Allergen-Induced Asthma is Decreased in Decorin-Deficient Mice

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“No man is an island, entire of itself” – John Donne

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ABSTRACT

Decorin, is an extracellular matrix proteoglycan with important biological functions. Decorin deficiency affects collagen fibrillogensis, airway mechanics, airway-parenchymal interdependence, and airway smooth muscle proliferation and apoptosis. We questioned whether decorin deficiency would alter allergen-induced asthma in a mouse model. Decorin⁻/⁻ and decorin⁺/⁺ mice (C57Bl/6) were sensitized and challenged with ovalbumin. Control animals received saline. Responsiveness was assessed at baseline and after delivery of increasing concentrations of methacholine. Histological analyses were also performed. Decorin deficiency resulted in more modest hyperresponsiveness. Respiratory resistance and elastance along with tissue damping and tissue elastance, were increased in ovalbumin decorin⁺/⁺ and decorin⁻/⁻, but more so in decorin⁺/⁺. Airway resistance was increased in ovalbumin decorin⁺/⁺ only. Inflammation and collagen staining within the airway wall, were increased in ovalbumin decorin⁺/⁺ mice only; whereas biglycan was significantly increased in ovalbumin decorin⁻/⁻ mice only. These results reflect the role of decorin in the development of allergen-induced asthma.
ABRÉGÉ

Le décorine est un protéoglycane présent dans la matrice extracellulaire avec des fonctions biologiques importantes. La déficience en décorine affecte la formation des fibrilles de collagènes, la mécanique respiratoire, l'interdépendance des voies respiratoires envers le parenchyme, ainsi que la prolifération cellulaire et l'apoptose des muscles lisses. Nous nous sommes demandé si la déficience en décorine modifiait le développement de l'asthme induit par un allergène dans un modèle expérimental murin. Des souris décorine−/− et décorine+/- ont été sensibilisées et exposées à l'ovalbumine en guise d'allergène. L'hypersensibilité des voies respiratoires fut étudiée en réponse à la métacholine et des analyses histologiques furent effectuées. La déficience en décorine engendre une sensibilité des voies respiratoires plus modeste que chez les souris décorine+/- . L'inflammation et la présence de collagène étaient aussi amplifiées dans les souris décorine+/- . D'autre part, biglycane était amplifiée avec l'ovalbumine dans les souris décorine−/− . Ces résultats reflètent le rôle de décorine dans le développement d'asthme par l'ovalbumine.
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<th>Description</th>
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<tbody>
<tr>
<td>AF</td>
<td>Asthmatic fibroblasts</td>
</tr>
<tr>
<td>AHR</td>
<td>Airway hyperresponsiveness</td>
</tr>
<tr>
<td>Alum</td>
<td>Aluminum hydroxide</td>
</tr>
<tr>
<td>ASM</td>
<td>Airway smooth muscle</td>
</tr>
<tr>
<td>α-SMA</td>
<td>Alpha-smooth muscle actin</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>Bgn</td>
<td>Biglycan</td>
</tr>
<tr>
<td>BM</td>
<td>Basement membrane</td>
</tr>
<tr>
<td>C&lt;sub&gt;RS&lt;/sub&gt;</td>
<td>Respiratory system compliance</td>
</tr>
<tr>
<td>CS</td>
<td>Chondroitin sulfate</td>
</tr>
<tr>
<td>Cys-LT</td>
<td>Cysteiny-Leukotrienes</td>
</tr>
<tr>
<td>D2</td>
<td>Maximum internal diameter</td>
</tr>
<tr>
<td>D1</td>
<td>Minimum internal diameter</td>
</tr>
<tr>
<td>Dcn</td>
<td>Decorin</td>
</tr>
<tr>
<td>Dcn&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Decorin replete</td>
</tr>
<tr>
<td>Dcn&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Decorin deficient</td>
</tr>
<tr>
<td>DS</td>
<td>Dermatin sulfate</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>E&lt;sub&gt;RS&lt;/sub&gt;</td>
<td>Respiratory system elastance</td>
</tr>
<tr>
<td>FB</td>
<td>Fibroblasts</td>
</tr>
<tr>
<td>FEV&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Forced expiratory volume in 1 second</td>
</tr>
<tr>
<td>FRC</td>
<td>Functional residual capacity</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycans</td>
</tr>
<tr>
<td>G&lt;sub&gt;ti&lt;/sub&gt;</td>
<td>Tissue damping/ tissue resistance</td>
</tr>
<tr>
<td>HASMC</td>
<td>Human airway smooth muscle cells</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
</tr>
<tr>
<td>HS</td>
<td>Heparan sulfate</td>
</tr>
<tr>
<td>H&lt;sub&gt;ti&lt;/sub&gt;</td>
<td>Tissue elastance</td>
</tr>
<tr>
<td>IgE</td>
<td>Immunoglobulin E</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>KS</td>
<td>Keratin sulfate</td>
</tr>
<tr>
<td>LAR</td>
<td>Late airway response</td>
</tr>
<tr>
<td>LR</td>
<td>Lamina reticularis</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucine-rich repeats</td>
</tr>
<tr>
<td>MBP</td>
<td>Major basic protein</td>
</tr>
<tr>
<td>MCh</td>
<td>Methacholine</td>
</tr>
<tr>
<td>MFB</td>
<td>Myofibroblasts</td>
</tr>
<tr>
<td>NF</td>
<td>Normal fibroblasts</td>
</tr>
<tr>
<td>OA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>PAS</td>
<td>Periodic acid Schiff</td>
</tr>
<tr>
<td>P&lt;sub&gt;bm&lt;/sub&gt;</td>
<td>Basement membrane perimeter</td>
</tr>
<tr>
<td>PC&lt;sub&gt;20&lt;/sub&gt;</td>
<td>Provocative concentration causing 20% decrease in FEV&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEEP</td>
<td>Positive-end expiratory pressure</td>
</tr>
<tr>
<td>PG</td>
<td>Proteoglycans</td>
</tr>
<tr>
<td>P(t)</td>
<td>Pressure</td>
</tr>
<tr>
<td>P-V</td>
<td>Pressure-Volume</td>
</tr>
<tr>
<td>R&lt;sub&gt;aw&lt;/sub&gt;</td>
<td>Airway resistance</td>
</tr>
<tr>
<td>R&lt;sub&gt;RS&lt;/sub&gt;</td>
<td>Respiratory system resistance</td>
</tr>
<tr>
<td>Sal</td>
<td>Saline</td>
</tr>
<tr>
<td>SLRP</td>
<td>Small leucine-rich proteoglycans</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>T&lt;sub&gt;H&lt;/sub&gt;2</td>
<td>T-Helper cells type 2</td>
</tr>
<tr>
<td>TLC</td>
<td>Total lung capacity</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>V(t)</td>
<td>Volume</td>
</tr>
<tr>
<td>V(t)</td>
<td>Flow</td>
</tr>
</tbody>
</table>
WA  Wall area
WT  Wild type
$Z_{in}/Z_{RS}$  Input Impedance/ Impedance of the respiratory system
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Chapter 1: INTRODUCTION
Chapter 1: Introduction

1.1 Asthma defined

The allergic asthma response, has increased in incidence in the western world\(^1\). Asthma is defined as an inflammatory lung disease, which is distinguished by specific features. Namely, asthma is characterized by episodes of airway obstruction, which are usually reversible, airway inflammation as well as airway hyperresponsiveness (AHR)\(^2\). Initially, asthma had been considered a reversible disease, in that the airway obstruction would abate over time or could be reversed by specific drugs; such as glucocorticoids, \(\beta\)-agonists, and leukotriene inhibitors. Recently, the structural changes, collectively termed ‘airway remodeling’, that occur in the airways as a consequence of asthma, have been given more importance in the pathogenesis of this disease\(^3\).

The development of asthma can be due to many factors, however, it is most likely to originate in genetically susceptible individuals as a result of the immune system reacting inappropriately to antigens\(^4\). Nevertheless, asthma itself is a very complex heterogeneous disease due to the multiple gene-environment interactions that may give rise to its characteristic features and respective symptoms\(^5\). Due to the variability of this disease, different individuals with asthma may experience diverse symptoms. However, the most common clinical symptoms associated with asthma include shortness of breath, wheezing, and the occurrence of asthmatic attacks which all contribute to breathing difficulty\(^6\).

Numerous studies have been directed at categorizing this disease and establishing clear mechanisms responsible for asthma pathogenesis. Clinical work, animal models, and in vitro research have all provided important pieces of information resulting in a greater understanding of this disease. It is through these important scientific advances, that more effective patient therapies or cures may be developed. Thus, it is crucial to identify the factors which play significant roles in the initiation, development and exacerbation of disease. In particular, considerable efforts have been directed towards clarifying the role of extracellular matrix (ECM) remodeling in the pathogenesis of asthma. Accordingly, the objective of this study was to evaluate the role of decorin (Dcn), an ECM proteoglycan (PG), in the development of allergen-induced asthma using animal
models of disease. In order to fully investigate the potential role of this PG in the development and progression of asthma however, it is important to understand the characteristic features of this disease and their functional significance in the clinical setting.

1.2 Inflammation

The inflammatory process present in asthma is a principal event in the pathogenesis of this disease, and is thought to play a significant role in its progression as well. The airways of asthmatics show increased immunoglobulin E (IgE) levels, increased levels of lymphocytes and mast cells, as well as eosinophilic inflammation\(^4\,7\). Various findings have confirmed the role of inflammation in promoting AHR, and have also suggested the possible implication of this process in initiating the remodeling response\(^2\,8\). Both AHR and airway remodeling will be discussed in further detail in subsequent sections.

There are numerous factors which contribute to the inflammatory response observed in asthmatics. The bone marrow as well as the vascular, lymphoid and nervous systems may all play a part in initiating and developing inflammation. Moreover, studies have proposed different pathways involved in the generation of airway inflammation. One particular pathway describes ‘allergic inflammation’ which is distinguished by a T\(_{\text{H}2}\)-mediated immune reaction characterized by increased IgE levels\(^8\). This pathway has been well established as a mediator in allergic diseases such as asthma. Research has suggested that there is a shift in the immune response which favours a T\(_{\text{H}2}\) phenotype and thus leads to an increase in T\(_{\text{H}2}\)-lymphocytes as well as an increase in the associated cytokines. CD\(_{4^+}\) T-cells are thought to play an important role in asthma as they are the cells that secrete T\(_{\text{H}2}\) cytokines (Interleukin (IL)-4, IL-5, and IL-13), which are responsible for the disease pathogenesis\(^9\). These cytokines influence the coordination of various events, resulting in the recruitment and activation of effector cells as well as many other contributing cells that initiate the allergic response. Although many studies have demonstrated an importance of IL-4 and IL-5 in IgE synthesis and eosinophilic inflammation respectively, recent work has suggested that these two cytokines are not essential in the asthmatic response. Alternatively, the critical importance of IL-13 in the
pathogenesis of asthma has been demonstrated and confirmed\textsuperscript{4}. The effects of IL-13 lead to the inflammation seen in allergic asthma, and furthermore can contribute to asthma pathogenesis by generating alterations in the airway epithelium\textsuperscript{10}.

1.2.1 Mast cells

There are a variety of other cells involved in promoting airway inflammation in asthmatics. Mast cells, for example, have an important role in allergic asthma. More specifically, mast cells, which are bound through their high-affinity receptors to IgE antibodies, will degranulate when these antibodies cross-link with the allergen. This will promote the release of pro-inflammatory mediators, such as histamine, proteases such as tryptase, as well as the production of prostaglandins, leukotrienes, and platelet-activating factor. In addition, release of these mediators will consequently result in increased mucus secretion, contraction of smooth muscle, and vascular permeability\textsuperscript{8}.

1.2.2 Eosinophils

The presence of eosinophils within the airways is one of the foremost features of asthma. In a study by Hamid et al.\textsuperscript{11}, lung tissue that was obtained from asthmatic patients demonstrated significant increases in inflammation, including both T-cells and activated eosinophils, when compared to non-asthmatic controls. Studies have also confirmed the presence of eosinophils in bronchoalveolar lavage (BAL) fluid and sputum samples of patients with varying severity of asthma\textsuperscript{5}. Eosinophils are effector cells which are regulated by various T\textsubscript{H}2 cytokines including, IL-13, IL-4 and IL-5\textsuperscript{2}. They have been shown to contain eosinophil cationic protein, eosinophil peroxidase, and major basic protein (MBP), and have demonstrated the ability to produce cysteinyi leukotrienes (cys-LTs) and eicosanoids. In addition, eosinophils release multiple cytokines, chemokines as well as superoxide which may have damaging effects to surrounding tissues (see figure 1)\textsuperscript{5,12}. Through the production of these numerous factors, eosinophils have the ability to bring about changes in the contractility of smooth muscle, and thus AHR, as well as influence different aspects of remodeling\textsuperscript{2}. A study by Lee and colleagues\textsuperscript{13} has effectively demonstrated the significant role of eosinophils in asthma pathology. In particular, their group developed a mouse line which is devoid of eosinophils, identified
as PHIL mice. In order to determine a contributing effect of eosinophils in allergen-induced asthma, PHIL mice were exposed to an allergen sensitization and challenge protocol. Exposure to ovalbumin (OA) resulted in a significant increase in airway and lung tissue eosinophilia in wild type (WT) mice only. Moreover, data from this work provided evidence for reduced airway pathology in OA-exposed PHIL mice. More specifically, OA-treated PHIL mice had decreased hypertrophy of the airway epithelium as well as decreased goblet cell and mucus production. An association was also observed between eosinophils and lung function, which was assessed during a methacholine (MCh)-response curve in both OA and control, PHIL and WT mice. Analysis of airway resistance measures revealed that OA-treated PHIL mice did not exhibit AHR, whereas OA-exposed WT mice did. Findings from this experiment thus provide convincing evidence for the causative role of eosinophils in asthma symptoms and pathology. There are however, conflicting results which question the role of these inflammatory cells in relation to lung function in asthma. In particular, clinical studies which employed anti-IL-5 antibodies, resulted in decreased eosinophil levels in the blood of mild asthmatics, but did not have any therapeutic effect on airway hyper-responsiveness or the late asthmatic response. An additional study by Flood-Page et al., however, demonstrated that despite the reduction of eosinophils in sputum and blood of mild asthmatics treated with anti-IL-5 therapy, airway eosinophilia was not completely abolished. In addition, tissue staining for MBP did not reveal any significant changes when comparing placebo and anti-IL-5 treated groups. The authors offer several suggestions to explain this observation, one which points to the ability of IL-3 and GM-CSF, which are both increased in asthma, in maintaining airway eosinophilia in the absence of IL-5. Therefore, the role of eosinophils in promoting airway disease cannot be completely discounted.
Fibrogenic or Growth factors
- TGF-α
- TGF-β
- Angiogenin
- FGF-2
- H3-EGF
- NGF
- PDGF
- VEGF
- SCF

Chemokines
- Eotaxin
- Rantes
- MCP-1, -2, -3, -4
- IL-6, MCP-1α

Lipids
- Cysteinyl leukotrienes
- PAF, PGE₁, PGE₂, 15-H ETE

Basic Granule Proteins
- MBP, EPO, EDN, ECP

Cytokines
- IL-1, 2, 3, 4, 5, 6, 9, 10
- IFN-γ, GM-CSF
- TNF-α

Others
- Reactive oxygen species
- Neuropeptides, MMP-9

**Figure 1:** Eosinophils have the ability to produce and secrete a variety of inflammatory mediators including chemokines, cytokines, basic granule proteins and a number of fibrogenic or growth factors. These mediators may all contribute to tissue damage.

