no swallowing.

OVA

For OVA treatment, and in accordance with published protocols [114], mice were sensitized using 200 µL OVA with aluminum hydroxide (Alum, Sigma-Aldrich) as an adjuvant dissolved in saline to a concentration of 2µg/mL, given via intra-peritoneal (I.P.) injection. Sensitizing injections were given on days 0 and 14 of the protocol. On days 19, 20 and 21 the mice were then challenged intra-nasally (I.N) using 50 µL of OVA (50 µg/mL in saline). Control treatment followed the same protocol timeline however only saline was delivered both I.P. and I.N.

HDM

Delivery of HDM was done exclusively by I.N. exposure. All mice were treated with HDM dissolved in saline, with each animal receiving 35 µL containing 0.71 µg HDM/µL delivered equally between both nostrils. Initially two different protocols tested for effectiveness in inducing airway inflammation and AHR (Figure 5):

Protocol 1, 2 & 3 (Short term challenge): on for 1 day (Protocol 1) , 2 days (Protocol 2), or 3 days (Protocol 3) consecutively mice received HDM I.N. and outcomes (i.e. airway inflammation and lung function, see details below) were measured either 24 or 48 hours after the final allergen challenge. Mice receiving control treatment consisting of saline only given at the same volume and time points as HDM were also assessed.

Protocol 4 & 5 (Extended acute challenge): for 5 consecutive days mice received HDM I.N., then after 48 hrs "rest", mice received a second round of 5 consecutive daily exposures. Note: in early interactions of this protocol some animals also received a 142 µg dissolved in saline sub-cutaneous sensitizing dose 7 days prior to first I.N. exposure. As per Protocol 1, inflammation and lung function outcomes were measured 48 (Protocol 4) or 72 hours (Protocol 5) after the final I.N. HDM challenge. Mice receiving control treatment consisting of saline only given at the same volume and time points as HDM were also assessed.

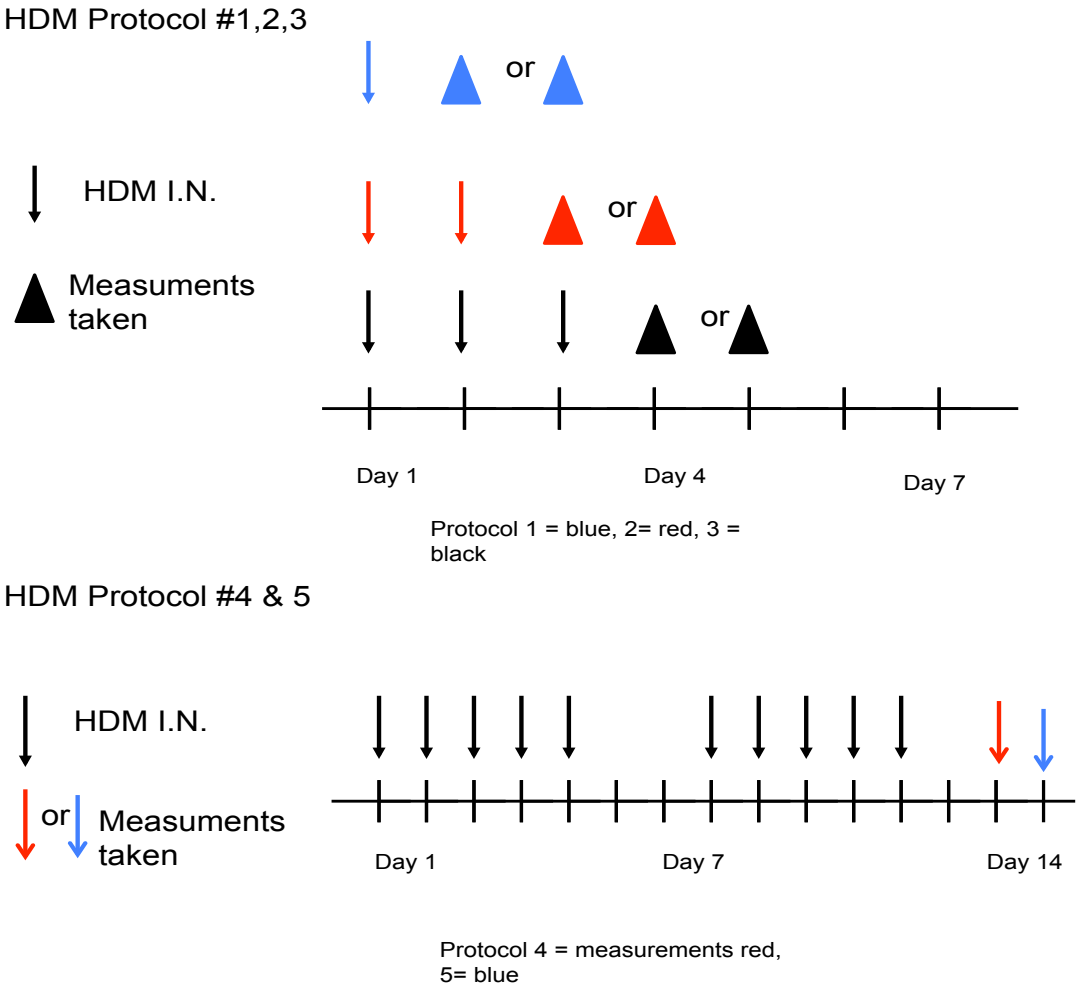

Figure 5 *– Visual representation of HDM sensitization protocols tested. Protocol 6 same as 5 with and intra-peritoneal HDM injection 7 days prior to first IN dosage.*

Airway Mechanics Assessment

Airway mechanics were assessed 72hrs post last allergen exposure. Using a small animal ventilator (Scireq, Montreal Canada) equipped with ultrasonic nebulizer, mice were exposed to increasing doses of methacholine (MCh), 0-50mg/mL. Thereafter, central airway resistance (R_{aw}), peripheral airway resistance (G) and peripheral tissue elastance (H) were determined.

To measure lung mechanics, mice were anesthetized with sodium pentabarbital (60 mg/kg), intra-peritoneal. When anesthesia was established (by lack of response to painful stimuli), the trachea was exposed and cannulated using an 8cm 20-gauge polyethylene catheter that was fitted to the ventilator. The ventilator deliverd a respiratory pattern of 150 tidal breaths per minute, at an inspiration/expiration time ratio of 1:1.5, with a tidal volume of 10 ml/kg of body weight. A constant positive end expiratory pressure (PEEP) of 3 cmH2O was achieved by submerging the expiratory outlet from the ventilator into a water trap.

Measurements were taken after nebulized administration of saline followed by sequential exposure to 3, 6, 12, 25 and 50 mg/ml of MCh, which was delivered over a period of 10 sec using an in-line ultrasonic nebulizer (approximately 150µL of MCh solution is delivered during this time). In order to have a uniform breathing volume the animal was subjected to two consecutive inspired total lung capacity (TLC) maneuvers followed by deflation to functional residual capacity (FRC) prior to delivering each dose of MCh. After delivery lung function was measured for 5 minutes.

The ventilator then implements a low frequency forced oscillation technique to derive changes in lung mechanics. Animal ventilation was interrupted and a perturbation signal was applied according to a preset protocol known as “Prime 8” which occured over 8 seconds during which a perturbation signal consisted of 18 serial sine waves ranging in frequency from

0.25 Hz to 19.625 Hz. Twenty such perturbations were performed in the 5 minute period after each MCh exposure. Respiratory mechanical impedance (Zin) was calculated from the pressure of the piston cylinder along with the displacement of the piston. Corrections were made for mouse size (weight), gas compressibility, and resistive and accelerative losses in ventilator, tubing and catheter were performed according manufacturer instructions, using dynamic calibration data obtained from volume perturbations applied to the system in an open and closed configuration. Using custom *flexi*Vent software calculation of R_{aw}, G, and H were completed by fitting the Z_{in} value of each to a constant phase model [74] (see literature review) and normalized to bodyweight, the mean of the 20 perturbation values was then calculated.

Airway Inflammation Assessment

After measurement of lung function, mice were removed from ventilator and bronchoalveolar lavage (BAL) was performed. For this purpose, 1 mL of ice-cold saline was flushed in and out of the lungs via the tracheal catheter twice using a 5mL syringe. The collected BAL fluid was then centrifuged (1500 rpm, 10 mins, 4^oC). The supernatant was collected and frozen at -80^oc for subsequent ELISA, whereas the pellet of cells was then resuspended in 1mL of ice-cold saline. A hemocytometer was used to determine the total cell count of the resuspended BAL sample.

To assess the immune cell composition of the BAL, slides were made by using cytospinning 100µl sample (1000 rpm, 5 min). Slides were allowed to air dry then were fixed by dipping in ice cold 150-200 uL methanol (95%), and finally treated with Giemsa-Wright stain (Fisher Biosciences). The number of eosinophils, neutrophils, macrophages and lymphocytes were counted based on standard morphological features. Cell counts were

completed for 6 individual fields of each sample and the percentage of each cell type for the entire sample was extrapolated from the average.

ELISA

To further characterize inflammation in the lungs, stored supernatant from BAL were used for standard ELISA for: IL-4, -5 and -6, TNFα, TGFβ1 & INFγ. Assays were performed according to published methods [115] in a blinded fashion using coded samples that were shipped on dry ice to Dr. John Gordon's lab at the University of Saskatoon.

Mucus Quantification

RT-PCR

After measuring airway mechanics lungs were removed and placed in RNA Later (Qiagen). Tissue samples were given two bursts using the maximum setting with a tissue homogenizer for 20 seconds and then centrifuged at 1500 rpm (377G) for 4 mins. Tissue samples were then sonicated for 2 bursts lasting 5 seconds each, centrifuged at 1500 rpm (377G) for 4 mins and then stored at -80^{oc}. Total RNA was extracted using a Qiagen RNeasy Kit following the protocol provided by manufacturer (Quiagen, Mississauga ON). RNA was then reversed transcribed using M-MLV reverse transcriptase (Promega, Madison, WI) for 1h at 37°C. RT-PCR reactions were carried out for cDNAs of interest in a thermal cycler (Mastercycler, Eppendorf, Germany): murine primers were used for MUC-5A (Table 2).

Primer	***Primer Sequence***
MUC-5A-5'	TGGACCTCAGGTATTTCACAC
MUC-5A-3'	TAGTTCTTAGCCCTGCATTGC

Table 1 – Primer sequences of MUC5a/c used for rt-PCR investigating mucus production in lung tissue samples

Morphometry

Left lungs were removed post lung function tests and were inflated using a 10% fomalin solution at a pressure of 21 cm H_2O, and kept for a further 24hrs suspended in formalin at room temperature. Inflated lungs were then processed and embedded in paraffin. Prior to embedding, samples were cut along the mid-line of the left lobe: the bottom half of lobe was used for subsequent sagittal sectioning, whereas the proximal half of the lobe was sectioned 90^o to the cut line. The two different orientations are used to look at both the main stem bronchus (bottom half sections but in cross section) as well as the peripheral airways (top half sections cut longitudinal to plane of the left lobe). Sections were cut to a thickness of 6 um and stained.

In order to visualize mucus producing goblet cells and the mucus they have secreted into the airway lumen, Alcian Blue staining was used; this utilizes the acidity of the mucus to generate a bright aqua blue that reveals both secreted mucous and that still harbored within individual epithelial goblet cells. Mucus staining was visualized using an upright light microscope equipped with a SPOT CCD camera using ImagePro software (Olympus, Center Valley PA). Analysis was done on airways selected using the following criteria: (i) the full circumference of the airway is in the field of view, (ii) airway perimeter was completely intact, (iii) the length to width ratio of the airway is less then 2 (to avoid airways that were cut obliquely), and (iv) regions of the airways analyzed were not directly between an adjacent blood vessel. Using ImagePro software macros, aqua blue staining could be automatically discriminated and the amount of mucus was estimated based on area of staining (pixel number converted to mm^2 based on prior calibration of the microscope). To allow for observations to be compared between animals and sections, total area of blue staining for mucous within the

airway lumen and in goblet cells was normalized to the length of the sub-epitheial basement membrane in the field of view (i.e generating staining area per μm of basement membrane).

Statistical Analysis

All results are expressed as mean ± S.E.M., where *n* equals the number of animals used. Results were compared using a standard t-test when comparing two conditions, and by analysis of variance when comparing between the all 4 groups, followed by the Tukey's *post-hoc* test using GraphPad 4 software. Differences were considered significant when the *p* value was less then 0.05.

Results

Optimizing HDM Exposure Protocols

Female Balb/C mice were exposed to whole HDM extract without any exogenous adjuvant for one, two or three days consecutively (see Figure 6, Protocols 1, 2 and 3). Twenty-four and forty eight hours after the final allergen exposure airway mechanics were measured in response to aerosolized MCh. In this triad of acute exposure studies, total BAL cell counts revealed no elevation in inflammatory cell number compared to saline exposed animals (Figure 6). As a measure to assess the impact of HDM exposure, some mice were also sensitized and challenged acutely with OVA, which as expected induced significant inflammatory cell infiltration (700,000 cells/mL). Consistent with the inability of acute HDM exposure for up to 3 days to induce lung inflammation, no animals showed any increase in response to any concentration MCh for central resistance (Raw), peripheral resistance (G) or tissue elastance (H) compared to saline exposed control mice (Figure 7). In contrast, but consistent with results of BAL cell counting, acute OVA sensitization and challenge markedly increased responsiveness to inhaled MCh for all parameters of lung function measured. Collectively, these results indicate that acute HDM exposure for up to only 3 consecutive days is not sufficient to induce significant lung inflammation or change physiological responsiveness to inhaled MCh.

