I, Naina Gour, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Immunology.

It is entitled:
Dectin-1 is a critical negative regulator of allergic asthma

Student's name: Naina Gour

This work and its defense approved by:

Committee chair: Marsha Wills-Karp, Ph.D.

Committee member: Ronald L. Schnaar, Ph.D.

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Dectin-1 Is A Critical Negative Regulator of Allergic Asthma

A dissertation submitted to the
Division of Research and Advanced Studies
of the University of Cincinnati
In partial fulfillment of the requirements for the degree of
Doctor of Philosophy (Ph.D.)
In the Graduate Program of Immunology
of the College of Medicine
August 2014
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Asthma is a chronic inflammatory disease of the airways, driven by an inappropriate Type-2 immune response to innocuous allergens in susceptible individuals. Among the allergens, sensitization to house dust mites constitutes a major risk factor for the development of asthma. Despite this recognition, the mechanisms driving initiation of skewed type 2 cell responses following allergen exposure in asthmatics remains unknown. Recent evidence suggests that the recognition of allergens by pattern recognition receptors (PRRs) expressed on lung epithelial cells and antigen-presenting cells (APCs) dictates the nature and magnitude of the effector T cell response. PRRs trigger the inflammatory response upon recognition of specific conserved structures known as Pathogen Associated Molecular Patterns (PAMPs) through germline-encoded PRRs expressed by the host. Recently, the carbohydrate moiety-sensing family of PRRs, the C-type lectin receptor family (CLRs) has been implicated in the development of Type 2 immune responses. Based on our earlier published findings that dust-mite contains an active non-mammalian expressed carbohydrate (β-glucan), we hypothesized that the putative β-glucan receptor, dectin-1 may mediate allergic airway responses. Contrary to our initial hypothesis, utilizing multiple approaches, we report that dectin-1 strongly inhibits the magnitude of asthmatic severity (AHR, eosinophilia and severe mucus plugging of the airways). Mechanistically, we report for the first time that dectin-1 expressed on lung structural cells, instead of on hematopoietic cells, inhibits the production of the type-2 promoting cytokine, IL-33, which in turns inhibits type 2
cytokine production from non-CD4+ innate lymphoid type 2 cells (ILC2s). Collectively, these results suggest that constitutive dectin-1 expression normally serves to dampen type 2 mediated immune responses at the airway surface-thereby maintaining homeostasis. Importantly, we demonstrate that this pathway is dysregulated in the bronchial epithelial cells of asthmatics as compared to healthy individuals suggesting that the loss of dectin-1-mediated inhibition of the IL-33-ILC2 pathway may be an important determinant of asthma susceptibility. These studies suggest that modulation of this pathway may provide a promising new therapeutic avenue for the treatment of this ever-increasing disease.
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Chapter 1: Introduction

1.1. Asthma History and Definition

Asthma is a complex chronic inflammatory disease of the lungs. The term asthma, which was first coined in ancient Greece, is derived from the verb ‘aazein’, which means to exhale with an open mouth, to pant, or to gasp [1]. The original term did not necessarily define the disease rather it was employed to describe the respiratory symptoms associated with the disease [2]. It was not until the nineteenth century that a comprehensive understanding of the disease was made by a British physician, Henry Hyde Salter [3]. Among his various seminal papers, the most commendable was “On Asthma: Its Pathology and Treatment” [4]. Based on his evaluation of hundreds of asthmatics he defined asthma as “Paroxysmal dyspnea of a peculiar character, generally periodic with intervals of healthy respiration between the attacks” [4].

1.2 Asthma Prevalence

Although asthma has been recognized for centuries, the incidence of disease has been dramatically increasing over the past few decades in developed nations [5]. Studies have shown that the number of people in the US with asthma has increased 2.9% each year between 2001 and 2010, from 20.3 million people in 2001 to 25.7 million people in 2010 [6]. Further, asthma prevalence is higher among children, females and those below the poverty line [7]. On a global scale, the World Health Organization (WHO) predicts that
around 300 million people are currently afflicted by asthma [8].

1.3. Asthma Pathophysiology

Asthma is a complex clinical syndrome characterized by recurrent episodes of chest-tightness, shortness of breath and wheezing. Although the etiology of asthma is unknown, both genetic and environmental factors are known to contribute to the development of the disease. Asthma has a strong family heritability, as individuals with a first degree relative with asthma are at a greater risk of developing asthma. Further support for a genetic basis of asthma is the fact that there is a greater concordance for asthma between monozygotic twins compared to dizygotic twins. However, the concordance is not 100%, suggesting that asthma is a polygenic disorder with strong environmental influences. One of the strongest identifiable predisposing factors for the development of asthma is atopy-the propensity to mount immunoglobulin E (IgE) responses, to otherwise innocuous environmental allergens [9]. Nearly 80–90% of children and 50–60% of adults with asthma have detectable allergen-specific IgE, usually directed against inhalant antigens [10]. Broadly, asthma can be distinguished as allergic (atopic or extrinsic) or non-allergic (non-atopic or intrinsic) [11, 12]. While atopic asthmatics have elevated levels of circulating IgE and develop positive skin tests to identifiable allergic triggers (house dust mite, pollens, animal dander) [13], non-atopic asthmatics have normal circulating levels of IgE and negative skin test reactivity to common allergens. Such IgE production is a tightly regulated process, part of a complex network of cellular
and molecular events necessary for the development of the allergic response.

The pathophysiological manifestations of asthma were initially thought to be bronchospasm and edema [14], however, several landmark studies reported in 1980’s and 1990’s reported the presence of inflammatory cells in the bronchoalveolar lavage (BAL), sputum and in bronchial biopsies from asthmatics [15-18]. These findings led to the hypothesis that inflammation is one of the major underlying causes of the disease. The current dogma is that in susceptible individuals, airway inflammation arises as a result of inappropriate immune responses to commonly inhaled allergens. Initiation of this response appears to occur with presentation of environmental allergens by antigen presenting cells residing in the mucosa to CD4+ T cells in the draining lymph nodes (a process referred to as sensitization). In allergic asthmatic individuals, responding allergen-specific T cells polarize to a Th2 pattern (IL-4, IL-5, IL-13) of cytokine production. However, the T cells from non-allergic, non-asthmatics, clearly recognize the same allergens, they develop tolerogenic immune responses to these innocuous antigens. The mechanisms controlling the Th2-polarized responses in allergic asthmatic individuals are not well understood.

The elaboration of Th2 cell cytokines sets into motion a series of events leading to IgE production and recruitment and activation of a variety of downstream effector cells such as mast cells, eosinophils, basophils, and effector T cells. In sensitized individuals, challenge with the specific allergen to which they are sensitized to results in the development of a rapid response within 15-30 min [19]. During the rapid response, mediators are released from mast cells
activated by IgE cross-linking with allergen, which result in bronchoconstriction and vascular leakage [20, 21]. The rapid response is followed by a late phase response that is characterized by the infiltration of other leukocytes such as lymphocytes and eosinophils [20]. The pathological consequences of chronic inflammatory reactions are associated with the migration of these inflammatory cells into the tissues. Below we will discuss the contribution of each of these inflammatory cells to the development of asthma.

