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Disruption of Mitochondrial Trafficking in Lung Epithelial Cells Potentiates Allergic Asthma Phenotypes

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DISRUPTION OF MITOCHONDRIAL TRAFFICKING IN LUNG EPITHELIAL
CELLS PONTENTIATES ALLERGIC ASTHMA PHENOTYPES

A Thesis Presented

by

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ABSTRACT

Allergic asthma is a serious socioeconomic and health issue that is characterized by chronic inflammation of the lungs and affects as many 300 million people worldwide of all ages. Inhaled environmental factors, such as allergens, can disrupt the airway epithelium, which is the physical barrier and the first line of defense against external irritants. Consequently, exposure to allergens can cause episodes of airway inflammation, increased mucus production and tissue remodeling, that leads to the obstruction and narrowing of the airways, which may present as difficulty breathing, wheezing, and persistent coughing. Unfortunately, there is no cure for allergic asthma, but allergen avoidance and prescription of medication can help manage symptoms. However, current treatment options fail in almost 10% of patients, making it necessary to study uncharacterized molecular causes to better understand this condition.

Evidence suggests that mitochondria play a vital role in lung health and disease. Mitochondria are dynamic organelles that can change in size, shape, and distribution depending on cellular demands and are transported through the cell on microtubules. Miro1, a calcium-binding membrane-anchored GTPase that is strongly associated with mitochondrial transport, is the primary adaptor protein required for the subcellular positioning of this organelle. While mitochondria play an important role in lung physiology, the role of localized mitochondrial function in response to allergen-induced inflammation remained unknown.

The main goal of this study was to investigate the effects Miro1-mediated mitochondrial trafficking in allergen-induced inflammation and provide a comprehensive phenotypic readout. To achieve this, our laboratory developed a novel tissue-inducible mouse model that allowed for the deletion of Miro1 from club cells, an abundant epithelial cell subtype in mice, and utilized this system with an established model of chronic allergen-induced inflammation. Our results show that epithelial deletion of Miro1 leads to a heightened inflammatory response, enhanced mucus metaplasia, pronounced tissue remodeling and smooth muscle levels that leads to increased airway hyperresponsiveness following chronic exposure to a complex allergen. Results from this study indicate a possible role for Miro1 in the development and progression of allergic asthma, providing insights into the role of Miro1-mediated mitochondrial positioning in allergic asthma severity. Future studies should be conducted to gain a better understanding of the mechanisms of Miro1 and mitochondrial positioning in asthma.

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CHAPTER 1: COMPREHENSIVE LITERATURE REVIEW

1.1. Allergic Asthma

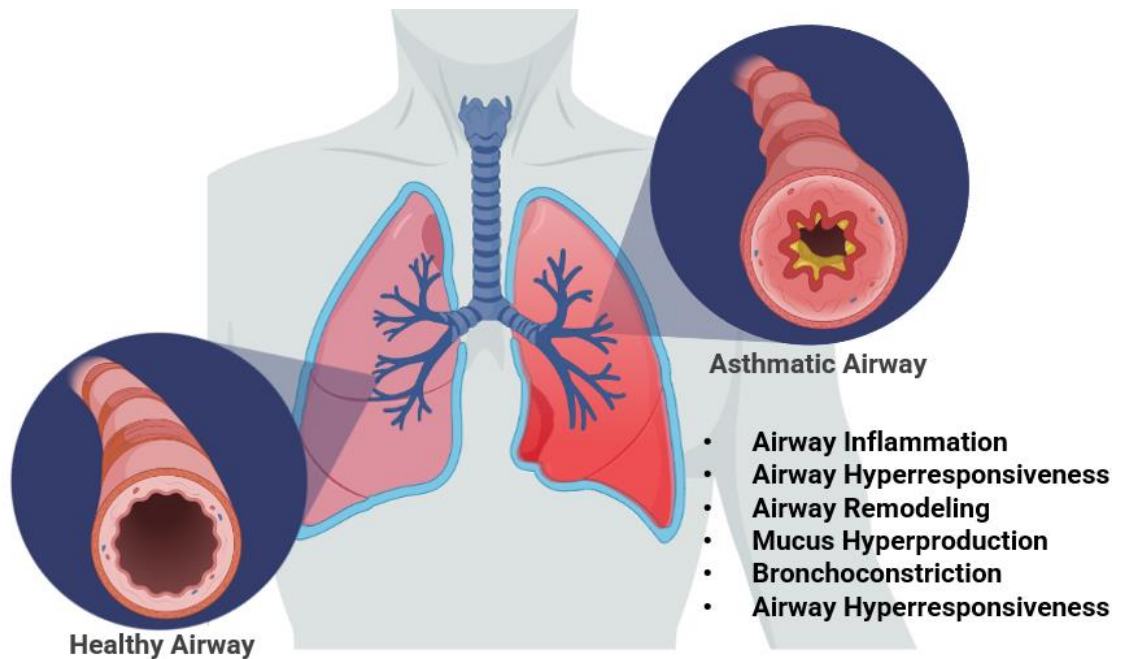
Asthma is a chronic inflammatory condition of the airways that affects as many as 300 million people of all ages worldwide, including 25 million Americans (about 8.5% of the United States' population), and accounts for approximately 400,000 deaths annually [1-3]. The incidence, prevalence, morbidity, and mortality of asthma varies among populations and ancestral origins possibly due to the influence of gene-by-environment interactions, concomitant atopy, and social determinants of health [4, 5]. Asthma imposes substantial burdens on healthcare systems and causes annual economic costs of over \$80 billion dollars in the United States [6]. Generally, asthma presents with intermittent and reversible episodes, or "attacks," of wheezing, cough, shortness of breath, dyspnea, and chest tightness often accompanied by expiratory airflow limitation [7]. Asthma exacerbations are often triggered by factors such as allergen exposure and viral respiratory infections [8]. Airflow limitation can be attributed to a combination of airway smooth muscle contraction, inflammation, edema, and fibrosis, and the presence of excess mucus in the airway lumen [9, 10]. Moreover, airway narrowing in response to bronchoconstriction and chronic inflammation are associated with asthma and often persist when a patient is asymptomatic, but may normalize with treatment [11, 12]. Unfortunately, there is no cure for asthma; avoidance of triggers and the use of bronchodilators and anti-inflammatory drugs such as inhaled rapid-acting β -2 adrenergic agonists and oral corticosteroids are commonly recommended for the management of symptoms. However, these current treatment options fail in 5% to 10% of patients or have serious side effects [13, 14].

Asthma is a complex, heterogenous condition as suggested by unbiased clustering-based approaches, which have identified subgroups of individuals with asthma that share certain characteristics such as age of onset, atopic status, body mass, and frequency of exacerbations [15-17]. The majority of asthmatics have co-existing atopic conditions such as allergic rhinitis, atopic dermatitis, or chronic rhinosinusitis [18, 19]. In addition to elevated concentrations of immunoglobulin E (IgE) associated with atopy, individuals with asthma can also present with elevated levels of eosinophils in serum, sputum, and airway tissue [20, 21]. Some asthma phenotypes include allergic (eosinophilic) asthma, obesity-related asthma, exercise-induced asthma, adult-onset asthma, and steroid-responsive asthma [22, 23]. Moreover, these phenotypes can be further subdivided based on specific biological mechanisms. Dysregulation of eicosanoid mediators and T helper type 2 (Th2) immune responses are examples of pathways important in specific endotypes [24, 25]. The best recognized asthma endotype is Th2-high asthma. In individuals with Th2-high asthma, type 2 cytokines such as interleukin-4 (IL-4), IL-5, and IL-13 are secreted by immune cells including innate lymphoid cells and CD4+ T cells [26]. These pro-inflammatory cytokines mediate the attraction of granulocytes like eosinophils, basophils, and mast cells, the secretion of IgE from B cells, and the activation of smooth muscle cells, fibroblasts, and epithelial cells to produce the characteristic features of allergic asthma [27].

Allergic asthma is the most common asthma phenotype, representing upwards of 80% of all persistent asthma cases [28]. This asthma phenotype is often induced by early life encounters with inhaled allergens such as house dust mite, animal dander, mold, and pollen [29]. Allergen sensitization is thought to have a significant role in the development

of allergic asthma [30]. Accordingly, the crosstalk between the innate and adaptive immune systems is crucial for the initiation and propagation of the allergic immune response [31, 32]. Although allergen-specific IgE responses play a significant role in allergic asthma, Th2 cytokines are considered the main drivers of the inflammatory response. Exacerbations in allergic asthma are typically associated with allergen exposure [8]. Repeated exposure to an allergen leads to the hallmark features of allergic asthma such as eosinophilic inflammation, bronchoconstriction, airway hyperresponsiveness, increased mucus production, and airway remodeling (**Figure 1**).

Figure 1. The Hallmark Features of Allergic Asthma



1.2. The Airway Epithelium in Allergic Asthma

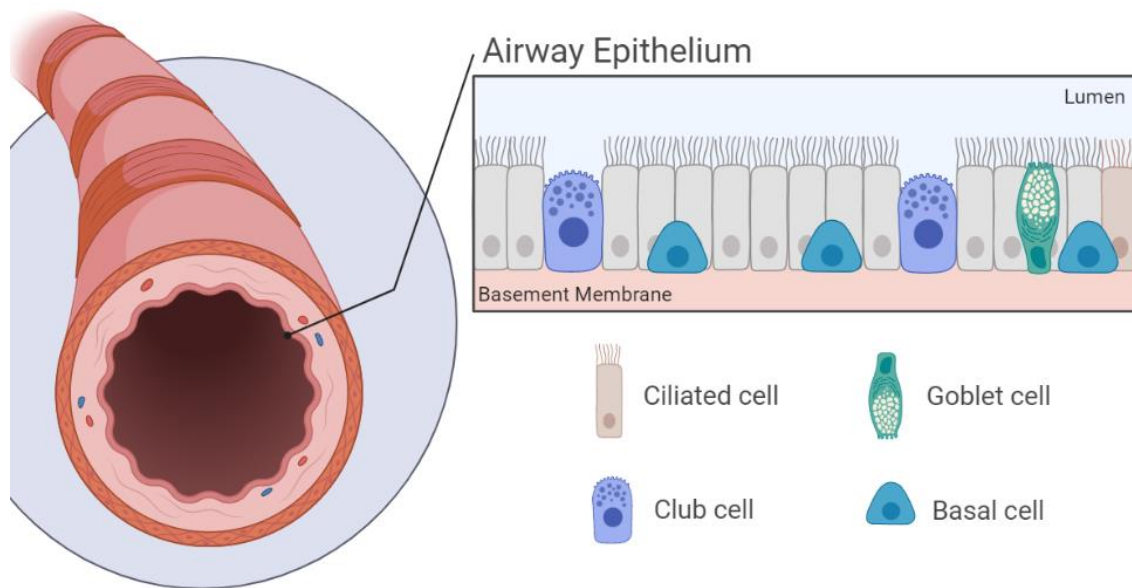
The lung's respiratory tract is a branching structure, dividing from the trachea to the alveoli. The lower airways, including the trachea, bronchi, and bronchioles, can be divided into the conducting airway zone, transporting inhaled air, and a respiratory acinar zone, involved mainly in gas exchange. Through the entirety of the airways structure there is a continuous lining, called the airway epithelium, which plays a significant role in maintaining the conduit of air to and from the alveoli [33]. It is often thought as a physical barrier and the first line of defense against inhaled microbes, noxious agents, and allergens between the host and its environment via secretion of substances such as surfactant proteins, mucus, and antimicrobial peptides and facilitation of mucociliary clearance [34]. However, due to its structure and diverse composition of cells, it also plays a role in lung fluid balance, temperature control, regulation of smooth muscle cells, and initiating innate and adaptive immune responses [33]. Moreover, the airway epithelium is the site of contact for both physical and inflammatory environmental stimuli. Consequently, airway epithelial damage can contribute to the bronchoconstriction, inflammation, and remodeling changes seen in allergic asthma [35].

1.2.1. Structure and Function of Airway Epithelial Cells

The airway epithelium is lined with an array of specialized cells that fulfil critical functions related to tissue homeostasis. There have been 10-12 morphologically distinct epithelial cell types described in the airway epithelium including long and small ciliated, basal, non-ciliated secretory (goblet, Clara cell, surface serous, submucosal serous, and submucosal mucous), pulmonary neuroendocrine, brush, and alveolar type I and type II

cell types [36, 37]. However, based on functional and biochemical criteria, airway epithelial cells can be classified generally as basal, ciliated, and secretory cells [33, 38]. The four airway epithelial cell types of interest are basal, mucus-secreting (Goblet), ciliated, and particularly club (Clara) cells (**Figure 2**).

Figure 2. The Airway Epithelium and its Cells



Basal Cells

Basal cells are ovoid in shape, have a prominent nuclei that fill most of the cell, and contain a sparse, electron-dense cytoplasm with bundles of low-molecular weight cytokeratin [39]. These cells are expressed ubiquitously throughout the conducting airway epithelium and are the only cell type within the airway epithelium to be rigidly attached to the basement membrane through hemidesmosome complexes [40, 41]. They form a monolayer along the basement membrane and provide the foundation for attachment to more superficial cells via desmosome attachments [40]. As such, they are

responsible for the pseudostratified appearance of the upper airways. However, the number of these cells decreases with airway size and the epithelium becomes columnar or cuboidal [42]. Functionally, basal cells can generate inflammatory responses by secreting cytokines, coordinate transepithelial water movement, and oxidant defense [43]. Still, the most important feature of basal cells is their primary stem cell potential in repopulating all the major epithelial cell types during regeneration and repair [44, 45].