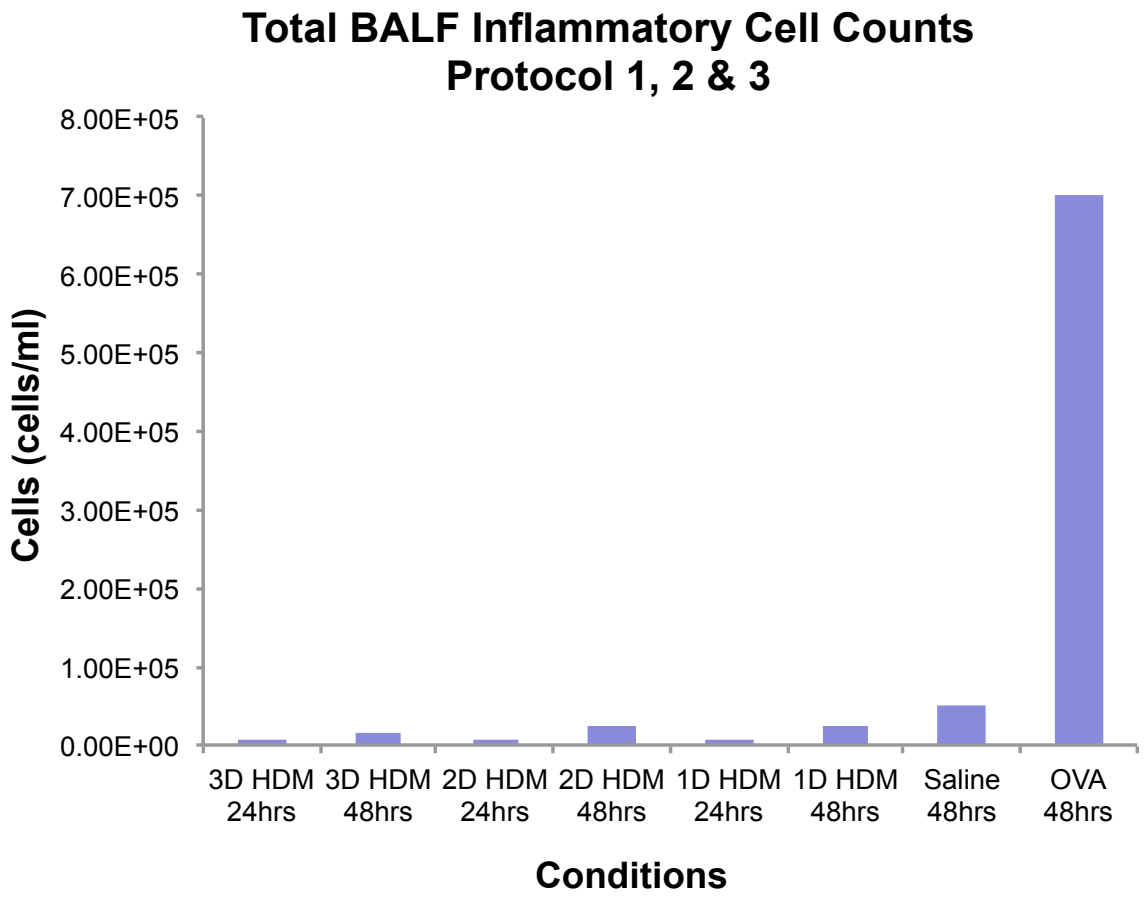

Figure #6 *- Exposing mice to HDM protocol 1, 2, 3 (1,2 or 3 days of consecutive HDM allergen exposure) did not induce any airway inflammation compared to saline exposed control groups and none were equivalent to OVA exposure protocol. N=2 for all groups.*

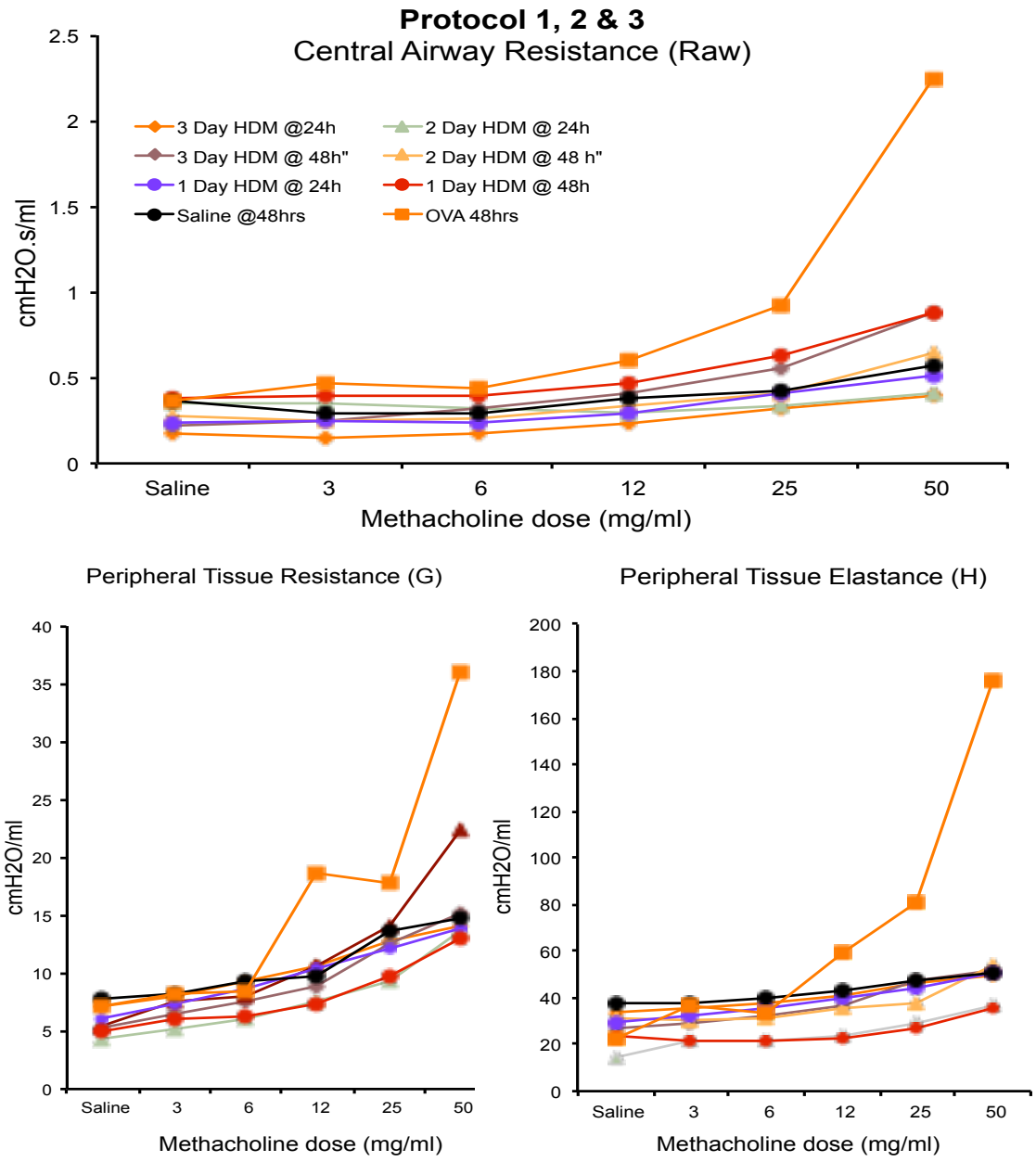

Figure #7 - Exposing mice to HDM protocol #1, 2, 3 (1,2 or 3 days of consecutive allergen exposure) showed no significant change in peripheral airway resistance, elastance or central airway resistance from saline exposed control groups. Furthermore was by no means equivalent to OVA exposure protocol. N=2 for all groups.

Based on the negative results of our initial acute HDM exposure protocols, we next assessed the impact of 5 consecutive days of I.N. HDM extract challenge followed by two days rest and a second round of 5 days of I.N. HDM; impact on inflammation and lung function was assessed 48 hours after final HDM challenge (see Figure 8 & 9, Protocol 4).

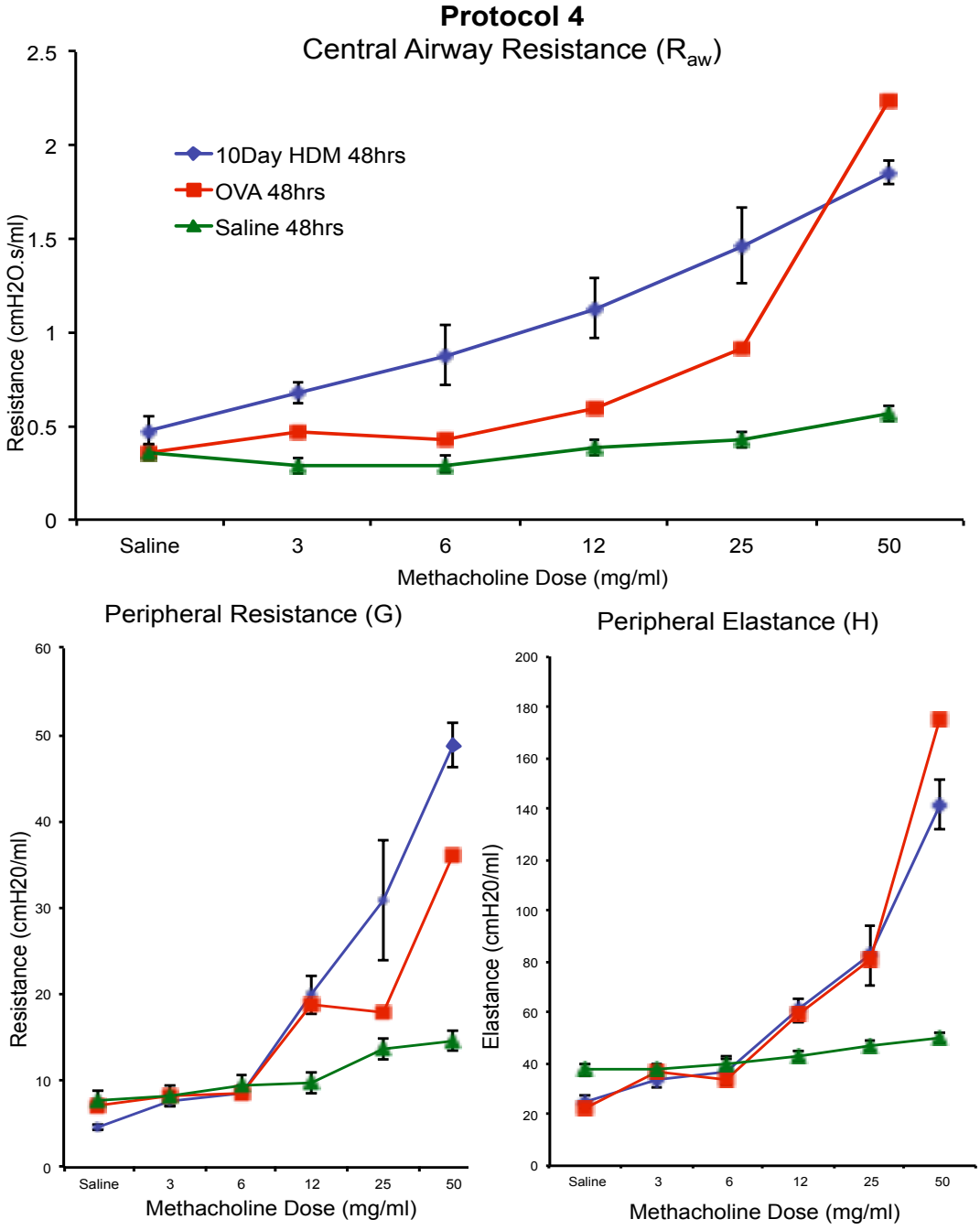

Figure 8 – HDM protocol #4 (blue) shows development of AHR well above saline control animals (green). Maximum development of central & peripheral resistance and peripheral resistance is equivalent to the OVA (red)sensitized animals. Furthermore R_{aw} shows greater specificity then the OVA animal at lower doses of MCh. (N=3 for HDM, N=2 for others)

HDM exposure led to development of a maximum R_{aw} of 1.85 ± 0.25 cmH2O.s/ml upon challenge with MCh (50 mg/mL). This response was significantly increased compared with that seen in the saline control group 0.55 ± 0.16 cmH2O.s/ml ($p<0.05$) (Figure 8). Interestingly, HDM exposure was nearly as effective in increasing max R_{aw} as in the OVA sensitized-challenged group (2.24 cmH2O.s/mL) indicating similar levels of airway hyperreactivity were attained. However, compared to OVA-challenged animals, HDM challenge more readily induced elevated R_{aw} in response to sub-maximum concentrations of MCh, indicating a greater impact on sensitivity to non-allergic provocation that is achieved with OVA exposure (Figure 8).

Other indices of respiratory function, G and H, exhibited similar trends to HDM and OVA exposure as was seen for R_{aw}. Upon maximum MCh challenge the HDM exposure group exhibited greatly increased peripheral airway resistance (G) and tissue elastance (H) compared to saline controls; 48.7 ± 2.63 vs 13.6 ±0.91 cmH2O/mL and 141.6 ±2.63 vs 34.4 ±7.09 cmH2O/mL ($P<0.001$), respectively. Equivalent reactions to that of OVA challenged mice were seen at all concentrations of MCh above 6mg/mL (Figure 8), with maximum responses being nearly equivalent in HDM and OVA challenged mice; 48.7±2.63 vs 35.9 cmH2O/mL for peripheral airway resistance and 141.6 ±2.63 vs. 175.1 cmH2O/mL for lung elastance, respectively.