1.3.1 Cellular Inflammatory response

1.3.1 Mast Cells

First discovered by Paul Ehrlich in 1878 on the basis of unique staining and granules, mast cells have been implicated in numerous inflammatory disorders [22]. Mast cells are derived from hematopoietic progenitors and circulate in the blood in an immature state. Their migration into the tissue is followed by maturation and differentiation under the guidance of stem cell factor (SCF) and other cytokines secreted by endothelial cells and fibroblasts [23]. Mast cells are found in all vascularized tissues, especially those that interface with the external environment [10]. The crucial step in mast cell activation is the cross-linking of the high-affinity immunoglobulin E receptor (FceRI) on their surface by antigen-bound specific IgE [24]. Activation results in exocytosis of pre-formed granules containing various inflammatory mediators such as histamine, proteases, cytokines and proteoglycans within minutes [10]. Activation also triggers the synthesis and release of lipid mediators such as prostaglandin
(PG)D$_2$, leukotriene (LT)B$_4$ and LTC$_4$ that are derived from the metabolism of endogenous arachidonic acids, such as prostaglandin (PG)D$_2$, leukotriene (LT)B$_4$ and LTC$_4$ [10]. Mast cell mediators such as histamine and LTs can induce bronchoconstriction, vasodilation, pulmonary edema, bronchial smooth-muscle hyperplasia and recruitment of leukocytes [10].

Studies in murine models have yielded mixed results regarding the role of mast cells in allergic-asthma. Utilizing a model of Ova+adjuvant sensitization and challenge, Takeda et al reported that mast cell-deficient mice [W/W(v)] and their congenic littermates develop similar eosinophilic airway inflammation and airway hyperresponsiveness [25]. However, studies by other groups reported that unlike WT mice, mast cell-deficient mice (W/W$^v$) are not able to mount AHR upon sensitization and challenge with Ova (without adjuvant) or with low doses of antigen [26, 27]. Unlike humans, the naïve mouse lung has few mast cell progenitors and lacks mature mast cells although they are enriched following airway inflammation [28]. Therefore, there are limitations to mouse models and caution must be taken when elucidating a role for mast cells in these models of asthma.

1.3.3. Basophils

The number of basophils are reported to be higher at baseline and after allergen challenge in lung biopsies from atopic asthmatic subjects compared with atopic control subjects or normal control subjects [29-31]. Unlike mast cells that mature exclusively in vascularized tissues, basophils mature in the bone marrow,
then circulate in the peripheral blood and cannot be found in lung tissue under homeostasis conditions [32]. However, some studies have suggested that basophils can rapidly infiltrate into the lung in response to inflammatory stimuli [33]. Various chemotactic receptors (c-kit, CRTH2, FPR, uPAR, C3aR, C5aR) are selectively displayed on basophils and are responsible for their recruitment into the asthmatic airways [34]. Basophils express the receptor for IgE, crosslinking of which results in release of inflammatory mediators such as histamines, tryptase and leukotrienes [35].

1.3.4. Eosinophils

One of the characteristic features of asthma is an elevated number of eosinophils in the airways, airway secretions and peripheral blood [36]. Numerous studies have linked eosinophilia with enhanced airway obstruction and airway reactivity to methacholine [36, 37]. However, studies by other groups have indicated that the severity of the disease does not necessarily correlate with tissue eosinophilia [38].

Eosinophils develop from bone-marrow precursors under the regulation of cytokines, IL-3, IL-5 and GMCSF [39]. Out of the three, IL-5 is more specific for the development of the eosinophil lineage and it is also involved in recruitment of eosinophils into the circulation [39]. Eosinophils are recruited to the tissues during inflammation under the guidance of various cytokines (IL-4, IL-5, IL-13), and chemokines (eotaxin) [40]. Eosinophils are characterized by cytotoxic granules containing specific cationic proteins such as major basic protein (MBP),
eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN), which induces tissue damage [40]. Apart from these classical cationic proteins, eosinophils can also secrete cytokines and chemokines such as IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IL-16, IL-18, RANTES, eotaxin-1 [40, 41]. Triggering of eosinophils by engagement of receptors for cytokines, immunoglobulins, and complement can lead to rapid secretion of such mediators leading to proinflammatory effects, including activation and regulation of vascular permeability, mucus secretion, and smooth muscle constriction [40].

1.3.5. Neutrophils

Neutrophils are polymorphonuclear leukocytes that are classically thought to serve as the first line of defense against bacterial and fungal infections [42]. Neutrophils arise from hematopoietic progenitors and are released into the circulation where they are short-lived [42]. During inflammation, neutrophils invade the airways under the influence of a variety of chemokines such as CXCL8 (or IL-8), TNF-α, and leukotriene B4 [43]. Following activation by microbial and/or host-derived factors, neutrophils begin the process of microbe killing through phagocytosis, oxidative burst, degranulation and formation of extracellular traps [44]. Degranulation results in release of mediators such as myeloperoxidase (MPO), defensins, lysozyme, elastase, cathepsin G, all of which can be detrimental to the host tissue [44, 45].

Both the numbers and activation status of neutrophils is elevated in peripheral blood and induced sputum of some asthmatics compared to controls
The number of neutrophils in the BAL has been shown to be positively correlated with the severity of asthma [49, 50]. As the levels of the cytokine IL-17A, which is an important regulator of neutrophil recruitment, have been positively correlated with disease severity, it is thought lung neutrophilia may play a role in the severe phenotype [51].

1.3.2. Airway remodeling

The cellular inflammatory response to allergen exposure is accompanied by structural changes, collectively known as airway remodeling. [52]. Chronic inflammation in the asthmatic airway is believed to be the cause of the structural damage that initiates the aberrant repair response seen in both large and small airways [53] [54].

Airway remodeling is characterized by thickening of the basement membrane, goblet cell hyperplasia, hyperplasia and hypertrophy of airway smooth muscle (ASM) cells and sub-epithelial fibrosis [55, 56] [57]. Excessive mucus secretion in the airways of asthmatics results in the formation of mucous plugs consisting of mucin, cellular debris from necrotic airway epithelial cells, inflammatory cells and plasma protein exudate [58].

1.3.2.1 Airway smooth muscle in asthma

The increase in ASM content observed in some asthmatic airways is of particular importance as it is thought to be a major contributor to airway luminal narrowing and reduced airway caliber in asthmatics [57]. ASM cells are
elongated cells comprised of various contractile proteins such as smooth muscle-specific α-actin, desmin, myosin heavy chain (MHC) and myosin light chain (MLC) kinase [59]. Actin and myosin are considered to be the primary constituents of the contractile apparatus in the smooth muscle cell [60]. ASM cells exist as bundles, in physical contact with the extracellular matrix and innervated by parasympathetic nerve fibers. Bronchoconstriction results due to the activation of the parasympathetic nervous system leading to release of acetylcholine that binds to muscarinic receptors (M3 type) on smooth muscle cells. Activation of M3 type receptors results in increases in intracellular calcium levels leading to contraction of smooth muscle [61]. In addition to the neurotransmitter (acetylcholine), inflammatory mediators such as histamine, cysteinyl leukotrienes and prostaglandins released from mast cells, eosinophils and basophils can induce ASM contraction [62].