An alternate pathway in the development of lung inflammation in asthma is a process which involves multiple growth factors, enzymes and inflammatory cytokines produced by the damaged epithelial lining or by cells of airways. This process will furthermore result in the development of structural changes or ‘remodeling’ of the lung.

1.2.3 Distribution of inflammation within the lung of asthmatics

The specific location of the inflammatory process within the lung may have important implications in the severity and associated pathology observed in asthma. As a result, numerous investigators have directed their work in order to obtain further information regarding this element. The proximal conducting airways have been demonstrated to be a major site of inflammation; however more recent studies have been able to define the inflammatory process that occurs within the distal airways as well. Hamid and co-workers have eloquently demonstrated an increased presence of MBP-positive eosinophils in both large airways, defined by internal diameters of greater than >2mm, and in distal airways, defined by internal diameters of <2mm, in asthmatic lung sections vs. non-asthmatic controls. Moreover, further analysis from this study reveals more significant inflammation, determined by the number of activated eosinophils, is
present within the distal airways of asthmatics compared to larger airways. Thus, more severe inflammation is thought to occur within the distal lung. These findings lend support to the principle that the distal airways are the main location of airway obstruction in asthma\textsuperscript{11}.

The location of the inflammatory process within specific regions of the airway wall was also evaluated. The area between the epithelium and the smooth muscle layer was defined as the inner wall, whereas the area between the smooth muscle and parenchyma was defined as the outer wall. Although there were more activated eosinophils within the inner wall of asthmatics, there were a greater number of total eosinophils present within the outer wall of these patients\textsuperscript{11}. Both may have functional consequences in terms of the mechanical behavior of the airways themselves. In the first instance, the presence of more activated eosinophils between the epithelium and smooth muscle would result in an increased tissue volume in this area. This would furthermore enhance the effect of a given smooth muscle contraction. Similarly, inflammation present between the smooth muscle and parenchyma, may serve to uncouple the airway from the parenchyma, which normally functions in tethering the airway open and thus providing a load against airway smooth muscle shortening\textsuperscript{11,18}.

1.3 Airway hyperresponsiveness

Airway hyperresponsiveness (AHR) is a very important pathological feature of asthma, which has been shown to be directly correlated with the severity of the disease\textsuperscript{19}. The responsiveness of airways in general, is defined as their ability to narrow in response to constricting agonists. In an asthmatic individual, however, this response is heightened, which is demonstrated by increased airway sensitivity and higher maximal responses to constricting agonists, as well as a steeper slope of the dose-response curve as shown in figure 2. In particular, asthmatic patients display a 20\% decline in 'Forced expiratory volume in 1 second' (FEV\textsubscript{1}) at lower provocative concentrations (PC\textsubscript{20}) of agonists, such as MCh, compared to normal individuals\textsuperscript{19}. Various stimuli, including chemical mediators, exercise, inhalation of cold air and/or environmental exposure to allergens may all directly or indirectly enhance AHR in these asthmatic individuals (for review see \textsuperscript{19}).
Figure 2: The percent change in FEV$_1$ is evaluated during a MCh response curve in patients with moderate and severe asthma, mild asthma, or healthy controls. This graph demonstrates that asthmatics possess increased airway sensitivity, illustrated by a response at a lower threshold (a); increased airway reactivity demonstrated by a larger slope (b); as well as a higher response depicted by the higher change in FEV$_1$ vs. baseline (c)\textsuperscript{19}.

Two different elements of AHR have been described in the literature; an episodic element and a persistent element\textsuperscript{20}, each of which have been causally related to certain disease factors. Studies evaluating the pathogenesis of AHR have alluded to the concept that inflammation present within the airways of asthmatics may determine the episodic component of AHR\textsuperscript{20}. Findings by De Monchy et al.\textsuperscript{21}, have in fact demonstrated that increases in eosinophils within the airways is associated with the allergen-induced late airway response (LAR)\textsuperscript{20,21}. The LAR is defined by airway obstruction which typically recurs 3-8 hours post allergen exposure\textsuperscript{20,22}. In addition, data from animal models, have also confirmed the role of inflammatory cells in eliciting allergen-induced AHR\textsuperscript{23}.

The persistent element of AHR has, on the other hand, been thought to be attributed to the structural changes that occur in the lungs\textsuperscript{19}. The rationale behind this notion stems from evidence showing that persistent AHR seems to be unresponsive to anti-inflammatory therapies. Thus, this component of AHR may be likely associated with disease progression and chronicity\textsuperscript{20}. Although the mechanism of this process is not
entirely clear, the association between thickening of the airway wall, a feature observed in many patients with asthma and changes in AHR, is very interesting\(^\text{20}\).

### 1.4 Airway Remodeling

Airway remodeling is a characteristic feature of asthma that has been recognized as increasingly important. The remodeling aspects of this disease include epithelial detachment, goblet cell hyperplasia, mucus hypersecretion, increases in airway smooth muscle (ASM) mass, expansion of the vascular network in the lungs, as well as subepithelial fibrosis caused by the deposition of collagens, tenascin and laminin. Given the potential physiological consequences that these structural changes may bring about, it has been suggested that the airway remodeling characteristic of asthma determines AHR and moreover, is associated with an accelerated loss of lung function\(^\text{24}\).

A Th\(_2\) type of inflammation, as is the case in allergic asthma, has been suggested to be the trigger that initiates tissue fibrosis \(^\text{3,25}\). In essence, Sime and colleagues\(^\text{26}\) have introduced the idea that when there is an imbalance in the immunologic response that favors a Th\(_2\) cytokine shift, fibrosis will occur. This is also known as the “type 2 cytokine hypothesis of fibrosis”.

#### 1.4.1 Epithelium

A common feature in the asthmatic airways is the detachment of the epithelium\(^\text{27}\). Histological studies have in fact confirmed epithelial shedding as well as damage to this lining\(^\text{28}\). In addition, sputum and BAL analysis of asthmatic patients have shown the presence of an increased number of epithelial cells in these samples\(^\text{28}\). Various findings have revealed an increased ability for epithelial cells originating from asthmatic patients to undergo apoptosis, when compared to normal controls\(^\text{27}\). Interestingly, a study by Jeffery et al.\(^\text{28,29}\) provided evidence for a correlation between the extent of the loss of epithelial lining, as observed in bronchial biopsies, and the degree of hyperreactivity. However, one must be careful in applying these results to different severities of disease.

There are certain potential factors which may contribute to the epithelial damage. They include the mechanical stress experienced by cells during bronchoconstriction as well as the negative effects due to ongoing inflammation\(^\text{27}\). There is also evidence to
suggest that asthmatic epithelial cells are intrinsically different from normal cells as they possess an increased ability for cytokine, growth factor and chemokine production.  

1.4.2 Goblet cell hyperplasia and mucus hypersecretion

A component of the remodeled epithelium in asthmatic airways consists of an increased number of goblet cells as well as an enlargement of mucus glands. Collectively, the increase in number of cells and size of sub-mucosal glands, result in excessive amounts of mucus being produced within the airways, leading to the partial obstruction of airflow. Mucus production is thus, an important contributor to the morbidity and mortality of asthma.

1.4.3 Airway smooth muscle

There have been multiple studies confirming the association between asthma and changes in ASM. The airway narrowing that occurs during an asthmatic attack is governed by the excessive contractility of ASM in these subjects. Several groups have reported evidence that asthmatic ASM is intrinsically different, which accounts for its altered contractile properties.

Moreover, findings have revealed an increased ASM mass present in the airways of asthmatics. There are numerous mechanisms which have been suggested to account for this increase in ASM mass. They include hyperplasia, resulting from increased division or reduced apoptosis of cells; hypertrophy or increased cell size; and migration of cells towards ASM bundles as well as differentiation of mesenchymal cells into smooth muscle-like cells. In addition, the increased mass of ASM cells may in part be due to increased deposition of ECM between and around the muscle fibres. These findings have also been substantiated by animal studies.

The ability of asthmatic ASM cells to proliferate at greater rates may be a consequence of increased mitogenic or inflammatory stimulation present in the asthmatic lungs. Furthermore, alteration in the ECM may also stimulate cells to proliferate. Data from Hirst and colleagues have in fact demonstrated an altered proliferative capacity of human ASM cells cultured on various ECM matrices. Their findings demonstrate that collagen and fibronectin will increase mitogen-stimulated proliferation of ASM cells.
whereas laminin will decrease the same process. These effects were also associated with respective changes in contractile phenotype of the cells. The functional significance of this increased ASM mass, as suggested by Lambert et al., is that a greater degree of ASM shortening is possible, against loads provided by the stiffness and elasticity of the airway wall as well as airway-parenchymal interdependence. Of interest, bronchial biopsy specimens of asthmatic patients have also shown the presence of ASM bundles closer to the epithelium compared to normal controls. Migration of cells towards the airway lumen as well as differentiation of mesenchymal cells may account for this characteristic finding.

### 1.4.4 Vascular changes

Two very important factors in the pathogenesis of asthma include airway remodeling and Th2-mediated airway inflammation. Angiogenesis may play an important role in these two processes. In fact, many investigators have reported increases in vessel number and size, as well as increases in vascular leakage and surface area of the vessels in asthmatic tissues. Many studies have demonstrated significant correlations between the asthma severity and the vascular changes that are seen. Thus, the assumption remains that alterations in the vasculature, which are stimulated by the underlying inflammation, will have a role in the generation of airway obstruction and/or hyperresponsiveness seen in asthma. Vascular Endothelial Growth Factor (VEGF) is in this case, very important, as it exhibits various functions in terms of regulating angiogenesis, and thus vascular permeability. This has subsequently led to the suggestion that VEGF plays a role in generating tissue edema in asthmatics.

### 1.4.5 Subepithelial fibrosis

One of the changes in the airway wall structure that has been consistently demonstrated in asthmatic subjects, and furthermore shown to correlate positively with number of asthma attacks, AHR, as well as fibroblast (FB) and myofibroblast (MFB) number, is the thickened subepithelial layer. More specifically, the lamina reticularis (LR) becomes thickened reaching depths of 10–15 μm in asthmatic conditions, equivalent to a two-fold increase, compared to control, non-diseased airways.
thickened LR consists of various ECM proteins including fibronectin, PGs, as well as a dense condensation of fibrillar type I, III and V collagens\(^3,4,24,44-46\). The structural remodeling occurring in this layer, has been correlated with the reduced distensibility of airways, along with increased limitation of airflow observed in asthma \(^47,48\). The excess deposition of matrix also occurs in other layers of the airway wall such as the smooth muscle layer, as previously mentioned, as well as the adventitia.

1.5 Proteoglycans

One of the principal components of the ECM is PGs. These macromolecules, which have been found to be present throughout the lung, are composed of a protein core as well as glycosaminoglycan (GAG) side chains. Various classes of PGs have been described in the literature and have been classified according to specific properties such as their localization and genetic and structural similarities\(^49\). These classes encompass the basement membrane PGs, such as perlecan and bamacan; large aggregating PGs, including aggrecan and versican; cell surface PGs, such as syndecan and; small leucine-rich PGs (SLRPs), comprising lumican, fibromodulin, biglycan (Bgn) and decorin (Dcn)\(^50,51\). Each PG core protein can also covalently bind different GAG side chains such as heparan sulfate (HS), keratin sulfate (KS), chondroitin sulfate (CS), and dermatin sulfate (DS)\(^50,51\).

The biological importance of these macromolecules, especially in the pulmonary setting, has recently been given further consideration. The lungs’ supporting framework which sustains the load of breathing is composed of collagen fibers, elastic fibers and PGs or “ground substance”\(^49\). PGs have been shown to have multiple biological functions ranging from their roles in lung mechanical properties to regulation of growth factors and cellular pathways\(^50,52,53\). The GAG side chains for example, which are characteristically hydrophilic, impart certain mechanical properties to the matrix itself. Thus, through their ability to attract water, GAGs can in turn modify viscoelasticity and turgor of tissues\(^52,54\). This was supported by the work of Al-Jamal et al.\(^52\), demonstrating that digestion of GAG side chains resulted in an alteration of lung parenchymal strip viscoelastic behavior. Versican, in this regard, is important in affecting the viscoelastic properties of the lung through its ability to control the water content of tissues. This is due to the presence of
multiple GAG chains as well as to its elevated ionic character\textsuperscript{50}. Basement membrane PGs have also been shown to be present in the basal laminae of lung capillaries, where they serve as barriers and delineate discrete functional areas of the lung\textsuperscript{50,55}. In addition, syndecan, a member of the cell surface PGs, has been observed to function as a receptor on the surface of lung epithelial cells, binding various ligands present within the matrix\textsuperscript{50,56}. Recent work in our laboratory has, furthermore, been directed towards the class of SLRP’s and their role in lung pathophysiology.