Mucus-secreting (Goblet) Cells

Mucus-secreting epithelial cells have a wide, enlarged apical portion with a narrow, tapered basal cytoplasm. The nuclei are typically compressed at the basal side of the cell. The cytoplasm in mucus-secreting cells is relatively dense and electron opaque due to the numerous membrane-bound acid-mucin granules located at the apical region of the cell. This cell type is often found in the trachea, bronchi, and bronchioles [46-48]. Their main functions include the secretion of mucus, antimicrobial peptides, surfactant proteins, chemokines, and cytokines [49, 50]. Under homeostatic conditions, mucus-secreting cells, along with the submucosal glands, secrete high-molecular weight mucus glycoproteins to trap foreign particles in the airway lumen and protect the epithelial surface. Proper regulation and production of the amount and viscoelasticity of mucin secretions is essential for normal functioning [51]. Overproduction of mucus can clog the airways, while underproduction can hinder mucociliary clearance [52]. Mucus-secreting cells are capable of self-renewal and can differentiate into ciliated epithelial cells [53].

Ciliated Cells

Ciliated cells are a major epithelial cell type, accounting for over 50% of all epithelial cells and lining the trachea and respiratory bronchioles [38]. These cells are columnar in shape and covered with cilia, with approximately 300 cilia found at the luminal surface of each cell [54]. Ciliated cells are attached to the basal lamina of the basement membrane via desmosomes and extend to the luminal side, where they are interconnected via tight junctions [37]. This cell type lacks mucus granules and secretory products. Many mitochondria are found at the apical pole of the cell, immediately under the row of basal bodies to which the cilia are attached, emphasizing the primary role of ciliated cells in the transport of mucus from the lungs to the throat [33, 55]. Ciliated cells play a significant role in repair of distal airway injury. As a result, ciliary dysfunction and ultrastructural abnormalities have been associated with asthma severity [56]. It has been shown that ciliated cells stem from basal or club cells, and until recently were considered to be terminally differentiated cells [33, 53].

Club (Clara) Cells

Club cells protrude above ciliated cells and are cuboidal in shape with a basally located nucleus, prominent Golgi apparatus, abundant organelles such as agranular and granular endoplasmic reticulum, and many membrane-bound electron-dense secretory granules. Atypical mitochondria with no cristae and an abundant pale matrix localize at the apical portion of club cells. These non-ciliated secretory cells are located in the terminal and respiratory bronchioles [46, 57]. Unlike mucus-secreting cells, the secretory granules do not contain glycoproteins. Club cells are metabolically active and can secrete

CC10 (or CCSP), a secreted protein important for regulating the inflammatory response, surfactant apoproteins A, B, and D, proteases, antimicrobial peptides, several cytokines and chemokines [58]. Moreover, they aid in several chemical and physical lung protective functions such as detoxifying xenobiotics and oxidant gases, regulating inflammation, and contributing to mucociliary clearance [57, 59]. In addition to their secretory role, club cells have the ability to self-renew and function as stem cells, serving as the progenitor for both ciliated and mucus-secreting cells to repopulate damaged airway tissue [60].

1.2.2. Allergen-induced airway responses

HDM-induced airway inflammation

Environmental allergens, such as house dust mites (HDM), are a the most relevant driver of disease in allergic asthma. HDM represent one of the most common sources of inhaled allergens, with more than 50% of atopic individuals sensitized to its allergenic peptides [61]. The primary allergenic particles of HDM are found within the fecal waste pellets, but HDM also contain an array of allergens packaged with bacterial DNA, endotoxin, and chitin that have proteolytic activity and can induce immune responses by acting as pathogen associated molecular patterns (PAMPs) [62]. Proteins found in allergens such as Der p1, a cysteine protease, and Der p2, a ligand binding to endotoxin receptors such as toll-like receptor 4 (TLR-4), have the highest rates of sensitization [62-64]. When in contact with the airway epithelium, the allergen-containing particles of HDM promote sensitization and atopic responses and changes to the airway epithelium [65].

Atopy arises as a consequence of a combination of both epithelial barrier dysfunction and a heightened immunologic response [66]. Being the first line of defense against allergens, the airway epithelium is equipped with protease-activated receptors (PARs) and pattern-recognition receptors (PRRs) to detect the active allergenic components of HDM such as proteases and PAMPs, respectively [65, 67]. HDM proteases, such as Der p1, directly stimulate PARs in the bronchial epithelium of asthmatics and disrupt the tight junctions between the airway epithelial cells, allowing the infiltration of the allergenic particles into the subepithelial tissue [68, 69]. Moreover, Der p2 and other components of HDM such as bacterial DNA, endotoxin, and chitin can initiate innate immune responses via PRRs such as TLR-4 [64]. Activation of PRRs in airway epithelial cells by HDM allergens leads to the recruitment and activation of antigen presenting cells, including dendritic cells and macrophages, through the release of chemokines and cytokines by epithelial cells such as CCL20, IL-25, IL-33, thymic stromal lymphopoietin (TLSP), and granulocyte-macrophage colony-stimulating factor (GM-CSF) [67, 70, 71]. Dendritic cells link the innate and adaptive immune responses, facilitating and promoting the inflammatory process in the airway epithelium. Infiltrated HDM allergens are processed by tissue resident antigen presenting cells. Once matured, these antigen presenting cells present the processed allergen particles to naïve T lymphocytes in the draining lymph nodes of the lung [72]. Furthermore, activated T lymphocytes signal to antigen presenting cells to produce Th2 cytokines that recruit, mature, and activate eosinophils such as IL-3, IL-4, IL-5, and IL-13 [61]. Secretion of Th2 cytokines promotes airway inflammation and the immunoglobulin isotype switching from IgG to IgE, leading to the production of allergen-specific IgE, which eventually

bind to receptors in mast cells, basophils, and eosinophils [67]. Consequently, later encounters with HDM allergens will result in an immediate hypersensitivity phase where mast cells and basophils are degranulated, a late inflammatory phase where newly synthesized pro-inflammatory mediators are synthesized and secreted, and a chronic phase with hypersensitive responses [61]. Prolonged immune responses are key drivers in allergic asthma and have detrimental effects to the airways such as hyperresponsiveness and remodeling [73].

HDM-induced airway remodeling

Airway remodeling is the repair process following chronic airway injury and subsequent airway inflammation. In allergic asthma, airway remodeling is considered the result of chronic bronchial inflammatory insults from an allergen exposure in sensitized individuals. Upon epithelial damage from exposure to allergens, the epithelial barrier is disrupted, promoting the release of cytokines and chemokines such as IL-4 and IL-13, as well as the release of growth factors including epidermal growth factor (EGF) [74], transforming growth factor beta (TGF- β) [75], and vascular endothelial growth factor (VEGF) [76]. Consequently, airway remodeling is characterized by changes in the molecular, cellular, and tissue composition of the airway epithelium. Some of the structural changes associated with airway remodeling include disrupted epithelial integrity and basement membrane thickening, goblet cell hyperplasia/metaplasia, smooth muscle hypertrophy/hyperplasia, subepithelial fibrosis, and angiogenesis [77]. Airway remodeling is an intractable problem in asthma, contributing to airway

hyperresponsiveness, airflow limitation, and progressive or irreversible loss of lung function.

Epithelial Barrier Dysfunction. Epithelial damage is a pathological feature observed in all phenotypes of asthma, with epithelial changes and disruption occurring during the pathogenesis of asthma [49]. Inability to regain epithelial barrier function as a result of E-cadherin degradation via EGFR activation can result in increased permeability to allergens, the propagation of downstream pro-inflammatory responses via secretion of CCL20 and GM-CSF, and abnormal repair through impaired re-differentiation of epithelial cells [78-81]. Moreover, continual loss of E-cadherin can activate β -catenin processes that cause additional loss of epithelial characteristics, the induction of more basal and mesenchymal phenotypes, as well as goblet cell hyperplasia with loss of ciliated cells [78].

Subepithelial Fibrosis. Fibroblasts are large, flat cells that reside around the lamina reticularis, just below the epithelial basement membrane. In inflammatory environments such as asthmatic airways, cytokines such as IL-4, IL-13, and GM-CSF, growth factors like TGF- β , PDGF, and bFGF, and other mediators such as histamine, tryptase, and thrombin can activate and induce fibroblast differentiation into myofibroblasts [82]. In asthmatic individuals, increased susceptibility to injury and abnormal repair responses result in persistent fibroblast activation [83]. Myofibroblasts are a key cell in the fibrotic response due to the secretion of pro-inflammatory mediators and extracellular matrix proteins, including collagens type I, III, and V, fibronectin, tenascin, lumican, and

biglycan [84]. Moreover, the synthesis and degradation of the extracellular matrix compartment in the airway wall is dynamic. A shift toward excess matrix deposition results in fibrosis, leading to alterations in the structure and function of the airways [85]. Subepithelial fibrosis has been linked to asthma severity with higher collagen expression in those with moderate to severe asthma when compared to those with mild disease [86-88]. Furthermore, the degree subepithelial fibrosis is inversely correlated with forced expiratory volume in one second (FEV1), a measure of lung function [86]. Increased subepithelial fibrosis in the small airways in asthma can lead to airway narrowing and obstruction [89].

Goblet Cell Hyperplasia/Metaplasia. IL-4 and IL-13 are key drivers in the Th2 immune responses of allergic asthma. IL-4 is mainly involved in Th2 cell differentiation, immunoglobulin class switching, and eosinophil trafficking, while IL-13 promotes IgE synthesis and goblet cell hyperplasia [90, 91]. In healthy lungs, goblet cells are present sparingly throughout the small airways. Goblet cell hyperplasia and metaplasia have been described in both the small and large airways of asthmatics [92-94]. Differentiation of club cells and ciliated cells into goblet cells is the main mechanism for goblet cell metaplasia [65]. Goblet cell changes in the peripheral, small airways contribute to a greater extent to disease because the clearance of mucus from the small airways appears to be more difficult when compared to the more central, larger airways [92, 95]. The key role of mucus in the airways is to form a protective layer between the external and internal environment [96]. The viscoelastic features of mucus are strongly associated with mucins, high molecular-weight glycoproteins, with MUC5AC and MUC5B being the

predominant mucins in airway secretions [97]. In healthy small airways, MUC5AC is not observed [98], but it is upregulated in the small airways of those with asthma [99]. The increased amount of mucus within the luminal space can also be attributed to the hindrance of the mucociliary escalator, with factors such as abnormal ciliary function resulting in reduced clearance [56]. Epithelial compression due to bronchoconstriction is also thought to induce goblet cell hyperplasia and metaplasia with subsequent mucus production [100].

Smooth Muscle Hypertrophy/Hyperplasia. Remodeling of airway smooth muscle (ASM), one of the main structural cells within the bronchi, is considered one of the causes of airway obstruction due to ASM hypertrophy and hyperplasia. Moreover, the migration of ASM cells toward the airway epithelium can also contribute to airway remodeling [101]. In addition to the structural changes, ASM cells participate in the inflammatory and remodeling process via the expression of cellular adhesion molecules, receptors for cytokines, chemokines, and TLRs [102, 103]. Inflammatory mediators such as TNF- α , IL-1 β , and IFN- γ have been shown to induce the expression of cellular adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) in cultured ASM cells, implicating ASM cells in regulating interactions with an array of inflammatory cells including eosinophils and T lymphocytes [104, 105]. In healthy airways, ASM play a significant role in regulating the respiratory airway tone and contractility. However, thickening of the ASM layer is a prominent feature of the asthmatic airway [93, 106]. Studies reviewed by Gosens and Grainge suggest that bronchoconstriction resulting from ASM contraction is sufficient to induce airway remodeling via processes triggered by

mechanical forces, independent of inflammatory status [107]. ASM hypertrophy and hyperplasia play a particular role in asthma symptoms in particular with regard to airway hyperresponsiveness, with enhanced contraction being one of the main causes [108].

Airway Hyperresponsiveness

Airway hyperresponsiveness (AHR), a cardinal feature of asthma, is the predisposition and increased ability of the airways to undergo bronchoconstriction, or excessive narrowing, in response to a stimuli that would produce little to no effect in apparently healthy individuals. Moreover, AHR is characterized by an increased airway smooth muscle sensitivity to airway constrictor agonists such as histamine or methacholine [109]. The mechanisms underlying AHR in asthma remain poorly understood likely due to the numerous pathophysiological pathways associated with the development of this condition. Genetic factors, airway inflammation and remodeling, as well as enhanced airway smooth muscle contractility have been linked as to the development of AHR [110]. Moreover, AHR is responsible for the recurrent episodes of wheezing and shortness of breath associated with asthma, with increased AHR being linked to more severe asthma [111]. The presence of AHR is associated commonly with an increased decline in lung function and is most frequent in those individuals with a late asthmatic response [112, 113]. Persistence of AHR can be short-lived or remain for up to several months after exposure to a stimulant [113]. Clinically, the use of reductions in spirometry parameters such as such as forced expiratory volume in one second (FEV1) are used to assess changes in AHR and used as a tool to diagnose, classify disease severity, and determine the management of asthma [114].

1.3. Mitochondrial Structure and Function in the Normal and Pathological Lung

Mitochondria are individual entities, or organelles, ubiquitously found within cells that arose from the fusion with another prokaryotic cell, referred to as the endosymbiotic theory [115]. Mitochondria are considered individual entities because they contain their own maternally inherited genome and have genetic continuity, but also require the import of over 1,000 nuclear encoded proteins for proper function [116, 117]. Structurally, mitochondria contain two phospholipid membranes, the outer mitochondrial membrane and the inner mitochondrial membrane, which form two distinct compartments within the organelle, the mitochondrial matrix and the intermembrane space. Its main function related to its structure is evident in the electron transport chain, where the electrochemical gradient between the mitochondrial matrix and intermembrane space allows cells to generate energy [118]. Moreover, mitochondria are also morphologically complex and polymorphic undergoing fission and fusion in response to external insults and metabolic cues [119, 120]. Furthermore, these double-membraned organelles are dynamically organized and strategically trafficked within cells to support an array of vital cellular functions [121].