More than 90 years ago, Huber and Kosser first reported that ASM mass is increased in patients that died of status asthmaticus compared to those who died from non-pulmonary conditions [63, 64]. Since that time, ASM has been extensively studied to investigate whether it is inherently defective in asthmatics. The results have been conflicting in that some ex vivo studies have reported enhanced force generation/contraction in asthmatics ASM cells compared to controls, while others have reported the opposite or no differences [64].

In addition to hyperplasia (i.e. increased numbers), asthmatic airways have been shown to have an increased deposition of extracellular matrix (ECM) proteins around the ASM. The ECM, which is comprised of components derived
from the basement membrane and connective tissues, forms a complex network of macromolecules that functions as a scaffold for the airways [65]. Histological analysis of asthmatic airways as compared to non-asthmatics has revealed that ECM proteins such as Collagen I, III, and V, fibronectin, tenascin, hyaluronan, versican, and laminin α2/β2 are increased whereas collagen IV and elastin are decreased [66]. ECM proteins are mainly secreted by activated fibroblasts whose numbers are reported to be increased in the sub-epithelial layer leading to thickening of the basement membrane in asthmatic airways [67]. Several inflammatory mediators (TNFα, IL-1β, TGFβ) that are reported to be higher in the BAL from asthmatics can promote the proliferative and synthetic potential of fibroblasts. Fibroblasts can also be a potential source of cytokines and chemokines such as IL-8, RANTES, eotaxin and GMCSF that can contribute to the recruitment of inflammatory cells into the asthmatic airway. In addition to fibroblasts, ASM cells can also secrete ECM proteins. Interestingly, ASM cells from asthmatics were shown to produce a different profile of ECM proteins than those from non-asthmatics [68]. Compared to ASM from non-asthmatics, those from asthmatics produced less of chondroitin sulfate and collagen IV but higher amounts of prelecan and collagen I in ex vivo [68]. In turn, ECM components can modulate the proliferation, survival and release of pro-inflammatory mediators from ASM [69]. Thus, there is a dynamic interaction between ECM and ASM whereby one can modulate the synthetic potential of the other.

Beyond its role as a contractile unit, ASM is now thought to also be involved in either driving/enhancing airway inflammation through the release of
various pro-inflammatory mediators. Chemokines and cytokines such as RANTES, IL-8, GMCSF, MCP-1, 2, eotaxin, IL-6, IL-5 have been shown to be secreted by ASM cells in response to TNFα, IL-1β, IL-4 and IL-13 [70]. Although there are numerous reports depicting the release of pro-inflammatory mediators in vitro, only a few have reported the expression of these mediators in vivo. Thus the contribution of ASM derived cytokines to the inflammatory process in asthma is not yet fully understood.

1.3.2.2 Goblet cell hyperplasia

The respiratory epithelium consists of four types of cells, goblet cells, ciliated cells, endocrine cells and basal cells [71]. While goblet cells secrete mucus that serves to trap allergens/pathogens that are being constantly inhaled, ciliated cells sweep the mucus out of the lungs through the movement of water and mucus up the mucociliary escalator [71]. Endocrine cells of lungs or pulmonary neuroendocrine cells (PNEC) secrete local mediators (serotonin etc.) that regulate mucus production, ciliary movement and smooth muscle contraction [71]. Basal cells are like undifferentiated stem cells that have the ability to differentiate into other types of lung epithelial cells following injury [71].

The mechanisms of mucus production are not completely understood, but the production of inflammatory mediators such as IL-13 and various growth factors (i.e. TGF-a, EGF) downstream of allergen sensitization promotes mucus production [72]. Mucin glycoproteins (mucins) found in the airway can be of two types, membrane-bound or secretory [73]. Secretory mucins produced by
epithelial cells form gel-like mucous in the airways. Out of more than 20 reported mucin genes (Muc), 12 are found in the respiratory epithelium [73]. Specifically, Muc5ac and Muc5b are the dominant secretory mucins in the airways. During allergic airway inflammation, the number of goblet cells increases (goblet cell hyperplasia) and hence mucus secretion is greatly enhanced. In healthy individuals, goblet cells constitute around 5% of airway epithelial cells. In contrast, autopsies from patients that died of fatal asthma have revealed that goblet cells represent up to 20-25% of the cells in the epithelium. This increase in goblet cells results in mucus clogging of small- and medium-sized airways obstructing the airways [73]. In addition to increases in the numbers (i.e. hyperplasia) of goblet cells, a change in the phenotype (i.e. metaplasia) is also thought to contribute to enhanced mucus production seen during inflammation [73].

1.3.3 Lung function alterations

Chronic lung inflammation is known to lead to alterations in lung function. Specifically in asthmatic airways, the inflammatory response which ensues upon exposure to an allergen triggers extensive airway remodeling (Changes in ASM, sub-epithelial cell fibrosis, extensive ECM deposition), enhanced contractile responses of the airways to bronchoactive substances (airway hyperresponsiveness), and heightened mucus production leading to obstruction of the lung respiratory tree and development of the characteristic symptoms of the disease (i.e. wheezing, coughing and breathlessness). Spirometry, the
measurement of maximum expired volume during a forced expiration is used to provide an assessment of the severity of airflow limitation, its variability and its reversibility [74]. In asthmatics, the amount of air that a person can exhale in 1 second, known as Forced Expiratory Volume in 1 second (FEV1) is decreased. A subject is considered to be asthmatic if following exposure to bronchoconstrictor (histamine or methacholine), the FEV1 is below 20% of predicted value when adjusted for size, weight and age. This characteristic of asthma is referred to as airway hyperresponsiveness (AHR). [75, 76].

1.4. Asthma Pathogenesis

1.4.1. T cells

As primary orchestrators of adaptive immune responses, T lymphocytes have been implicated in the pathogenesis of asthma. Although asthma is a complex disease involving a plethora of immune cells, the fundamental role of T lymphocytes in the disease pathogenesis remains unchallenged. Based on the differential expression of surface proteins, T cells are grouped into either CD8+ T (expressing CD8 protein) subset or CD4+ T (CD4 protein) cells [77]. CD8+ T cells or cytotoxic T lymphocytes are primarily involved in killing of infected (microbe) or tumor cells [77]. CD4+ T cells or helper T lymphocytes play a crucial role in promoting the humoral (B cells) and cell-mediated (macrophage) immunity [77]. CD4+ T cells are paramount in instructing B cell mediated antibody (IgE) production and recruitment of inflammatory cells (eosinophils, basophils, neutrophils), all the hallmarks of allergic asthma [78].
Tremendous evidence supports a role for CD4+ T cells in asthma pathogenesis. The numbers of CD4+ T lymphocytes in BAL fluids and bronchial biopsies have been found to be consistently elevated in asthmatics as compared to healthy individuals [9, 79-82]. CD4+ T cells are actively recruited to the airways following allergen challenge as a simultaneous drop in their number is observed in peripheral blood [83]. In addition to an increase in the numbers of T cells in the lungs, the CD4+ T cells from the BAL and bronchial biopsies from asthmatic patients have an activated phenotype. Gavett et al. were the first ones to show that CD4+ T lymphocytes are required for antigen-induced airway hyperreactivity and pulmonary eosinophilia in a mouse model [84]. They showed that depletion of CD4+ T cells with depleting anti-CD4 mAb, prior to antigen (sheep red blood cells, SRBC) sensitization and challenge resulted in diminished asthmatic airway responses. Taken together these data support a role for CD4+ T cells in disease pathogenesis [9].