1.5.1 Small leucine-rich proteoglycans

Biology

The small leucine-rich PGs (SLRPs) subclass is composed of nine PGs divided into three separate classes according to their gene and protein arrangement. All three classes have a characteristic N-terminal region containing cysteine-rich clusters, as well as leucine-rich repeats (LRRs) located in their central domains. Class I, which includes the PGs Dcn and Bgn, have been the focus of our studies\textsuperscript{51,53}.

Dcn and Bgn reveal 57\% homology, the highest among any members of each class. They both possess a pro-peptide which has been suggested to serve as an enzyme recognition signal in order to promote GAG synthesis. Dcn contains one chondroitin/dermatin sulfate GAG side chain adjoining the N-terminal domain, whereas Bgn contains two chondroitin/dermatin sulfate side chains. In addition, the foremost feature of Dcn and Bgn is the presence of 10 LRRs which are surrounded on either side by cysteine-rich clusters\textsuperscript{51,53}. Although similar in structure, Dcn and Bgn, are primarily located in different areas within tissues. Bgn has been observed in the pericellular space and near the surface of cells, whereas Dcn is present within the collagen network\textsuperscript{57,58}. Within the lung framework, the distribution of these two PGs is also observed to be different. Immunostaining and immunofluorescence data in rodents, provided evidence for Bgn’s presence within the airway smooth muscle layer, while Dcn seemed to spare the smooth muscle bundles\textsuperscript{59} and was located primarily in the adventitial layer of the airway wall\textsuperscript{60}. Their differential location has thus been suggested to be a reason for their variation in biological functions\textsuperscript{58}. 
Decorin

The SLRP Dcn, has been demonstrated to have several important biological functions. Dcn plays a predominant role in the development and assembly of tissues, and has been shown to be involved in the stability and formation of collagen fibers. A study by Cavalcante et al. addressed how the mechanical interactions between collagen and PGs affect the stability of lung tissue. PGs play a critical role in lung mechanics due to their influence on the extent of folding and stretching of the collagen fibers. Dcn has been shown to bind the surface of fibrillar collagen affecting fibrillogenesis in vitro and in vivo. Given the characteristic horse-shoe shape of many SLRPs, Dcn is able to acquire a close interaction at the d and e bands of the collagen fibrils, stabilize them and moreover regulate fibrillogenesis. Accordingly, Dcn determines the overall stiffness of the collagen fibers by acting as a cross-linking molecule present between collagen fibrils; hence modifying the mechanical properties of the lung. Moreover, Danielson et al. addressed the role of Dcn in collagen fibrillogenesis, in Dcn-deficient (-/-) mice, and demonstrated that a lack of Dcn resulted in abnormal morphology of the collagen fibrils which displayed irregular outlines and size distribution, and were organized in a loosely packed network when compared to WT animals. In addition, Dcn null (-/-) fibrils in the skin exhibited decreased tensile strength. This data suggests that changes in Dcn may in turn affect collagen fiber mechanical properties and thus the mechanical properties in the airway wall. Further studies by Geng et al. have demonstrated Dcn’s capacity to inhibit cleavage of collagen fibrils by collagenases such as MMP-1 or MMP-13, thereby adding a protective role as one of its functions.

An alteration in the mechanics of the lung in Dcn-/- mice has been demonstrated by Fust and coworkers. In vitro and in vivo studies were employed to study the behavior of the lung tissue of Dcn-/- mice compared to WT Dcn+/+ mice. In particular, parenchymal tissue strips were used in order to evaluate in vitro complex impedance as well as length-stress connections. Lung mechanical behavior was also assessed in vivo by evaluating lung complex impedance and quasistatic pressure-volume (P-V) curves. Results of in vivo dynamic measurements demonstrated a decrease in airway resistance (R_{aw}), whereas tissue elastance (H_t) and tissue damping (G_t) were not significantly altered between the two groups. In addition, in vivo P-V curves as well as in vitro length-stress curves of
Dcn$^{-}$ mice displayed increased compliance compared to the Dcn$^{++}$ group$^{68}$. Recent studies have also suggested that the lack of Dcn produces abnormal collagen fibers in the lung, which was shown to affect the airway-parenchymal interdependence. More specifically, airway-parenchymal interdependence during induced constriction was observed to be enhanced in Dcn$^{-}$ mice$^{60}$. This may be due to the altered elastic and resistive properties of the lung, attributable to the abnormal collagen fibrils in Dcn$^{-}$ mice$^{60}$. This modification could be further enhanced during induced bronchoconstriction in an asthmatic model, given that changes in the mechanical properties of the lung will alter the degree of AHR. Collagen type I is the major ECM protein in the lungs, and it is found to be significantly increased in the subepithelial layer of the asthmatic airway wall. Given these observations, changes in the collagen fibril morphology, assembly and tensile strength caused by a lack of Dcn could in turn influence the asthmatic response of these airways; i.e. AHR.

Dcn has also been shown to affect lung FB morphology and cytoskeleton, causing increased cell migration. In particular, Tufvesson and Westergren-Thorsson’s$^{69}$ study demonstrated that lung FB cells in culture that were stimulated with Dcn displayed stress fiber formation, and enhanced alpha-smooth muscle actin (α-SMA) expression throughout the whole cell. In addition, Dcn was shown to increase the activation of factors RhoA and Rac1, which are both implicated in the migration of FBs. Given that Dcn is suggested to play a role in cell migration and induction of contractile phenotype in FBs, this data implicates Dcn as a potential regulator of ECM remodeling$^{69}$.

Dcn’s influence on cells also extends to its effects on human airway smooth muscle cells (HASMC) in culture. D’Antoni et al.$^{70}$ has recently shown that when culturing HASMC on a Dcn matrix, a decrease in cell number is observed. This decrease was revealed to be due, in part, to a decrease in proliferation, measured by BrdU cell proliferation assays, and more so by an increase in apoptosis.

Other developments in the area of PG research have pointed to their ability to bind and modulate the activity of different growth factors. Dcn, has been demonstrated to have a high affinity for transforming growth factor-β (TGF-β), and will primarily bind the active form of this growth factor$^{61}$. The binding occurs through Dcn’s core protein and in turn, results in the reduction of TGF-β’s bioavailability$^{61,71,72}$. As the binding is
reversible, Dcn can function as a reservoir of this growth factor. An additional complexity in the Dcn–TGF-β interaction is that TGF-β has been shown to stimulate Dcn production in several cells. This has led to the proposition that Dcn may be part of a negative feedback mechanism, which results in the regulation of TGF-β’s activity\(^{71,72}\). In vivo studies have also corroborated Dcn’s ability to impede TGF-β’s activity. Kolb and colleagues\(^{58}\) have used a mouse model of pulmonary fibrosis, where they have over-expressed TGF-β using adenoviral transfers. Results from their study revealed that the simultaneous transfer of both TGF-β and Dcn adenoviral genes resulted in a considerable decrease in BAL TGF-β levels, as well as a significant reduction in fibrosis, assessed by hydroxyproline assays of lung tissue and analysis of general lung histology, when compared to mice treated with TGF-β adenoviral gene alone.

1.5.2 Proteoglycans in asthma

Various studies have revealed that PG deposition is altered in asthma. A report by Roberts\(^{73}\), which examined postmortem lung tissues from severe asthmatic patients whose death was caused by their disease, revealed significant staining for PGs Bgn, versican, Dcn as well as hyaluronan, in the airway wall, particularly in the submucosa and smooth muscle layers. Huang and colleagues\(^{45}\) also showed increases in lumican, Bgn and versican in the sub-epithelial layer of the airways of mild asthmatics as compared to normal subjects. In addition, the increases in both lumican and Bgn were shown to be significantly correlated with airway responsiveness of asthmatic patients, as assessed by \(\text{PC}_{20}\). A report by de Kluijver and co-workers\(^{74}\), which examined bronchial mucosal biopsies of mild asthmatics vs. controls, demonstrated an increase and decrease in the mean density of Bgn and Dcn, respectively, in the asthmatic group.

A subsequent study from our laboratory, comparing PG deposition in severe and moderate asthmatics as well normal controls, further exemplifies the potential impact of PG alteration in disease\(^ {75}\). This study presented further evidence that PGs, lumican, Bgn and versican, are increased in the asthmatic airway wall. Moreover, analysis of the pattern of deposition was shown to be different in moderate vs. severe asthmatics. The sub-epithelial layer of the airway wall did not display any differences in PG deposition between moderate and severe asthmatic groups. Alternatively, increases in Bgn and
lumican were found to be significantly higher in moderate asthmatics compared to severe, when the smooth muscle layer was examined. The authors have thus postulated that the differential distribution of PGs, which are increased in asthma, may in fact alter the degree of airway narrowing.

In another study by De Medeiros et al., PG deposition in both small and large airways of individuals who died of fatal asthma was evaluated in comparison to control subjects. The data revealed significant increases in versican in both the internal area of the small and large airways, as well as a significant decrease in lumican and Den in the external area of the small airways of fatal asthmatics. Taken together, these data have provided evidence which confirms an altered deposition of PGs in asthma. Nevertheless, some uncertainty still remains regarding the type of PG deposition within the airway wall of asthmatics and the role of these PGs in determining the severity of disease.

In vitro work has also provided information regarding alterations in PG deposition in asthma. Cell culture studies by Westergren-Thorsson and co-workers have compared the production of PGs from primary FB cell lines of mild asthmatic patients to those of normal controls. In addition, they have shown evidence for correlations between PG production and airway responsiveness as evaluated by PC<sub>20</sub>. It was observed that the amount of total PG production was significantly higher in cell cultures from subjects experiencing the greatest hyperresponsiveness. When further analyzed, production of Bgn, versican and perlecan was found to be significantly elevated in FB cell cultures from asthmatics compared to normal controls. Moreover, negative correlations were displayed between Bgn, as well as perlecan, and the PC<sub>20</sub>. Together, these results have suggested that altered PG deposition may in fact contribute to the increased hyperresponsiveness observed in asthma. Work from our group has also demonstrated an increase in PG message in asthmatic fibroblasts (AF) compared to FBs isolated from normal patients (NF). In this study, Ludwig et al. confirmed the presence of increased mRNA message for Den in AF vs. NF. Additional studies by Johnson et al. have described that asthmatic HASMCs display increased production of perlecan compared to non-asthmatic cells.

Data obtained from animal models have also corroborated important information regarding PG deposition in asthma. One such study, carried out by Pini and colleagues,
demonstrated that in the Brown Norway rat model of allergic asthma, Dcn and Bgn deposition were both increased in the airway wall of OA-challenged rats. The distribution of these PGs was also found to differ. While Bgn was located within the ASM layer, Dcn was primarily detected external to the ASM, in the adventitial layer. In a murine model of allergic asthma, Reinhardt et al.\textsuperscript{80}, similarly confirmed increases in PG deposition and in particular, increased subepithelial Dcn staining in OA-challenged mice vs. controls.

Thus, there is considerable evidence that not only supports the potential role of PGs in asthma, but also demonstrates that alteration in their deposition and distribution may result in functional consequences.

1.6 Mechanisms for remodeling

The fundamental molecular mechanisms responsible for the development of tissue fibrosis comprise a significant evolving field of research\textsuperscript{81}. Normal tissue homeostasis depends on complex yet balanced cell–ECM interactions, which occur in cooperation with, and entail the action of various cytokines. Thus, a disruption of this balance will consequently result in disease\textsuperscript{82}. It has become evident that fibrogenesis is a result of normal biological events of tissue repair that occur in immoderation. In particular, TGF-β, the main cytokine shown to be involved in the initiation and termination of wound healing and repair, when over-expressed, has been demonstrated to have important roles in the development of tissue fibrosis\textsuperscript{81,83}. This is due to the ability of TGF-β to control proliferation, differentiation as well as apoptosis of numerous cells\textsuperscript{84-86}.

1.6.1 Transforming growth factor-β

The cytokines belonging to the TGF-β group participate in numerous functions, ranging from regulation of cell growth, development and differentiation, to roles in tissue remodeling and repair (figure 3)\textsuperscript{87}. TGF-β\textsubscript{1} may play different roles within different settings. TGF-β\textsubscript{1} can promote anti-inflammatory functions and fibrotic effects, whereas in other conditions, TGF-β\textsubscript{1} can be pro-inflammatory (for review see\textsuperscript{87}). In the context of T\textsubscript{H}2 pathologies such as asthma, TGF-β\textsubscript{1} can stimulate angiogenesis as well as the deposition of matrix components\textsuperscript{87}. Several studies have pointed to TGF-β as the central mediator of fibrogenesis\textsuperscript{88}. As previously mentioned, this is due to its capacity to act as a
chemotactic agent\textsuperscript{89}, as well as its ability to regulate cell proliferation and differentiation, and stimulate synthesis of many matrix components, such as collagens, PGs, and fibronectins\textsuperscript{88,90}. Accordingly, in the tissue remodeling phase of several lung diseases, increases in both TGF-\(\beta\) mRNA and protein expression have been described\textsuperscript{84,91}. Asthmatic patients have demonstrated increased BAL fluid levels of TGF-\(\beta\) following segmental allergen challenge. Levels of this growth factor within the submucosa and epithelial layer of airways, have also been shown to correlate with thickness of the basement membrane, thereby confirming a potential role for TGF-\(\beta\) in the remodeling process\textsuperscript{84,92}. Many animal models have also reinforced the role of TGF-\(\beta\) as a central mediator of pulmonary fibrosis\textsuperscript{89}. In a mouse model of fibrosis, Kenyon and colleagues\textsuperscript{93} demonstrated that intra-tracheal instillation of TGF-\(\beta\) induced subepithelial fibrosis, evaluated by the increased presence of collagen I and III mRNA, as well as increased total collagen deposition observed in lung histology.

1.6.2 Sources of TGF-\(\beta\) in lung disease

In the pathogenesis of lung diseases involving fibrosis, many cellular sources of TGF-\(\beta\) seem to be activated. In particular, eosinophils, macrophages, mast cells, endothelial cells, epithelial cells, FBs and MFBs have been revealed to have the capacity to release this growth factor. In turn, TGF-\(\beta\) can carry out its biological effects on most cells\textsuperscript{89}. In particular, TGF-\(\beta\)'s effect on FBs and MFBs is of interest. Several characterizing observations of FBs and MFBs \textit{in vivo} and \textit{in vitro} have indicated a chief role of these cells in the production and deposition of ECM components such as collagens, fibronectins, PGs and elastic fibres. These functions are thus, fundamental in the characteristic ECM remodeling seen in fibrotic diseases such as asthma (for review see \textsuperscript{94}).
Figure 3: The pleiotropic effects of TGF-β result in the accumulation of ECM and alteration of lung architecture characteristic of pulmonary fibrosis.