1.3.1. Mitochondrial Function

Mitochondria are commonly referred to as the “powerhouses of the cell,” due to their prominent role in energy production [122]. However, mitochondria have been characterized also as a signaling organelle involved in numerous physiological functions such as programmed cell death, innate immunity, autophagy, redox signaling, and calcium homeostasis as well as metabolic reactions such as steroid hormone synthesis,

lipid metabolism, and the interconversion of amino acids [123-125]. The main outputs associated with mitochondria include adenosine triphosphate, reactive oxygen species, and calcium. Mitochondria are key organelles in cells, as they are mainly involved in the production and sequestering of these molecules.

Adenosine Triphosphate

Adenosine triphosphate (ATP) is the principal cellular energy source in aerobic cells since its structure contains a high-energy chemical bond that releases free energy when hydrolyzed and converted to adenosine diphosphate (ADP). ATP synthesis, or oxidative phosphorylation, is dependent on the structural integrity of the mitochondrion, particularly the mitochondrial matrix. The mitochondrial matrix contains enzymes necessary for the tricarboxylic acid cycle and fatty acid beta-oxidation pathways and is enclosed by an inner membrane that contains the four complexes (I-IV) of the electron transport chain, ATP synthase (complex V), and specific metabolite carriers. During oxidative phosphorylation, electrons are donated from reduced coenzymes such as NADH and FADH₂ and delivered to molecular oxygen via complexes I-IV. Complexes I, III, and IV are redox pumps that establish a proton gradient across the inner mitochondrial membrane and an electrochemical energy gradient that is used to transform ADP into ATP by ATP synthase [126]. Moreover, in the lungs, mitochondria are found in several populations of highly specialized cells to facilitate high energy-consuming functions such as airway clearance via phagocytosis and ciliary movement, bronchial gland secretion, constriction of airways, and production of pulmonary surfactant [127,

128]. Although oxidative phosphorylation is a vital part of metabolism, it produces by-products such as ROS.

Reactive Oxygen Species

Reactive oxygen species (ROS) are highly reactive chemicals, such as peroxides, superoxide radical anions, and hydroxyl radicals, formed from oxygen, an ideal terminal electron acceptor [129]. ROS are produced from various endogenous sources and compartments of the cell including peroxisomes, phagocytic cells in the endoplasmic reticulum, and as a by-product of inflammation [130]. However, mitochondria are the major contributor of ROS, as they generate upwards of 90% of the total number of cellular ROS [131]. As a result, mitochondria represent the key source of ROS production in the majority of cell types. Nevertheless, at least ten sites within mitochondria have been shown to play a role in ROS production. However, oxidative phosphorylation and complexes I and III are thought to be the major contributors to generation of unpaired electrons or radicals [132-134]. It has been proposed that mitochondria regulate their activity to maintain a membrane potential that balances ATP synthesis against ROS production, as oxidants can uncouple proteins that reduce membrane potential [135]. To counteract these effects, cells contain multiple antioxidant enzymes such as superoxide dismutase, catalase, and peroxiredoxins [136]. Mitochondrial ROS are implicated in cell function, as they participate in multiple networks that control the cell cycle, stress responses, energy metabolism, and redox balance as well as a variety of degenerative processes [137].

Calcium

Calcium ions play a crucial and universal role as a second messenger [138]. Calcium is a versatile signaling molecule with an array of physiological functions including muscle contraction, neuronal excitability, cell proliferation, and cell migration. Moreover, calcium is an important regulator of mitochondrial function [139]. Within the mitochondrion, calcium acts at several levels to stimulate oxidative phosphorylation through the allosteric activation of various calcium-sensitive dehydrogenases and the stimulation of ATP synthase [140, 141]. Consequently, mitochondrial ATP outputs can be modified through calcium buffering to meet the cellular energy demands [142]. Likewise, calcium influx within mitochondria can occur when there is an increase in both the endoplasmic reticulum calcium release and cytosolic calcium concentrations. The mitochondrial membrane aids in regulating calcium concentrations by functioning as a reservoir and a buffer depending on local cellular conditions [143]. An elevation of mitochondrial matrix calcium results in the upregulation of the respiratory chain machinery, resulting in higher ATP outputs. Additionally, mitochondrial matrix overload can lead to changes in the mitochondrial membrane potential and increased ROS, triggering the permeability transition pore, and cytochrome c release, ultimately leading to apoptosis [144]. Overall, calcium is a positive global effector of mitochondrial function, with a delicate balance between the positive and negative effects of calcium.

1.3.2. Mitochondrial Dynamics and Trafficking

Mitochondria are highly dynamic organelles that can change in size, shape, and distribution [122, 145]. Mitochondria undergo coordinated cycles of fission and fusion,

termed ‘mitochondrial dynamics,’ to adapt morphologically. Mitochondria are trafficked on microtubule tracks for spatial and temporal control of cellular processes such as the cell cycle, immunity, apoptosis, mitochondrial biogenesis, mitochondrial quality control (mitophagy) and cell migration [146, 147]. Mitochondrial dynamics are mediated by several large GTPases and their combined effects lead to the formation of the fluid mitochondrial networks in many cell types. Imbalances in mitochondrial dynamics leads to structural changes and dysfunction. It is believed that mitochondrial dynamics allows the appropriate distribution of mitochondrial metabolites to localized cytosolic regions.

Mitochondria Fusion

Mitochondrial fusion is the physical merging of the outer and inner mitochondrial membranes of two distinct mitochondria. In mammals, mitochondrial fusion is tightly regulated by three dynamin-related GTPases proteins namely mitofusin 1 (Mfn1), mitofusin 2 (Mfn2), and optic atrophy 1 (OPA1) [148]. Mfn1 and Mfn2 reside in the outer mitochondrial membrane, while OPA1 localizes to the inner mitochondrial membrane. Because mitochondria are double-membraned organelles, the activation of these GTPases leads to the three-step mitochondrial fusion process. To initiate mitochondrial fusion, mitofusins tether to adjacent copies of themselves on nearby mitochondria and form a ring structure around the contact point between the outer membranes [149]. Subsequent GTP hydrolysis triggers the outer membrane fusion event, followed by fusion of the inner membranes by OPA1 rendering a new mitochondrial network [146]. Alterations in expression of these proteins can lead to distinct mitochondrial morphologies. Overexpression of Mfn1 and Mfn2 leads to perinuclear

mitochondrial clustering, while underexpression of Mfn1 and Mfn2 leads to mitochondrial fragmentation [150]. Likewise, knockdown of OPA1 induces mitochondrial fragmentation and overexpression of OPA1 leads to mitochondrial elongation [151, 152]. Fusion is necessary to help alleviate mitochondrial stress by mixing the contents of partially damaged mitochondria with healthy mitochondria to prevent the loss of essential components [153].

Mitochondrial Fission

Mitochondrial fission is the process where mitochondria divide into two distinct organelles, an important process for remodeling and rearrangement of mitochondrial networks during development [154], apoptosis [155], acute organ injury [156], as well as the segregation of mitochondria during cell division [157]. The large GTPase dynamin-related protein 1 (Drp1) is a central player in mitochondrial fission. Although Drp1 is not localized to the mitochondrial membrane and described as being mostly a cytoplasmic protein, it can associate with the mitochondrial membrane via interactions with mitochondrial receptors [158-161]. Drp1-mediated fission is an intricate process involving the translocation of Drp1 to the mitochondrial outer membrane, followed by its high-order assembly, GTP hydrolysis, and its disassembly [159]. At the mitochondrial outer membrane, Drp1 binds to receptors and forms a functional complex that is transported from the cytoplasm to the fission site [148]. Moreover, the transfer of calcium from the endoplasmic reticulum into the mitochondrion results in the recruitment of Drp1 to the mitochondrial surface [162]. Reduced Drp1 activity, mainly through impaired calcium signaling and intercellular communication, is seen in aged mice leading to

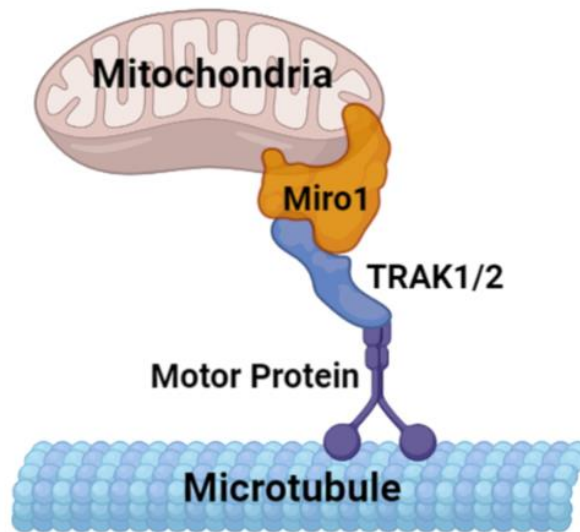
defective organelle morphogenesis in oocytes [163]. Genetic loss of Drp1 leads to pronounced mitochondrial elongation in multiple cell lines and animal models [154, 164]. Additionally, downregulated or inactivated Drp1 can lead to genomic instability, autophagy escape, reduction of invasion and migration, and reduction of cell viability [165]. Conversely, Drp1 overexpression does not lead to enhanced mitochondrial fission since Drp1 activity relies on its activation by various post-translational modifications, as well as the translocation from the cytosol to mitochondria [166]. Upregulation and activation of Drp1 has been associated with increased cell proliferation, cell invasion and migration, and mitophagy [165]. Mitochondrial fission is required for mitophagy as it enables the separation of depolarized mitochondria from the mitochondrial network, ultimately allowing the engulfment of autophagosomes [167, 168].

Mitochondrial Trafficking

Subcellular mitochondrial positioning has emerged as a critical component for an array of cellular processes including cell division [167], migration [169, 170], signaling [171], and PINK1/Parkin-dependent mitophagy pathways [172]. Calcium-binding mitochondrial Rho 1 and 2 (Miro1/2) GTPases are key regulators of mitochondrial transport along microtubules [173]. Miro1 and Miro2 are C-terminally-anchored proteins in the outer mitochondrial membrane with two GTPase domains and two calcium binding embryonic fibroblast (EF) hand motifs facing the cytoplasm [174-178]. Evidence suggests that Miro proteins also function as mitochondrial calcium sensors via the EF hand motifs [179, 180]. Moreover, Miro proteins are the only known surface receptors that tether mitochondria to the trafficking adapter proteins 1 (TRAK1) and TRAK2 that

associate with the microtubule-associated molecular motors kinesin and dynein for long-range mitochondrial transport [174-176, 181-183] (**Figure 3**). Miro1 also associates with the actin motor protein myosin-19 for shorter distance movements [184]. A study using Miro1/2 protein double knockout cell lines suggest that TRAK proteins localize to mitochondria in the complete absence of Miro proteins, while myosin-19 mitochondrial transport is dependent on Miro1 proteins [173]. Moreover, Miro1 knockout, but not Miro2, in cells show that mitochondria localize and concentrate in the perinuclear region of cells [173, 185]. Miro1/2 protein double knockout in cells results in a more accentuated mitochondrial perinuclear accumulation when compared to Miro1 knockout cells [173]. However, it has been shown that the number of microtubule-dependent mitochondrial trafficking events is significantly reduced in Miro1 knockout cells when compared to the unaltered trafficking events in Miro2 knockouts in cells [173]. Miro1 and Miro2 are roughly 60% identical and have non-redundant roles in regulating mitochondrial trafficking and distribution, with Miro1 being the key adaptor protein in regulating subcellular microtubule-dependent mitochondrial trafficking [174, 175]. Defects in Miro1-mediated mitochondrial trafficking has been implicated and thoroughly described in cancer [186, 187], metabolic conditions [188, 189], and especially neurological disorders such as Parkinson's disease [172, 190-194], as it can disrupt membrane dynamics and energy levels in the distant cortical cytoskeleton.

Figure 3. Miro1 is the Primary Adaptor Protein for Microtubule-Dependent Trafficking



1.3.3. Mitochondrial Dysfunction in Asthma

In asthma, the airway epithelium is frequently injured by aeroallergens, environmental pollutants, and oxidants, leading to inflammation via the secretion of pro-inflammatory molecules and immune cells. This response promotes oxidative stress in the airways through the release of various ROS including superoxide anions, hydroxyl radicals, hydrogen peroxide, and nitric oxide. ROS can damage the surrounding airway epithelial cells and amplify the associated inflammation leading to structural changes such as thickened basement membranes, shedding of the surface epithelium, smooth muscle cell hypertrophy/hyperplasia, goblet cell hyperplasia/metaplasia, and angiogenesis surrounding the airways [14]. These structural changes alter airway function, leading to the signs and symptoms associated with asthma. Moreover, following cellular stress or injury, mitochondrial membranes can be damaged and become dysfunctional. Mitochondrial dysfunction is characterized by ROS accumulation, loss of

membrane potential, mitochondrial calcium overload, mitochondrial DNA (mtDNA) mutation or release, and mitophagy dysregulation [195].