T cells originate in the thymus from common lymphoid progenitors (CLPs) that migrate from the bone marrow to the thymus via the blood [85, 86]. The differentiation of T cells from a CLP population is complex wherein the precursor cells lose the potential to differentiate into other lineages like B cells or NK cells in a highly orchestrated manner and therefore become developmentally committed to give rise to naïve T cells [87-90]. Clonally, these T cells further differentiate into subsets that will either express T cell receptors (TCR) that recognize antigenic peptide on either MHC I –CD8 complex giving rise to CD8+ T cells or on MHC II-CD4 complex, giving rise to CD4+ T cells. CD8 T cells
principally recognize pathogen-derived cytosolic peptides on the surface of infected cells and hence are effective in clearing such infected cells during bacterial and viral infections [91]. While there are several reports that suggest a role for CD8+ cells in asthma, their contribution to disease pathogenesis remains controversial [92-94].

The differentiation of naïve CD4+ T cells into functional effector subsets is guided through its interaction in the periphery with antigen-presenting cells (APCs) like dendritic cells and macrophages. Upon coming in contact with antigen-loaded APC in lymphoid organs, three major interactions between APCs and naïve T cells occur, facilitating the differentiation of naïve T cells into a functional effector subset. Briefly, these interactions include; presentation of antigenic peptide on MHC II molecule by APC to TCR, co-stimulatory receptor ligation on both cells and secretion of specific cytokine by APCs that shapes T cell differentiation program. The initial reports of the existence of different subsets of CD4 T cells came from seminal findings by Mosmann & Coffman [95] and later by Bottomly and colleagues [96] wherein they showed the existence of two major clones of mouse CD4 T cells, namely Th1 and Th2 cells [97]. CD4+ T cell subsets are defined by the profile of cytokines they produce, in which Th1 cells primarily secrete IFNγ, while the cytokine signature Th2 cells is IL-4, IL-5 and IL-13. A third major effector population that differentiates from naïve CD4+ T cells was discovered [98-100] in 2003 and later designated as Th17 cells as they primarily secrete IL-17A, IL-17F along with IL-22 [97, 101-103]. Recently new subsets of CD4+ T cells have been recognized namely, Th22 [104, 105] and Th9
Th22 cells primarily secrete the cytokine IL-22, but no IL-17A. Th9 cells are major producers of IL-9. Other types of lymphocytes such as NKT cells [107] and natural regulatory T cells or nTregs [108] also exists, but these cells are not generated in the periphery from naïve CD4+ T cells, rather they originate in the thymus. Although, induced Tregs (iTregs) can be derived from naïve CD4+ T cells in the periphery [109].

Cytokines produced by APCs play a critical role in differentiation of effector CD4+ T cells from naïve CD4 T cells [110]. For example, IL-12 secreted by APCs was shown to be essential for Th1 differentiation [111]. Although the source of IL-4 remains controversial, it was first thought that IL-4 was essential for generating IL-4-producing T cells [112-114]. While TGFβ is critical for differentiation of Tregs [115, 116], a combination of TGFβ and IL-6 is necessary for differentiation of Th17 cells [103, 117, 118]. In addition, Th17 cells require IL-23 for the effective priming and maintenance of their phenotype [99, 119]. The differentiation of Th22 cells requires IL-6 and TNFα [104]. Th9 cells develop from naïve T cells in the presence of TGFβ and IL-4 [120].

Transcription factors and the signaling transducer and activator of transcription (STAT) proteins play an essential role in determining and maintaining the fate of effector CD4 T cells. Transcription factors are unique proteins that induce transcription of specific gene(s) in a cell and while their activity is dependent on expression levels, STATs are regulated by cytokine-mediated post-translational modification [97]. Activated STAT proteins, in collaboration with master transcription factors regulates the production of the key
cytokines by Th cells and also play an important role in the induction of the master transcription factors [97]. For the majority of CD4+ T effector subsets the master transcription factor is known, these include T-bet/Eomes (Th1), GATA3 (Th2), RORγt (Th17), PU.1 and IRF4 (Th9) and FoxP3 for Tregs [121-127] but the transcription factor for the Th22 lineage remains to be defined.

1.4.1.2 Th1 cells in asthma

Based on the cytokine profile secreted by the effector CD4+ T cells, they are able to participate in distinct immune responses. IFNγ, lymphotoxin alpha (LTα) and IL-2 are the principal cytokines secreted by Th1 cells. These cells are classically associated with immune responses against intracellular pathogens through activation of macrophages, CD8+ T cells and B-cell mediated antibody (IgG) production. In humans, Th1 cells have been shown to be indispensable for resistance against mycobacterial infections [128, 129]. IFNγ produced by Th1 cells potentiates macrophage's anti-microbial activity that helps fight against Toxoplasma gondii infection [130]. LTα, a member of TNF super family is known to be pathogenic in autoimmune diseases such as experimental autoimmune encephalitis (EAE), an animal model of multiple sclerosis. [131, 132]. The role of Th1 cells in asthma pathogenesis remains controversial as there are studies suggesting both pathogenic and protective roles in mouse models of asthma [133, 134]. Nonetheless, there is growing evidence that an IFNγ-mediated Th1 response is associated with neutrophilic inflammation and AHR in severe asthmatics. Several human studies also indicate that IFNγ levels correlate with
asthma severity [135] as IFNγ+ cells were shown to be elevated in the airways of severe asthmatics [136]. Moreover, in a longitudinal birth cohort study it was reported that IFNγ positively correlates with both atopic and non-atopic AHR [137]. In a murine model of asthma, adoptive transfer of antigen-specific Th1 cells was shown to mediate development of steroid–resistant AHR upon subsequent allergen and endotoxin (LPS) challenge [138]. This was dependent upon IFNγ produced by Th1 cells, as mice treated with neutralizing IFNγ antibodies prior to the cell transfer did not develop AHR. Interestingly, antigen-specific Th1 cells alone could not induce AHR, however in combination with a second signal, LPS, a steroid-refractory airway response ensues. Therefore, a secondary signal (LPS) is required to drive Th1 cells in promoting allergen-driven AHR.

While a Th1 response is initiated to combat intracellular pathogens, a Th2 immune response is useful in mounting appropriate immune responses against extracellular parasites, such as helminths [139]. A protective anti-helminth Th2 immune response is associated with the formation of granulomas. For example, during Schistosoma mansoni infection, the antigens released from worm eggs induce a marked Th2 immune response that orchestrates the development of granulomas in the liver [140]. These granulomas surround and segregate the eggs from surrounding healthy tissue thereby protecting against dissemination of the parasite. Similarly, Th2 responses have been shown to be essential in the expulsion of intestinal nematodes like Trichuris muris, Heligmosomoides polygyrus and Nippostrongylus brasiliensis [141]. Although protective against
worms, an excessive Th2 immune response can damage infected host tissues due to the excessive fibrosis, granuloma formation and tissue lesions [142].