1.7 Animal models of asthma

Asthma is a multifactorial disease which is not yet entirely characterized. Many investigators have expended vast efforts in determining the various mediators and dissecting the pathways which are believed to be responsible for the pathology of this disease. Studies which are performed in humans have many limitations due to ethical reasons, and may not fully elucidate the complex in vivo interactions that may contribute to the development of asthma. Similarly, although very important, in vitro studies are also restricted in terms of identifying the exact mechanisms of disease. Therefore, the use of in vivo animal models has been a valuable tool needed to more thoroughly investigate human disease. There are many advantages in using animal models, such as the mouse, to study asthma. Murine models are readily available, inexpensive and offer
numerous inbred strains which have already been genetically characterized. There is the possibility of genetic manipulation, in terms of over-expressing or deleting entirely a specific gene or protein. Transgenic and knockout (KO) mice are thus, a valuable tool which offers the ability to further examine disease pathways as well as determine the functional role of different proteins in vivo. Moreover, experimental factors, in terms of environmental exposures, can be highly controlled.

Interestingly, one of the greater risk factors for the development of asthma is an individual’s genetic susceptibility for IgE-mediated reactions against allergens. The term associated with this predisposition is referred to as ‘atopy’. Essentially, animal models of allergic asthma attempt to replicate what is observed in atopic individuals. Multiple studies have successfully shown using antigen sensitization and challenge protocols, that mice do in fact develop phenotypic aspects typical of human asthma. These murine models of asthma demonstrate allergic inflammation and hyperresponsiveness, as well as increased IgE levels, and Th2 driven responses mediated by cytokines such as IL-4, IL-5, and IL-13. The use of animal models can therefore help address important scientific questions.

However, in studying diseases such as asthma, the use of animal models has been questioned. There has been some controversy as to whether these models are in fact appropriate in comparison to the pathologies observed in humans. A common concern is the difference in the architecture of the respiratory system of mice compared to that of humans. For example, the lung structure of the mouse contains six to eight branching airway generations, whereas humans have twenty to twenty-three airway generations. Furthermore, the terminal bronchioles open directly into the alveolar ducts in mice, whereas humans possess respiratory bronchioles which separate the terminal bronchioles and alveoli.

The availability and use of different mouse strains in modeling allergic asthma has also raised some concerns. On the one hand, one can argue that given the variability in the data obtained from experiments utilizing different strains, it becomes very difficult to interpret results and apply them to the human condition. On the contrary, the availability of different strains may represent the diversity also seen in humans. This may in fact support the role of genetic factors in asthma and may help to discover which genes are
responsible for increased disease susceptibility. In this regard, different strains, as different individuals, may have diverse susceptibilities in the development of asthma\textsuperscript{96}.

The concept of strain susceptibility is important in the development and characterization of animal models of asthma. Work by Shinagawa and Kojima\textsuperscript{99}, has led to a characterization of four different mouse strains in terms of their susceptibility to the development of the asthmatic phenotype. In particular, they studied A/J, Balb/c, C57Bl/6 and C3H/HeJ strains and evaluated their asthmatic reactions following an antigen challenge protocol. The advantage of this study is that all strains received the same antigen challenge protocol and could thus be compared. After evaluating eosinophilic inflammation, ASM, mucus cell layers, collagen deposition and hyperresponsiveness, data from their experiments demonstrated that A/J followed by Balb/c mice are the highest responders after intranasal administration of OA. C57Bl/6 and C3H/HeJ mice on the other hand, were considered very low responders as they did not exhibit pronounced histological changes such as increased collagen deposition, or smooth muscle mass as observed in the other strains. In addition, lung function measurements did not show any significant increases in airway resistance in either of these two strains. Several other investigators have also arrived at similar conclusions in the evaluation of strain susceptibility. Slight variations do exist however, and highlight the importance of the different antigen sensitization and challenge protocols used.

1.8 Induction of experimental allergic asthma

Various research groups have employed different experimental protocols to induce airway allergy in murine models. A commonly used protocol includes a systemic sensitization via intraperitoneal (i.p.) injection followed by airway challenge with antigen\textsuperscript{100}. The sensitization often includes an adjuvant such as aluminum hydroxide (alum) in order to artificially induce a T\textsubscript{H}2 response by the immune system\textsuperscript{101}. These two protocol phases have been shown to induce IgE-specific antibodies, eosinophilic inflammation as well as AHR. Repeated challenges with allergen may then cause certain structural changes in the airways of these mice, including mucus hypersecretion, basement membrane thickening and ASM hypertrophy. This protocol thus serves as a useful tool in investigating human disease as it produces many of the hallmark features of
human asthma\textsuperscript{100}. The allergen itself can be delivered by different methods and routes, including via aerosol, providing inhalation exposure, or by intra-nasal or intra-tracheal delivery of the allergen dissolved in solution. It is interesting to note that the deposition of the allergen within the respiratory tract will differ depending on the method of challenge employed\textsuperscript{100}. In addition to the application of numerous methods, routes and durations of antigen challenges, different antigens have been employed in inducing an allergic response. OA, a constituent of egg protein\textsuperscript{102}, is an antigen which is extensively used by many investigators studying experimental models of allergic asthma. This is due to both its ready availability and its capacity to induce the characteristic features of disease previously mentioned\textsuperscript{103}. Many studies have collectively established that systemic sensitization followed by intra-nasal instillation challenges of OA reproduce the asthmatic phenotype in mice. The reason that many investigators adopt the intra-nasal route of administration of OA as part of their challenge protocol is that this method is most analogous to the route of exposure of airborne allergens in humans; in essence via inhalation. Additionally, this method of exposure is noninvasive and can reach and affect the upper and lower tracts of the respiratory system of these animals\textsuperscript{101}.

1.9 Respiratory mechanics

The assessment of respiratory mechanics in murine models of asthma is crucial in determining the presence of altered airway behavior as well as allowing the assessment of structure–function correlations. Appropriate methodologies are therefore required to give an accurate representation of lung function in these animals. In particular, total respiratory resistance ($R_{RS}$) and compliance ($C_{RS}$) can provide a general evaluation of lung function in smaller animals such as the mouse. Compliance, which can also be expressed as the reciprocal of elastance ($E_{RS}$) of the lung, is a functional measure of lung stiffness. Alternatively, the $R_{RS}$ parameter reflects airflow resistance and is dependent on airway lumen. It also includes a component due to resistance of the lung tissues. Of note, one of the characteristics of the mouse respiratory system that differs relative to humans is that the chest wall of these animals is more compliant. Consequently, mice possess a lower functional residual capacity (FRC) and will therefore decrease the fluctuations of gas concentrations in the alveoli by breathing at higher frequencies\textsuperscript{95}. 
There are various techniques available to study lung function in small animals. The classical view, known as the single compartment linear model, can provide information on both $R_{RS}$ and $E_{RS}$ by acquiring measurements of pressure at the airway opening ($P(t)$), flow ($\dot{V}(t)$), and volume ($V(t)$). This model is described using the equation of motion given by:

$$P(t) = RV(t) + EV(t) + P_0$$

where $P_0$ corresponds to the resting applied pressure (positive end-expiratory pressure). A principal concept associated with this equation is that $E_{RS}$ is determined based on the changes in lung volume, whereas resistance is dependent upon the flow within the airways. This model samples lung mechanics at a single frequency of breathing only. It is also important to note that $R_{RS}$ and $E_{RS}$ are dependent on a variety of factors, namely frequency, flow, tidal volume, mean lung volume, as well as volume history. In order to control for all these factors during experimental conditions, different methodologies have been employed. For example, by anesthetizing and paralyzing the animals prior to data collection, one is able to eliminate the spontaneous effort of these mice. In addition, tracheostomizing the mice will essentially eliminate the involvement of upper airways. The mechanical ventilator itself may then be set to control parameters such as frequency, tidal volume as well as volume history by initially inflating the lungs of all subjects by providing a set volume of air. Moreover, the mean lung volume of subjects during the experiment may also be controlled by setting the positive end-expiratory pressure (PEEP).

The dependence of resistance and elastance on frequency is attributable to the viscoelasticity of the lung tissues along with potential heterogeneities of mechanical function present in different areas of the lung. As a result, simply determining the resistance and elastance at a single frequency does not give a complete portrayal of the respiratory system. Additional information may therefore be obtained by sampling the respiratory system at a range of frequencies and measuring input impedance ($Z_m$) using the forced oscillation technique. This paradigm provides an additional and very significant characterization of respiratory mechanics in that it allows the partitioning of $R_{RS}$ into airway and tissue components. This is described by the constant phase model which includes the airway compartment of resistance ($R_{aw}$) and the viscoelastic tissue...
compartment distinguished by two properties: (1) tissue damping ($G_t$) referring to tissue resistance; and (2) tissue elastance ($H_t$). Due to high breathing frequencies in mice, under homogeneous conditions, $G_t$ or tissue resistance will contribute a relatively small amount to the total $R_{RS}$. On the other hand, during induced constriction due to the administration of a bronchoconstrictor, $G_t$ as well as $R_{aw}$ are significantly increased. Moreover, the increase in $G_t$ is greater than or equal to the increase in $R_{aw}$. In addition, it is important to note that under induced constriction, the parameter for $G_t$ includes the resistance of the small airways as well as the lung tissue.

1.10 Hypothesis

Given the evidence demonstrated by several studies, we reasoned that development of allergen-induced asthma would be altered in Dcn-deficient mice due to effects of Dcn on ASM mass, and/or the mechanical properties of the airway wall and lung tissues. Further, the effects of Dcn on TGF-β may influence remodeling and inflammation indirectly. Thus, the hypothesis of this study was that the development of allergen-induced asthma would be enhanced in Dcn-deficient mice.
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2.1 Determination of genotype

C57Bl/6 Dcn⁻/⁻ mice were obtained courtesy of Dr. R. Iozzo (Thomas Jefferson University). The generation of these nullizygous animals, as described previously by Danielson et al.⁶⁴, was achieved by introducing a pgk-neo cassette within exon 2 of the murine Dcn gene leading to disruption of the gene locus.

In order to generate additional Dcn⁻/⁻ mice, breeding was initiated between female C57Bl/6 mice, heterozygous for decorin (Dcn⁺/-) and C57Bl/6 Dcn⁻/- males. The genetic makeup of the offspring was then determined by extracting DNA from a piece of tissue originating from the tail. Briefly, 150µl of lysis buffer solution for tissue digestion containing 1µl Proteinase K/100µl of lysis reagent were added to each DNA sample. Samples were heated for up to 24 hours at 55°C, after which temperature was increased to 85°C for 45 minutes. Samples were then stored at -20°C until ready for use.

Polymerase chain reaction (PCR) was used for DNA analysis of mouse tail tissues. As described by Danielson et al.⁶⁴, sense and antisense primers (5'-CCTTCTGGGACAAGTCTCTTG-3' and 5'-TCGAAGATGACACTGGCATCG-3', respectively) which correspond to exon 2 of murine Dcn, were employed. These primers displayed a 161-bp fragment, which reveals a lack of homologous recombination, and corresponds to the wildtype Dcn gene. Presence of homologous recombination, on the other hand, was visualized by using a third primer corresponding to the Pgk-promoter of the pgk-neo cassette (5'-TGGATGTGGAATGTGTGCGAG-3') and yielded a 250-bp fragment, signifying the knocked out Dcn gene. The total reaction mix of 13.21µl consisted of 1µl of DNA, 1µl of sense primer, 1µl of antisense primer or 1µl of pgk-primer, 5.35µl of H₂O, 2µl of 10X rxn buffer, 2µl of MgSO₄ (Invitrogen, Ontario, Canada), 0.8µl of dNTP mix (containing all 4 dNTPs; Onbio, Ontario Canada) and 0.06µl of Taq polymerase (Invitrogen, Ontario, Canada). The reaction conditions comprised: melting for 5 min at 95°C; 35 cycles of 1 min at 94°C, 30 sec at 57°C, and 30 sec at 72°C; followed by 10 min at 72°C; and cooling at 4°C. The products of the PCR reaction were then confirmed to be the correct size by running the samples on a 2.5% agarose gel (Agarose 'B' Low EEO, LAB MAT, Montreal, Canada) at 120V for 25-30 min.
2.2 Animal preparation

Male and female wildtype (Dcn\textsuperscript{+/+}) C57Bl/6 mice were obtained from Charles River Laboratories (Saint-Constant, Quebec, Canada). Dcn\textsuperscript{+/+} and Dcn\textsuperscript{−/−} animals were studied between the ages of 6-8 weeks. Mice were then separated into four groups: Dcn\textsuperscript{+/+} saline (Sal) controls, Dcn\textsuperscript{+/+} OA, Dcn\textsuperscript{−/−} Sal controls and Dcn\textsuperscript{−/−} OA mice. All animals were housed in an animal facility at McGill University and were cared for in compliance with the Canadian Council of Animal Care’s guide; protocols and procedures were evaluated and accepted by the animal ethics committee of McGill University. For ethics certificate please refer to the ‘APPENDIX’ section.

2.3 Allergic sensitization and challenge protocol

The sensitization and challenge protocol used in this study is shown in figure 4. Briefly, experimental groups were sensitized with an i.p. injection of a saline solution containing 10 µg OA (Sigma Chemical Co., St Louis MO) with 1 mg of adjuvant aluminum hydroxide, Al(OH)\textsubscript{3} (Fisher Scientific Ottawa, Ontario). Sensitization occurred on two separate days; day 0 and day 7. One week following the second sensitization, mice were challenged via an intra-nasal instillation of 10 µg OA in 50 µl of saline three times per week for three weeks under light anaesthesia. The anaesthetic agent halothane was used in order to lightly anaesthetize mice before each challenge. Two days following the final challenge, experiments were performed. Control mice were sensitized and challenged with sterile saline solution.