In addition to measuring lung function we also assessed lung inflammation after ten days of HDM exposure (Protocol #4 in Figure 9). Balb/C mice showed a marked increase in total inflammatory cell number in BAL fluid 48 hours after receiving final HDM challenge (5.47 ±0.14 x 10^5 cells/mL) compared to saline challenged controls (1.3 ± 0.17 x 10^5 cells/mL)($p<0.001$)(Figure 9). For the time point at which BAL was collected in these studies

(48 hrs post allergen challenge), OVA-exposed animals showed a slightly higher level of total inflammatory cells than HDM challenged mice; 7.0×10^5 vs $5.47 \pm 0.14 \times 10^5$, respectively (Figure 9). We also determined the differential distribution of inflammatory cell types in BAL: whereas in naïve mice the predominant cell type was the alveolar macrophage (71.65 ±1.50%) with minimal contribution of eosinophils (4.68 ±0.93%) or neutrophils (9.17 ±1.36%). In the HDM group the fractional contribution of phagocytic macrophages was markedly decreased to 24.45 ±14.84%, with a concomitant rise in the percentage of eosinophils (40.21 ±12.29%) and neutrophils (15.86 ±1.7%) (Figure 9). While both HDM and OVA-exposed mice both exhibited recruitment of eosinophils and neutrophils to the lung, interestingly, for our protocols the primary inflammatory cells differed between antigens, because while eosinophils predominated in HDM animals, for OVA-challenged animals neutrophils (34%) made up the largest percentage of cells seen (Figure 9).

As these findings show that 10 days of allergen exposure was sufficient to elicit a respiratory inflammation and dysfunction, we next performed studies to better determine temporal patterns of response after HDM challenge. To this end we compared lung function and inflammation 72hrs after allergen challenge with results seen 48 hrs post-allergen (Protocol #4 in Figure 6, data shown in Figures 10 and 11).

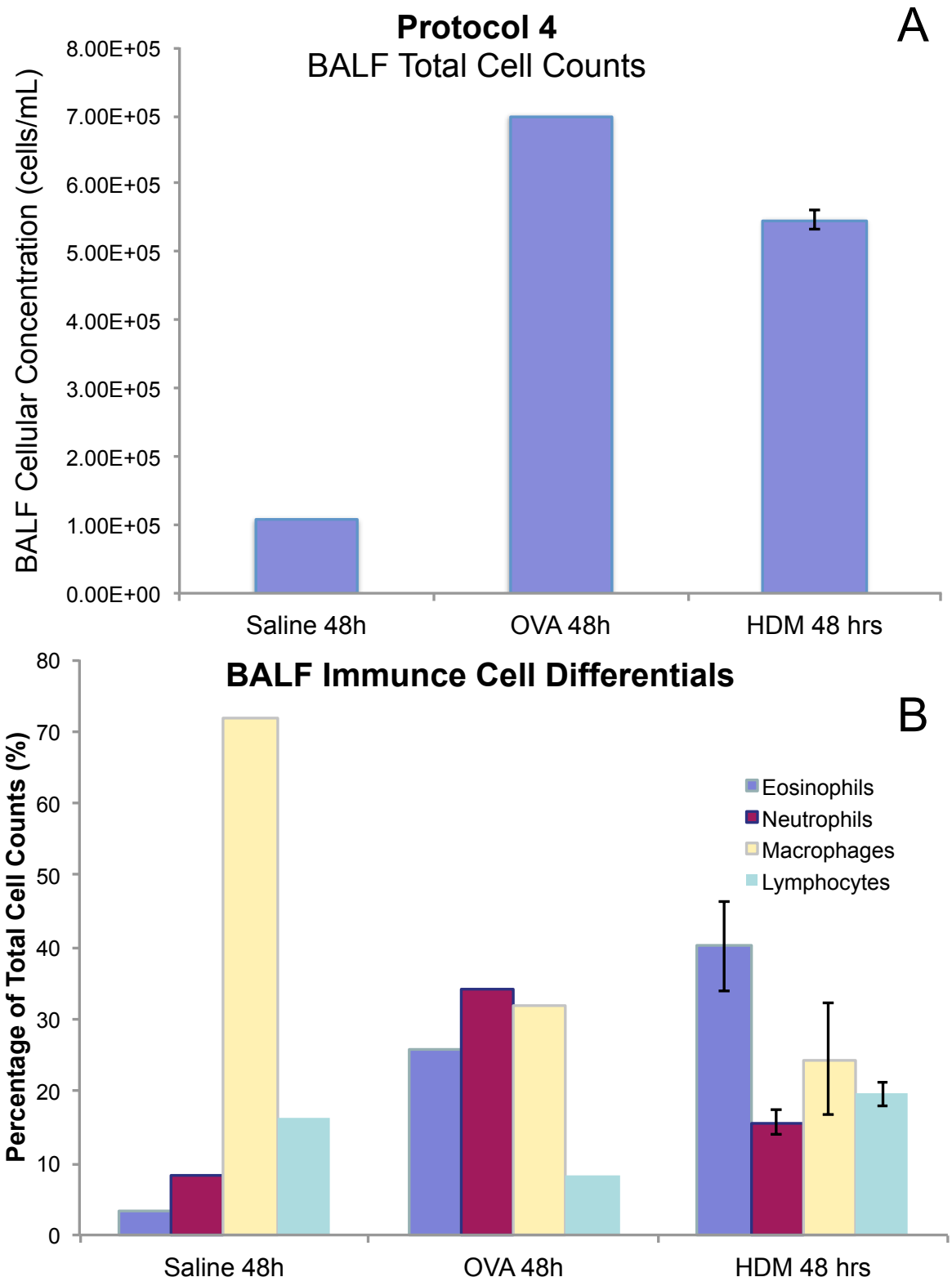

Figure #9 – HDM protocol 4 shows a total BALF inflammatory cell count largely increased compared to the saline control, however slightly less robust then that of the OVA model (A). The inflammatory reaction of the HDM sensitized mice was eosinophilic, while the greater contributor to the OVA model inflammation was neutrophilic (B)

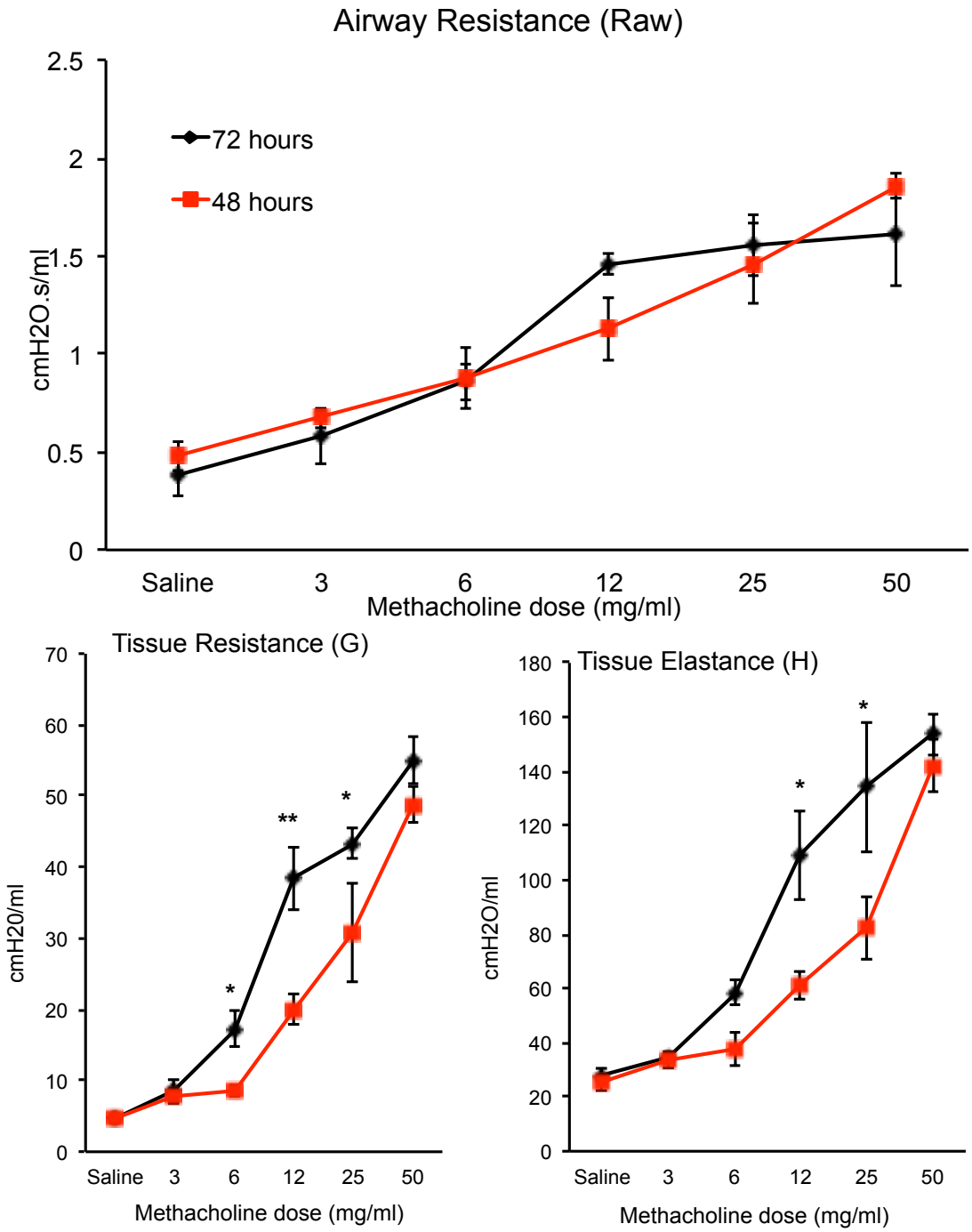

Figure 10 - There was no discernable difference in maximum resistance or elastance whether measurements were taken 48 or 72 hrs post final intranasal, however there was a significant trend of an increase in sensitivity seen at 6,12 & 25 mg/ml of MCh in peripheral mechanics in 72hrs animals. (*= p<0.05 b/w HDM groups, **=p<0.01 b/w HDM groups)(n=3 for HDM groups, n=2 for saline groups)

Performing MCh challenge on mice 72hrs post allergen administration (Protocol #5 - see Figure 6) did not reveal any in difference in the maximum central airways (R_{aw}) response

or sensitivity to MCh indicating 48 hrs was sufficient to develop airway hyperresponsiveness (Figure 10). In contrast to R_{aw}, 72hrs post-allergen we did observe increased sensitivity to MCh with respect to tissue resistance and elastance compared to that measured 48hrs post allergen administration. This effect is evident from the leftward shift of the MCh concentration-response curves for G and H parameters (Figure 10). Despite this shift in sensitivity, no significant increase in G (55.02 ±3.29 vs. 48.77 ±2.63 cmH20/mL; P =0.22) or H (153.56 ±7.53 vs. 141.84 ±9.77 cmH2O/mL, P=0.76) was evident.

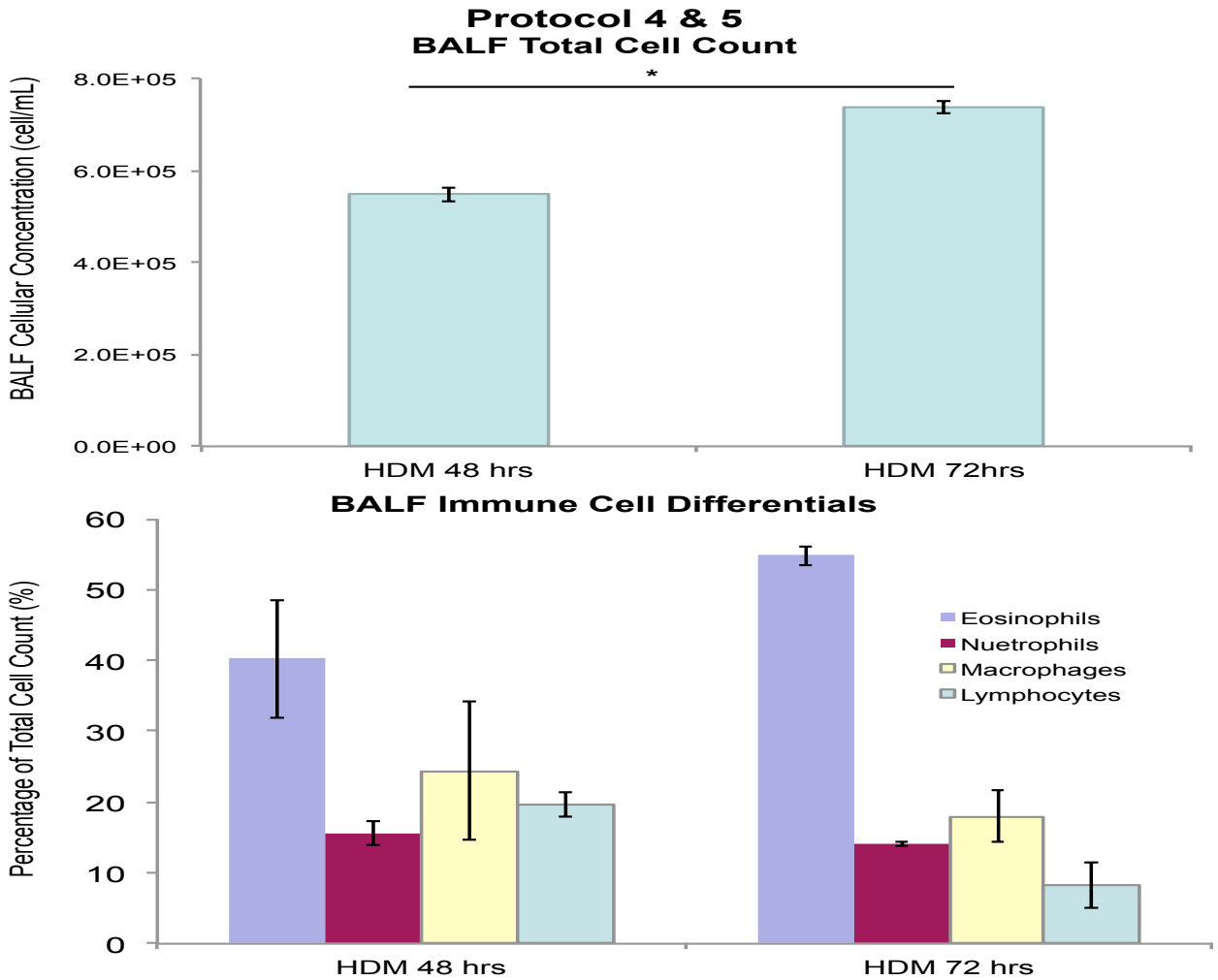

Figure 11 – Protocol 5. 72 hours showed a significant increase in total inflammation. Exaggerated eosinophilia was also seen that however was not significant compared to 48 hrs (protocol 4). (* = $p < 0.05$)

We also compared airway inflammation 48hrs and 72hrs post HDM-challenge (Figure 11). We observed a significant 34% increase ($p < 0.05$) in the total inflammatory cells in BAL fluid collected 72hrs after allergen challenge ($7.4 \pm 0.31 \times 10^5$ cells/mL) compared to fluid taken from animals at 48hrs ($5.5 \pm 0.15 \times 10^5$ cells/mL). The nature of inflammation also differed between sampling time points, with a greater fraction of eosinophils at 72hrs compared to 48hrs ($55. \pm 2.81$ % vs 40 ± 12.29%, $p < 0.05$).