The literature suggests that mitochondrial dysfunction has been implicated in the pathogenesis of asthma. A murine model study of ovalbumin-induced asthma by Jaffer *et al.* showed that mtROS, TGF- β , and collagen deposition are increased when compared to mice treated with saline. Moreover, these authors also show that Mito-TEMPO, a mitochondria-targeted antioxidant, significantly decreases mtROS in ovalbumin-challenged mice. Human airway epithelial cells from the same study treated with IL-13 showed an increase in mtROS and collagen expression, which were also diminished by the Mito-TEMPO treatment [196]. Other studies suggest that antioxidant enzymes are decreased in the peripheral tissue of asthmatic adult patients when compared to control subjects, showing that oxidative stress and mitochondrial dysfunction are present in asthmatics [197-201]. Other studies have shown increased mtROS in the venous blood of severe asthmatics when compared to apparently healthy individuals [202]. Furthermore, polymorphisms in mitochondrial encoded genes associated with mtROS production have been found in asthmatic children, indicating a genetic predisposition to increased asthma risk [203]. Other studies have shown that increased production of mtROS can lead to loss of membrane potential and ATP depletion, leading to the initiation of cytochrome c and caspase-3 apoptotic pathways [204]. Regarding mitochondrial membrane potential alterations, another study suggests that fibroblasts from severe asthmatics have reduced membrane potential with reduced membrane activity, as well as the increased mitophagy as suggested by an increase in PINK1 and Parkin [205]. Evidence also suggests that mitochondrial mass and oxygen consumption are higher in the bronchial smooth muscle

of those with asthma. This finding has been linked to an enhanced extracellular calcium influx and mitochondrial biogenesis. These changes might lead to the proliferation of bronchial smooth muscle cells and remodeling changes within the airway [206]. Another study shows that a mitochondrial calcium uniporter deficiency suppresses mitochondrial calcium uptake (MCU) and ROS production. Suppression of the MCU also maintained the mitochondrial membrane potential and had protective effects against apoptosis in response to IL-13 [207]. The literature also shows that allergen-induced ROS generation leads to the activation of calcium/calmodulin-dependent kinase II (CaMKII), which plays a role in mitophagy regulation. Activation of this kinase can result in airway hyperresponsiveness, ROS generation, and Th2 inflammatory responses in the lung. Inhibition of CaMKII reduces allergen-induced mitophagy, mitochondrial dysfunction, and cytokine production in the airway epithelium. These changes reduce the subsequent airway hyperresponsiveness and inflammation that leads to the pathogenesis of asthma [208]. Finally, an *in vivo* model of asthma using wild-type and Parkin deficient mice showed that inoculation with IL-13 leads to increased numbers of neutrophils and eosinophils in the BALF of wild-type mice when compared to Parkin deficient mice. IL-13 also enhanced mtDNA release and chemokine levels in the BALF of wild-type mice when compared to Parkin deficient mice. Similarly, an *in vitro* experiment from the same study demonstrated that Parkin deficient human bronchial epithelial cells inhibit mtDNA release when exposed to IL-13. Data from this study suggest a link between mitophagy, mtDNA, and inflammation in the development and progression of asthma [209].

1.4. Miro1-mediated Mitochondrial Positioning and Chronic Inflammatory Lung Conditions

Mitochondria are the primary source of cellular ATP and produce substantial amounts of ROS, which play a significant role in the lung epithelium in response to an external stimuli [14, 210, 211]. The fluid reorganization of mitochondrial networks enables the positioning of functional mitochondria to sites requiring mitochondrial metabolites [121]. Mitochondria are transported and strategically repositioned throughout the cell by the microtubule-associated molecular motors kinesin and dynein and function as subcellular barriers that allow the compartmentalization of signaling and amplification of responses [212-214]. The attachment of mitochondria to these molecular motors is primarily mediated by a protein complex that includes TRAK1/2 and Miro1/2 [174-176, 181-183]. In polarized lung epithelial cells, mitochondria localize at the apical and basolateral poles of cells, which allows the compartmentalization of calcium signaling following ipsilateral receptor activation in the plasma membrane [215, 216]. Furthermore, the uncoupling of mitochondrial oxidative phosphorylation with FCCP prevents the compartmentalization of calcium signaling in polarized lung epithelial cells [215]. Our laboratory has shown that deletion of Miro1 in mouse embryonic fibroblasts confines intracellular ATP and ROS gradients to the perinuclear space and disrupts membrane reorganization and redox dependent signaling [217, 218]. The mechanism regulating mitochondrial positioning in the lung epithelium remains unknown.

Sundar *et al.* [219] looked at the role of Miro1 in cigarette smoke extract-induced mitochondrial dysfunction *in vitro*. This study showed that cigarette smoke extract treatment leads to mitochondrial dysfunction associated with increased concentrations of

mtROS, increased mitochondrial mass, and reduced membrane potential in primary lung epithelial cells. Moreover, cigarette smoke extract exposure altered the abundance of oxidative phosphorylation protein complexes, which was correlated with changes in the mitochondrial respiratory chain as evidenced by decreased basal and maximal respiration, diminished ATP production, and reduced spare capacity. Furthermore, chronic cigarette smoke extract exposure in lung epithelial cells showed an increase in mitochondrial fragmentation and perinuclear clustering around the nucleus. These structural changes were linked to increased Drp1 and reduced Mfn2 protein abundances. Cigarette smoke extract treatment significantly reduced Miro1 and PINK1 protein levels in primary lung epithelial cells.

An *in vivo* study from the same laboratory used an epithelial-specific partial and complete Miro1 knockout mouse model to assess the effects of acute and chronic exposure to cigarette smoke extract. Sharma *et al.* [220] found that cigarette exposure in mice with Miro1 ablation from CC10 positive cells leads to increased expression of pro-inflammatory and inflammatory mediators in the BALF. Moreover, total cell counts, macrophages count and neutrophil counts were also significantly elevated in the BALF. Histopathological changes observed with Miro1 deletion included foamy macrophages and epithelial hyperplasia. Although some structural changes were noted, no changes in the lung mechanics were appreciated. Findings from both studies strengthen the concept that Miro1 is a key player in exerting the inflammatory responses due to cigarette smoke in the lungs, which is in part mediated by cigarette smoke-induced mitochondrial dysfunction and impaired quality control.

CHAPTER 2: DISRUPTION OF MITOCHONDRIAL TRAFFICKING IN LUNG EPITHELIAL CELLS POTENTIATES ALLERGIC ASTHMA PHENOTYPES

2.1. Introduction

Allergic asthma is a complex, chronic inflammatory condition of the small airways that affects as many as 300 million people of all ages worldwide, including 25 million Americans (about 8.5% of the United States' population), and accounts for approximately 400,000 million [1-3]. Generally, a combination of environmental and genetic factors can cause allergic asthma [4, 5]. However, allergen exposure is the single most important cause of allergic asthma and the main driver of acute episodes, or “attacks,” of airway inflammation, increased mucus production, and obstruction and narrowing of the airways [221]. These disruptive changes to the airway epithelium, the first line of protection against external insults and pathogens, can lead to some of the classical symptoms associated with allergic asthma such as chest tightness, difficulty breathing, wheezing, and persistent coughing [7]. In allergic asthma, the airway epithelium is frequently injured by allergens, leading to the secretion of pro-inflammatory cytokines and chemokines, as well as the recruitment and activation of innate and adaptive immune cells [222]. Chronic activation of these pro-inflammatory molecules potentiates structural changes in the airways such as thickened basement membranes, shedding of the surface epithelium, subepithelial fibrosis, mucus hyperplasia and metaplasia, and increased angiogenesis surrounding the airways [77]. These structural changes to the airways lead to functional changes, making it difficult to breathe.

Currently, there is no cure for allergic asthma. Avoidance of triggers and the use of bronchodilators and anti-inflammatory drugs such as inhaled rapid-acting β -2

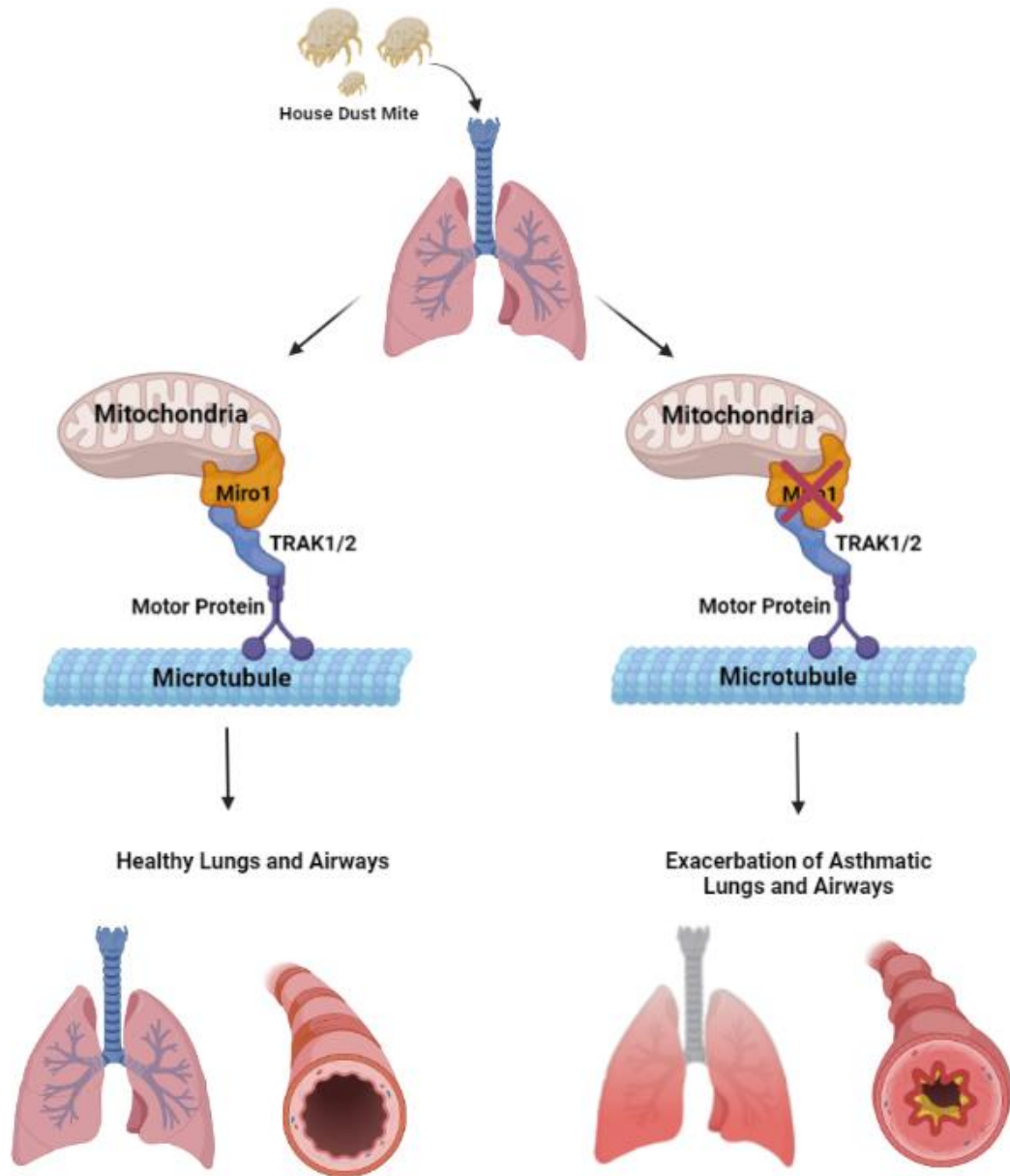
adrenergic agonists and oral corticosteroids are commonly recommended for the management of symptoms. However, these current treatment options fail in upwards of 10% of patients or have serious side effects [13, 14], making it necessary to study novel molecular targets to better understand the pathophysiological nature of allergic asthma and to explore alternate therapeutic options.

Ample evidence suggests that mitochondria play a key role in lung health and pathophysiology [223-226]. Mitochondria, the “powerhouses of the cell,” are the main producers of adenosine triphosphate (ATP). Moreover, mitochondria are a primary source of reactive oxygen species (ROS), and can actively buffer calcium (Ca^{2+}) [144]. Following cellular stress, mitochondrial membranes can be damaged and may become dysfunctional. Mitochondrial dysfunction is characterized by ROS accumulation, loss of membrane potential, mitochondrial Ca^{2+} overload, mitochondrial DNA mutation or release, and mitophagy dysregulation [195]. Increased production of ROS has been implicated in the development of chronic inflammatory lung conditions such as asthma [14, 210]. As a result, these double-membraned organelles can change in size, shape, and distribution depending on intracellular mitochondrial metabolite demands [121, 122]. Within the cell, mitochondria are transported on the microtubule-associated molecular motors kinesin and dynein [212-214], and tethered to these motors by a protein complex composed of trafficking kinesin protein 1 and 2 (TRAK1/2) and Mitochondrial Rho GTPase 1 and 2 (Miro1/2) [174-176, 181-183]. Miro1 shares approximately 60% homology with Miro2, but evidence shows Miro1 is the primary adaptor protein required for the subcellular positioning of mitochondria within differentiated cells [173, 185]. The dynamic reorganization of mitochondrial networks is important in highly active cells,

such as airway epithelial cells, due to their increased need in energy to maintain the functionality of the airways. Evidence suggests that mitochondria are positioned at the apical and basolateral poles of polarized airway epithelial cells to allow calcium signaling [215], and provide energy for mucus to be secreted and cleared [227-230], which becomes critical during high energy demand events such as stress or injury. Recent *in vitro* and *in vivo* models of COPD suggest that Miro1 plays a contributing role to this pathology [219, 220]. Until now, the role of Miro1-mediated mitochondrial positioning in the context of allergic asthma remained unexplored.

Herein, we have developed a novel mouse model to study Miro1 biology in the context of allergic asthma, where we hypothesize that epithelial-deletion of Miro1 leads to exacerbated inflammatory responses following chronic allergen exposure (**Figure 4**). We find that epithelial ablation of Miro1 following chronic exposure to house dust mite (HDM) leads to significant changes in tissue architecture and organization, as well as changes in lymphocyte infiltration and airway hyperresponsiveness.

Figure 4. Schematic Representation of Miro1 Expression Leading to Appropriate or Exacerbated Airway Responses Following Allergen Challenge



2.2. Materials and Methods

2.2.1. Study Approval

All mouse studies were approved by the Institutional Animal Care and Use Committee of the University of Vermont, Burlington, VT, USA under protocol number PROTO202000216.

2.2.2. House Dust Mite

HDM (XPB70D3A2.5, Stallergenes Greer, Lenoir, NC, USA) was suspended in Phosphate Buffered Saline (PBS). HDM concentration was determined by total protein concentration in HDM vial.