2.4 Measurement of \textit{In vivo} lung mechanics

Mice were injected i.p. with xylazine (10 mg/kg) and pentobarbital sodium (30 mg/kg). Mice were tracheostomized and an 18-gauge metal canula was inserted into the trachea and firmly tied. Subsequently, the animal was connected to a computer controlled small animal ventilator (Flexivent, Scireq Inc. Montreal, Canada) and normal tidal breathing was initiated. Mice were mechanically ventilated at 150 breaths/minute, and at a PEEP of 3 cmH\textsubscript{2}O. Animals were then paralyzed with pancuronium bromide (1.2 mg/kg i.p.), administered i.p., prior to the initiation of mechanical ventilation.
Respiratory mechanics were measured using the forced oscillation technique. Briefly, the computer generates a volume signal that is delivered at the airway opening. This volume signal which interrupts normal ventilation, is composed of a two-second pseudo-random perturbation comprising waveforms of mutually primed frequencies. For these experiments, two different measurements were used to assess lung physiology: the single compartment model and the complex impedance model. This first model uses a single frequency perturbation to measure \( R_{RS} \) and \( E_{RS} \). The complex impedance model uses multiple frequency perturbations, which permits the partitioning of resistance into \( R_{aw} \) and \( G_t \). Furthermore, \( H_t \) is obtained as well. This approach allows assessment of more central (\( R_{aw} \)) and peripheral (\( G_t, H_t \)) lung responses. Measurements of volume or piston displacement as well as the cylinder pressure are recorded during this signal perturbation and are furthermore used to derive the impedance data.

The experiment begins with 5 seconds of default ventilation followed by a ‘Total Lung Capacity’ (TLC) breath of 30 cmH\(_2\)O to standardize volume history. Default ventilation is resumed for 3 minutes after which a second TLC is given. Measurements are obtained at baseline as well as after the delivery of increasing concentrations of MCh aerosols (Sigma, Louis, MO). The increasing concentrations (6.25, 12.5, 25 and 50 mg/ml) of the airway constricting agent MCh are initially dissolved in sterile saline and delivered to the airways via an ultrasonic nebulizer for a period of 10 seconds (Hudson RCI, Teleflex Medical). Perturbations were delivered every 10 seconds for 1 minute, for baseline measurements, and every 15 seconds for 5 minutes, following MCh aerosol delivery. The perturbations alternated between the single frequency oscillation, which was delivered first, and the complex impedance perturbation. A total of 6 measurements were acquired for each model during the MCh challenge.

2.4.1 Calculation of mechanical parameters

The mechanical ventilation employed in these experiments incorporates two separate perturbations or volume/pressure waveforms, which allow assessment of mechanics using the single compartment and complex impedance models.
2.4.2 Single Compartment Model

The first model employed utilizes a single frequency oscillation. More specifically, the single compartment model signal consists of 3 periods of sinusoidal waveforms given at a constant frequency of 2.5 Hz. This model relies on the equation of motion which is given by:

\[ P(t) = R \dot{V}(t) + EV(t) + P_0 \]  

where \( P \) is pressure, \( R \) is the flow-dependant resistance, \( \dot{V}(t) \) corresponds to flow of the gas, \( E \) is the volume-dependant elastance, \( V \) signifies volume, and \( P_0 \) is simply an added pressure term. Both independent variables, \( V \) and \( \dot{V}(t) \), are linearly correlated with pressure. This as a result, allows for the calculation of values reflecting ‘Resistance’ (\( R_{RS} \)) and ‘Elastance’ (\( E_{RS} \)) or stiffness of the total respiratory system.

2.4.3 Complex Impedance Model

The constant phase model is a broadband signal that applies multiple frequency perturbations at the airway opening ranging from 1Hz to 20.5Hz. Using the ‘Forced Oscillation Technique’, input impedance spectra can be derived from pressure and volume measurements made at the airway opening. The impedance of the respiratory system (\( Z_{RS} \)) was calculated using the following equation:

\[ Z_{RS}(\omega) = \frac{P(\omega)}{[dV(\omega)/dt]} \]  

where \( P \) denotes pressure and \( V \) is volume (ml) corresponding to piston displacement. Both these parameters are dependant on angular frequency (\( \omega \)). Input impedance data are then fit to the equation of the ‘Constant Phase Model’ (Hantos et al.106) using a multiple linear regression.

\[ Z_{RS} = R_{aw} + J\omega I + (G_{ti} + jH_{ti})/\omega^\alpha \]  

where \( R_{aw} \) signifies airway resistance, \( I \) is airway inertance, \( G_{ti} \) is tissue damping, \( H_{ti} \) corresponds to tissue elastance, \( \alpha = (2/\pi)\tan^{-1}(H/G) \), and \( J \) represents the imaginary part. For each parameter measured, the peak response having a coefficient of determination (C.O.D.) greater than 0.8 was determined and used in the statistical analyses.
2.5 Bronchoalveolar lavage

Following lung mechanics, BAL was carried out twice by instilling, and subsequently withdrawing 1 ml of cold saline via the tracheal canula. Supernatant of the first BAL sample for each subject studied, was collected and stored at -80°C. A cytospin centrifuge was then used to deposit the BAL cell suspension on a glass slide, which was then fixed and stained with Diff-Quick (Fisher Scientific, Kalamazoo, MI). Differential cell counts were then determined by analyzing the percentage of macrophages, eosinophils, neutrophils, and lymphocytes out of a total of three hundred cells that were counted on BAL samples slides.

2.6 Tissue preparation and fixation

Following the in vivo measurements, the chest wall was opened and lungs were excised. Right lobes of the lungs were snap-frozen in liquid nitrogen and stored until use for protein analysis. The left lung was inflated with 10% phosphate-buffered formalin at a transpulmonary pressure of 25 cmH₂O for 25 minutes. The lungs were then placed in 10% formalin overnight. The following day, the left lobes of the lungs were placed in the tissue processor. Subsequently, lungs were embedded in paraffin blocks. Sagittal tissue sections with a thickness of 5µm were cut, and staining performed.

2.7 Histochemistry

2.7.1 Hematoxylin and Eosin (H&E) – In order to visualize tissue inflammation, H&E staining was performed on paraffin embedded tissue sections. Tissue sections from both Sal control and OA-challenged mice were rehydrated. Sections were then immersed in Hematoxylin (Gill I hematoxylin, Sigma Aldrich) for three minutes. Subsequently, slides were rinsed with water, followed by saturated lithium carbonate, and then water again. Next, slides were immersed in 1% Eosin (Sigma Aldrich) for one minute, after which the tissue sections were dehydrated and mounted in resinous media (Cytoseal TM60 mounting medium, Low viscosity, Co. Richard-Allen Scientific) and cover slips applied.

2.7.2 Periodic Acid Schiff (PAS) – Mucus hypersecretion was visualized using Periodic-acid Schiff staining. Paraffin embedded lung tissue sections were de-waxed and hydrated.
Following the rehydration period, tissue sections were treated with Periodic acid Solution (Aqueous solution, 1g/L, Sigma Aldrich) for ten minutes. After washing repeatedly in water, slides were covered with Schiff’s reagent solution (pararosaniline HCl, 1% (w/v) Sodium bisulfite, 4% (w/v) in HCl, 0.25mol/L, Sigma Aldrich) for a period of fifteen minutes. Slides were washed in water and immersed in Harris Hematoxylin, followed by a differentiation in acid alcohol (HCl conc. 4ml; 95% EthOH – 396ml). Sections were washed in water, followed by lithium carbonate, followed by water once more. The tissue sections were then dehydrated through different concentrations of alcohol, and sections were mounted in Cytoseal (Cytoseal TM60 mounting medium, Low viscosity, Co. Richard-Allen Scientific). Finally cover slips were applied.

2.7.3 Picro-sirius red – The Picro-sirius red method was used to visualize collagen fibrils within lung tissue sections. Tissue sections from both Sal control and OA-challenged animals were de-waxed and hydrated. The tissue sections were subsequently immersed in picro-sirius red solution (Sirius Red F3B 0.5g; saturated aqueous solution of picric acid-500ml; solid picric acid; Sigma Aldrich) for one hour. The tissue sections were then washed in two changes of acidified water (0.5% glacial acetic acid), followed by dehydration through alcohol. Sections were then cleared in xylene, mounted in a resinous medium (Cytoseal TM60 mounting medium, Low viscosity, Co. Richard-Allen Scientific) and cover slips were applied.

2.8 Immunohistochemistry

2.8.1 Alpha-smooth muscle actin (α-SMA) – Antigen retrieval was initially performed on tissue sections using sodium citrate buffer 0.01M (pH 6.0). Sections were then incubated overnight at 4°C in a humid chamber with the primary antibody α-SMA (1:250 dilution, rabbit anti-mouse α-SMA, Abcam Laboratories, Cambridge MA). The secondary antibody applied was biotinylated goat anti-rabbit (1:100 dilution, Vector Laboratories), and immunoperoxidase was used for detection. Slides were counterstained using hematoxylin (Gill I Hematoxylin, Sigma Aldrich). All immunostaining was developed using DAB (DAKOCytomation, Carpinteria, CA, USA). Positive stain for α-SMA was visualized to be brown under a light microscope (Olympus BX51, Ontario, Canada).
2.8.2 Decorin and biglycan – Tissue sections were incubated overnight with the primary antibody for Dcn (rabbit anti-Dcn 1:800 dilution), or Bgn (rabbit anti-Bgn 1:500 dilution, both provided by Dr. Larry Fisher, NIH). The biotinylated secondary antibody used was goat anti-rabbit (1:100 dilution, Vector Laboratories). Immunoperoxidase was used for detection. Slides were then counterstained using hematoxylin (Gill I Hematoxylin, Sigma Aldrich). All immunostaining was developed using DAB (DAKOCytomation, Carpinteria, CA, USA).

2.9 Morphometry and Measurements

The airway selection criteria employed for morphometric analyses included an intact epithelial lining as well as a minimum to maximum internal diameter (D2/D1) greater than or equal to 0.5. Measurements of internal diameters allowed for a quantitative measure of airway roundness and permitted the selection and analysis of only these airways. This is of importance for morphological assessment as sectioning biases, which result in airways cut at different angles, can alter the data. Stained slides were viewed under the Olympus light microscope (Olympus, Ontario, Canada) and images were captured with either the Cool-Snap PROcf color camera (Media Cybernetics, Baltimore MD) or the Olympus Qcolor5 Camera (Olympus, Center Valley PA). Measurements were obtained using the Image pro-PLUS software (Version 5.2, Media cybernetics L.P., Silver Spring, MD, U.S.A). All results were calibrated according to the magnification of the captured image. A total of n=8 mice were sampled at random from each group (i.e. 8 Sal control, 8 OA-challenged). Generally, 5 airways were examined per mouse.

2.9.1 Tissue inflammation – Morphometric analysis of tissue inflammation consisted of randomly choosing 5 airways per mouse that fit the above criteria. In order to quantify tissue inflammation, the number of inflammatory cells was counted within a wall area (WA) around the airways. The inflammatory cells counted included both granulocytes and mononuclear cells, and the WA was characterized as the area extending from the basement membrane to the peribronchial area around the airways, defined by the alveolar
attachments. Tissue inflammation was expressed as ‘the number of inflammatory cells per WA. Averages of the 5 airways/mouse were calculated.

2.9.2 Mucus staining – Periodic acid Schiff staining was used to visualize mucus within the airway epithelium. Using 20X magnification, the tissue section was scanned and 3 airways selected at random that would fall within a (3x3) line grid placed upon the image. The spacing of the grid was calibrated to a horizontal and vertical spacing of 460 and 344 units, respectively. The airways were then selected according to their location in the grid, in that the three airways falling in the diagonal squares of the grid were chosen. If more then one airway was found within the same square, the airway closest to the edges of the grid was chosen. Once this selection had been made, a picture of each airway selected was taken at a total magnification of 100X. Positive staining appeared pink under a light microscope, and was quantified by outlining the area of the positive stain within the epithelial area. Results were therefore expressed as the percent (%) positive area divided by total epithelial area. An average of the three airways for each mouse was calculated and the average for all Sal controls vs. OA-challenged mice was represented graphically.

2.9.3 Airway smooth muscle – Once again, a maximum of 5 airways per mouse that fit the above criteria were examined. The following measurements were made: basement membrane perimeter (P_{bm}); and total area of smooth muscle bundles present around the airway (ASM). Average values were calculated for each mouse. Results were then expressed as the ‘area of smooth muscle divided by basement membrane perimeter squared’ (P_{bm}^2). Airway smooth muscle was quantified by two separate operators between which a correlation was established (R^2 value = 0.9788).

2.9.4 Collagen, decorin, and biglycan – A maximum of 5 airways matching the above criteria were examined per mouse. For each airway, a WA was delineated, and encompassed the area between the basement membrane (BM), out towards the end of the adventitia. Analysis of positive staining for collagen, Dcn and Bgn was limited to this area. Using the Image Pro-Plus software, the color of the positive stain was selected and
an area measurement was generated. Results were expressed as the area of positive stain / WA.

2.10 Data Analysis

Respiratory physiology data for the single compartment model as well as the constant phase model were generated using the Flexivent software. A two-way analysis of variance (ANOVA) for repeated measures was used to verify whether in vivo mechanical parameters were altered during a MCh response curve, in OA-challenged mice compared to Sal controls and in Dcn$^{+/}$ vs. Dcn$^{+/+}$ groups. A one-way ANOVA with Bonferroni post-tests was used for all histological analyses. A p-value < 0.05 was considered statistically significant. Values are depicted as the means ± SE.
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3.1 Genotyping

Figure 5 illustrates sample genotyping results for Dcn\(^{+/+}\), Dcn\(^{+/-}\) and Dcn\(^{-/-}\) mice. Following isolation of DNA from tail tissue, samples were incubated with three different reaction mixes; (1) a reaction mix containing the 3' WT primer; (2) a mix containing the 3' KO primer designed to include the pgk-neo cassette or; (3) a combination reaction mixture containing both 3' WT and KO primers. As can be seen in figure 5, heterozygous (+/-) mice display a band at 161-bp in lane (1), as well as a band at 250-bp in lane (2). This was further confirmed by the presence of both these bands in the third lane where a combination of WT and KO primers was used. On the other hand, Dcn\(^{-/-}\) mice displayed only one band at 250-bp, determining the presence of the pgk-neo cassette. Finally, Dcn\(^{+/+}\) mice displayed only one band at 161-bp.