1.4.1.3. Th2 cells in asthma

CD4+ Th2 cells are considered to be the main drivers of asthma pathogenesis as the cytokines produced by these cells regulate the induction of the hallmark pathological features of asthma i.e. IgE production, eosinophilia, goblet cell hyperplasia and AHR. Several human studies in the early 1990’s indicated that asthmatics, compared to non-asthmatics, displayed a distinct pattern of cytokines that mirrors a Th2 phenotype in lung tissue and/or biological fluids [83, 143, 144]. CD4+ T cells isolated from the BAL of asthmatics express elevated levels of mRNA for IL4, IL5 and IL13. Furthermore, GATA-3 expression is reported to be higher in atopic asthmatics compared to controls [145]. Moreover, genetic linkage studies in humans have shown an association between polymorphisms in IL13, IL-4 and STAT6 and asthma [146-148].

As human studies are correlative, more definite evidence supporting the role of Th2 cells in asthma has come from murine models. Studies by Cohn et al first dissected the role of CD4+ Th2 cells in antigen-induced asthmatic response [149]. They isolated and transferred antigen-specific Th1 or Th2 cells from DO11.10 mice into syngeneic BALB/c mice and then exposed them to OVA by aerosol. Mice receiving antigen-specific Th2 cells, but not Th1 cells developed lung eosinophilia and mucus production. They showed that the phenotype was dependent on IL-4 as Th2 cells from Il4-deficient mice were not recruited to the
lungs and did not induce the phenotype. Interestingly, neutralizing IL-4 with specific monoclonal antibodies prior to sensitization effectively inhibited airway hyperreactivity, however, when given after antigen priming they had no effect [150]. Interestingly, it was shown that blockade of the IL-4R even at the time of allergen challenge was effective at inhibiting the allergic phenotype. The discrepancies between these studies led to the hypothesis that the IL-4R may have another ligand, which plays a more important role in the effector phase of the allergic responses. Based on the fact that IL-13 and IL-4 are structurally similar and share a receptor (i.e. composed of the IL-4Rα and the IL-13Rα1) [151], it was hypothesized that IL-13 may mediate the effector phase of the allergic response. Indeed, two landmark studies by Wills-Karp et al [152] and Grunig and colleagues [153] showed that IL-13, not IL-4, is the central mediator of allergic asthma. Utilizing A/J mice exposed to ovalbumin (OVA), Wills-Karp and colleagues showed that blockade of IL-13 utilizing soluble IL-13Ra2, prior to OVA sensitization and challenge resulted in complete reversal of allergen-induced AHR [152]. Interestingly, IL-13 blockade did not significantly affect pulmonary eosinophilia or the ova-specific IgE response, but reversed allergen-induced increases in mucus secretion. Further, they showed that not only is IL-13 necessary for allergen-induced AHR, but it is also sufficient to mediate all the phenotypic changes associated with allergic asthma as administration of recombinant IL-13 (rIL-13) to the airways of naïve A/J mice lead to the induction of AHR accompanied by mucus cell metaplasia and a mild influx of eosinophils in the BAL. Significant time-dependent increases in serum-IgE levels were also
observed following administration of rIL-13. Subsequent studies utilizing transgenic mice overexpressing IL-13 in the lungs confirmed these results [154]. Specifically lung specific IL-13 transgenic mice developed AHR to methacholine, pulmonary eosinophilia and mucus cell metaplasia [154].

The importance of Th2 cytokines has also been supported in human asthma. *IL13* mRNA expression has been repeatedly reported to be significantly enhanced in bronchial biopsies, and sputum of asthmatics as compared to controls [155]. Moreover, compared to controls, IL-13 is enhanced in the BAL fluids of asthmatics following allergen challenge [156]. Based on the knowledge gained from human and murine studies, several clinical trials targeting either IL-13 directly or IL-13 signaling molecules were initiated [157-159]. Recent trials utilizing the neutralizing IL-13 antibody, lebrikizumab in poorly controlled asthmatics showed an improvement in FEV1 in a group of individuals displaying a Th2 inflammatory phenotype, but not in a group with a low Th2 phenotype [160]. These studies suggested that not all cases of asthma are exclusively IL-13-dominated, and that other CD4+ T cells and cytokines (IFNγ, IL-8, IL-17A) might also contribute to asthma pathogenesis.

Apart from IL-4 and IL-13, there are other Th2 cell-derived cytokines that affect the allergen-induced asthma phenotype. As IL-5 is critical for eosinophil development from bone-marrow precursors, their survival and activation [161-165], its role in asthma pathogenesis has been extensively studied. Indeed, bronchial biopsies from asthmatic patients show increased *IL5* mRNA expression in activated T cells and elevated levels of IL-5 in serum and BAL fluid [83, 166].
While IL-5 serum levels correlate with eosinophil numbers in the peripheral blood [167], the number of activated IL5 mRNA-expressing T cells correlates with both numbers of lung eosinophils and asthma severity (AHR) [143, 168]. Utilizing a monoclonal antibody to murine IL-5 (TRFK-5) in a model of ova-induced pulmonary inflammation, Chapman & colleagues [120] showed that IL-5 is crucial for lung eosinophilia as mice receiving TRFK-5 prior to allergen challenge had reduced number of eosinophils in the BAL and lung-tissue compared to the isotype-treated control mice. Concurrent with low eosinophils in the lungs, TRFK-5 treated mice had more eosinophils in the bone-marrow suggesting that IL-5 is crucial for the exit of eosinophils from the bone-marrow into the lungs during active pulmonary inflammation.

While the role of IL-5 in allergen-induced pulmonary eosinophilia is clear, the role of IL-5 and eosinophils in allergen-induced AHR remains controversial. Utilizing transgenic mice (expressing diphtheria toxin under eosinophil peroxidase promoter) devoid of eosinophils (PHIL mice), Lee et al demonstrated that eosinophils are crucial in allergen induced AHR [169]. However, Humbles & colleagues utilizing eosinophil-lineage ablated mice (Δdbl GATA mice) reported that while eosinophils promote airway remodeling (ASM hyperplasia, collagen deposition), they are not required for allergen-induced AHR [170]. While PHIL mice were generated on the C57BL/6 background, ΔdblGATA mice utilized by Humbles & colleagues were on the BALB/c background. Similarly depending upon the strain of mice used in the experiment, varying roles of IL-5 in experimental allergic airway disease have been observed. While IL-5 is reported
to be crucial in allergen-induced AHR in C57BL/6 mice [171] it is not essential in BALB/c mice [172, 173].

Several clinical trails examining the effects of IL-5 neutralization have been conducted. While, the anti-IL-5 antibody, Mepolizumab was effective in decreasing eosinophil numbers in the lung, blood and sputum, it did not necessarily impact airway physiology in mild to moderate asthmatics [174-177]. In contrast, there has been some success with mepolizumab treatment in severe asthmatics with recurrent exacerbations suggesting that eosinophils might impact asthma pathophysiology during acute attacks in severe patients [178]. Thus, while eosinophils do not appear to be necessary for initiation of the disease, they may play a role in the exacerbation of disease suggesting that targeting of IL-5 or eosinophils more directly might improve disease outcomes in some types of asthma patients.