2.2.3. Epithelial Tissue-Specific Transgenic Mice

To achieve conditional airway epithelial specific deletion, C57B/L6 mice containing floxed Miro1 alleles (Miro1^{flx/flx}) were crossed with double transgenic mice containing a Clara Cell secretory protein (CCSP) promoter fused to a reverse tetracycline trans activator (rTetA) and a Tet operon fused to Cre recombinase (TetOP-Cre), termed CCSP-rTetA/TetOP-Cre, to generate triple transgenic (CCSP-rTetA/TetOP-Cre/Miro1^{flx/flx}) mice, referred to as Δ Epi-Miro1. Mice containing the three gene inserts were used to conditionally delete Miro1 in CCSP positive airway epithelial cells upon exposure to doxycycline-containing food for 7 days. Littermates missing one of the three gene inserts (CCSP-rtTA, TetOP-Cre, or Miro1^{flx/flx}) also on the doxycycline-containing food were used as control mice.

2.2.4. Experimental Design

An established model of allergic asthma was used for all experiments. In this model, 25 µg of HDM or PBS was administered to control and ΔEpi-Miro1 mice via nasopharyngeal aspiration. Mice were sensitized to the allergen 7 days after the start of the doxycycline-containing food. In this chronic model of allergen challenge, the allergic response in mice was boosted 7 days after sensitization and challenged for five consecutive days 14 days after sensitization with HDM or PBS. Mice were sacrificed 24 hours following the last allergen challenge.

2.2.5. Tissue Processing

Following euthanization, left lung lobe tissue was collected, inflated, and fixed in 4% paraformaldehyde for 24-48 hours at 4° C. Formaldehyde-fixed lung tissue was sent to the University of Vermont Medical Center for paraffin embedding. Tissue blocks were serially sectioned at a 5 µm thickness using a Leica 2030 manual paraffin microtome (Microscopy Imaging Center, University of Vermont). Tissue sections were mounted onto glass slides and dried in an oven at 52° C for 60 minutes. De-paraffinization and tissue rehydration for all staining procedures was achieved by immersing the glass slides through the following solutions: three 15 minute xylene washes, two 5 minute washes for 100% ethanol and 95% ethanol, and a 5 minute wash for 70% ethanol, 50% ethanol, and dH₂O.

2.2.6. Mucus Metaplasia Quantification

Periodic Acid Schiff (PAS) staining was conducted to assess mucus metaplasia. Lung tissue slides were immersed in 0.5% periodic acid for 10 minutes, followed by three 5 minute rinses in dH₂O, and 30 minutes in Schiff reagent. Next, two 1 minute washes in 0.55% potassium metabisulfite, followed by a 10 minute rinse under running water. Tissue was counterstained by immersing slides for 10 minutes in hematoxylin, rinsed under running water for 5 minutes, and 2 dips in 0.5% lithium carbonate. Images were captured at 20x magnification by Leica VERSA8 whole slide imager (Microscopy Imaging Center, University of Vermont). Mucus metaplasia was measured in the airways by measuring positive PAS stained area using the Positive Pixel Count algorithm of Leica Aperio ImageScope Software (Aperio Technologies, San Diego, CA, USA). The Positive Pixel Count algorithm outputs used to determine the positive PAS stained area were number of strong positive pixels normalized to lung tissue area.

2.2.7. Remodeling Quantification

Masson's trichrome staining was conducted to assess remodeling changes. Lung tissue slides were immersed for 1 hour in Bouin's solution at 56 ° C and cooled to room temperature. Tissue was rinsed under running water until stain disappeared. Tissue was stained for 10 minutes in Wiegert hematoxylin and washed under running water for 10 minutes. Next, tissue was stained for 2 minutes in Biebrich Scarlet-Acid Fuchsin solution, followed by a rinse in dH₂O, and then immersed for 10-15 minutes in a phosphomolybdic/phosphotungstic acid solution. The last stain was 5 minutes in aniline blue, followed by a rinse in dH₂O, and 3-5 minutes in a 1% acetic acid solution. Nuclei

were stained in black; cytoplasm, keratin, and muscle fibers were stained in red; and collagen was stained in blue. Images were captured at 20x magnification by Leica VERSA8 whole slide imager (Microscopy Imaging Center, University of Vermont). Representative images of the small airways were captured for each experimental group. Images were de-identified and blindly scored using an arbitrary unit (A.U.) scale ranging from 1-8 by ~6 separate individuals. The arbitrary unit scoring scale assessed changes in remodeling including collagen deposition, immune cell infiltration, epithelial layer thickening, and alveolar space changes. Lower scores signified little to no remodeling changes and higher scores signified increased remodeling changes. Mean A.U. scores per evaluator were obtained and these were averaged per experimental condition.

2.2.8. α -Smooth Muscle Actin Immunohistochemistry and Quantification

Immunohistochemical staining was used to determine changes in airway smooth muscle levels. Lung antigen retrieval was achieved by submersing slides in a DAKO antigen retrieval solution for 20 minutes at 95° C, slides were then allowed to cool down for 20 minutes at room temperature, and rinsed in three 5 minute PBS washes. Lung tissue slides were blocked in a 10% H₂O₂ in methanol solution for 15 minutes, followed by seven 5 minute PBS washes. A 2.5% normal goat serum protein block (Vector Laboratories) was put on the slides for 15 minutes, followed by overnight incubation of primary α -smooth muscle actin antibody (Abcam, ab5694) diluted to a 1:2000 concentration in PBS at 4° C. Following overnight incubation, slides were rinsed in seven 5 minute PBS washes and a ImmPRESS polymer reagent (Vector Laboratories) was put on the tissue for 30 minutes at room temperature. Tissue was rinsed in seven 5 minute

PBS washes and exposed to a diaminobenzidine (DAB) peroxidase solution (Vector Laboratories) for immunohistochemical staining. Tissue was rinsed in dH₂O and counterstained with hematoxylin and ammonium hydroxide.

2.2.9. Airway Hyperresponsiveness Assessment

Mice were anesthetized using sodium pentobarbital (90 mg/kg) via intraperitoneal injection and tracheotomized using 18-gauge cannulas. Mice were mechanically ventilated at a rate of 200 breaths/minute using FlexiVent computer controlled small-animal ventilator (SCIREQ, Montreal, QC, Canada). Airway hyperresponsiveness parameters such as Newtonian resistance (R_n), tissue dampening (G), and tissue elastance (H) were measured in the mice after exposure to increasing concentrations (12.5 mg/ml, 25 mg/ml, and 50 mg/ml) of aerosolized methacholine. Measurement of lung mechanics presented are the average of three peak measurements.

2.2.10. Bronchoalveolar Lavage Fluid Collection and Processing

Bronchoalveolar lavage fluid (BALF) was collected by washing the airways with 1 mL of sterile PBS. Cells were isolated via centrifugation and total cell counts were determined using a hemocytometer (3110, Hausser Scientific, Horsham, PA, USA). Cytospins were conducted and cells were stained using Hema3 stain reagents (Fisher Scientific, Waltham, MA, USA) to obtain differential cell counts. A minimum of 300 cells were counted to determine differential cell counts.

2.2.11. ELISAs

Right side lung lobes were flash frozen immediately after harvest and crushed to make lysates in buffer containing 137 mM Tris HCL (pH 8.0), 130 mM NaCl, and 1% NP-40. Samples were normalized to total lung protein and used to assess expression levels of IL-6, IL-33, CCL20, Eotaxin-1 (DuoSet ELISA Kits, R&D Systems, Minneapolis, MN, USA), IL-4, IL-13 (eBioscience Kits, Thermo Fisher Scientific, Waltham, MA, USA), and MUC5AC (Novus Biologicals, Littleton, CO, USA) per manufacturer's instructions.

2.2.12. Caspase Assay

25 µg of tissue lysates was diluted to 25 µL in dH₂O. Following dilution, tissue lysates were incubated with 25 µL Caspase-Glo 3/7 assay reagent (Promega) in an opaque plate in a dark room for 30 minutes at room temperature. Total luminescence was measured using a Synergy HTX plate reader (Biotek) and values were recorded as relative activity.

2.2.13. Statistical Analyses

Normal data were analyzed by one-way ANOVA and Tukey's multiple comparisons posttest. A p-value <0.05 was considered significant. Data were averaged and expressed as the mean ± SEM.

2.3. Results

2.3.1. Conditional Deletion of Miro1 *In Vivo* Enhances the Pro-Inflammatory Response following Chronic HDM Exposure in a Mouse Model of Allergic Airway Disease

To assess changes in the epithelial pro-inflammatory response to HDM following the conditional deletion of Miro1, we generated a CC10-rTetA/TetOP-Cre/Miro1^{flx/flx} mouse model to delete Miro1 in CCSP positive lung epithelial cells in the presence of doxycycline (**Figure 5**). Mice were given doxycycline-containing chow 7 days before the start of the chronic allergic airway disease protocol. The doxycycline diet was maintained until the end of the sensitization exposure and chronic allergen challenges. Mice were sensitized intranasally to HDM on days 0 and 7 with 25 µg of HDM, followed by five consecutive day challenges with 25 µg of the allergen or the phosphate buffered saline (PBS) control. Mice were sacrificed 24 hours after the last allergen challenge to assess the role of Miro1 in the allergic airway response (**Figure 6**). BALF was analyzed for immune cell infiltration into the lung. This analysis revealed no statistically significant differences in the number of total cell infiltrates, as well as select immune cells such as macrophages, eosinophils, and neutrophils among control and ΔEpi-Miro1 mice exposed to HDM. However, a significant increase in the number of lymphocytes in the lungs of ΔEpi-Miro1 mice challenged with HDM was observed when compared to control mice exposed to HDM (**Figure 7**). Moreover, whole lung tissue lysates were analyzed for several epithelial-secreted pro-inflammatory cytokines, chemokines, and Th2 adaptive cytokines via enzyme-linked immunosorbent assay (ELISA). There were no statistically significant differences observed in the production of the pro-inflammatory cytokines IL-6

and IL-33 or the Th2-specific cytokines IL-4 and IL-13 between the Δ Epi-Miro1 mice challenged with HDM, when compared to the HDM-challenged control mice. Conversely, Δ Epi-Miro1 mice challenged with HDM expressed a statistically significant increase in the levels of the chemokines CCL20 and Eotaxin (**Figure 8**). These results are suggestive of a potential role for Miro1-mediated mitochondrial positioning in the regulation of specific epithelial pro-inflammatory signaling.

Figure 5. Experimental mouse model using CCSP-rTetA/TetO-Cre/Miro1^{flx/flx} mice to achieve epithelial deletion of Miro1 following doxycycline exposure.