3.2 Dynamic measurements of in vivo lung mechanics

Lung physiology measurements using the 'Single Compartment' model are shown in figure 6 and Table 1. Analysis of these results during a MCh response curve revealed that R\(_{RS}\) was significantly increased in OA-challenged Dcn\(^{+/+}\) mice at MCh doses of 25 and 50 mg/ml (p<0.001), as well as in OA-challenged Dcn\(^{-/-}\) mice at MCh 50 mg/ml (p<0.01), in comparison with their respective Sal controls. Interestingly, Dcn\(^{+/+}\) and Dcn\(^{-/-}\) Sal controls showed similar increases in R\(_{RS}\) across the MCh response curve. In contrast, Dcn\(^{-/-}\) animals challenged with OA demonstrated significantly lower R\(_{RS}\) values at the highest MCh dose (50mg/ml) when compared to OA-challenged Dcn\(^{+/+}\) animals (p<0.01; 8.57 ± 0.76 vs. 12.64 ± 1.25 respectively).

Total respiratory elastance (E\(_{RS}\)) was, in the same way, significantly increased in OA-challenged Dcn\(^{+/+}\) (p<0.001 at MCh 25, 50mg/ml) and Dcn\(^{-/-}\) (p<0.001 at 50mg/ml) mice compared to Sal controls. Once again, responses of Dcn\(^{+/+}\) and Dcn\(^{-/-}\) Sal controls groups were not different in response to increasing concentrations of MCh. Conversely, E\(_{RS}\) measurements of OA-challenged animals were significantly lower in Dcn\(^{-/-}\) compared to Dcn\(^{+/+}\) mice at MCh concentrations of 25 mg/ml (p<0.05; 136.95 ± 25.04 vs. 211.45 ± 28.01) and 50 mg/ml (p<0.05; 233.62 ± 29.63 vs. 311.13 ± 30.25) (see Table 1).
Further assessment of the lung mechanics was achieved by partitioning this response into central (R_{aw}) and distal (G_{t}, H_{t}) lung components as shown in figure 7A-C. When analyzing R_{aw}, only OA-challenged Dcn^{+/+} mice showed a significant increase in this parameter compared to control Sal challenged Dcn^{+/+} mice (p<0.05 at MCh 25mg/ml). Alternatively, OA challenge did not induce AHR in Dcn^{-/-} animals in terms of R_{aw}. Furthermore, OA Dcn^{-/-} mice had a significantly lower R_{aw} response at a dose of 25mg/ml of MCh compared to OA-challenged Dcn^{+/+} mice (p<0.05; 0.81 ± 0.18 vs. 1.45 ± 0.36, respectively) (see Table 2). Dcn^{-/-} mice show significantly less OA-induced distal lung hyperresponsiveness than Dcn^{+/+} mice as well. G_{t} and H_{t} measurements, shown in table 2, were significantly increased in both Dcn^{+/+} as well as Dcn^{-/-} OA-challenged mice in comparison with their respective Sal controls. Despite these significant increases, responses for G_{t} and H_{t} were significantly lower in the OA Dcn^{-/-} mice when compared to Dcn^{+/+} mice at MCh doses of 25 (G_{t} and H_{t}, p<0.001) and 50 mg/ml (G_{t}, p<0.001; H_{t}, p<0.01).

3.3 Histological analyses

3.3.1 Airway inflammation and BAL analysis

Peribronchial inflammation was assessed in lung histological sections and photomicrographs are shown in figure 8(A-D). Qualitatively, these photomicrographs depict a notable increase in inflammatory cells present around airways of OA-challenged Dcn^{+/+} mice (8B) when compared to the Dcn^{+/+} Sal (8A) group. Alternatively, OA Dcn^{-/-} airways, as seen in figure 8D, do not demonstrate any differences in terms of inflammatory infiltrate compared to Dcn^{-/-} Sal mice (8C). The quantitative data for tissue inflammation are shown in figure 9A, and are expressed as the number of inflammatory cells/ WA. Inflammation was found to be significantly increased in the OA-exposed Dcn^{+/+} group vs. Dcn^{+/+} Sal (p<0.001; 12832.25 ± 1097.78 vs. 7810.98 ± 558.36, respectively), whereas OA challenge does not induce tissue inflammation in the Dcn^{-/-} mice. Moreover, the amount of inflammation present within the peribronchial areas of OA Dcn^{+/+} mice was significantly higher than that observed around OA Dcn^{-/-} airways (p<0.001; 12832.25 ± 1097.78 vs. 8039.48 ± 521.45, respectively).
Quantitative analysis of the BAL cell differential is seen in figure 9B. Tissue inflammation was not increased with OA challenge compared to Sal controls in Dcn+/− mice, however, cells present within the BAL of OA-challenged Dcn+/− mice were predominantly eosinophils (p<0.001; 73.17 ± 4.36% in OA vs. 1.17 ± 0.55% in Sal Dcn+/−). The pattern of cell differential was essentially similar between the Dcn+/+ and Dcn+/− groups; where OA challenge resulted in a significant increase in the proportion of BAL eosinophils, along with a significant decrease in macrophages (p<0.001). Neutrophils and lymphocytes proportions were similar across all four groups.

3.4 Remodeling

3.4.1 Goblet cell hyperplasia

Micrographs depicting airway mucus expression are shown in figure 10A-D. Positive staining for mucus expression can be visualized as dark pink color on stained lung tissue sections. As shown in figures 10A and C, Sal challenged Dcn+/+ and Dcn+/− groups respectively, do not display any positive staining within the epithelial lining of airways. On the other hand, a clear positive stain can be seen with OA challenge in both Dcn+/+ and Dcn+/− groups (10B and D, respectively). In order to determine the extent of this increase in mucus expression, the area of PAS-positive staining was quantified within the epithelium. Tissue sections stained with PAS demonstrated an increase in mucus expression within the bronchial epithelium of both Dcn+/+ and Dcn+/− OA-challenged mice. As shown in figure 10E, the area of positive stain was significantly increased in OA-challenged Dcn+/+ as well as Dcn+/− mice in comparison to their Sal controls (p<0.001; Dcn+/+ 19.21 ± 2.91% vs. 0.43 ± 0.22%; and Dcn+/− 16.69 ± 3.44% vs. 0.187 ± 0.15%, respectively). Mucus expression was similar between OA Dcn+/− and Dcn+/+ mice.

3.4.2 Airway smooth muscle

Another prominent feature of asthma includes the remodeling that occurs within the ASM layer. In order to determine the presence of these changes, immunohistochemistry, using the α-SMA antibody, was performed on various tissue sections. The photomicrographs shown in figure 11A-D, demonstrate the amount of smooth muscle, depicted by the brown stained bundles, that surrounds the airways. As
depicted in figure 11E, ASM mass was not increased with OA in either Dcn<sup>+/+</sup> or Dcn<sup>−/−</sup> mice. A trend towards an increase, however, in the area of ASM per Pbm<sup>2</sup>, was seen with OA challenge in Dcn<sup>+/+</sup> mice (p = 0.1489 vs. p = 0.3550 for Dcn<sup>−/−</sup>).

### 3.4.3 Collagen deposition

Collagen deposition within the WA of the airways was also evaluated across the four groups. Figure 12A-D shows Dcn<sup>+/+</sup> and Dcn<sup>−/−</sup> tissue sections stained with picrosirius red, which was used to visualize the extent of collagen remodeling present. Sal control Dcn<sup>+/+</sup> and Dcn<sup>−/−</sup> mice showed relatively similar amounts of collagen staining. As illustrated in figure 12E, collagen deposition was found to be significantly increased in OA-challenged Dcn<sup>+/+</sup> mice in comparison to Dcn<sup>+/+</sup> Sal controls (p<0.05; 0.523 ± 0.02 vs. 0.401 ± 0.032, respectively).

Analysis of the positively stained area in OA-challenged Dcn<sup>−/−</sup> tissue sections on the other hand, depicted no increase in collagen deposition compared to Sal controls (0.367 ± 0.032 vs. 0.353 ± 0.032, respectively). Of note, the amount of collagen deposition observed in Dcn<sup>+/+</sup> OA-exposed mice was significantly higher in comparison to OA Dcn<sup>−/−</sup> mice (p<0.01; 0.523 ± 0.02 vs. 0.367 ± 0.03).

### 3.4.4 Biglycan

As suggested by the study of Fust et al.<sup>68</sup>, alterations of the tissue matrix may in fact be responsible for the diverse mechanical properties exhibited by the Dcn<sup>−/−</sup> mice. Thus, the deposition of Bgn, a PG with high homology to Dcn, was examined. Micrographs of Bgn deposition are shown in figure 13A-D. The amount of Bgn present within the WA was not statistically different in Dcn<sup>+/+</sup> OA-challenged mice with respect to their Sal controls (0.504 ± 0.02 vs. 0.384 ± 0.03, respectively). Alternatively, Bgn staining was significantly increased with OA challenge in Dcn<sup>−/−</sup> mice (p<0.001; 0.532 ± 0.05 vs. 0.285 ± 0.05, respectively) (Figure 13E).

### 3.4.5 Decorin

To clarify whether Dcn deposition is actually altered with OA challenge in Dcn<sup>+/+</sup> mice, immunostaining for this PG was performed. In effect, as shown in figure 14, OA
challenge significantly increased the amount of Dcn present within the WA of airways in these mice (p<0.001; 0.500 ± 0.03 vs. 0.187 ± 0.04). The absence of Dcn was also verified using immunohistochemical techniques on Dcn−/− tissue sections (data not shown).
TABLES and FIGURES
### TABLES

**Table 1:**

<table>
<thead>
<tr>
<th>MCh dose</th>
<th>Dcn&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>Dcn&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td>Sal</td>
<td>OA</td>
</tr>
<tr>
<td>25 mg/ml</td>
<td>2.67 ± 0.44</td>
<td>8.31 ± 1.22 *</td>
</tr>
<tr>
<td>50 mg/ml</td>
<td>5.14 ± 1.04</td>
<td>12.64 ± 1.25 *&lt;sup&gt;†&lt;/sup&gt;</td>
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</table>

<table>
<thead>
<tr>
<th>MCh dose</th>
<th>Total Respiratory Elastance (E&lt;sub&gt;RS&lt;/sub&gt;; cmH&lt;sub&gt;2&lt;/sub&gt;O/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 mg/ml</td>
<td>73.52 ± 9.86</td>
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<tr>
<td>50 mg/ml</td>
<td>127.56 ± 20.88</td>
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</tbody>
</table>

* OA vs. Sal  
† Dcn<sup>−/−</sup> vs. Dcn<sup>+/+</sup>
### Table 2:

**Airway Resistance (Rₘ; cmH₂O·s/ml)**

<table>
<thead>
<tr>
<th>MCh dose</th>
<th>Dcn⁺⁺⁺</th>
<th>Dcn⁻⁻⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 mg/ml</td>
<td>Sal</td>
<td>0.83 ± 0.19</td>
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<tr>
<td></td>
<td>OA</td>
<td>1.13 ± 0.27</td>
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</table>

**Tissue Damping (Gₜ; cmH₂O/ml)**

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<thead>
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<th>MCh dose</th>
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<th>Dcn⁻⁻⁻</th>
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</thead>
<tbody>
<tr>
<td>25 mg/ml</td>
<td>Sal</td>
<td>20.60 ± 3.46</td>
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<tr>
<td></td>
<td>OA</td>
<td>21.24 ± 3.11</td>
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<tr>
<td>50 mg/ml</td>
<td>Sal</td>
<td>31.06 ± 5.06</td>
</tr>
<tr>
<td></td>
<td>OA</td>
<td>23.84 ± 2.19</td>
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**Tissue Elastance (Hₜ; cmH₂O/ml)**

<table>
<thead>
<tr>
<th>MCh dose</th>
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<th>Dcn⁻⁻⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 mg/ml</td>
<td>Sal</td>
<td>60.66 ± 8.83</td>
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<tr>
<td></td>
<td>OA</td>
<td>57.47 ± 9.35</td>
</tr>
<tr>
<td>50 mg/ml</td>
<td>Sal</td>
<td>102.76 ± 18.62</td>
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<tr>
<td></td>
<td>OA</td>
<td>72.36 ± 8.66</td>
</tr>
</tbody>
</table>

* OA vs. Sal
⁺ Dcn⁻⁻⁻ vs. Dcn⁺⁺⁺
**Figure 4:**

Antigen sensitization and challenge protocol. Briefly, experimental groups were sensitized on two separate occasions, one week apart, with OA and adjuvant aluminum hydroxide. This was followed by total of (9) challenges of an intra-nasal instillation of OA in saline (3days/wk for 3wks). Physiological assessments were subsequently carried out on day 35. Control mice were sensitized and challenged with saline alone.
Figure 5:

PCR products of mouse DNA from Dcn⁺/⁻, Dcn⁻/⁻ and Dcn⁺/+ mice, respectively, run on a 2.5% agarose gel. Reaction mixes used include: (1) WT mix consisting of sense and antisense primers of exon 2 of the murine Dcn gene; (2) KO mix consisting of sense and pgk primers; or (3) WT/KO mix containing sense, antisense and pgk primers. Bands identified at 161-bp depict the wildtype Dcn⁺/+ allele, whereas bands present at 250-bp represent the disrupted Dcn gene.
Figure 6:

**A**

- Dcn+/+ Sal
- Dcn+/+ OA
- Dcn+/ Sal
- Dcn+/ OA

**B**

- Dcn+/+ Sal
- Dcn+/+ OA
- Dcn+/ Sal
- Dcn+/ OA
Figure 6 Lung physiology measurements evaluating changes in total respiratory system resistance ($R_{RS}$)(figure 6A) and elastance ($E_{RS}$)(figure 6B) during MCh aerosol challenges in OA-challenged and Sal control Dcn$^{+/+}$ and Dcn$^{-/-}$ mice. A: At MCh doses of 25 and 50 mg/ml, $R_{RS}$ was significantly increased in OA-challenged Dcn$^{+/+}$ mice compared to Sal controls; OA-challenged Dcn$^{-/-}$ mice exhibited a significant increase in $R_{RS}$ only at the highest dose of MCh (50mg/ml) in comparison to Sal controls; furthermore, $R_{RS}$ values at the highest MCh dose were significantly lower in the OA-challenged Dcn$^{-/-}$ group then in the OA-challenged Dcn$^{+/+}$ group. B: $E_{RS}$ was significantly increased in both OA Dcn$^{+/+}$ and Dcn$^{-/-}$ compared to Sal groups; this response was evident however, at a lower concentration of MCh in the OA Dcn$^{+/+}$ groups; again, the increase in response in the Dcn$^{+/+}$ OA group was greater than the Dcn$^{-/-}$ OA group at both MCh dose of 25 and 50mg/ml. **, p<0.01 OA vs. Sal controls; ***, p<0.001 OA vs. Sal controls; †, p<0.05 Dcn$^{-/-}$ vs. Dcn$^{+/+}$; ††, p<0.01 Dcn$^{-/-}$ vs. Dcn$^{+/+}$. 
Figure 7:

- **$P_{\text{aw}}$, cmH$_2$O s/ml**
  - Baseline
  - Mch 6.25
  - Mch 12.5
  - Mch 25
  - Mch 50

- **$G_u$, cmH$_2$O/ml**
  - Baseline
  - Mch 6.25
  - Mch 12.5
  - Mch 25
  - Mch 50

- **$H_u$, cmH$_2$O/ml**
  - Baseline
  - Mch 6.25
  - Mch 12.5
  - Mch 25
  - Mch 50

Legend:
- Dcn$^{+/+}$ Sal
- Dcn$^{+/+}$ OA
- Dcn$^{-/-}$ Sal
- Dcn$^{-/-}$ OA

Significance:
- *: p < 0.05
- **: p < 0.01
- ***: p < 0.001
- †: p < 0.001
- ††: p < 0.001
Figure 7 Changes in central airway resistance ($R_{aw}$), tissue damping ($G_t$) and tissue elastance ($H_t$) during MCh aerosol challenges in OA-challenged and Sal control Dcn$^{+/+}$ and Dcn$^{-/-}$ mice (figure 7A-C). A: $R_{aw}$ was significantly increased in the Dcn$^{+/+}$ OA group only compared to Sal challenged mice; Moreover, the $R_{aw}$ response observed in OA Dcn$^{-/-}$ mice was significantly lower than OA-challenged Dcn$^{+/+}$ mice at MCh dose of 25mg/ml.

B,C: $G_t$ and $H_t$ responses were significantly increased in both Dcn$^{+/+}$ as well as Dcn$^{-/-}$ OA-exposed mice compared to Sal, although this response was apparent at a lower dose of MCh (25mg/ml) in the Dcn$^{+/+}$ group; in addition, $G_t$ and $H_t$ responses were significantly higher in Dcn$^{+/+}$ than in Dcn$^{-/-}$ OA groups at MCh 25 and 50 mg/ml. *, p<0.05 OA vs. saline; ***, p<0.001 OA vs. saline; †, p<0.05 Dcn$^{-/-}$ vs. Dcn$^{+/+}$; ††, p<0.01 Dcn$^{-/-}$ vs. Dcn$^{+/+}$; †††, p<0.001 Dcn$^{-/-}$ vs. Dcn$^{+/+}$. 
Figure 8: Photomicrographs depicting tissue inflammation (H&E) (A-D). **A**: Dcn\(^{++}\) Sal control; **B**: Dcn\(^{++}\) OA-challenged; **C**: Dcn\(^{-/-}\) Sal control; and **D**: Dcn\(^{-/-}\) OA-challenged. Tissue inflammation, determined by the presence of inflammatory cells as displayed by the **arrow**, is present in greater amounts in the OA-challenged Dcn\(^{++}\) (B) group in comparison to Sal control (A). Furthermore, no significant inflammation is seen with OA in Dcn\(^{-/-}\) (D).
Figure 9: A: Tissue inflammation quantified in the airway wall as the number of inflammatory cells/WA (mm²). Tissue inflammation was shown to be significantly increased in OA-challenged Dcn⁺/⁺ mice only, when compared to Dcn⁺/⁺ Sal mice (**p<0.001). The increase observed in OA-exposed Dcn⁺/⁺ mice was, in addition, significantly higher than the level of tissue inflammation present in the OA Dcn⁻/⁻ group (†††p<0.001). B: BAL cell differential. Proportion of eosinophils, macrophages, neutrophils and lymphocytes determined out of a total 300 cells counted. OA-challenged mice demonstrated significant increases in eosinophil proportions (***p<0.001), along with concomitant decreases in the proportion of macrophages (***p<0.001). No significant changes were observed in the proportion of neutrophils or lymphocytes across all four groups.
Figure 10:

A  

B  

C  

D  

E  

- PAS+ve / total epi area (%)
Figure 10  Airway mucus expression (PAS). Panels A-D show photomicrographs of lung sections from (A), $\text{Dcn}^{+/+}$ Sal controls; (B), $\text{Dcn}^{+/+}$ OA; (C), $\text{Dcn}^{-/-}$ Sal controls; and (D) $\text{Dcn}^{-/-}$ OA-exposed mice. Mucus expression was significantly increased with OA in both $\text{Dcn}^{+/+}$ and $\text{Dcn}^{-/-}$ mice ($***p<0.001$) (E). Arrows in panel (B) and (D) identify goblet cells positive for mucus expression.
Figure 11:

E

area of SM/μm²

Dcn⁺/⁺ Sal
Dcn⁺/⁺ OA
Dcn⁻/⁻ Sal
Dcn⁻/⁻ OA
Figure 11  α-Smooth muscle actin (α-SMA) immunohistochemistry. Photomicrographs shown in panels A-D depict α-SMA expression demonstrated by the positive brown stained bundles present around the airways (arrow in B). Dcn<sup>+/+</sup> Sal (A), Dcn<sup>-/-</sup> Sal (C) and OA-exposed mice (D), demonstrated approximately the same amount of positive staining, whereas OA-challenged Dcn<sup>+/+</sup> mice exhibited somewhat increased α-SMA staining. This was quantified, as shown in (E), by calculating the area of smooth muscle corrected for the basement membrane perimeter squared (Pbm<sup>2</sup>). Smooth muscle was not significantly increased with OA in either the Dcn<sup>+/+</sup> or Dcn<sup>-/-</sup> mice, although a trend for an increase was present in the OA Dcn<sup>+/+</sup> mice vs. Dcn<sup>+/+</sup> Sal controls.
Figure 12:

A

B

C

D

E

**area of collagen/ WA**

- **Dcn+/+ Sal**
- **Dcn+/+ OA**
- **Dcn+/- Sal**
- **Dcn+/- OA**

* Significant difference

** Significant difference
Figure 12  Collagen deposition evaluated using picro-sirius red (PSR). Photomicrographs (A-D) reveal positive staining for collagen fibres shown in red, present around the airways. OA challenge significantly increased collagen deposition around the airways of Dcn\(^{+/+}\) mice only (B), compared to Dcn\(^{+/+}\) Sal controls (A) (*p<0.05). Collagen deposition was not observed to be altered between OA (D) and saline (C) Dcn\(^{-/-}\) mice. These results were further quantified by calculating the area of positive stain present within a given WA as shown in (E). In addition, the amount of collagen deposition present in OA Dcn\(^{+/+}\) mice was significantly higher than that present in OA Dcn\(^{-/-}\) mice (\(^{†}\)p<0.01).
Figure 13:
Figure 13  Biglycan Immunostaining. Representative micrographs demonstrating Bgn deposition in OA-challenged (B, D) and Sal control (A, C) mice. Positive staining for Bgn displayed in brown, shows increased Bgn deposition with OA challenge in both Dcn\textsuperscript{+/+} (B) and Dcn\textsuperscript{−/−} (D) mice compared to their respective saline controls (A and C, respectively). This increase however, was found to be statistically significant when comparing OA vs. Sal Dcn\textsuperscript{+/+} mice only (***p<0.001) as quantitatively demonstrated in (E).
Figure 14 Decorin immunostaining. Staining for Dcn using the Dcn primary antibody was performed on various lung sections. OA challenged Dcn^{+/+} mice (B) exhibited significant increases in Dcn deposition compared to Sal controls (A) (***p<0.001). This was quantified by measuring the area of positive stain corrected for a given wall area (WA) (C).
Chapter 4:
DISCUSSION
Chapter 4: Discussion

4.1 Summary of findings

The main finding of this work is that the development of allergen-induced asthma in C57Bl/6 mice is significantly altered in the absence of Dcn, as evidenced by significant differences in lung mechanics, inflammation and airway remodeling in OA-challenged Dcn<sup>-/-</sup> compared to Dcn<sup>+/+</sup> mice. In particular, after OA challenge, Dcn<sup>-/-</sup> mice demonstrate more modest hyperresponsiveness than decorin replete mice. Furthermore, OA challenge did not induce significant tissue inflammation in Dcn<sup>-/-</sup>, and the number of inflammatory cells/ peribronchial area was in fact considerably lower in OA Dcn<sup>-/-</sup> mice vs. Dcn<sup>+/+</sup> mice. Another finding of this study is that, smooth muscle and collagen deposition was not altered with OA challenge in the Dcn<sup>-/-</sup> mice. The more modest airway remodeling observed in the Dcn<sup>-/-</sup> animals may reflect the relatively decreased inflammation. We hypothesize this may be explained by the relative bio-availability of TGF-ß, with its attendant anti-inflammatory effect.

4.2 Discussion of results

Recently, the presence of airway remodeling has been recognized by a number of investigators as an important feature of asthma. Changes in airway structure, characterized by mucus hypersecretion, increases in smooth muscle as well as ECM deposition, have significant functional consequences that influence the clinical symptoms and severity of disease. Furthermore, data has provided evidence for the alteration of several PGs in the asthmatic airway wall. In particular, the SLRP Dcn has been shown to be differentially altered in this disease. In the current study, we extend these observations to evaluate the effects of Dcn in a murine model of allergic asthma.

Asthma has been described as a multifactorial disease involving many complex interactions. Thus, the use of animal models of asthma has been employed to investigate human disease<sup>95,96</sup>. In the present study we evaluated the asthmatic response in C57Bl/6 Dcn<sup>+/+</sup> mice exposed to OA. The C57Bl/6 background, commonly used for genetic manipulation, was chosen with the purpose of comparing the results obtained to Dcn<sup>-/-</sup> mice, also on a C57Bl/6 background. When assessing AHR during a MCh-response
OA-challenged Dcn<sup>+/−</sup> mice displayed significant increases in R<sub>RS</sub> and E<sub>RS</sub> compared to Dcn<sup>+/+</sup> Sal controls. Further assessment of lung mechanics was achieved by partitioning this physiologic response into central and distal lung components. The complex impedance model, allowed a separation of the total response into the central (R<sub>sw</sub>) and distal lung mechanical parameters, including tissue damping (G<sub>t</sub>) and elastance (H<sub>t</sub>), which represent the responses in the distal airways as well as the parenchymal tissues<sup>106,107</sup>. It is essential to note that during induced constriction, the increased response in G<sub>t</sub> also reflects small airway constriction, as peripheral airway inhomogeneities as well as airway closure become increasingly significant<sup>108,109</sup>. This is of particular importance in regards to asthma, as this disease is largely characterized by a heterogeneous constriction of the airways. In addition, there has been collective agreement on the notion that the heterogeneous lung responses exhibited in asthma occur within the distal lung<sup>11,110</sup>. Thus, separation of the lung response into central and peripheral indices, adds an additional level of understanding into the contribution of diverse factors in development of disease. Significant increases in R<sub>sw</sub> were observed in the Dcn<sup>+/−</sup> mice exposed to OA. Similarly, OA-challenged Dcn<sup>+/−</sup> mice also exhibited substantial increases in both G<sub>t</sub> and H<sub>t</sub> when compared to Sal Dcn<sup>+/−</sup> controls.

Assessment of inflammation present within the peribronchial area of airways was also performed, and was expressed as ‘the number of inflammatory cells/ WA’. Results obtained demonstrated that OA challenge significantly increased the inflammatory cell number/ WA in Dcn<sup>+/−</sup> mice. Analysis of BAL cell differential highlighted certain characteristic features of asthma, in that, relative proportions of eosinophils were considerably higher, and conversely, macrophage proportions were significantly lower in OA-challenged Dcn<sup>+/−</sup> mice vs. Sal controls. The sensitization and challenge protocol employed also resulted in significant increases in goblet cell and mucus production, evaluated by PAS-positive staining present within the epithelial layer in OA Dcn<sup>+/−</sup> mice. Furthermore, OA challenge also induced airway remodeling in these mice. Although ASM was not significantly increased with OA in the Dcn<sup>+/−</sup> mice, there was a trend to increase when compared to Dcn<sup>+/+</sup> Sal controls. In addition, the deposition of collagen was found to be considerably increased in the Dcn<sup>+/−</sup> OA-exposed mice. This corresponds well with other animal studies showing that OA challenge induces many characteristic
features of human asthma including increases in AHR, inflammation and airway remodeling. It is interesting to note, however, that various studies have characterized C57Bl/6 mice as low responders to allergic sensitization. In particular, in a study by Shinagawa and Kojima, C57Bl/6 mice challenged with OA did not exhibit AHR, in terms of airway resistance, when compared to Sal controls. In addition, OA challenge did not induce eosinophilia or thickening of the airway wall in this strain. However, slight increases in collagen deposition were seen. The differences observed may be explained by differences in antigen sensitization and challenge protocols employed. In the study by Shinagawa and Kojima, mice were challenged 5 times a week for a range of durations via OA aerosol. This differs from the current study whereby intra-nasal instillations of OA in saline solution were used. Different methods of allergen delivery may thus play an important role in determining the allergic response in this strain.