1.4.4.4 Th17 cells in asthma

By the late 1990’s it had become clear that severe asthmatics might not only exhibit a maladaptive Th2 immune response, but that other T cell subsets might also be involved. In the early 2000’s several studies led to the discovery of a subset of effector T cells that were defined by the production of IL-17 and hence referred to as Th17 cells. CD4+ Th17 cells primarily secrete IL-17A and IL-17F, in addition to IL-22 and IL-21 [179, 180]. Classically, Th17 cells are best known to induce the production of neutrophil-chemoattractants, IL-8 and GROα, by tissue resident cells [181]. IL-17 has also been shown to promote neutrophil
granulopoiesis, recruitment and activation during inflammation [182]. Th17 cells have also been shown to be pathogenic in autoimmune disease like EAE [183]. Several studies showed that auto-reactive CD4+ T cells producing IL-17 (Th17) were not induced in IL-23 deficient mice in EAE and collagen-induced arthritis (CIA) and that these cells are necessary for the pathophysiology seen in these diseases [98, 184]. Apart from autoimmunity, Th17 cells are primarily associated with mounting of an immune response against extracellular bacteria and fungi such as *Staphylococcus aureus* [185], and *Candida albicans*, respectively [186].

As airway neutrophilia correlates with asthma severity and IL-17A can promote neutrophilia, several studies have investigated the role of IL-17A in severe forms of the disease [37, 187]. Indeed, the levels of IL-17A in sputum have been shown to correlate with methacholine responsiveness in humans [188]. Moreover, cells expressing IL-17A were increased in the sputum and BALs from asthmatic patients and both IL-17A and IL-17F have been shown to be highly expressed in bronchial biopsies from severe asthmatics as compared to mild asthmatics [189]. Similar findings have been reported in a mouse model of allergic asthma wherein IL-17A has been shown to correlate with the severity of the disease [190]. Specifically it has been shown that individual strains of mice that are differentially susceptibility to house-dust mite (HDM) -induced allergic asthma display differences in Th17 cytokine production [190]. Specifically, A/J mice develop a robust airway hyperresponsive phenotype (AHR), while C3H/HeJ mice develop a milder form of the disease. Surprisingly both strains of mice mount an equipotent Th2 immune response to HDM as the levels of Th2
cytokines, IgE and eosinophilia are comparable in both strains. However, they differ in their ability to mount a strong Th17 response. While A/Js have elevated IL-17A levels after HDM stimulation, C3H/HeJ mice have significantly lower levels of IL-17A and blockade of IL-17A prior to HDM challenge results in diminished AHR in A/J mice. Conversely, exposure of HDM along with recombinant IL-17A results in more severe AHR in C3H/HeJ mice. However, rIL-17A was shown not to be sufficient in inducing an asthmatic response by itself, only in conjugation with IL-13 administration can IL-17A exacerbate an asthmatic response. However, other groups have demonstrated that Interleukin-17A (IL-17A) enhances contractile force generation by both mouse and human airway smooth muscle [191]. Moreover, IL-17A secreted from Th17 cells has also been shown to stimulate the production of mucins, MUC5AC and MUC5B from cultured primary human epithelial cells in vitro [192] suggesting that IL-17A may promote mucus production in vivo. Further, IL-17A has been shown to promote the production of pro-fibrotic cytokines like IL-6 and IL-11 from human fibroblasts ex vivo [193]. Therefore, in addition to inducing airway hyperreactivity, IL-17A might also contribute to airway remodeling during asthma severity. Mouse studies of RSV infection have also suggested that IL-17A may inhibit rather than enhance type 2 immune responses [194]. Surprisingly, a recent clinical trial targeting the IL-17 receptor with the human anti-IL-17A monoclonal mAb (Brodalumab) in moderate to severe asthmatics showed no improvement in the disease symptoms [195]. However, a Phase II clinical trial targeting the cytokine directly (human, anti-IL-17A, Secukinumab) in severe asthmatics is ongoing
As clinical trials aimed at neutralizing either IL-13 or the IL-17 receptor alone have not been widely successful, clinical trials targeting both IL-13 and IL-17A might prove to be more beneficial in controlling severe asthmatics.

1.4.1.5 Th9 and Th22 cells in asthma

Although the role of the newly discovered subsets of CD4+ T cells, Th9 and Th22, is not as well studied, their role in asthma is currently being explored. Th9 cells are found in the peripheral blood of allergic patients however; this population is rarely seen in non-allergic individuals [197]. PBMCs isolated from atopic infants produce more IL-9 compared to those from non-atopic infants upon dust-mite or cat allergen exposure [198]. Similar responses from PBMCs have been shown in adult asthmatics upon dust-mite and pollen exposures [199-201]. IL-9 has been reported as a candidate gene for atopy, bronchial hyper-responsiveness and asthma [202, 203]. Initially Th2 cells along-with mast cells and eosinophils were considered to be the primary source of IL-9.

The detection of IL-9 secreting T cells has been challenging in mouse models as their appearance seems to be transient [120]. However, using flow cytometry IL-9-positive CD4+ T cells have been identified in various mouse models of allergic inflammation. In these models, IL-9 secreting Th9 cells have been shown to be pathogenic as they promote eosinophil recruitment, goblet cell metaplasia and IgE production [127, 197]. Further, IL-9 enhances the asthma phenotype as mice overexpressing IL-9 in the lungs had marked increases in AHR compared to control mice upon methacholine inhalation [204]. Although this
report indicated a pathogenic role for IL-9 in asthma, the authors did not identify the cellular source of IL-9. However, they hypothesized that the source of IL-9 was CD4+ Th2 cells. In an adoptive transfer model, Staudt et al. demonstrated that transfer of in vitro skewed Th2 and Th9 cells into a Rag2-deficient host induced the symptoms of asthma including AHR, goblet cell hyperplasia and eosinophilia following OVA-challenged mice [127]. Further they showed that anti-IL-9 mAb treated mice receiving Th2 cells had a slightly decreased asthma phenotype whereas mice receiving Th9 cells and neutralizing IL-9 antibody had a profoundly reduced asthma phenotype. Their data indicates that while Th2 cells are crucial, IL-9 produced by Th9 cells and not Th2 cells drives asthma pathogenesis.

Th22 cells have been shown to express the skin-homing receptors, CCR4 and CCR10 [104] and can be induced by skin-resident dendritic cells, referred to as Langerhans cells (LCs) [205]. These studies suggest that Th22 cells preferentially home to the skin and thus may be important in skin homeostasis and diseases. Indeed IL-22 expression is increased in skin lesions of patients with psoriasis and atopic dermatitis [206] and IL-22 was shown to be pathogenic in murine models as transgenic overexpression of IL-22 lead to acanthosis. There are only a few reports depicting the role of IL-22 in allergic disease. IL-22 and IL-22R expressing infiltrating cells have been observed in inflamed skin from nickel-challenged allergic subjects [207]. No direct evidence or reports investigating the role of Th22 cells in asthma pathogenesis have been published. Provided that Th17 cells can also secrete IL-22 and that Th17 cells have been
shown to exacerbate asthma, careful studies depicting the role of IL-22 or other mediators from Th22 cells remains to be done.