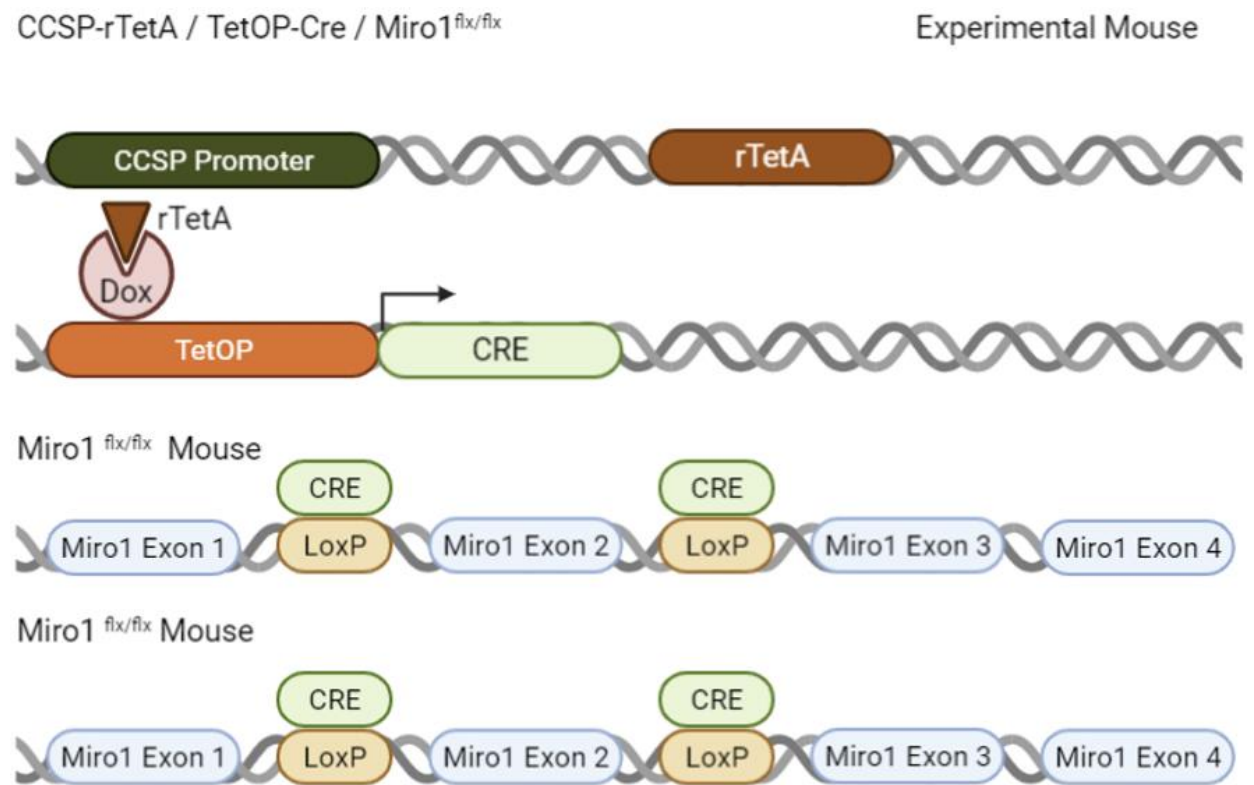
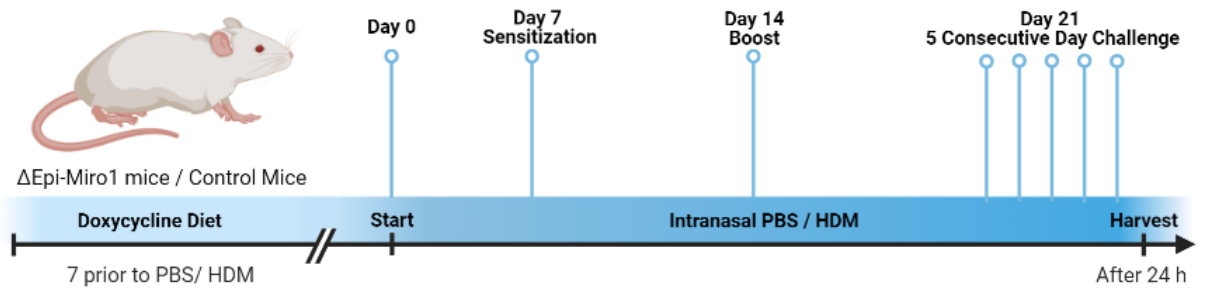
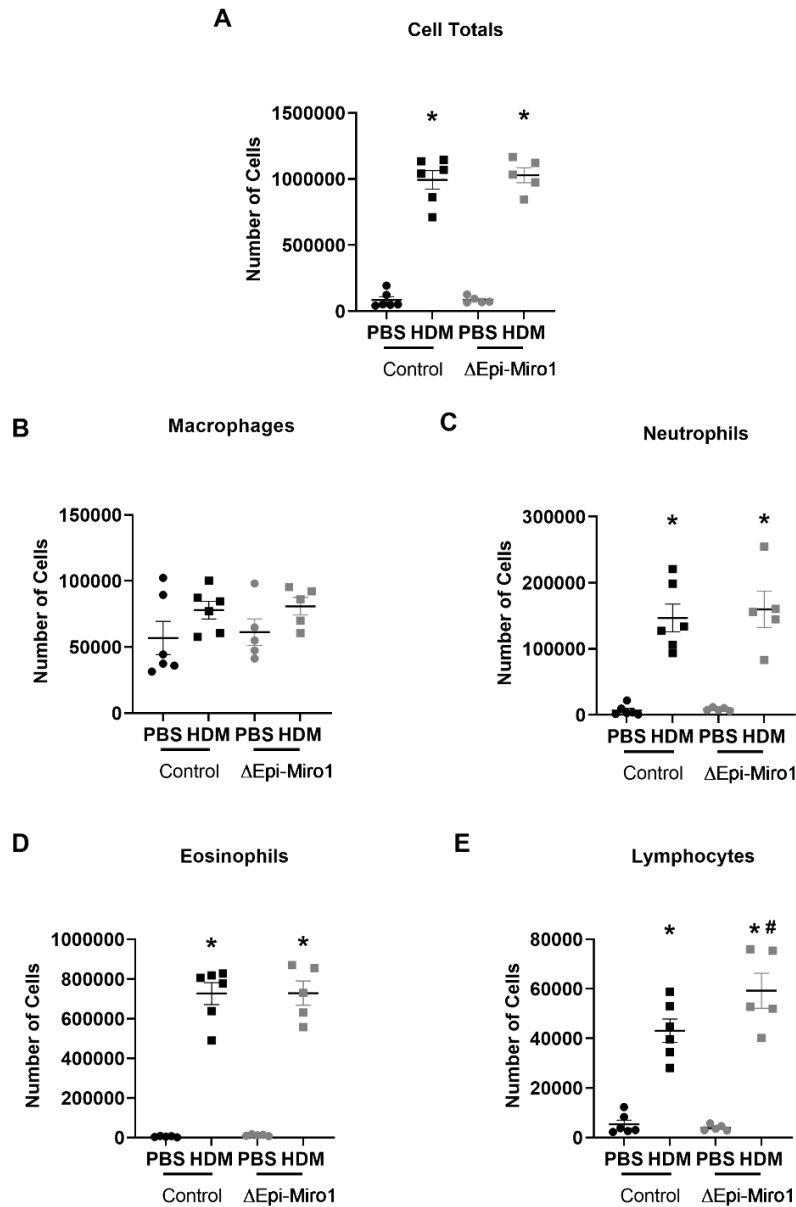


Figure 6. Deletion and HDM exposure protocol.



Δ Epi-Miro1 mice and control mice were given a doxycycline-containing diet 7 days prior to the start of experiments. Δ Epi Miro1 mice were sensitized to HDM on day 7, boosted on day 14, and serially challenged for 5 consecutive days starting on day 21.

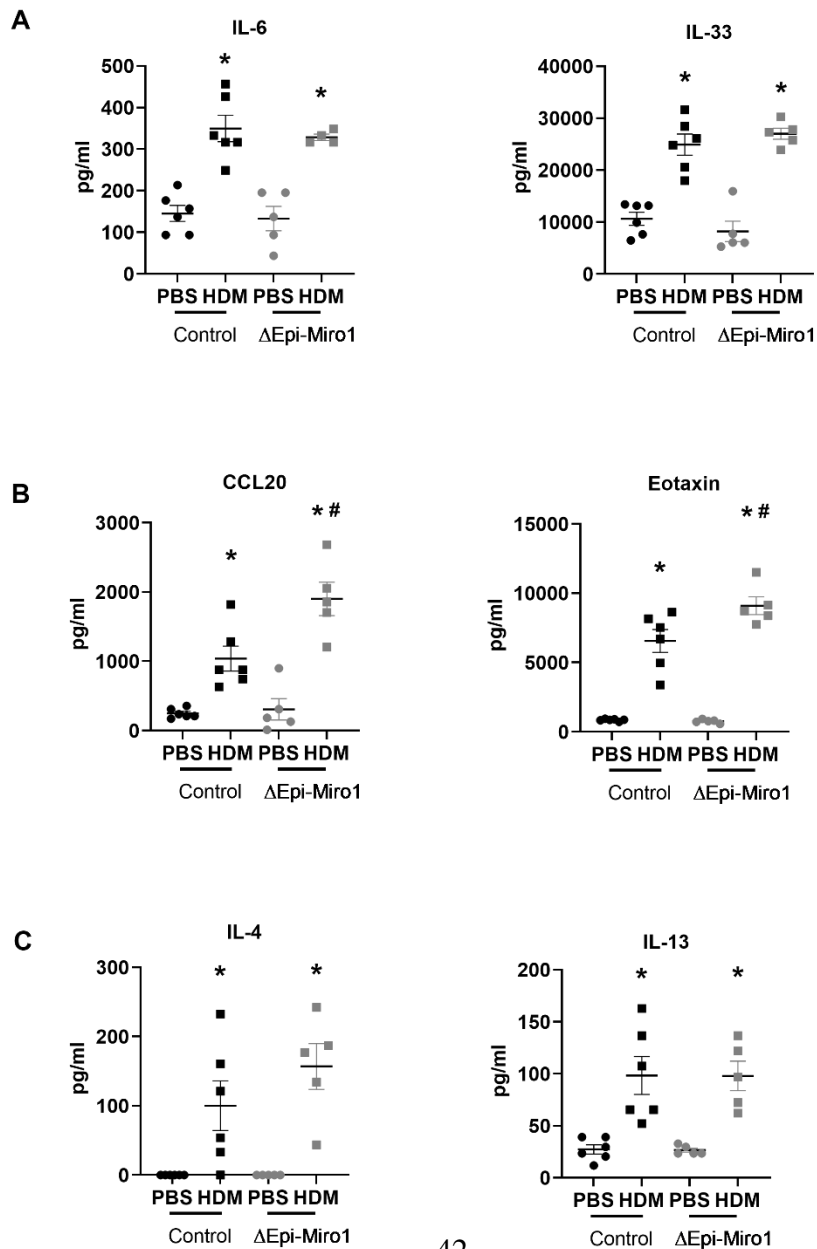
Figure 7. Conditional deletion of Miro1 from airway epithelial cells enhances lymphocyte levels in the mouse lungs following chronic exposure to HDM.



(A) Total inflammatory cells present in the BALF, n = 5 – 6 mice per group from one experiment. (B-E) Inflammatory cell-specific totals in the BALF, n = 5 – 6 mice per

group from one experiment. (A-E) * p-value < 0.05 vs. corresponding PBS group, # p-value < 0.05 vs. Control HDM group. Error bars represent mean \pm SEM. Data collected in collaboration with Sierra Bruno, PhD from the Anathy Lab at the University of Vermont.

Figure 8. Conditional deletion of Miro1 from airway epithelial cells enhances chemokine expression in mouse lungs following chronic exposure to HDM.

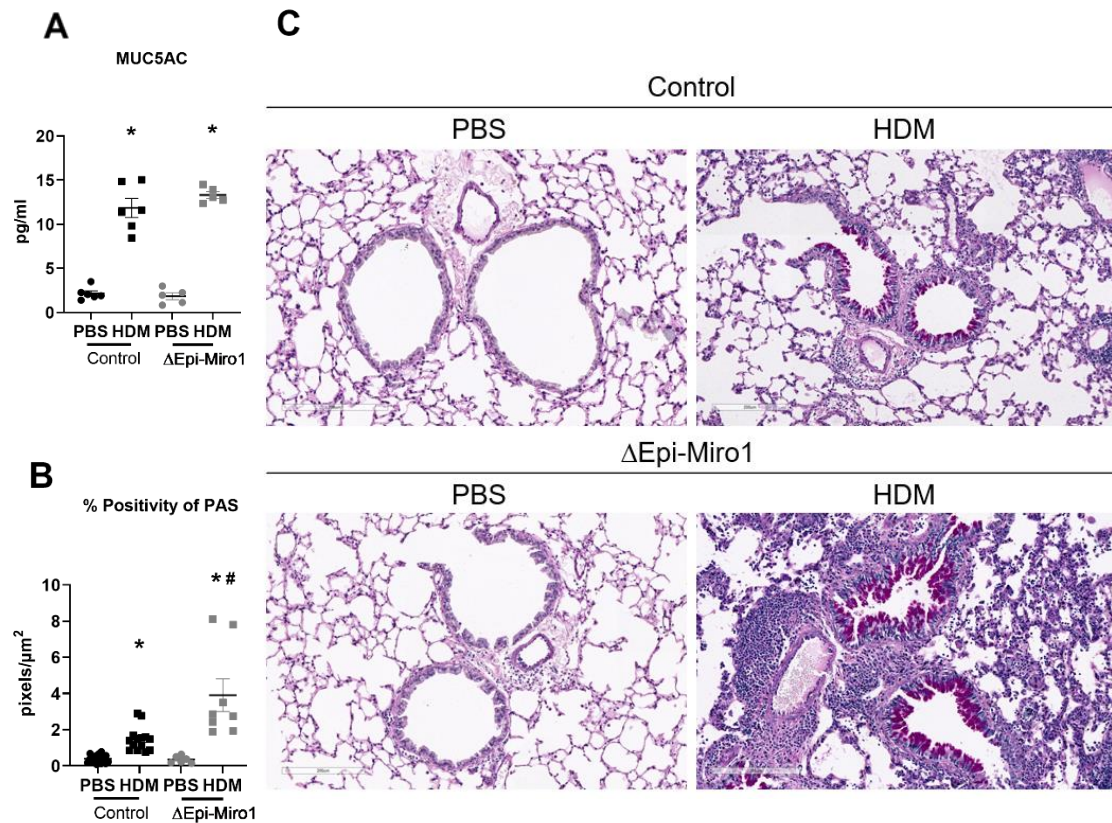


(A) ELISAs of pro-inflammatory cytokines in the whole lung lysates, n = 5 – 6 mice per group from one experiment. (B) ELISAs of pro-inflammatory chemokines in the whole lung lysates, n = 5 – 6 mice per group from one experiment. (C) ELISAs of Th2 adaptive cytokines in the whole lung lysates, n = 5 – 6 mice per group from one experiment. (A-C) * p-value < 0.05 vs. corresponding PBS group, # p-value < 0.05 vs. Control HDM group. Error bars represent mean \pm SEM. Data collected in collaboration with Sierra Bruno, PhD from the Anathy Lab at the University of Vermont.

2.3.2. Conditional Deletion of Miro1 *In Vivo* Augments Mucus Metaplasia

Mucus metaplasia is a hallmark of allergen-induced asthma and an indicator of severity [231-233]. Thus, we examined mucin secretion into the BALF in the lungs after Miro1 deletion from the airway epithelium via ELISA. There were no statistically significant differences in the MUC5AC secretion between Δ Epi-Miro1 mice and control mice exposed to HDM in the BALF (**Figure 9A**). To assess mucus levels histologically, lung tissue was stained using the PAS stain. Intriguingly, mucus staining was significantly increased in the airways of Δ Epi-Miro1 challenged with HDM when compared to the HDM-challenged control mice (**Figure 9B**). These data suggest that epithelial Miro1 may regulate mucin secretions, further suppressing severity of the allergic response to HDM.

Figure 9. Conditional deletion of Miro1 from airway epithelial cells enhances mucus metaplasia following HDM exposure.