Subsequently, the data obtained in the Dcn+/+ mice were compared to the development of the asthmatic phenotype in Dcn-/- mice. Lung mechanics have been shown to be influenced by Dcn, in that the lack of this PG has been shown to affect airway resistance, compliance and airway-parenchymal interdependence under baseline conditions. In previous studies from this laboratory, Dcn-/- mice were shown to exhibit lower baseline Raw compared to Dcn+/+ mice. Moreover, the speculation that the airways of these mice have increased airway compliance was further supported by results demonstrating increased changes in length of Dcn-/- tracheal rings for a given change in tension, when compared to Dcn+/+ controls. In the current experiment, responsiveness of the airways during a MCh-response curve was assessed in both Dcn-/- OA-challenged and Sal controls and compared to the responsiveness demonstrated by Dcn+/+ OA-challenged and Sal mice. Analysis of the total respiratory system confirmed an increase in resistance and elastance in Dcn-/- animals challenged with OA compared to their respective Sal controls. Surprisingly, OA-challenged Dcn-/- mice demonstrated more modest hyperresponsiveness in terms of both RRS and ERS when compared to Dcn+/+ OA-exposed mice. In agreement with the study by Salerno et al., there was no difference in the MCh-induced bronchoconstriction between Sal Dcn+/+ and Dcn-/- control groups.

Increases in central Raw were observed only in OA-challenged Dcn+/+ mice compared to Sal controls at the highest MCh concentration. Once again, Dcn-/- mice
exposed to OA demonstrated a significantly lower airway response compared to Dcn+/+ mice. These results may suggest that central airway resistance does not contribute to the total resistance of the respiratory system observed in Dcn+/+ mice exposed to OA. Dcn−/− mice also show significantly less distal lung hyperresponsiveness than Dcn+/+ mice after OA exposure. Although Gti and Hti were both significantly elevated in the Dcn+/+ OA-challenged group compared to its respective Sal control, these indices were significantly lower in the OA Dcn−/− vs. OA Dcn+/+ group, at the two highest concentrations of MCh.

There are certain technical issues which warrant discussion. The in vivo studies were performed using the Flexivent small-animal ventilator, which has certain mechanical limitations. In partitioning the responsiveness of the airways into central and distal lung components, the power detection threshold of the system did not allow us to obtain representative values for Raw at the highest concentration of MCh (50mg/ml). One of the main reasons for this is that, at higher levels of MCh the degree of bronchoconstriction in OA-challenged groups was considerable. As a consequence, the volume signal was no longer equally distributed across the lung, and the assumptions underlying the quantification of Raw using the constant phase model, no longer apply. As a result, we obtained negative values for Raw which were excluded from our analysis.

There are many possible explanations for the physiologic results obtained in this study. The differences in physiological responsiveness between Dcn+/+ and Dcn−/− OA-exposed groups may be explained by the observed differences in inflammation. The evaluation of tissue inflammation, demonstrated an increase in inflammatory infiltrate in OA-exposed Dcn replete mice, as previously described. Alternatively, OA challenge did not induce tissue inflammation in Dcn−/− mice, which also exhibited significantly lower levels of inflammation when compared to OA-challenged Dcn+/+ mice. The absence of a significant change in inflammation in the OA Dcn−/− group may also be a potential explanation for the observed differences in AHR in these mice. The consequence of inflammatory exudates present within the peribronchial space has important implications in the mechanics of the airways themselves. This has been effectively elucidated by different analyses which show that the presence of edema or inflammation will uncouple the airways from the surrounding parenchyma, resulting in a decreased elastic load on ASM. The load acting in opposition to ASM shortening, and the way in which this load is
altered during constriction of the airways, are critical determinants of airway narrowing. Furthermore, it has now been widely accepted that the presence of airway inflammation may contribute to the pathogenesis of AHR by means of inflammatory mediator release. Examples of such mediators include IL-1, IL-2, IL-3, IL-4, IL-5, IL-9, IL-13, and tumor necrosis factor (TNF)-α as well as various other chemokines. Among the pleiotropic effects of these mediators, there is evidence to suggest that one of their more important actions, in the context of asthma and AHR, is their influence on ASM function.

The increased sensitivity and maximal response of ASM, characteristic of asthma, may be influenced in part by the inflammatory mediators present around smooth muscle bundles. Several studies have provided evidence for the ability of inflammatory mediators, and more specifically cytokines, to increase the response of normal ASM to contractile agonists such as histamine. For example, TNF-α has been demonstrated to enhance HASMC responsiveness in vitro. Furthermore, IL-13 has been shown to indirectly enhance ASM contractile state by inhibiting inducible nitric oxide synthase, which results in decreased nitric oxide production, an important bronchodilator. Increased levels of surrounding inflammatory mediators has been speculated to not only enhance responsiveness of ASM cells to contractile agonists, but also increase the stiffness and the contraction velocity of ASM, as well as decrease smooth muscle plasticity, a term used to define the ability of ASM to alter their phenotype and potentially their mechanical functions. Accordingly, lower levels of inflammation in the Dcn−/− OA-exposed mice, may imply lower levels of inflammatory mediators released and thus, a lower degree of AHR.

An additional explanation for the more modest AHR in Dcn−/− mice is that the lack of Dcn may result in more bio-available TGF-β. TGF-β has been demonstrated to function as both anti-inflammatory as well as pro-remodeling, depending on different settings. We suggest that the more modest inflammation observed in Dcn−/− OA-exposed animals reflects the relative bio-availability of TGF-β. In addition, it seems that in this model, the anti-inflammatory effect of uninhibited TGF-β may be more important than the pro-remodeling effects. An interesting study by Alcorn and co-workers has also demonstrated that in a Balb/c model of allergic asthma, the presence of anti-TGF-β antibodies resulted in an enhancement of AHR to inhaled MCh, in mice exposed to...
aerosolized OA. They therefore concluded that TGF-β may have a suppressive role in the development of AHR induced by antigen. Although a different mouse strain and challenge protocol were used compared to the study presented here, the evidence regarding TGF-β’s role in ameliorating AHR may be a possible mechanism responsible for the more modest AHR observed in the OA-exposed Dcn^{-} mice. This is an interesting hypothesis to be further examined. In particular, future studies evaluating whether the levels of TGF-β in lung tissue homogenates differ in OA-challenged Dcn^{-} vs. Dcn^{+/+} mice are warranted.

Analysis of BAL cell differential revealed a similar pattern of cell distribution between Dcn^{+/+} and Dcn^{-} groups. OA did significantly increase the percentage of eosinophils in Dcn^{-} mice compared to Sal controls, as was also observed in Dcn^{+/+} mice; furthermore, significant decreases in macrophages were also observed in Dcn^{-} OA-challenged groups similar once again to Dcn^{+/+} mice. The observation that eosinophils were the predominant cells present within the BAL of both OA-exposed Dcn^{+/+} and Dcn^{-} groups, may account for the observed increase in PAS positive staining within the epithelial layer of these airways. Eosinophils have been shown to secrete various mediators including cys-LTs, which may promote epithelial damage. Studies have presented evidence for the ability of these mediators to affect airway vascular permeability as well as promote the secretion of mucus. Moreover, the role of eosinophils in promoting increased mucus and goblet cell hypersecretion is corroborated by Lee et al., in a study using PHIL mice, which are deficient in eosinophils. This work provided in vivo evidence that OA-exposed PHIL mice exhibited less airway pathology including reduced epithelial hypertrophy as well as goblet cell and mucus production. Recent data has also suggested that eosinophils maintain a ‘T_{H2} environment’ and thus the vast category of T_{H2} cytokines, including IL-13. IL-13 has itself been demonstrated to be implicated in the development of airway mucus. In vivo studies involving IL-13 blockade, achieved through gene targeting techniques or the administration of the soluble IL-13Rα2-Ig, show prevention or reversal of the mucus response as well as an inhibition in AHR. Moreover, Dudley et al. have shown through their studies of primary cells (human or mouse) grown at an air-liquid interface, that IL-13 directly induces the production of mucus. In fact, IL-13’s effect on epithelial cells is such that it
may alter airway mucus content and viscosity. Thus, it is also through indirect mechanisms that eosinophils may contribute to airway mucus production and consequently the observed increases in PAS positive staining. Mucus hypersecretion may play a role in establishing airway obstruction and thus increases in respiratory resistance, which may account for the observed increases in airway responsiveness in Dcn−/− OA-exposed vs. Sal mice. However, the more modest response in these animals when compared to Dcn+/+ OA-challenged mice suggest that increased mucus production is not sufficient to drive the entire lung response.

Another factor which may contribute to the more modest hyperresponsiveness in the OA-challenged Dcn−/− group may be the reduced degree of remodeling present within the airways of these mice. Firstly, a trend to an increased area of ASM was shown in OA-exposed Dcn+/+ mice only, whereas Dcn+/+ mice challenged with OA did not exhibit any changes in ASM area compared to Sal controls. In addition, OA Dcn−/− mice did not show any increases in the deposition of collagen, which was found to be significantly increased in the Dcn+/+ OA-exposed mice. The amount of collagen present in Dcn+/+ mice was also significantly higher than that observed in Dcn−/− OA-challenged mice. The lack of enhanced remodeling in the OA-exposed Dcn−/− group, may therefore explain the differences in AHR between Dcn+/+ and Dcn−/− experimental groups.

Interestingly, the amount of Bgn was significantly increased with OA in Dcn−/− mice only, which suggests an upregulation of this structurally similar proteoglycan in the absence of Dcn. In the study by Fust et al., no observable differences were found when the amount of Bgn was measured in both Dcn+/+ and Dcn−/− lung tissues. However, it is important to note that this study evaluated mice which were neither sensitized nor challenged. Our evidence suggests that exposure to OA in Dcn−/− mice may trigger increased Bgn deposition to compensate for the lack of Dcn. This may have important implications in the generation of AHR. Although not evaluated in this study, Pini et al. have shown that Bgn and Dcn are differentially located within the airway wall of a rodent model of asthma. Whereas Dcn is observed within the adventitia, Bgn is present primarily within the ASM layer. The presence of increased ECM components such as Bgn within the ASM layer and surrounding the ASM bundles themselves may provide a parallel elastic impedance or radial constraint to ASM shortening and consequently a limitation in
the narrowing of airways (for review see 42). The data demonstrating that Dcn is increased in OA-exposed Dcn replete mice also corroborates evidence that Dcn deposition is altered in an animal model of asthma and may therefore exhibit important biological functions.

Another possible explanation for the reduced airway and tissue responses seen in the Dcn+/− OA-challenged group may be that the absence of Dcn within the adventitia, where it is primarily located, results in a better coupling of the airways and parenchyma due to more moderate remodeling within this layer of the airway wall. Various investigators have in fact suggested that increased remodeling or thickening of the adventitial layer results in reduced transmission of forces of interdependence acting against the ASM shortening. This will consequently lead to an increased luminal narrowing and airflow resistance. It is the parenchymal tethering which provides an elastic load against smooth muscle shortening42. Thus, if this tethering effect is enhanced due to the absence of Dcn and accordingly, the lack of adventitial thickening, a more modest increase in resistance will be observed for the same degree of ASM shortening.

4.3 Conclusion

In conclusion, this study has demonstrated that lung responsiveness, inflammation as well as certain indices of remodeling, are more modest in Dcn−/− mice vs. Dcn+/+ exposed to OA. Dcn−/− OA mice exhibited more modest AHR, accompanied by a lack of significant increases in inflammatory infiltrate. Furthermore, these animals did not display any increase in smooth muscle or collagen deposition as compared to Dcn+/+ OA mice. The data in the current study therefore provides important information on the role of Dcn in regards to the development of allergic asthma. Along with previous studies demonstrating a significant role for Dcn in the determination of airway mechanical properties60,68, our results which show that the lack of Dcn results in more modest airway disease, may have important implications in understanding the disease process. An additional avenue which would be of interest to pursue would be to investigate the effect of up-regulating Dcn, in the context of the allergic asthmatic model presented here, and evaluate its impact on AHR. Further studies to document increased active TGF-β and the effects of uninhibited TGF-β in this model would also be of interest.
REFERENCES


Include here ALL procedures described in the original protocol. New and changed procedures in CAPS (was section 10a in main protocol); Please only attach SOPs related to new and changed procedures to this renewal form.

### 7. Endpoints

a) For B and C level of invasiveness,

The procedures are the same as the original protocol: YES ☒ NO ☐

IF NO, supply new endpoints that are different from the original protocol:

Experimental endpoints:

Clinical endpoints:

b) For D level of invasiveness,

Include here ALL endpoints, including the ones described in the original protocol as well as new and changed endpoints in CAPS:

Experimental endpoints:

Clinical endpoints:

### 8. Hazards

(check here if none are used: ☒)

a) Are the hazards different from original protocol? (Infectious radioactive, toxic, carcinogen, tumours)

YES ☐ NO ☐ if yes, supply details (material, risks, precautions):

b) Have the cell lines been tested for human and animal pathogens? YES: ☐ NO: ☒ None used: ☒

### 9. Description of Animals to be used in the coming year (only):

Quality Control Assurance: To prevent introduction of infectious diseases into animal facilities, a health status report or veterinary inspection certificate may be required prior to receiving animals from all non-commercial sources or from commercial sources where animal health status is unknown or questionable. Quarantine and further testing may be required for these animals. If more than 6 columns are needed, please attach another page.

<table>
<thead>
<tr>
<th>Species</th>
<th>Sp/strain 1</th>
<th>Sp/strain 2</th>
<th>Sp/strain 3</th>
<th>Sp/strain 4</th>
<th>Sp/strain 5</th>
<th>Sp/strain 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplier/Source</td>
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<td>Thomas Jefferson U</td>
<td>Harlan labs</td>
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<td>m/f</td>
</tr>
</tbody>
</table>
We have already established successful breeding of decorin deficient and wild type animals and experiments are underway in these 2 populations. We have completed some experiments using the acute exposure (sensitization and challenge over 35 days) and require 5 more animals in both the ovalbumin (OA) and saline control groups. In future studies we will examine the effects of a more chronic exposure (over 57 days) to determine if additional airway remodeling occurs and whether this contributes further to the disease process. We will need 20 animals of each genotype - 10 for OA and 10 for saline control in order to make meaningful statistical comparisons.

In breeding these animal, we have obtained a number of mice which are heterozygotes. Once genotyping establishes the precise genotype, heterozygotes will be euthanized.

We will use 3 breeding pairs to generate 30 animals in 6 months time. Breeders will be retired by euthanasia at the age of 12 months.

For the study in Brown Norway rats, experiments are underway. We will need 10 additional animals in the ovalbumin challenged and saline challenged groups to complete the analyses.

Submit to your local Facility Animal Care Committee. Please note that after two renewals, a full protocol needs to be submitted.

This approval does not imply that space will be made available. If a major increase of space needs is anticipated, please contact the appropriate animal facility manager.