In a final series of experiments we assessed whether a more robust lung and inflammatory response at 72hrs could be induced with 10-day I.N. HDM challenge using a sub-cutaneous, sensitizing injection of allergen (200 μL, 0.71μg HDM/mL) 7 days prior to first I.N This was called "Protocol 6". The MCh dose response curve for R_{aw} 72 hrs after final I.N. HDM challenge was unchanged from that measured in mice that received only I.N. HDM (Figure 12). In contrast, addition of a pre-sensitizing injection of HDM actually decreased both G and H at all concentrations of MCh. We also examined the effect of a pre-sensitizing injection of HDM on airway inflammatory cell infiltration, and found that there was no impact on total cell number in BAL ($6.3 \pm 1.89 \times 10^5$ cell/mL) compared to mice that received I.N. challenge only ($7.4 \pm 0.31 \times 10^5$ cell/mL)($p=0.26$)(Figure 13).

Collectively, data from all of the aforementioned studies using Balb/C mice indicate that HDM challenge is equal or superior to OVA sensitization and challenge at provoking airway inflammation and hyperresponsiveness. Furthermore, we noted that maximum impact of HDM challenge on lung inflammation and all indices of lung function required administration of 10 I.N doses in a two-week period, with outputs being assessed 72hrs after the final challenge with HDM. On this basis we used this approach (Protocol 5 in Figure 6) in all subsequent experiments to determine the impact of allergen challenge on Cav-1 KO mice.

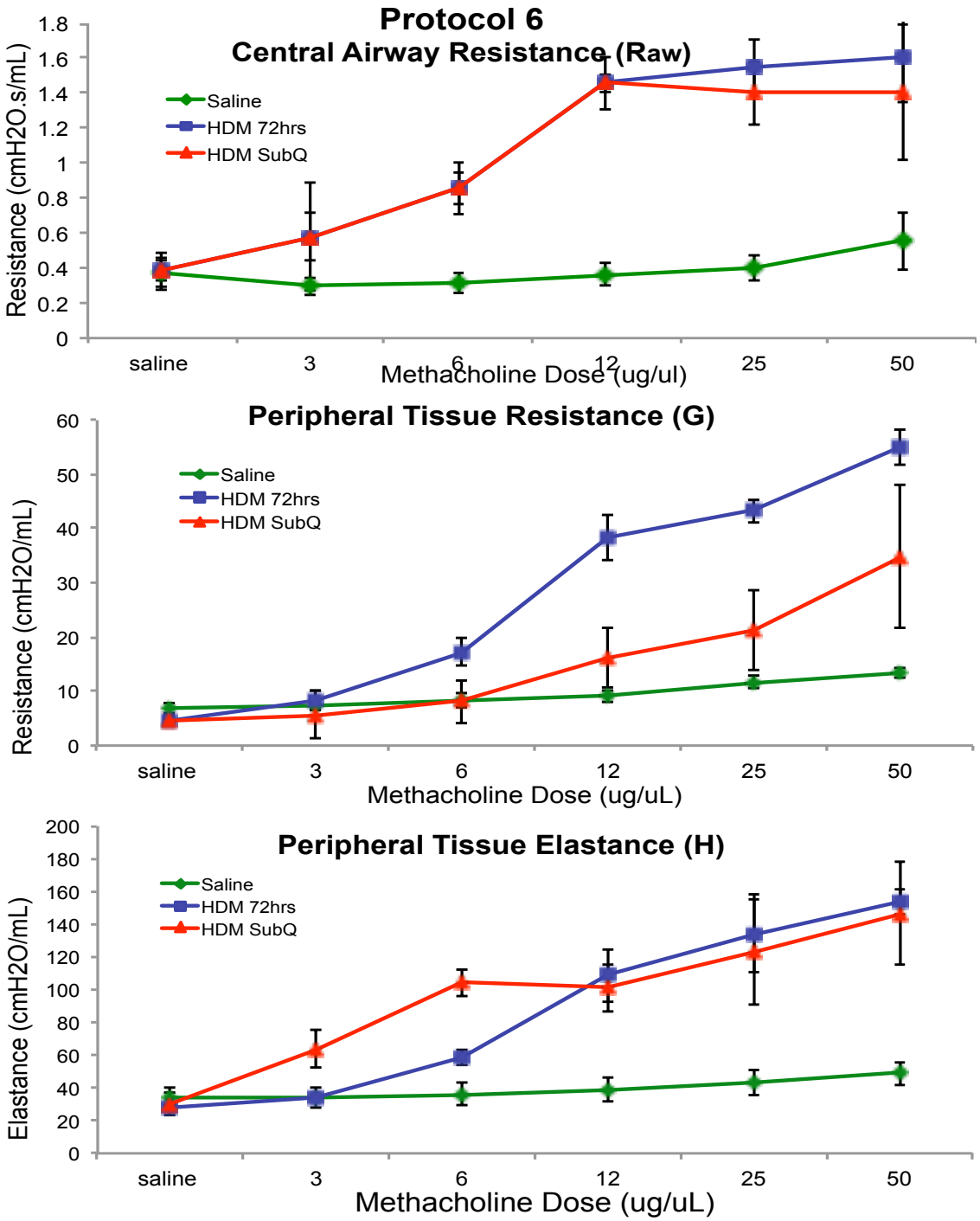

Figure 12 – Adding a sub-cutaneous sensitizing injection(protocol 6) to the developed 10 day HDM protocol with measurements taken at 72hrs showed no augmentation of dysfunction in airway mechanics compared to no injection (saline n=4, SubQ & HDM n=3)

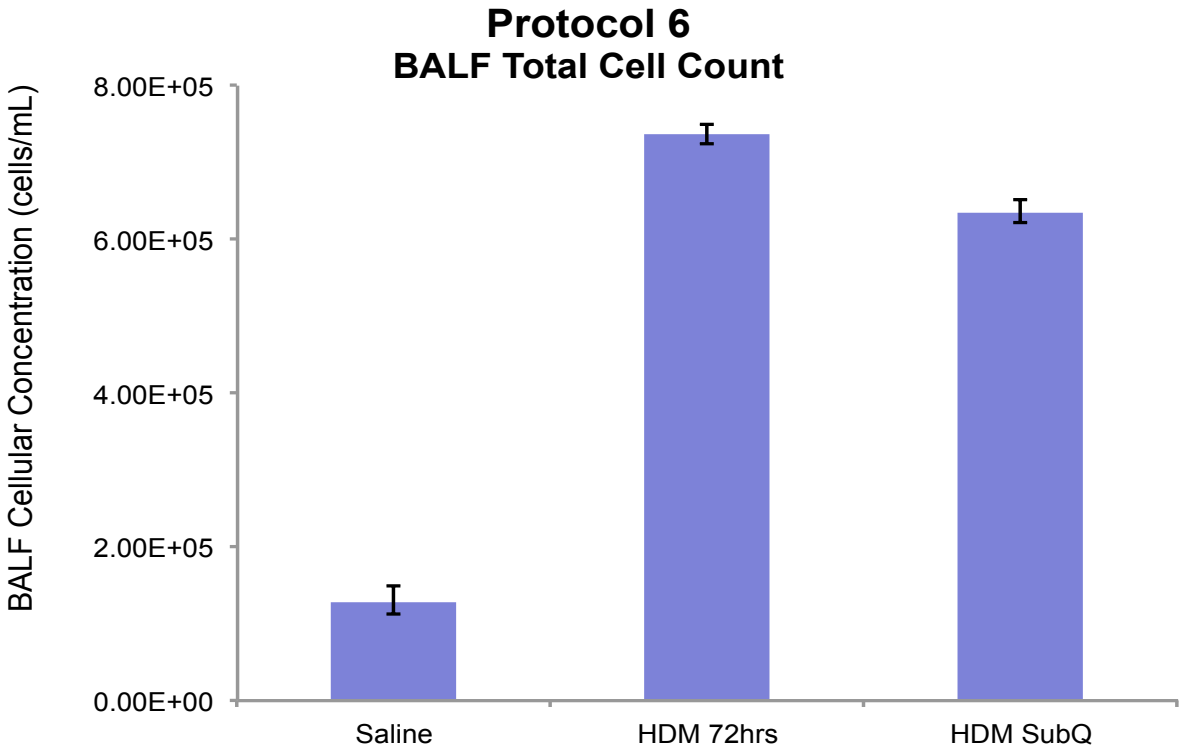

Figure 13 – Protocol 6 - with the addition of a sub-cutaneous sensitizing injection to the developed 10 day HDM protocol showed no significant difference in the extent of airway inflammation was seen (saline, OVA, Sub Q n=2 HDM n=3)

Effects of HDM Challenge in Cav-1 KO Mice

For all studies 8-10 week old female Cav-1 KO mice ($Cav^{tm1Mls/J}$) were used. As the animals have a mixed background, age- and gender-matched B6129SF2/J mice served as a genetic control since they most closely resemble the Cav-1 KO line. Genotype was confirmed using PCR (not shown) with primers according to instructions from the animal supplier (Jackson Laboratories, Barr Harbor Maine). We also performed transmission electron microscopy to confirm the absence and presence of caveolae in tracheal smooth muscle from Cav-1 KO and B6129SF2/J mice, respectively (Figure 14). In addition, we screened tissues using immunoblotting to assess expression of Cav-1, -2 and -3 and confirmed the absence of Cav-1 in all tissues from KO mice that otherwise express the protein in genetic control animals (Figure 14). Notably, for all tissues, including lung and tracheal smooth muscle that

are devoid of Cav-1 in KO animals, we noted that Cav-2 was markedly decreased in abundance compared to genetic (wild type) controls. In contrast, Cav-3 content was unaffected in tissues from Cav-1 KO animals.

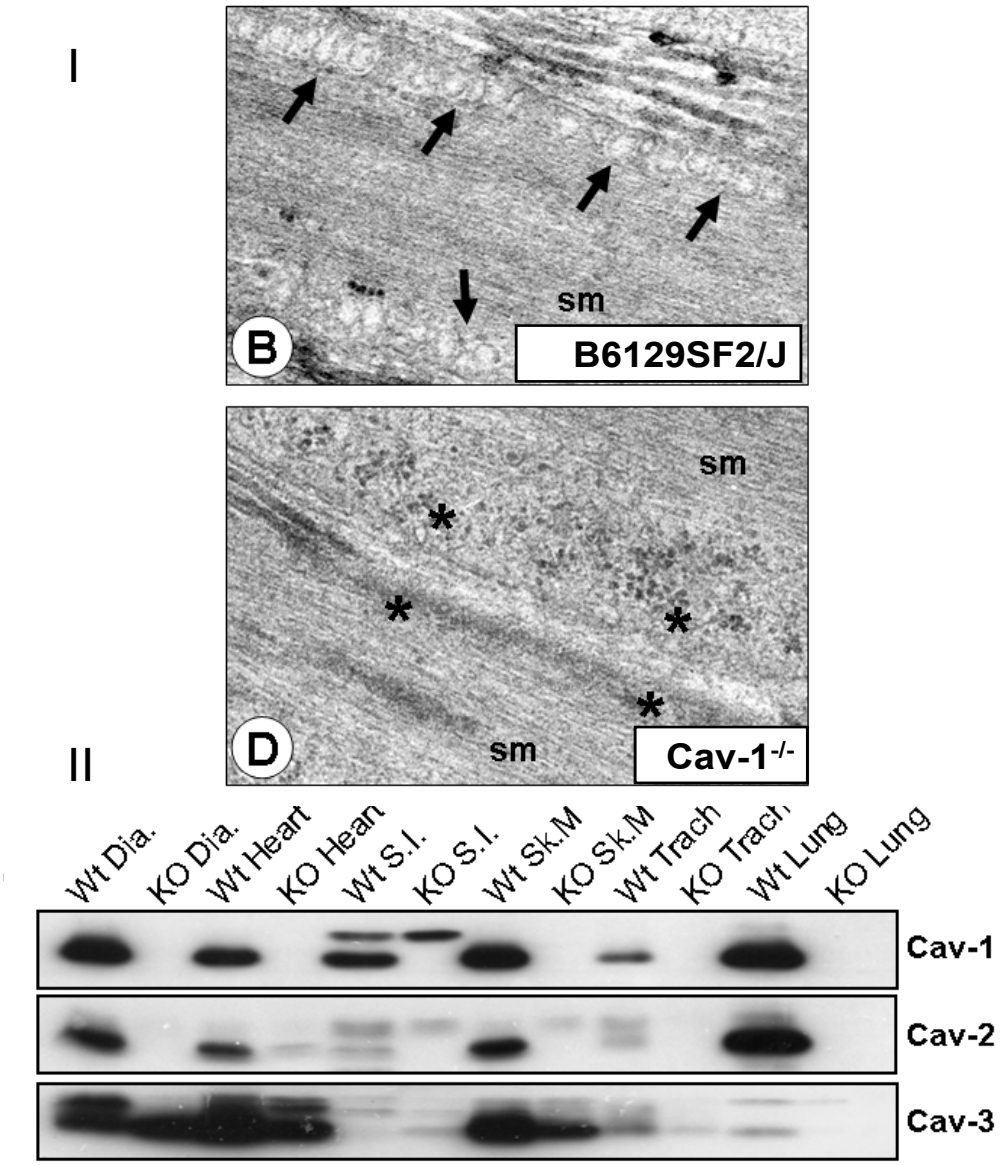

Figure 14 – I) electron micrograph showing the presence of caveolae on cell membrane of wildtype animal (B) and the absence of caveolae on same cell type Cav-1 null mice (D). II) Western blot shows Cav-1, 2 & 3 expression in wildtype and Cav-1 KO mice in different tissues.