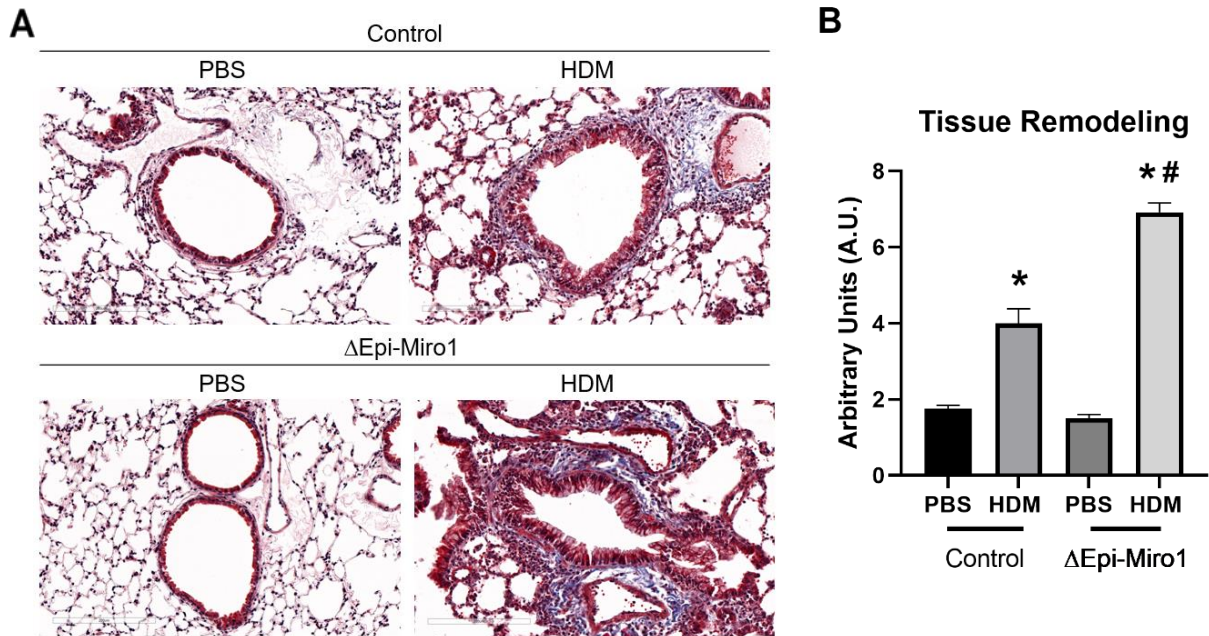


(A) ELISA of MUC5AC in the BALF, $n = 5 - 6$ mice per group from one experiment. (B) Quantification of PAS staining, $n = 3 - 6$ mice per group from one experiment. (C) Representative images of PAS staining in lung tissue sections from one experiment. (A-B) One-way ANOVA, * p -value < 0.05 vs. corresponding PBS group, # p -value < 0.05 vs. Control HDM group. Error bars represent mean \pm SEM. (C) Scale bars are 200 μ m. MUC5AC data collected in collaboration with Sierra Bruno, PhD from the Anathy Lab at the University of Vermont.

2.3.3. Conditional Deletion of Miro1 *In Vivo* Enhances Tissue Remodeling

To assess airway tissue remodeling, changes such as collagen deposition, immune cell infiltration, epithelial layer thickening, and alveolar space size in the lung tissue were examined using the Masson's trichrome tissue stain. There were marked remodeling changes observed, as indicated by higher AU scores, in the lung tissue of Δ Epi-Miro1 mice challenged with HDM, when compared to control mice challenged with HDM (**Figure 10**). These data suggest that Miro1 might help prevent severe remodeling changes in the airway epithelium following allergen exposure.

Figure 10. Conditional deletion of Miro1 from airway epithelial cells enhances tissue remodeling following HDM exposure.



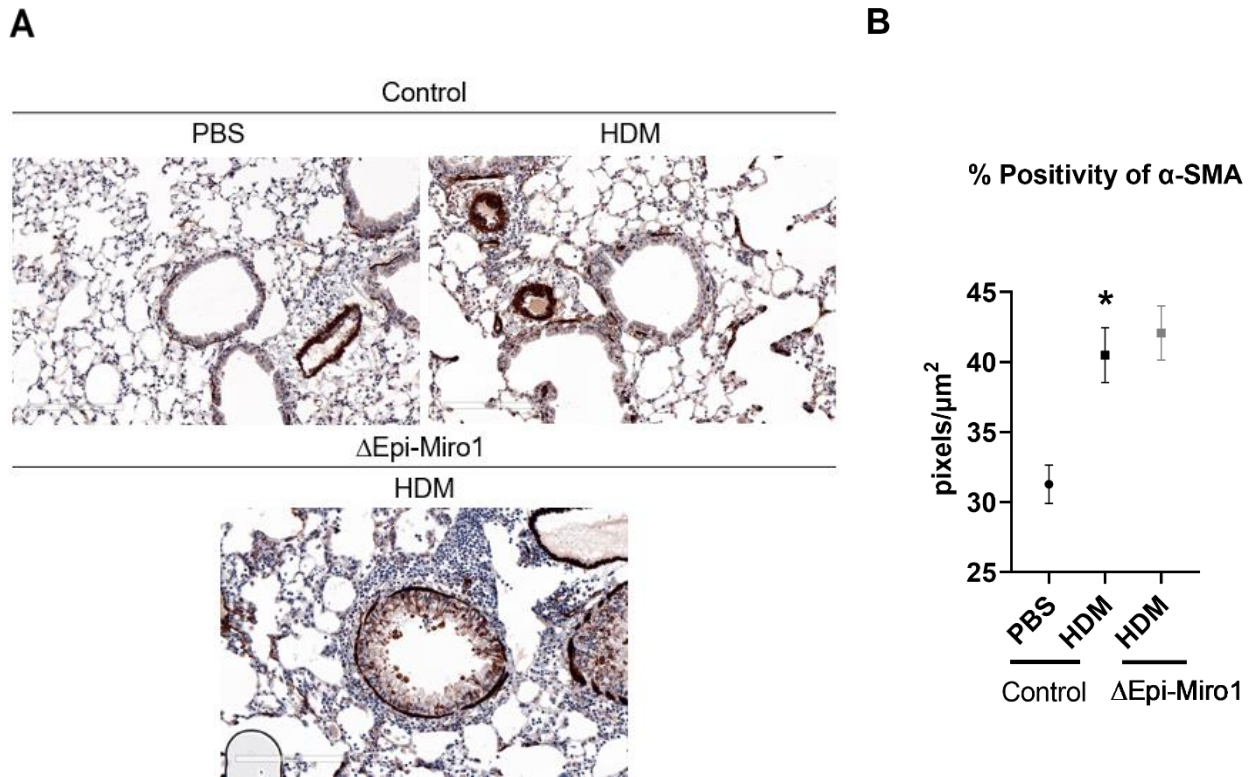
(A) Representative images of Masson's trichrome staining in lung tissue sections, n = 3 – 6 from one experiment. Scale bars are 200 μ m. (B) Quantification of Masson's trichrome

staining. The arbitrary unit scoring scale assessed changes in remodeling including collagen deposition, immune cell infiltration, epithelial layer thickening, and alveolar space changes. Lower scores signified little to no remodeling changes and higher scores signified increased remodeling changes. Error bars represent mean \pm SEM. One-way ANOVA, * p-value < 0.05 vs. corresponding PBS group, # p-value < 0.05 vs. Control HDM group.

2.3.4. Conditional Deletion of Miro1 *In Vivo* Augments Airway Smooth Muscle Levels

To assess airway smooth muscle changes, lung tissue was stained for α -smooth muscle actin via immunohistochemistry. Although there were no statistically significant differences between α -smooth muscle actin levels in the lung tissue of Δ Epi-Miro1 mice and control mice exposed to HDM, a marked phenotypic difference can be seen in the lung tissue of Δ Epi-Miro1 mice and control mice exposed to HDM (**Figure 11**).

Figure 11. Conditional deletion of Miro1 from airway epithelial cells increases smooth muscle levels following HDM exposure.



(A) Representative images of α -smooth muscle actin immunohistochemical staining in lung tissue sections, $n = 2$ mice per group from one experiment. Scale bars are $200 \mu\text{m}$.

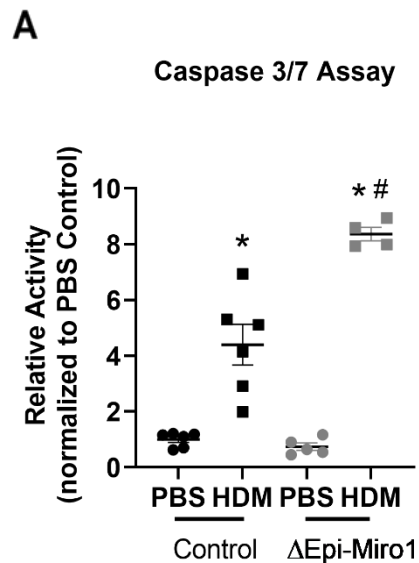
(B) Quantification of α -smooth muscle actin staining. Data points shown are the mean of $n = 79 - 111$ airways from each mice per group. Error bars represent mean \pm SEM. One-way ANOVA, * p -value < 0.05 vs. corresponding PBS group.

2.3.5. Conditional Deletion of Miro1 *In Vivo* Enhances Epithelial Apoptosis

Activation of the apoptosis regulator, Caspase-3, in the mouse lung tissue was examined using a Caspase Glo assay. A statistically significant increase in the activity of caspases in tissue lysates of Δ Epi-Miro1 mice exposed to HDM mice was observed, when

compared to control mice exposed to HDM (**Figure 12**). These data suggest an increase in apoptosis in the airway epithelium. These data indicate a worsened allergic airway response to HDM after conditional Miro1 deletion with enhanced epithelial cell death.

Figure 12. Conditional deletion of Miro1 from airway epithelial cells enhances epithelial cell death following HDM exposure.

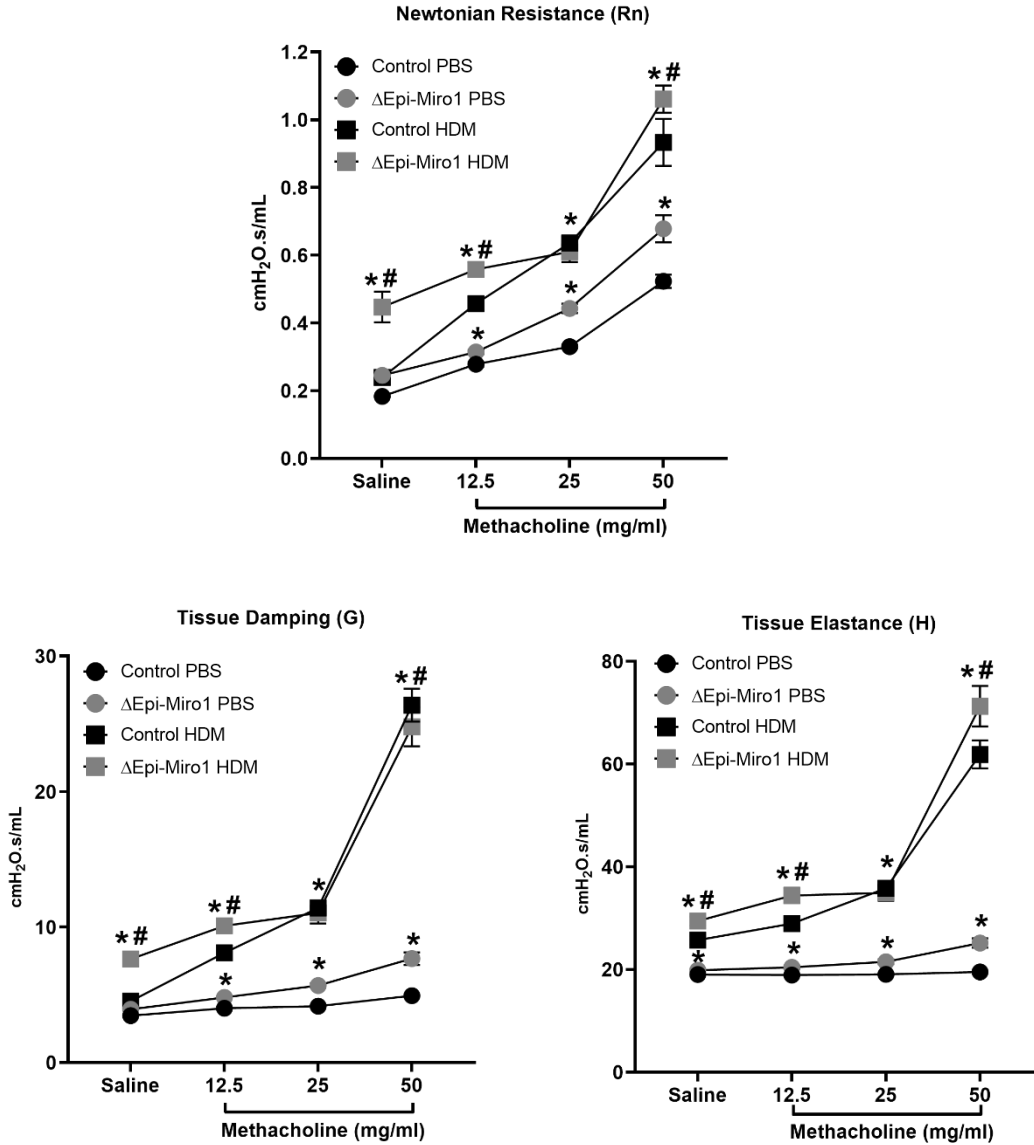


(A) Luminescence activity assay of Caspase 3 and 7 (Caspase Glo Assay) in whole lung lysates, $n = 4 - 6$ mice per group from one experiment. One-way ANOVA, * p -value < 0.05 vs. corresponding PBS group, # p -value vs. Control HDM group. Error bars represent mean \pm SEM. Data collected in collaboration with Sierra Bruno, PhD from the Anathy Lab at the University of Vermont.

2.3.6. Conditional Deletion of Miro1 *In Vivo* Enhances Airway Hyperresponsiveness following Methacholine Challenge

Methacholine-induced airway hyperresponsiveness in an established chronic HDM-induced allergic airway disease model was assessed. Δ Epi-Miro1 mice exposed to both PBS and HDM show slightly increased, but significant, Newtonian resistance and tissue dampening and elastance (**Figure 13**). These alterations in the AHR parameters, together with an increase in inflammation, mucus secretions, and smooth muscle, are suggestive of increased airway constriction and collapse following exposure to HDM after Miro1 deletion, leading to worsened breathing.