1.4.1.6. T-regulatory (Tregs) Cells in Asthma

The immune system has many mechanisms to control destructive inflammatory responses, one of the most critical of these is mediated via regulatory T cells or Tregs. As their name suggests T regulatory cells are known to regulate or dampen inflammatory responses. Tregs have been shown to be critical for protecting against autoimmune diseases like type 1 diabetes, multiple sclerosis and limiting chronic inflammatory diseases like inflammatory bowel disease (IBD) and asthma [208]. In vitro and in vivo studies have shown that Tregs can suppress the activation, proliferation and effector functions of various immune cells including CD4+ T cells, CD8+ T cells, NK cells, NKT cells and APCs [209]. Suppression mechanisms utilized by Tregs to dampen an immune response can be grouped into four basic “modes of action”: production of inhibitory cytokines (IL-10 and TGFβ), inducing cytolysis, initiating metabolic disruption and controlling maturation (in DCs) [208].

The frequency of IL-10 producing Tregs has been reported to be lower, while the frequency of IL-4 producing T cells is enhanced in the blood of atopic allergic donors as compared to healthy donors [210]. These findings suggested that CD4+CD25+ T regulatory and IL-10-producing T cells have the potential to suppress pathogenic Th2 responses and that this suppression may be defective or overridden in patients with allergic disease [211]. Moreover, peripheral blood
CD4+CD25+ cells from HDM allergic asthmatic children have been shown to be less competent than those from non-atopic donors in suppression of CD4+CD25-cells [212]. Hartl et al demonstrated that the percentage of BAL T cells that were CD4+CD25+ and expressed FOXP3 was lower in children with asthma as compared to healthy controls. Moreover, these parameters were positively correlated with FEV₁ for children with asthma [211, 213].

The role of Tregs in controlling asthma pathogenesis has been extensively studied in murine models. Depletion of Tregs before allergen sensitization was shown to exacerbate airway inflammation and AHR in a model of dust-mite induced asthma [214]. Conversely, adoptive transfer of Tregs was shown to be sufficient to suppress established lung eosinophilia, Th2 cell infiltration, expression of Th2 cytokines (IL-5, IL-13) and airway remodeling in chronic allergen-induced inflammation model [215]. Further studies have demonstrated that the suppressive ability of transferred Tregs is dependent on IL-10 produced by these cells [216]. Moreover, deletion of IL-10 specifically in Tregs was shown to enhance AHR and inflammation in mice exposed to inhaled allergen [217]. In another report, suppression of allergic airway disease by Tregs was shown to be dependent on both IL-10 and TGFβ production [218].

1.4.2 Antigen-Presenting Cells (APCs)

Although the exact steps leading to the aberrant expansion of CD4+ Th2 cells in asthma are unknown, signals provided by APCs are critical for CD4+ T cell differentiation. APCs serve as sentinels in pulmonary immune responses by
recognizing inhaled antigens and initiating T cell responses [219]. An effector T cell response can only develop after the interaction of a naïve T cell with an APC. For this reason, APC play a crucial role in asthma pathogenesis. In susceptible individuals, allergen sensitization in the airways occurs when an airway-resident APC takes up and processes inhaled allergens and presents these to naïve T cells leading to initiation of a Th2 immune response [220]. APCs express a diverse set of innate receptors referred to as Pattern Recognition Receptors (PRRs) that can recognize specific patterns in allergens or pathogens. Recognition through PRRs facilitates uptake of allergen by APCs, which then process the allergen into small antigenic peptides. Antigenic peptides are generated through proteolysis and there are two major proteolytic systems operating within a cell that contribute to antigen presentation [221]. While cytosolic protein undergoes active proteolysis by the proteasome, internalized proteins or proteins from an exogenous source are degraded by lysosomal proteolysis. Briefly, the peptides generated in the cytosol are translocated to the endoplasmic reticulum (ER) where they are loaded onto the Major Histocompatibility Molecule I (MHC-I) molecule with the help of transporter associated with antigen processing (TAP) proteins [222]. The MHC-I-peptide complex is then transported from the ER to the golgi apparatus and eventually to the cell surface for presentation to CD8+ T cells. Antigens from an exogenous source can be internalized by multiple pathways, including phagocytosis, endocytosis, macropinocytosis and eventually traffic to the lysosome or the late endosomal compartment, often called the MHCII compartment or MIIC [221]. The
MHC-II complex is assembled within the ER, followed by functional maturation in the acidic environment of the MIIC in the presence of exogenous antigenic peptides. Eventually the MHCII-peptide complex is shuttled to the surface to be presented to CD4+ T cells [223]. However, cross-presentation can occur wherein the MHC-I complex can bind to peptides internalized by endocytosis or phagocytosis, this might be critical for initiation of primary responses by naïve CD8 T cells. Similarly, in some cases, MHCII can bind to endogenous peptides generated in lysosomes. In several human studies, the MHC region on chromosome 6 has been linked to asthma-associated phenotypes with antigen presentation through specific MHC-II alleles (HLA-DR) predominating in response to certain allergens [224].

While MHCI is expressed on all nucleated cells, MHC-II expression is restricted to APCs, facilitating antigen uptake and presentation by these cells. There are 2 major populations of professional APCs in the lungs, namely, alveolar macrophages (AM) and dendritic cells (DCs). At steady state, AMs are mainly found in alveolar spaces of the lungs, while DCs are found in the lung parenchyma. Although AM have high phagocytic activity, they are thought to be poor T cell stimulators and even immunosuppressive as their deletion resulted in severe pulmonary inflammation following antigen exposure in mice [225]. Therefore, DCs are chief APCs in the lungs following pathogen/allergen exposure. Immunostaining of bronchial biopsies has demonstrated the accumulation of DCs in the lungs and concomitant decrease in circulating DCs post-allergen challenge in atopic asthmatics [226]. Following allergen challenge,
DCs also increase in BALF from asthmatic patients [227]. Another study in asthmatic patients revealed a reduction in blood DC numbers as early as 3 hours after allergen challenge with ragweed and dust mite allergen, suggesting a rapid recruitment of these cells from the blood to the lungs in response to allergen challenge [228].