Respiratory Mechanics

The lack of Cav-1 was associated with a significant increase in MCh sensitivity and MCh-induced maximum R_{aw} in allergen naïve mice compared to genetic controls (Figures 15 and 16). Indeed, naïve Cav-1 null mice showed a higher maximum R_{aw} of 1.40 ±0.14 compared to 0.79 ±0.11 cmH20.s/mL ($p < 0.01$) for the genetic controls (Figure 15). In both allergen naïve and challenged animals we saw significant difference in maximal peripheral tissue resistance (G) and elastance (H) reached with MCh challenge. The Cav-1 KO animals reached 19.81±5.47 and 63.80±12.32 for tissue resistance (G) and elastance (H) respectively compared to 8.12±0.86 and 36.32±4.03 for the naïve animals, comparing these numbers gives p-values less then 0.01 and 0.05 for resistance and elastance respectively (Figure 15). Indeed, this increase was evident at all concentrations of MCh challenge, resulting in a lower PC20 value in Cav-1 KO mice (5.29±0.26 mg MCh/mL) compared to genetic controls (9.91±1.23 mg MCh/mL)($p < 0.01$) (Figure 16). These data are consistent with earlier reports on baseline lung mechanics in adult Cav-KO mice[105] that the lungs of Cav-KO mice have thickened alveolar septae and increased collagen deposition in large airways [104, 116]

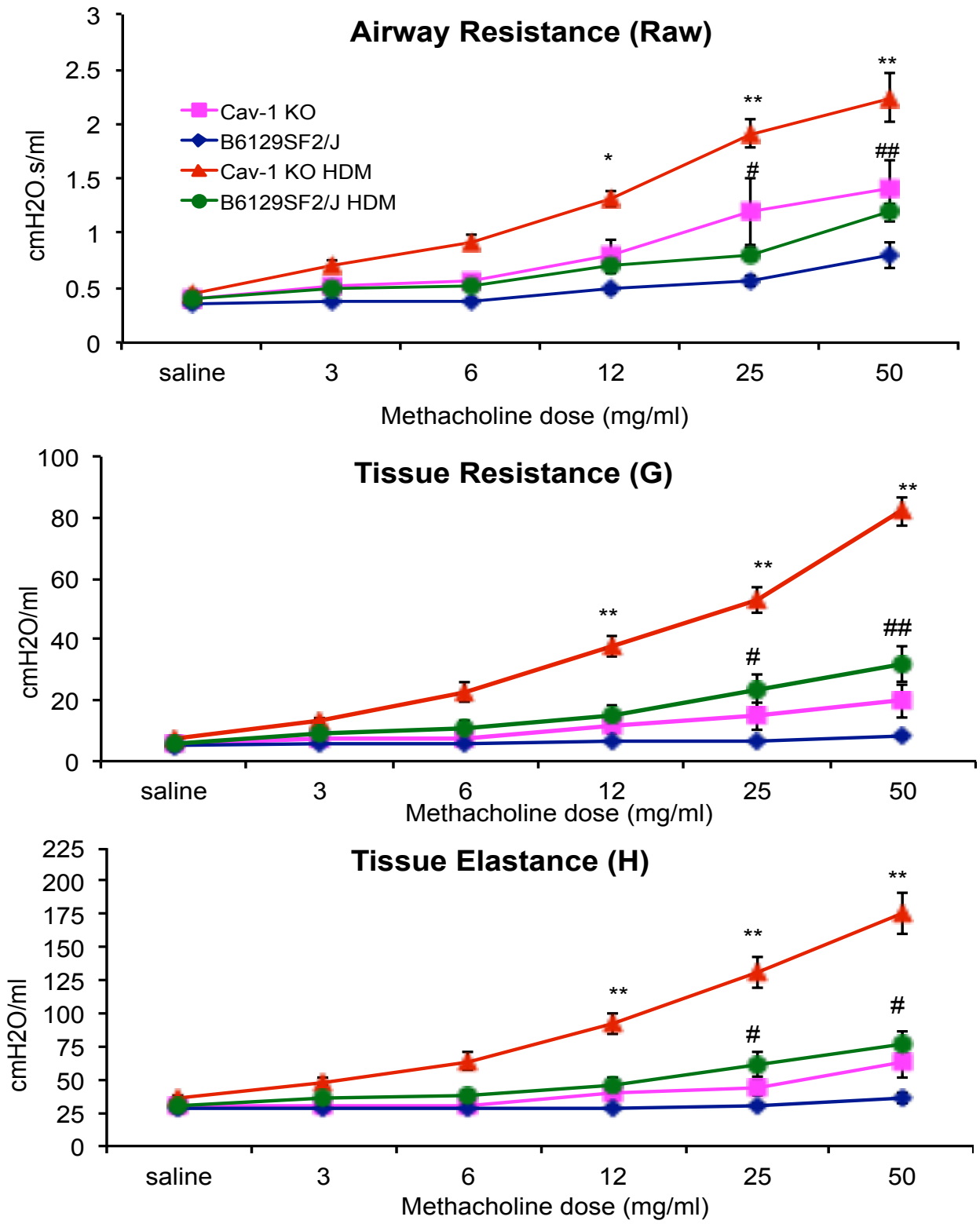

Figure 15 – Cav-1 null mice show increased central (a) & peripheral (b) resistance and elastance (c) at all doses of MCh during challenge indicating a greater airway responsiveness and sensitivity. HDM group n= 16, cont. n= 12, * comparison b/w HDM groups, # comparison b/w control groups, * = $p<0.05$, ** = $p<0.01$

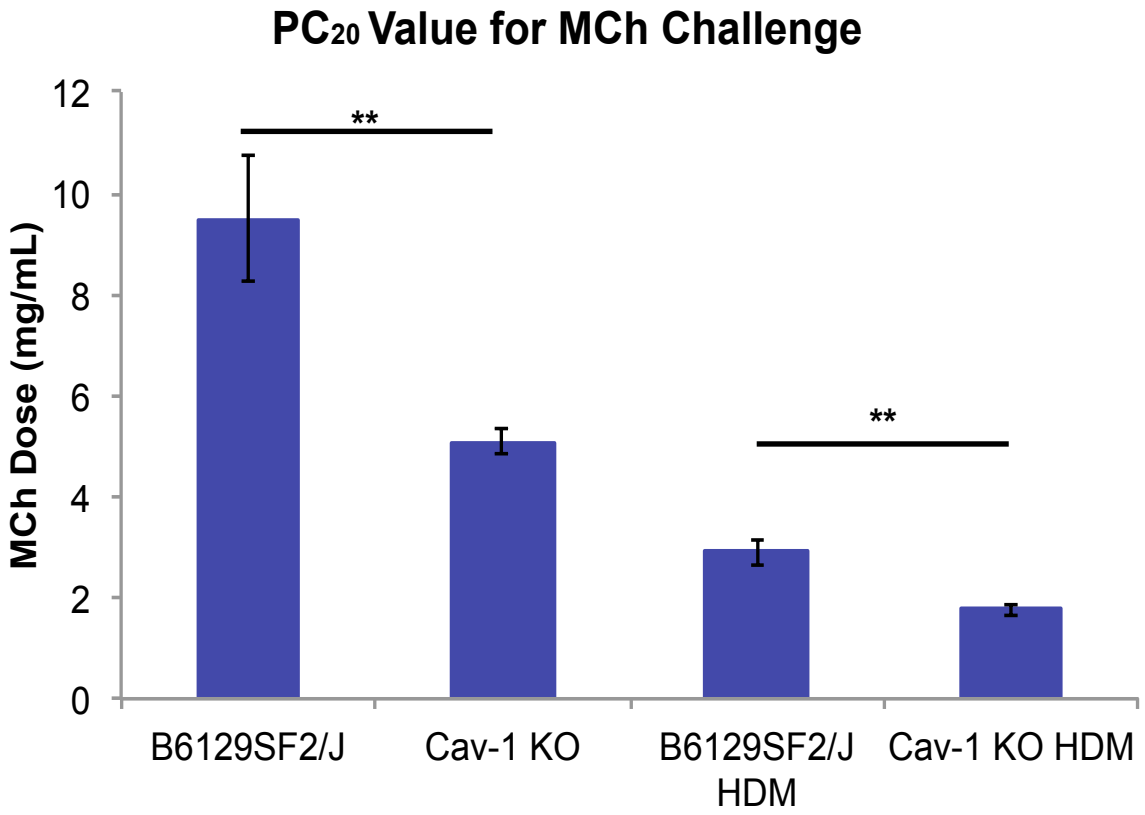

Figure 16 – The amount of MCh needed to produce a 20% augmentation of central airway resistance (Raw) is significantly decreased in both HDM sensitive and naïve Ca-1 null animals compared to their respective controls. HDM groups n=16 control group 12, ** = p< 0.01

As already noted, Cav-1 KO mice and their wild type genetic controls received a two-week regime consisting of 10 I.N. HDM challenges, and 72hrs after final allergen exposure respiratory mechanics were assessed using a small animal ventilator. We compared the impact of HDM challenge on respiratory mechanics in Cav-1 null and B6129SF2/J mice (Figures 15 and 16). Enhanced R_{aw} that was evident in naïve Cav-1 KO mice became more pronounced after allergen challenge resulting in Cav-1 null animals exhibiting nearly double maximal R_{aw} compared to genetic control mice (2.24 ± 0.22 vs. 1.19 ± 0.08 cmH2O.s/mL, p< 0.001)(Figure 15). Furthermore, MCh PC20 for R_{aw} in Cav-1 KO mice decreased >65% to 1.77 ±0.11 mg/mL after HDM challenge, whereas the PC20 value for genetic control mice after HDM challenge, though decreased by ~70% compared to allergen naïve B6129SF2/J,

was 2.90 ±0.25 mg/mL. This was nearly double that for Cav-1 KO mice ($p< 0.001$), demonstrating the significantly greater sensitivity to inhaled bronchoconstrictor that KO animals exhibited after allergen challenge (Figure 16).

Excess pulmonary dysfunction in Cav-1 KO mice was also clearly evident in indices of peripheral lung mechanics: HDM challenged Cav-1 null mice exhibited ~200% higher maximum tissue resistance (G) compared to genetic controls, 82.01±4.66 vs. 31.71±5.63 cmH2O/mL respectively ($p<0.001$). A similar difference existed for maximum lung elastance (H) between Cav-1 KO (175.66±15.84 cmH2O/mL) and B6129SF2/J mice (76.15 ±10.94 cmH2O/mL)($p<0.001$) (Figure 15). The increase in peripheral lung reactivity in Cav-1 KO mice was accompanied by increased sensitivity to MCh, as HDM-exposed genetic control mice showing significantly lower G and H at each dosage of methacholine (Figure 15).

Airway Inflammation

Cellular Infiltration of the Airways

Cav-1 KO and B6129SFJ/2 mice exposed to HDM exhibited lung inflammation consistent with that seen in earlier studies in Balb/c mice in which we established our allergen exposure protocol (protocol 5). Indeed there was a striking increase in total inflammatory cell count in BAL fluid after HDM exposure (Figure 17). Prior to HDM challenge, there was no difference in total BAL cell counts between Cav-1 KO and genetic control mice (1.21 ±0.47 x 10^5 vs 1.20 ±0.49 x 10^5 cells/mL, respectively), indicating no pre-existing baseline difference in this index of airway inflammation (Figure 17). However, after HDM exposure, Cav-1 null mice exhibited markedly higher total inflammatory cells in BAL

(1.10±0.19 x 10^6 cells/mL) compared to HDM-challenged genetic controls (6.37 ±0.59 x 10^5 cells/mL) ($p < 0.05$) (Figure 17).

We assessed the inflammatory cell profile in BAL and found no differences between strains, neither before nor after HDM exposure (Figure 18). Indeed, alveolar macrophages predominated (~70%) in naïve mice, whereas eosinophils and neutrophils collectively accounted for ~80% of all cells in BAL fluid after HDM. Of note, although the fractional abundance of each cell type did not differ between KO and wild type strains after HDM exposure, as noted above there was nearly 73% more cells in total in Cav-1 KO BAL fluid, thus total eosinophil and neutrophil numbers were similarly higher in HDM-exposed Cav-1 null mice compared to B6129SFJ/2 mice.