Figure 13. Conditional deletion of Miro1 from airway epithelium enhances airway hyperresponsiveness in mouse lungs following chronic exposure to HDM.



Results from airway hyperresponsiveness initiation experiment, $n = 6$ mice per Δ Epi-Miro1 HDM group and $n = 4$ for all other experimental groups from one experiment. * p-value 0.05 vs corresponding PBS group, # p-value 0.05 vs Control HDM group. Error bars represent mean \pm SEM.

2.4. Discussion

The results from this study show that deletion of Miro1 from club cells leads to an increased inflammatory cell influx and enhanced secretion of pro-inflammatory cytokines from epithelial cells, as well as the activation of pro-apoptotic markers following chronic HDM exposure. Miro1 epithelial deletion also led to augmented mucus metaplasia and pronounced remodeling in the airways. Finally, Miro1 deletion altered lung mechanics following chronic allergen insult. The role of Miro1-mediated mitochondrial trafficking upon exposure to a complex allergen in airway epithelial cells, and their associated inflammatory responses, had not been demonstrated in previous literature. Our results provide compelling evidence for the role of Miro1 in airway epithelial cells by mediating inflammatory responses associated with HDM exposure. However, the mechanistic details underlying the observed phenotype remains unclear.

Chronic inflammation of the lungs caused by aeroallergens such as HDM is a hallmark of the pathophysiology associated with allergen-induced asthma [234]. In this study, Miro1 was conditionally deleted from CCSP positive airway epithelial cells in C57B/L6 mice. CCSP is predominantly expressed in club cells, a non-ciliated secretory epithelial cell subtype that is ubiquitously expressed in murine lungs [58, 235]. Δ Epi-Miro1 and control mice were sensitized, boosted, and exposed for 5 consecutive days in a chronic model of allergen-induced inflammation to determine the role of Miro1 in HDM-induced lung inflammation. Our results show that epithelial deletion of Miro1 leads to an inflammatory cell influx in the BALF, especially lymphocytes, as well as the increase of pro-inflammatory mediators such as CCL20 and eotaxin in the whole tissue lysate following HDM exposure. It is possible that the increase of CCL20, a lymphocyte and

dendritic cell chemoattractant, and eotaxin, an eosinophil chemoattractant, leads to the striking increase in inflammatory cells following Miro1 deletion in the lung tissue. Specialized staining on the lung tissue would be beneficial to determine the inflammatory cell profile in the observed sub-epithelial inflammation. In agreement with our work, another study demonstrated that epithelial deletion of Miro1 leads to heightened pro-inflammatory responses following exposure to cigarette smoke [220]. However, the inflammatory response in allergen-induced asthma is characterized by eosinophilia associated with an increased number of T lymphocytes and mast cells. CD4+ T lymphocytes regulate chronic inflammation in asthma via the release of Th2 adaptive cytokines [236, 237], which can lead to the observed changes in airway hyperresponsiveness, mucus production, and airway remodeling. Moreover, Miro1 plays a critical role in signaling for mitophagy and interacting with the Pink1/Parkin mitochondrial quality control system [238, 239], and compromised degradation of Parkin has been shown to promote inflammation and the release of mtDNA [209]. However, mitophagy can activate apoptotic signaling pathways [240-242], an observation we made via the increase in the activity of caspases 3/7. Although we see non-significant increases in the pro-inflammatory cytokines, Th2 adaptive cytokines, and inflammatory cell types *in situ*, these should be measured in the serum to assess global changes of these markers. Our data suggest that Miro1 regulates inflammatory responses, primarily lymphocyte recruitment, following HDM exposure.

Airway remodeling has long been considered a cardinal feature of asthma that results from longstanding inflammation and can lead to airway hyperresponsiveness [243]. Structural changes within the airway wall such as epithelial membrane thickening,

hypertrophy of smooth muscle cells, and peribronchial fibrosis result in the pathology associated with asthma [244]. In this study, Miro1 deletion from epithelial cells led to prominent airway remodeling changes resulting in increased collagen deposition, heightened immune cell infiltration, and epithelial layer thickening, as demonstrated via Masson's trichrome staining. Miro1 deletion also led to increased smooth muscle surrounding the perimetry of the airways, as shown through immunohistochemical staining for α -smooth muscle actin. The observed increase in eotaxin in the whole tissue lysate could be attributed to the increase in airway smooth muscle following Miro1 deletion, as airway smooth muscle has been shown to produce eotaxin and recruit eosinophils from systemic circulation [245]. Moreover, other studies suggest that altered calcium homeostasis that leads to increased mitochondrial biogenesis results in increased bronchial smooth muscle mass [206]. It is possible that lack of appropriate mitochondrial positioning following Miro1 deletion could lead to calcium level dysregulation and increased airway smooth muscle. Together with our inflammatory cell profiles, these data are suggestive that Miro1 helps attenuate inflammation-associated remodeling changes.

Mucus is primarily produced and secreted by goblet cells, a specific subset of airway epithelial cells [51, 246]. Goblet cell hyperplasia and metaplasia has been associated with asthma severity [99, 232], with mucus plugs being the primary cause of death in asthma due to asphyxiation from intraluminal airway obstruction [233]. Our data suggest that club cell specific deletion of Miro1 leads to augmented mucus metaplasia as shown through the accumulation of mucus following exposure to HDM in the airway epithelium. However, there were no increases in mucin protein levels in the BALF after HDM exposure. Therefore, we hypothesize that mucins may remain in the secretory

vesicles and not secreted into the intraluminal space following Miro1 deletion. Studies have shown that ATP is required to initiate a signaling cascade that results in a Ca²⁺-triggered fusion of the mucin granules to the membranes [227-230]. Recent literature examining the role of Miro1 during alveolar formation observed a similar phenomenon where loss of epithelial Miro1 compromised the release of platelet-derived growth factor [247]. We suspect that Miro1 expression leading to the appropriate positioning of mitochondria in lung epithelial cells may be necessary for the secretion of mucins from airway epithelial cells, preventing more severe asthma phenotypes. However, it is unclear whether Miro1 deletion from club cells changes the functionality of mucus producing cells if transdifferentiated, as murine asthma models have shown a dramatic shift in cell phenotypes in the epithelium resulting from club cell differentiation to mucus cells [248].

The functional consequence of asthma is reversible airflow limitation [249]. Airway inflammation alone may cause airflow limitation or through inflammatory mediators that act directly on airway smooth muscle [250]. Enhanced tissue remodeling and mucus hyperplasia have been associated with increased airway hyperresponsiveness [251, 252]. Our data show that Miro1 deletion from epithelial cells leads to changes in airway mechanics independent of HDM exposure, suggesting intrinsic alterations within the epithelium. The observed changes in airway hyperresponsiveness in this study could be attributed to the heightened immune cell infiltration, mucus obstruction as a result of goblet cell metaplasia, and enhanced smooth muscle levels and remodeling changes.

Results from this study suggest that Miro1-mediated mitochondrial trafficking plays a role in the regulation of pro-inflammatory responses in the airway epithelium. Deletion of Miro1 was shown to augment chronic HDM-induced inflammatory responses

in the lungs, associated with pronounced inflammatory cell infiltration and remodeling changes in the mouse lungs leading to altered lung mechanics. These results indicate a possible role for Miro1 in the development and progression of inflammatory responses and provide insights for the role of Miro1 in allergic airway diseases. Additional studies should be conducted to elucidate the mechanisms leading to disease. Altogether, these findings might have implications for the pharmacological targeting of Miro1 for the management and treatment of allergic airway diseases.

CHAPTER 3. DISCUSSION AND FUTURE DIRECTION

The goal of this study was to investigate the effects Miro1-mediated mitochondrial trafficking in allergen-induced inflammation and provide a comprehensive phenotypic readout. The results of this study show that epithelial deletion of Miro1 leads to a modest heightened inflammatory response, enhanced mucus metaplasia, pronounced tissue remodeling and increased smooth muscle levels that result in increased airway hyperresponsiveness following chronic exposure to HDM. Altogether, our results indicate a possible role for Miro1 in the development and progression of allergic asthma and provide insights into the role of Miro1-mediated mitochondrial positioning in asthma severity.

Our results show that Miro1 deletion from the epithelium results in a modest increase of lymphocytes in the BALF following exposure to HDM. Likewise, an increase in sub-epithelial inflammation was observed in the tissue. Specialized tissue staining should be done to determine if the cell profiles present in the tissue correlate with the cell populations seen in the BALF. Histological confirmation of lymphocyte populations could be determined through CD45 staining of the lung tissue. Moreover, staining for specific epithelial cell markers should also be conducted to assess if there are any changes in the airway epithelial cell subtypes present before and after Miro1 deletion and exposure to HDM. Staining tissue for KRT5, MUC5AC, β -tubulin IV, and CC10 for basal cells, goblet cells, ciliated cells, and club cells – respectively – could provide information about the role of Miro1 in cell trans-differentiation following HDM exposure.

Furthermore, signs of epithelial barrier dysfunction, mainly pronounced

invaginations stemming from the apical to the basolateral side of the epithelium, were noted in the tissue of mice lacking Miro1 independent of HDM exposure. These pronounced invaginations in the tissue could be due to disrupted adherens junctions, which are responsible for cell-cell adhesions. E-cadherin, a Ca^{2+} -dependent adhesion molecule, is the primary adherens junction protein in the airways. Furthermore, E-cadherin is critical for epithelial barrier formation and for maintaining the apical-basolateral polarization and adhesion to neighboring cells [81]. Evidence suggests that E-cadherin is disrupted in human pulmonary epithelial cell lines following HDM exposure via the activation of PAR1 and/or PAR2 [253]. Other studies have shown that E-cadherin deficient murine airways had an increased expression of α -smooth muscle actin, as well as the progressive loss of airway epithelial cells, spontaneous mucus hypersecretion, and eosinophilic airway inflammation [254]. We suspect that E-cadherin levels would be decreased in mice lacking Miro1 exposed to HDM and that inappropriate mitochondrial positioning following Miro1 deletion could alter the local calcium gradients leading to the pronounced invaginations observed in the tissue. Moreover, proteins like CCSP, which are abundant proteins in the extracellular lining fluid of the airways [255], could be measured in the serum to see if there is any infiltration into systemic circulation as an indirect measure of barrier dysfunction.

Our data also shows that Miro1 deletion from airway epithelial cells results in subtle changes in lung mechanics that are suggestive of increased airway hyperresponsiveness. These data show that there is a change in the lung tissue independent of HDM exposure following Miro1 deletion. Unlike our results, a study by Sharma *et al.* [220] suggests that epithelial deletion of Miro1 from airway epithelial cells

led to changes in the mechanical properties in mice lacking Miro1 following cigarette smoke exposure, as well as no significant airspace enlargement. Although our Flexivent experiments provide valuable data regarding airway mechanics following Miro1 deletion, other parameters such as respiratory capacity should be measured to assess any changes in lung volumes. A study by Nguyen *et al.* [185] showed that global deletion of Miro1 in mice caused postnatal lethality associated with unexpanded lungs, with post-mortem examination of the lung tissue revealing a lack of alveoli expansion. Similarly, a recent study by Zhang *et al.* [247] suggests that epithelial deletion of Miro1 results in the disruption of alveolar formation. As alveolar numbers have been associated with total lung volumes [256], we speculate that Miro1 deletion could result in reduced respiratory capacity that is further altered following HDM exposure. However, it remains unknown whether epithelial deletion of Miro1 affects lung volumes and the respiratory capacity of mice following HDM exposure.

Moreover, given the severe phenotypes observed in our mouse model following Miro1 deletion, it would be of interest to assess the expression of Miro1 in populations of mild, moderate, and severe asthmatics to see if there are any correlations between Miro1 expression and disease severity. We suspect that Miro1 expression levels will be diminished in those with more severe asthma. However, confirmation using cell cultures should be done to see if Miro1 levels are reduced in our model. A study by Sundar *et al.* [219] showed that Miro1 levels are significantly reduced in primary human lung epithelial cells following chronic cigarette smoke exposure. Other studies using mesenchymal stem cells have shown that overexpression of Miro1 leads to more efficient mitochondrial transfer to epithelial cells, as well as epithelial tissue repair when

compared to wild-type mesenchymal stem cells. Conversely, Miro1 knocked-out mesenchymal cells lose their mitochondrial transfer capacity, and, as a result, lose their healing abilities. Moreover, Ahmad *et al.* also demonstrated that overexpression of Miro1 in mesenchymal stem cells was linked to better therapeutic efficacy, as suggested by reversal of airway hyperresponsiveness and remodeling in three separate models of allergen-induced asthma [257].

Finally, screening for Miro1 mutations in severe asthmatics and other asthma endotypes could also provide additional information about the role of Miro1 in the development of asthma. A study by Grossmann *et al.* [193] showed heterozygous mutations in the gene encoding for Miro1, *RHOT1*, in four individuals with Parkinson's disease. These mutations were located in highly conserved protein domains of Miro1. The authors suggest that because of their position, calcium sensing and binding, GTP hydrolysis, and localization features of Miro1 could be affected.

Altogether, our findings support a role for Miro1 in the exacerbation of inflammatory responses and tissue reorganization in the context of allergic airway diseases. Therefore, continued evaluation into the possible mutations to the Miro1 gene and altered expression of the Miro1 protein in asthmatics is warranted in asthmatics. Additionally, investigation into the regulation of the Miro1 gene via chromatin modifications and transcription factor activation in the context of allergic asthma may inform us of upstream control of Miro1 expression. In conclusion, Miro1 expression in lung epithelial cells mediates inflammatory responses and tissue reorganization in a mouse model of allergic asthma providing strong data and rationale for further investigation into Miro1 and mitochondrial dynamics in allergic airway diseases.

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