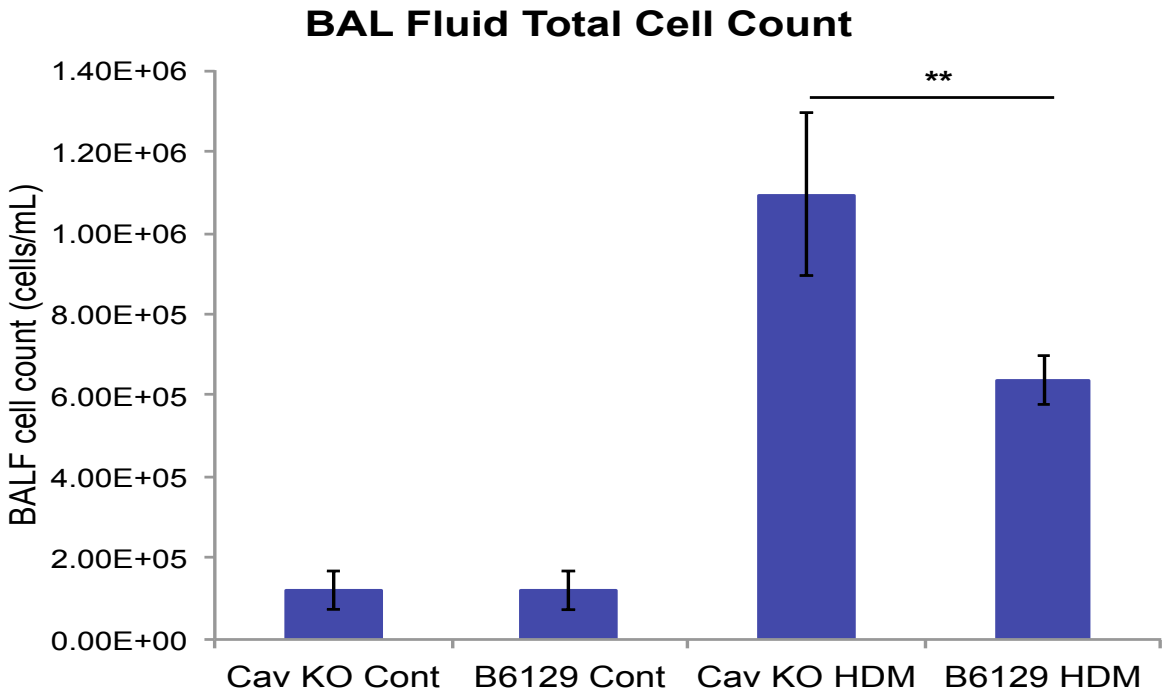

Figure 17 – With exposure to HDM Cav-1 null group developed a greater amount of airway inflammation; as seen by an increase in inflammatory cells found in BAL fluid, when compared to there genetic controls. HDM groups n=16 control group 12, * = p< 0.05

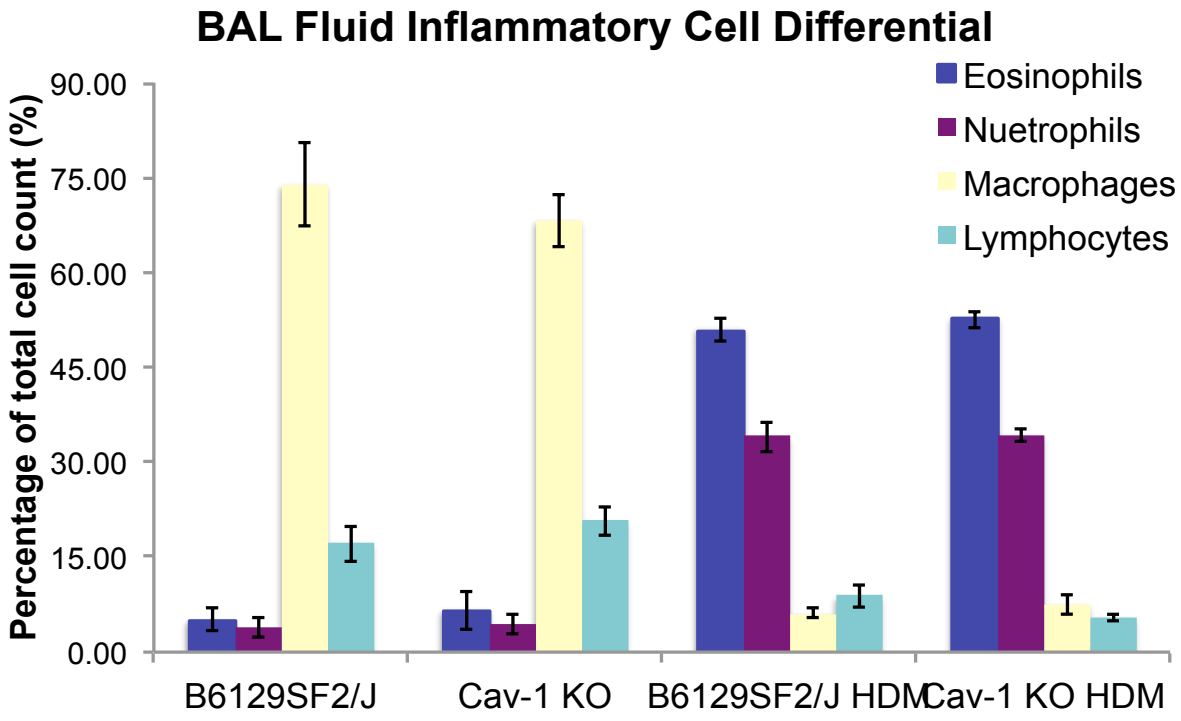

Figure 18 – Shows no differences In the immune cell profile between the Cav-1 KO mice and their genetic controls both with and without allergen challenge. HDM group = 16, Control groups = 10

Cytokine Profile of the Airways

As cellular infiltration of the airways of Cav-1 KO mice was seen to be increased, we next used ELISA of BAL supernatants to assess the inflammatory mediator profile in the airways pre- and post-allergen exposure. For this purpose we selected mediators associated with Th-1 type inflammation (INFγ) and Th-2 type inflammation (IL-4, -5, and -6) as well as TGF-β1 and TNF-α, which are associated with allergic airway inflammation and airway remodeling in human asthma.

Caveolin-1 null animals that had not been exposed to allergen displayed significantly higher baseline levels of TNF-α, TGF-β1 and IL-5 compared to allergen-naïve genetic controls (Table 2 and Figure 19). This suggests that in Cav-1 KO mice there is local

inflammation in the absence of inflammatory cell infiltration prior to HDM exposure (the latter was shown in Figures 17 and 18).

Airway Cytokine levels

	TNF-α	TGF-β,	INF-γ	IL-4	IL-5	IL-6
B6129 Cont	11. 17 * (1.5 - 20.7)	15.19 * (2.8 - 27.5)	29.01 (21.9- 36.2)	12.99 (6.0 -19.9)	5.27 * (0 -13.0)	4.63 (0.0 – 11.27)
Cav-1 KO Cont	50.30 (25.7- 75.0)	57.01 (22.9-91.1)	35.91 (26.7 - 45.1)	15.32 (5.1 - 25.5)	74.19 (35.6 -112.7)	16.19 (3.2 - 29.1)
B6129 HDM	34.16 (10.9-57.4)	92.93 (34.7 - 151.1)	64.63 (30.7 - 98.5)	98.16 (64.5- 131.6)	267.70 ** (242.2-293.2)	52.33 (45.9 -58.6)
Cav-1 KO HDM	57.55 (26.7 -88.3)	94.21 (32.2 -156.2)	54.62 (27.6 -81.54)	40.81 (0.0 - 82.2)	121.72 (85.1-158.3)	21.38 (15.4 -27.3)

Table 3 *– Inflammatory cytokine levels in BALF as measured by ELISA. All measurement in pg/mL, in brackets are the 95% CI. * = p<0.05 and **= p<0.01 when compared to Cav-1 KO of same allergen exposure*

We also compared the impact of repeated HDM challenge on the inflammatory mediator milieu in the lungs of Cav-1 KO and B6129SF2/J mice (Table 2 and Figure 19). The genetic control strain exhibited significant increase in all mediators measured, whereas in Cav-1 null mice we observed no statistically significant increase in any mediator upon HDM exposure. This resulted in there being equivalent mediator abundance in HDM-exposed animals regardless of strain, except for IL-5 which was significantly higher in wild type mice (267.70 ± 12.75 pg/mL) compared to Cav-1 KO mice (121.72 ± 18.32 pg/mL)($p < 0.01$). A trend of this nature also existed for IL-6 as the Cav-1 KO mice exposed to HDM generated 21.37 ± 2.95 pg/mL in BAL fluid supernatant compared to HDM-exposed genetic control animals (52.33 ± 3.17 pg/mL) ($p < 0.01$). For all other cytokines, we observed no significant difference between knock out and genetic control animals after HDM challenge.

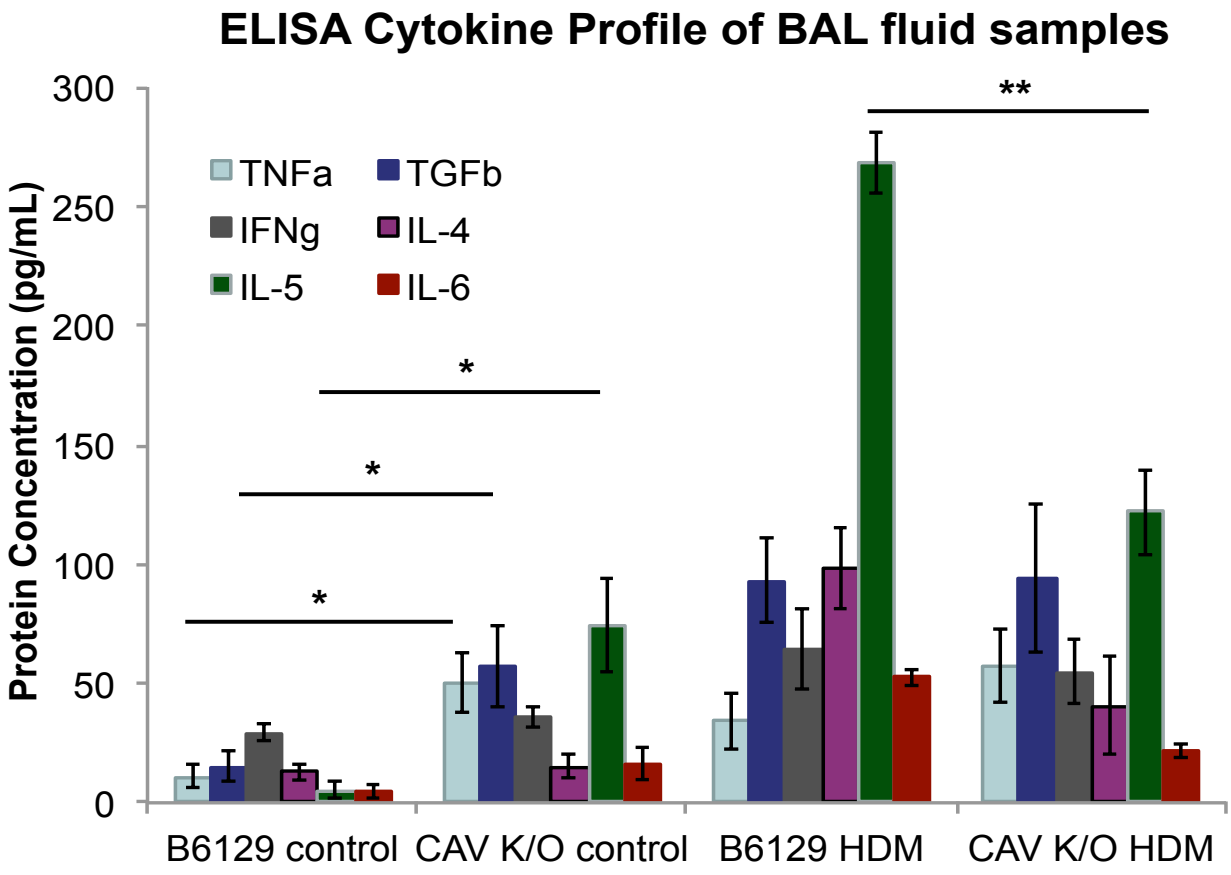

Figure 19 – Using ELISA to elucidate the inflammatory environment, it is shown that naïve Cav-1 KO mice have increased cytokine release. However the augmented response to produce sensitization and challenged by HDM was not as robust as that seen in their genetic controls. NDM groups n= 9 , control groups n = 7; **= p<0.01, * = p<0.05.

Mucus Production and Secretion

Airway goblet cell hyperplasia and mucus secretion

As a hallmark response to allergen challenge is increased number of airway goblet cells, expression of mucins and mucus secretion, we next compared the impact of HDM challenge on these parameters in wild type and Cav-1 KO mice. We used Alcian Blue staining to assess mucus production and goblet cell number in the airways of naïve and allergen-challenged mice. In naïve mice we observed very little staining in the luminal space or the epithelial lining, however, in striking contrast both mouse strains showed augmented Alcian blue staining after allergen exposure (Figure 20).

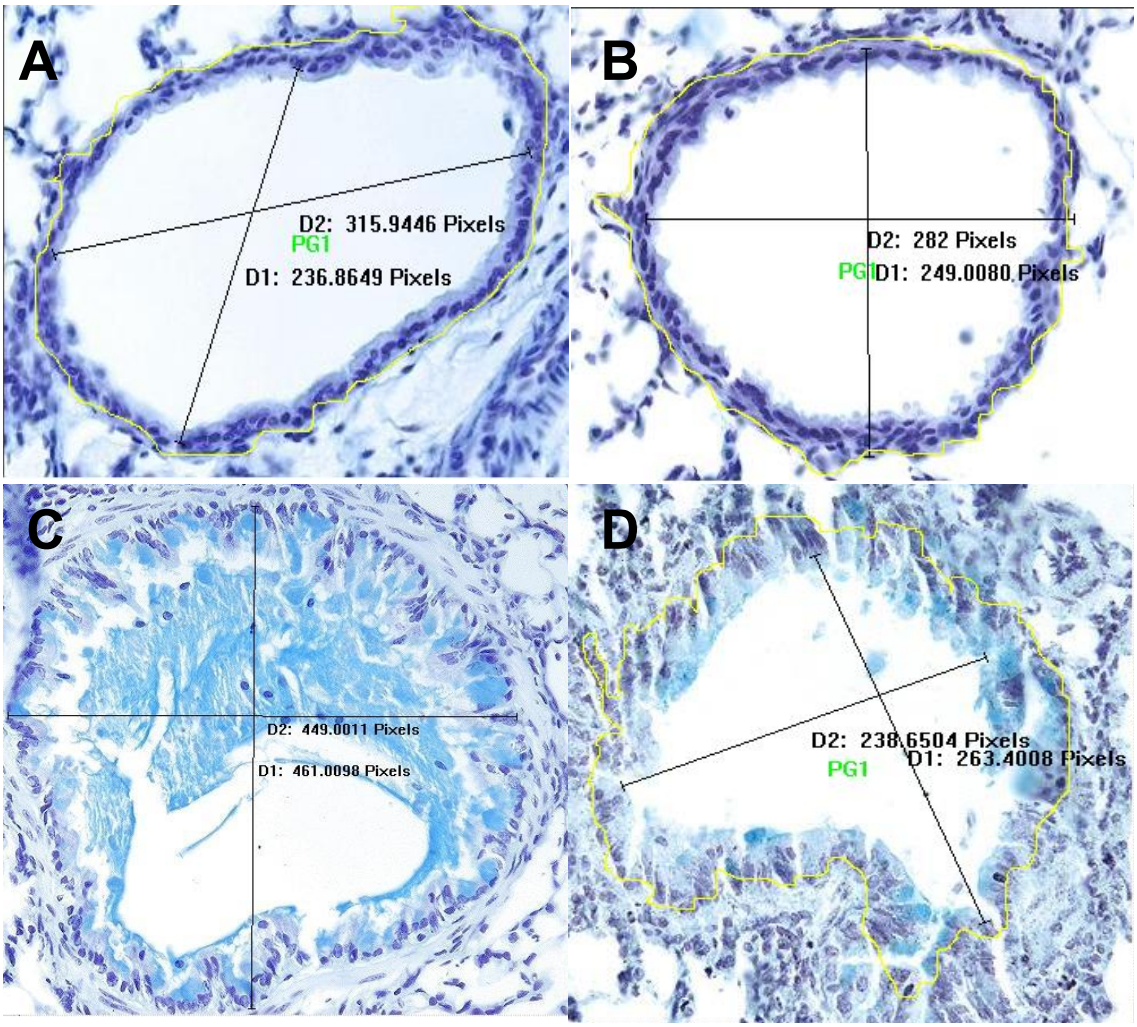

Figure 20 – Representive pictures of naive B6129 (A) Cav-1 (B) & HDM exposed B6129 (C) Cav-1 (D) animal airways stained for mucus presence with alcian blue stain.

After challenge with HDM, B6129SF2/J mice displayed significant staining for total mucus, which we determined by the sum of that within the epithelial lining cell and the airway lumen (indicating exaggerated secretion) which was normalized to the length of basement membrane for comparison 0.31 ±0.049 pixels/µm basement membrane, (n=9) (Figure 21). Of this total, we observed significant staining in the airway epithelium, indicating increased goblet cell number (0.079 ± 0.009 um^2/ um of basement membrane) (Figure 22). Interestingly, with HDM-challenge Cav-1 KO mice showed markedly less induction of total mucus (sum of epithelial cell and lumen mucous) (0.085 ±0.013 pixels/µm basement membrane, n=6). This trend was also evident in epithelium cell-associated mucous (0.063 ±0.010 pixels/µm basement membrane, n = 9) compared to HDM-exposed genetic control

mice ($p > 0.01$, $p=0.29$ respectively) (Figures 21 & 22). Interestingly, the distribution of Alcian blue staining for mucus between the mouse strains differed markedly, with the majority of the mucus in Cav-1 KO mice found within the goblet cells of the airway epithelial lining (74.01 ±4.7%), whereas the majority of staining in B6129SF2/J mice was found within the airway lumen (72.04 ±4.43%).

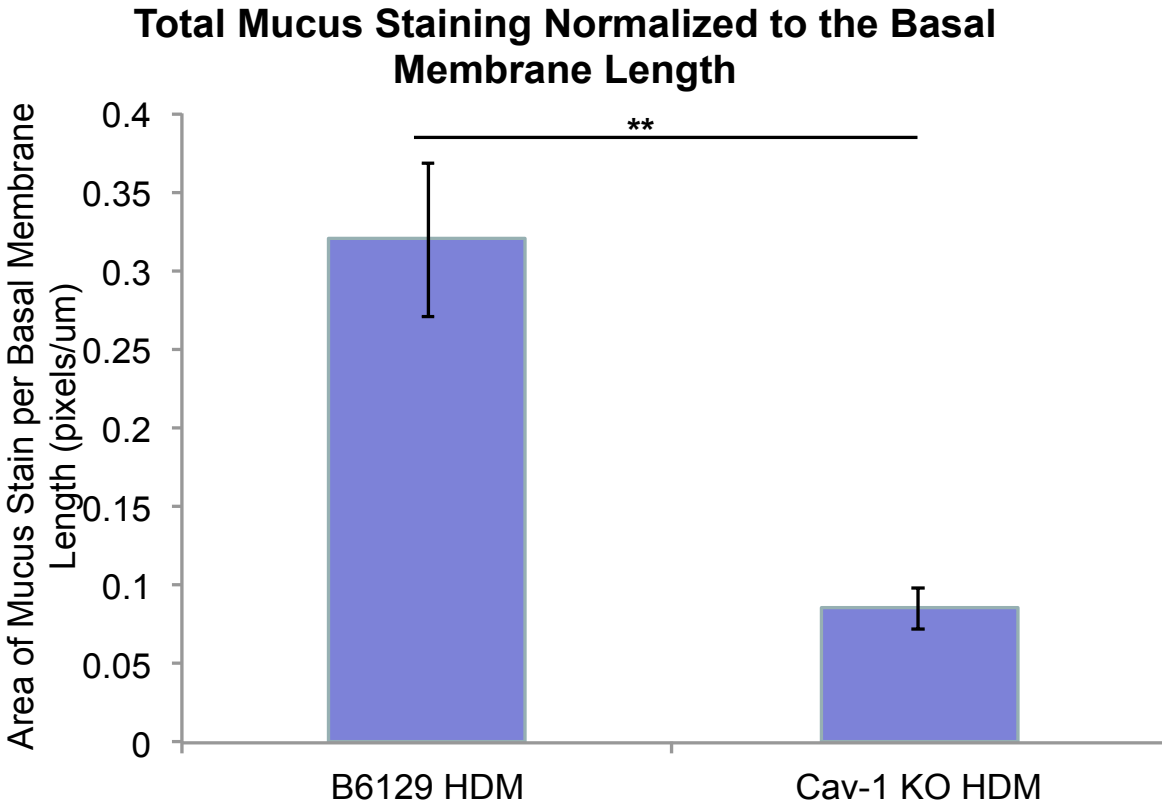

Figure 21– Total alcian blue stained pixel area normalized to the length of airway basement membrane allowing comparison between all airways. Results show that B6129 mice exposed to HDM secrete significantly more mucus then Cav-1 null animals. B6129 n=7, Cav-1 KO n=9; **= p <0.01. (Naïve mice not shown as no alcian stain was seen in airways)

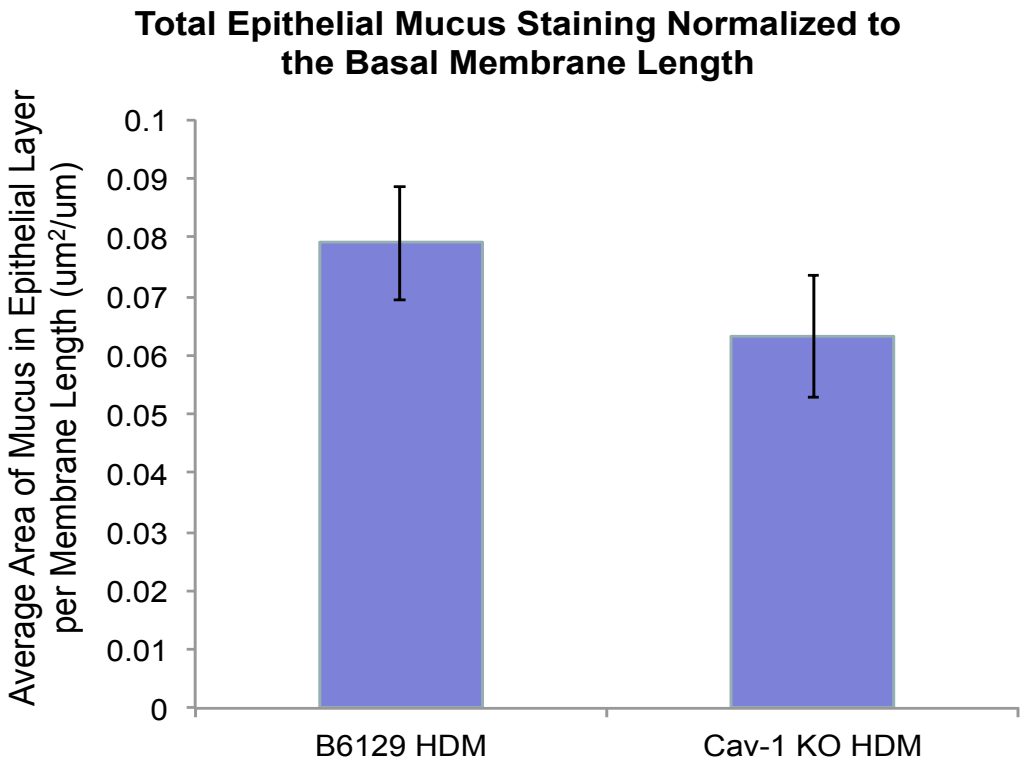

Figure 22– Total alcian blue stained pixel area within the epithelial lining normalized to the length of airway basement membrane allowing comparison between all airways. Results show that B6129 mice exposed to HDM produce more mucus then Cav-1 null animals. B6129 n=7, Cav-1 KO n=9;

Mucus mRNA expression

To further investigate mucus production within the lungs we also used quantitative rt-PCR to evaluate the abundance of mRNA for MUC5a/c. In allergen-naïve animals, Cav-1 KO mice, MUC5a/c expression was less than that seen for allergen-naive B6129SF2/J animals (1.09 ± 0.09 vs 0.35 mRNA levels). After exposure to allergen, there was a marked increase in MUC5a/c mRNA in both strains, but the relative abundance in Cav-1 KO mice was nearly 3.5 times greater than in genetic control animals (1.09 ± 0.093 vs 6.39 ± 0.91 mRNA levels)($p<0.05$) (Figure 23).

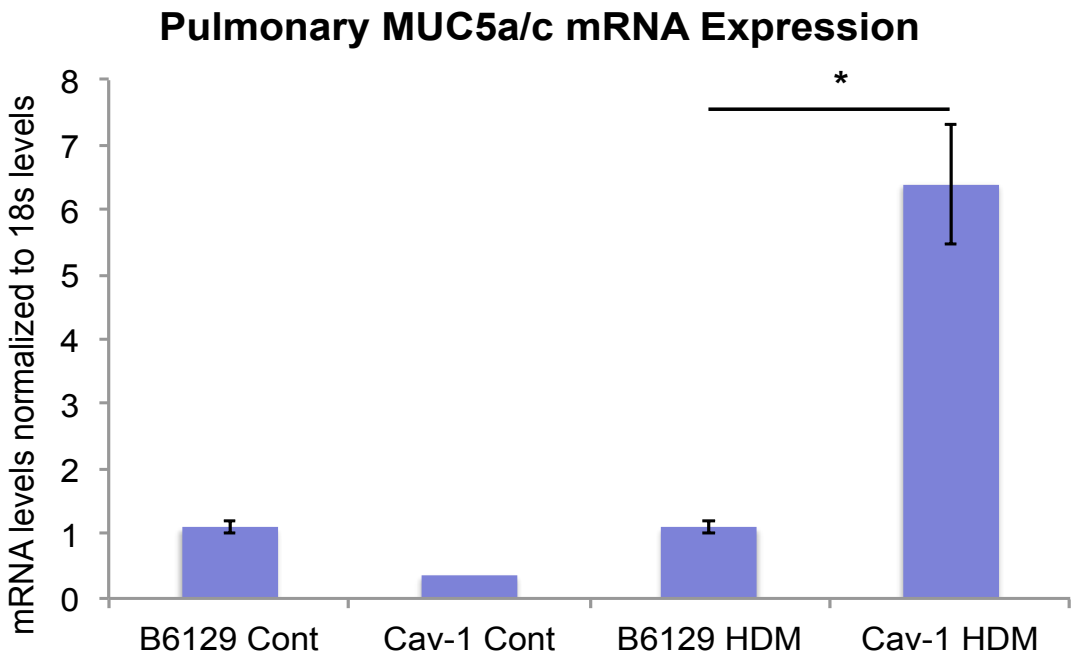

Figure 23 - mRNA expression is shown to be significantly up regulated in Cav-1 KO mice sensitized and challenged with HDM compared to their genetic control counterparts (Cav-1 cont. n=2, rest of groups n=3; * $p < 0.05$)

Discussion

HDM model of acute allergic asthma

Although murine models using OVA as a sensitizing and challenging agent have been widely used and accepted as a platform to study atopic asthma, the development of a protocol using the more relevant allergen, HDM, was imperative to the accuracy and quality of this project. House dust mites are a perennial allergen, and sensitized asthmatics can suffer daily from exposure because the allergen facilitates the development of acute exacerbation of symptoms [117], while OVA is an egg white protein that the majority of asthmatics will never encounter via an inhaled route. HDM as an allergen in animal models more closely mimics the immune, inflammatory and sensitivity reactions seen in humans. Furthermore, as HDM does not require a subcutaneous sensitizing injection or the use of an adjuvant protein, as does OVA, it more directly reveals mucosal immunity and local lung inflammatory and wound repair processes, independent of atopic status.

Although relevance of an allergen may be enough to consider HDM a preferred option over OVA for a murine model of allergic asthma, our studies also show that HDM causes a greater development of lung inflammation and functional symptoms. First and foremost an increase in reactivity and sensitivity to inhaled MCh was seen in both peripheral and central airways. Components of HDM, DerP1, 3, 5 and 9 are cysteine and serine proteases and as such have been shown to break down the airway epithelial [118]; this could allow allergen to penetrate the basement membrane with great ease and possibly reach the smooth muscle layer, causing direct stimulation of the ASM leading to its activation. Additionally Jordana et al. [65] showed that greater mucus secretion occurred in the airway of mice challenged with HDM compared to those with OVA, this increase in viscoid substance within the airway most

certainly would cause increase the resistance to air flow and occlude airways, thereby increasing elastance.

Another possibility for increased mechanical dysfunction in response to HDM challenge is a greater degree of induced airway inflammation. This leads to increased airway wall thickness and exaggerated effects of constriction ASM in a chronic exposure model [119]. Additionally, the type of inflammation is key: it can be seen in our data that HDM-sensitized mice had a greater degree of eosinophilia measured in BAL fluid samples compared to that of the OVA sensitized animals. A major eosinophil secretory molecule is major basic protein, which has been linked to the development of augmented AHR response [120]. As mentioned above, a greater degree of eosinophila was seen with HDM exposure compared to OVA sensitization and challenge. This may indicate that HDM extract is more efficient at eliciting a Type 2 immune response, a well-known characteristic of asthma inflammation. It has been hypothesized that HDM up regulates an esinophilic/Th-2 immune response due its ability to induce granulocyte macrophage colony stimulating factor (GM-CSF) mediated pathways [119]. The cytokine GM-CSF functions to induce bone marrow proliferation specifically the production of granulocytes and macrophages. Evidence to further support this idea comes from findings that HDM exposure significantly up regulates interleukin-4, -5 and -13 [65], all of which are Th-2 type cytokines. Johnson et al. [65] used flow cytometry to show that CD4+ T-cells with T1/ST2 (an activation marker for Type 2 T-helper cells) were readily up regulated in animals exposed to HDM.

Another hallmark characteristic of the acute exacerbations of atopic asthma is that they are a Type I hypersensitivity reactions in which IgE mast cell surface antibodies become crossed linked by the allergen leading to their widespread degranulation. House dust mite

extract has been shown to be a potent stimulator of IgE production [114] which would support or augment the production of the local immune reactions of asthma.

Role of Caveolin-1 in acute episodes of atopic inflammation and airway Hyperresponsiveness

In this study we investigated the role of caveolin-1 in the pathogenesis of mechanical dysfunction of the lung and lung inflammation, features of acute atopic asthmatic events. Our work shows that Cav-1 has a protective effect against the magnitude of airway inflammation and bronchospasm, as both were increased in Cav-1 KO mice.

In allergen-naïve Cav-1 null mice airway resistance and peripheral lung tissue damping (resistance) & elastance were increased compared to genetic controls. This may indicate that Cav-1 has a role in fundamental pulmonary tissue development and structure that underpins lung function. It has been shown that Cav-1 plays a role in extracellular matrix deposition and that without Cav-1 matrix deposition is increased [91, 121]. Such an upsurge in extracellular matrix may increase the stiffness of lung tissue, leading to increasing total pulmonary resistance. The mechanism behind upregulation of extracellular matrix production in the absence of Cav-1 is believed to be linked with the ability of Cav-1 to inhibit TGF-β1 signaling[55]. Thus, absence of Cav-1 increases transduction of canonical TGF pathways involving regulatory Smads that promote increased tropoelastin and col1agen-α2 and -α1 gene expression in lung tissues [121].

There are other theories involving Cav-1 and its contribution to increased airway responsiveness in genetically altered mice. The lack of Cav-1 directly alters ASM contractility via the protein's ability to contribute to intracellular Ca++ control. Within caveolae of ASM there is a high concentration of L-type Ca++ channels, plasma membrane Ca++ pumps and Ca++ binding proteins such as calsequestrin and calreticulin [122]. Loss of

Ca++ control could increase intracellular concentrations leading to exaggerated contraction, or dysfunctional re-sequestration of intracellular Ca++ could compromise ASM relaxation. Work in Dr. Halayko's lab has shown that Cav-1 plays a crucial role in ASM phenotype conversion from a synthetic to a contractile state [123] thus increasing abundance of myocytes designed to contract. Furthermore, muscarinic receptors that mediate ASM constriction are Cav-1 dependent, as M2 and M3 muscarinic receptor-mediated signaling proteins that support contraction are associated with and regulated by Cav-1 [124]. Any combination of the above mechanisms may offer an explanation for Cav-1 effects on airway mechanics, as the protein's full effect has yet to be elucidated. Therefore from the work presented it is evident that its protective role is significant and understanding this will be crucial to develop full understanding of the asthmatic syndrome and effectiveness of current or future treatments.

We also showed that the absence of caveolin-1 was linked with augmented development of airway inflammation after HDM challenge. This may be key as developing and sustaining AHR is associated with the inflammatory reaction, in which the cellular infiltration exaggerates ASM contraction and/or the inflammatory mediators induce the AHR itself [42]. Caveolin-1 has been demonstrated as a modulator of both acute and chronic inflammation in a wide variety of conditions, including inflammatory bowel disease, atherosclerosis, and severe sepsis reactions [80, 125, 126]. The role of Cav-1 as a down regulator of inflammation was suggested by demonstrating that during inflammation Cav-1 expression is down-regulated in whole lung lysate from mice exposed to OVA [127]. Cav-1 KO mice have been shown to have higher degrees of platelet adhesion, inflammatory cell migration and angiogenesis in murine colitis models [128]. Additionally in sepsis, Cav-1 null

mice exhibit decreased survival and increased levels of serum TNF-α and IL-6 [125], potent inflammatory cytokines. Additionally Cav-1 confers anti-inflammatory properties to cultured airway macrophages in LPS-induced inflammatory reactions [101]. It is clear that caveolin-1 is an important protein that is critical to many prongs of the immune/inflammatory response. Its role in acute airway inflammatory response was confirmed by our primary data, as the Cav-1 KO mice demonstrated exaggerated inflammation at baseline and post allergen exposure, however these data reveal that considerable mechanistic work remains to fully understand how Cav-1 exerts these effects.

The role of caveolin-1as an inflammatory modulator has been implicated to be mediated by its effects on cell signaling pathways, however these pathways are poorly understood. Numerous pathways have been implicated to be attenuated by Cav-1 during inflammation, including eNOS/NO/NF-kappa B [102], the p42/44 MAPK cascade [95], cyclooxygenase-2, vascular endothelial growth factor and Ca++ mediated pathways [129]. It has also been demonstrated that Cav-1 is down regulated by IL-4 [105], suggesting that inflammation can modulate Cav-1 expression to dampen its suppressor role and promote an exaggerated inflammatory response.

Further investigation of the inflammatory cytokine medium in Cav-1 KO mice exposed to HDM yielded some interesting results. In BALF taken from allergen naïve animals we observed the expected; from previous work [105, 125], increased baseline cytokines levels in Cav-1 KO mice were similar to the naïve genetic controls. However as inflammation was induced with HDM-challenge, the expected augmentation in inflammatory cytokines was not seen in the BAL fluid from Cav-1 null animals; rather the magnitude of the increase from baseline was greater in genetic control mice exposed to HDM. The unforeseen effect may

reflect our finding that at baseline cytokine levels in Cav-1 KO mice we already elevated, without the need of allergen provocation. Interestingly, with HDM challenge, though there were a larger number of eosinophils in the airway of Cav-1 KO mice, the increase in IL-4 & 5 levels that usually occur concomitantly with eosinophilic inflammation were not observed. One possible explanation for this observation would be a malfunction of the endo- or exocytosis cellular processes. If endocytosis were affected inflammatory inducers such as IL-1 or -2 would be unable to reach their receptors and induce their cascade effects to produce further cytokines. It has been shown that lipid rafts are crucial in the endocytotic progression, particularly caveolae. Endocytosis in endothelial cells was shown to be mediated by activating gp60 binding protein which leads to Src kinase activation of the CSD whose downstream effects causes dynamin-2-mediated fission and directed migration of caveloae vesicles [130]. Exocytosis malfunction could also lead to our observed decrease in cytokine levels in Cav-1 KO mice but in this case the cytokines are being produced but not being released into the environment. Ikonen et al. [131] showed that the presences of Cav-1 homooligomer and Cav-1 & 2 heterooligomers were crucial in directing vesicles to either the apical or basolateral aspects of epithial cells and further more anti-Cav-1 antibodies inhibited the apical delivery of these vesicles. Also Cav-1 has been shown to act a chaperone molecule across the cellular membrane during the exocytotic process [131-133]. Interestingly, preliminary real time PCR studies showed that there was indeed an increase in mRNA production of IL-5 and TGFβ1 (data not shown), a finding consistent with the idea that exocytosis dysfunction may exist in Cav-KO mice. Furthering this hypothesis, TGF-β has been shown to be increased by allergen exposure in Cav-1 KO mice in the past [105] and IL-5 should be increased due to the eosinophilia.

Recently a new framework of asthmatic inflammation has been gaining notoriety, which is known as the Th-17 immune pathway; an immune response distinct from Th-1 or -2 mediated processes. This branch of the immune system has been shown to be mediated by IL-21 [134] and IL-22 [135] that stimulate T-cells to begin producing IL-17. Interestingly it has been shown to involve TGF-β [136], which was one of the only cytokines that showed any increase with HDM exposure in the Cav-1 null animals. Therefore this Th17 paradigm produces the interesting idea that the lack of Cav-1 pushes the immune system towards a Th17 pathway. Thus future experiments investigating Th17-associated cytokines in Cav-1 KO mice may needed to understand the proteins role in airway inflammation.

A further hypothesis for our cytokine discrepancy is the possibility that an overwhelming cascade of cellular infiltration leads to attempts by the system to 'balance the ship'. Caveloin-1 has been shown to modulate cell adhesion, and lack of Cav-1 leads to weaker epithelial cellular junctions that allow more allergen to gain easier access to underlying tissues and augment the inflammatory response [137]. Moreover it has been shown that Cav-1 modulates inflammatory cell activation, adhesion and migration [102] along with decreasing vascular permeability [102], thus leading to greater airway cellular infiltration. Is it possible by hindering the release of pro-inflammatory mediators, the body is attempting to offset the overactive immune response? In the end the lack of Cav-1 seems to have great impact on the development of airway inflammation after allergen exposure, and may in fact be the underlying cause of airway mechanics dysfunction. In total, my findings and those of others demonstrate Cav-1 has significant importance in pathogenic pathways associated with inflammation, and further work is required to fully understand the mechanistic features of this affect.

Beyond the impact of loss of Cav-1 on airway function and inflammation, we also noted the over production of mucus in Cav-1 KO mice after HDM challenge. Increasing amounts of viscous mucus within the airways leads to increased resistance to airflow. However, in Cav-1 null animals although their airways showed increased resistance, there was less mucus within airway lumen than seen for genetic control counterparts exposed to allergen. This finding is not inconsistent with the published literature, as it has been demonstrated that Cav-1 and caveolae are important in mucus production. Pachter et al. [138] demonstrated that within caveolae, the monocyte chemotactic protein-1 receptor, CCR2B, is abundant. Stimulation of CCR2B induces both MUC5A and -B expression and increases secretion of mucus by the bronchial epithelium [139]. Moreover, work done in signet ring cancer (a mucus secreting tumor of the bladder) showed that increased mucus secretion is associated with increased expression of caveolin-1 by tumor cells [140]. Interestingly, our data reveal that although there was less mucus within the airways of Cav-1 KO mice after HDM exposure, MUC5A/C mRNA abundance was in fact increased in whole lungs from Cav-1 KO mice. An explanation for this finding may lie in the inflammatory reaction, as it is known that an increased Th-2 inflammatory response leads to increased mucus production [45, 141-143]. Importantly, within the Cav-1 KO animals an exaggerated Th-2 response was seen via the inflammatory cellular profile. Paradoxically, ELISA of BAL fluid for Th2 mediators, IL-4 and -5 revealed them to be lower in HDM-exposed Cav-1 KO mice compared to genetic control mice. These cytokines are believed to increase mucus production during a Th2 response [46, 143], thus another mechanism for amplifying mucus expression in Cav-1 KO mice must exist. One of these possibilities lies with IL-13, which was not one of the cytokines examined in this work but has been shown to induce mucus production [46,

142]. Although the majority of literature indicates that inflammation contributes to mucus production, some of the literuature point to human eosinophils and it's release of major basic protein having the ability to inhibit mucus secretion [144]. Using our model we found elevated eosinophils in Cav-1 KO BAL fluid after HDM challenge. One other possibility to explain paradoxical data regarding mucous secretion and expression in Cav-1 KO mice is the "dysfunction in exocytic abilities hypothesis", due to the lack caveolin-1. This may be a reasonable hypothesis as Cav-1 integral role in exocytosis was discussed above. From the work presented above and the literature discussed this area is extremely complicated and warrants more work to fully understand the effects of caveolin-1 on airway mucus production and secretion.

Summary and Future Direction

This work has shown that an acute exposure protocol using house dust mite extract is a potent inducer of airway hyperresponsiveness and inflammation in mice. More specifically the inflammation seen was characterized by eosinophilia, which is characteristic of a Th2 immune response, a hallmark of atopic asthmatic inflammation. Not only was maximal airway resistance and elastance increased, there was also an increase in sensitivity to muscarinic agonist, a feature also characteristic of the airways of human asthmatics. This data plus the relevant characteristics of the protein make this model a useful tool in the further investigation of atopic asthma.

It is clear that caveolin-1, the protein responsible for forming and stabilizing membrane caveolae in cell types throughout the lung, plays an important protective role in the pathogenesis of an acute atopic exacerbation of asthmatic airways. We showed that the lack of caveolin-1 underpinned increased airway hyperresponsiveness and inflammation after HDM challenge. However, the exact mechanisms for these effects remain an area needing further research. The current work showed that Cav-1 provides protective effects against airway hyperresponsiveness and inflammation, but whether these are independent events or interrelated remains to be seen. Possible points of overlap in the development of both these pathologies may relate to the role of Cav-1 as a signal modulator, particularly in its control of intracellular Ca++ concentration in ASM and dysfunction in exocytosis and/or endocytosis.

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