Dendritic cells develop in the bone marrow from a precursor population known as granulocyte-monocyte precursors (GMPs), which arise from the parent population referred to as the common myeloid progenitors (CMP). Hence these DCs are referred to as myeloid DCs (mDCs). However, some populations of DCs found particularly in lymphoid organs (CD8α+ DCs) are thought to be lymphoid in origin as they differentiate from common lymphoid progenitors (CLP). DC progenitors in the bone marrow give rise to circulating precursors that home to tissues, where they reside as immature cells with high phagocytic capacity [229]. Resident immature dendritic cells can be found in dense networks throughout the epithelium of the respiratory tract, positioned both above and beneath the basement membrane, positioning these cells as first responders to inhaled antigen [228]. Immature DCs extend their processes between epithelial cells into the airway lumen, sampling the airway lumen for inhaled antigens [228]. Also, upon allergen encounter, the bronchial epithelium secretes the DC chemoattractant CCL20 that guides the accumulation of immature DCs expressing the CCL20 receptor, CCR6 [230, 231]. CCR6-expressing immature DCs can also be recruited from circulating DC precursors and thus can accumulate rapidly (within hours) in areas adjacent to the bronchial epithelium.
Antigen capture induces immature DCs to undergo a series of phenotypic and functional changes that culminate in the complete transition from an antigen-capturing cell to a mature APC [229]. DC maturation occurs in parallel with their migration from peripheral tissues to the draining lymphoid organs where they interact with naïve T cells. Maturation results in loss of phagocytic capacity, upregulation of co-stimulatory molecules (CD80, CD86 etc.), cytoskeleton rearrangements and acquisition of high motility [229]. Antigen-bearing DCs leave the non-lymphoid organs (lungs) following downregulation of CCR6 and upregulation of CCR7 receptor that binds to the chemokine CCL21 found abundantly in lymphatic vessel [233]. The CCR7+ DCs in the draining lymph nodes home to the CCL19 rich paracortex/ T cell region where DCs receive further maturation signals upon T cell interaction [234]. As mentioned before, three major interactions between DCs and naïve T cell are necessary to drive T cell differentiation into a specific effector subset. First; antigen peptide presentation on MHC complex by DCs to TCR, second; co-stimulatory molecule interaction between cells, third; cytokine secreted by DCs signaling through receptors on T cells. The amplification and modification of the MHC-TCR signal by the co-stimulatory signal enables the antigen-specific T lymphocyte to proliferate, secrete cytokines, and express cell-surface molecules for further cell–cell interactions [235].

While antigen-presentation is not reported as compromised in asthmatics, the degree of co-stimulatory molecule interactions via CD28 (T cells) with CD80
(B7.1), CD86 (B7.2) may be altered in asthma. Numerous studies have reported higher expression of CD80 and CD86 on B-lymphocytes, alveolar macrophages, and monocytes from asthmatic patients over that observed in controls. The levels of soluble CD86 are elevated in asthmatic children and they correlate with the severity of AHR [236]. In concordance with human studies, studies in mouse models have reported that the CD28-CD86 interaction is crucial to generate IL-4-producing Th2 cells [237]. However, the contribution of CD80 to allergen-induced Th2 response is debated as some studies suggests that CD80 favors the generation of Th1 over Th2 immune responses [235]. Another ligand for CD80 and CD86, CTLA-4, which is expressed by activated T cells, provides a negative signal, promoting Treg responses and limiting inflammatory immune responses [235]. Polymorphisms in the CTLA4 gene have been linked with elevated serum IgE and bronchial hyper-responsiveness in patients with asthma [238].

Several other co-stimulatory molecule interactions such as Ox40L (APC): Ox40 (T cells) and CD40 (APC): CD40L (T cells) have also been implicated in promoting Th2 differentiation, IgE class switching and AHR in allergic airway disease [239, 240]. While there are reports supporting the role of these co-stimulatory molecules (mentioned above) in driving Th2 immune responses, there are also reports suggesting the opposite [235]. The discrepancies in the data might be due to either differences in the mouse models used (genetic composition) or the experimental protocols utilized in the individual studies. Furthermore, as costimulatory molecule interactions are not antigen specific, blockade of other specific signals such as those involved in antigen-presentation
in conjunction with costimulatory molecule blockade might prove to be more effective in controlling Th2 immune responses.

While antigen presentation and co-stimulation are necessary to properly activate T cells, cytokines secreted by APC or the surrounding tissues promote the skewing of T cells to a particular lineage (Treg, Th1, Th2, Th9, or Th17). As IL-4 is widely known to be required for differentiation a naïve T cell into a Th2 effector lineage, it’s cellular source still remains to be defined. In vivo, as DCs have never been found to produce IL-4 or a Th2-skewing cytokines, it had been thought that Th2 responses might develop by default, in instances in which differentiating factors driving Th1, Th17, and Tregs are absent [241]. In 2009, three independent studies utilizing helminth infection and a protease allergen (papain) were published suggesting that basophils were the early source of IL-4 that guides the generation of Th2 immune responses [242-244]. However, a year later studies by Lambrecht & colleagues showed that basophils could not perpetuate Th2 allergic immune responses in a model of HDM-induced experimental asthma. Moreover, they reported that the anti-FcεR1 antibody (MAR-1) used to deplete basophils in the earlier reports, could also deplete a subset of DCs expressing FcεR1. Following airway exposure FcεR1+ DCs take up significantly more OVA antigen (10%) than basophils (3%) and only FcεR1+DCs, not basophils, drove CD4+ T cells and cytokine production. Therefore, although basophils likely don’t present antigen to T cells due to their low expression of MHC-II, they can amplify Th2 responses [241].

The role of DCs in asthma pathogenesis has been extensively studied in
murine models of asthma. Similar to humans, studies in murine and rat models have shown that DCs increase significantly in the lungs after exposure to allergen [245, 246]. Transfer of OVA-pulsed DCs directly into the airways of naïve animals results in the development of the cardinal features of allergic asthma, i.e. eosinophilia, goblet cell hyperreactivity and AHR upon subsequent exposure to OVA [247]. As DCs express the surface marker, Cluster of Differentiation 11c (CD11c), mice expressing the diphtheria toxin receptor under the control of the CD11c promoter (CD11c –DTR) can be depleted of DCs upon exposure to diphtheria toxin. However, in addition to DCs, lung alveolar macrophages are also known to express CD11c and hence CD11c-DTR mice are also deficient in alveolar macrophages upon inhalation of diphtheria toxin. Utilizing such transgenic mice Rjit et al showed that the conditional deletion of CD11c+ cells in OVA-sensitized mice abolished the development of Th2 allergic response upon subsequent allergen challenge [248]. Further, they showed that adoptive transfer of CD11c+ DCs and not alveolar macrophages restores eosinophilic airway inflammation in CD11c-depleted mice. Moreover, they showed that not only are DCs necessary to establish a Th2-driven asthmatic immune response but they are also critical in maintaining the asthma phenotype as DC depletion during an ongoing Th2 inflammation resulted in diminished features of allergic asthma.

Depending on the subset of DCs engaged by an allergen either a tolerogenic or allergic inflammatory response is initiated. There is a significant overlap in the expression of surface markers among lung DCs subsets, however
certain subsets can be defined using unique markers and their role in murine models of asthma pathogenesis varies. Unlike conventional DCs (cDCs) that express high level of integrin CD11c, plasmacytoid DCs (pDCs) have very low CD11c but high expression of B220 and CD317 (PDCA-1). cDCs can be further divided into CD103+ CD11c+ CD11b- intraepithelial DCs and CD103-CD11c+CD11b+ myeloid DCs (mDCs). While mDCs are effective T cell stimulators and can induce a variety of effector T cell response (Th1, Th2, Th17) depending on the pathogen/allergen, pDCs can induce a strong Treg response thus dampening the inflammatory response [249]. Lambrecht et al first demonstrated that mDCs are critical in the induction of allergic inflammation as transfer of OVA-pulsed mDCs, but not saline treated-mDCs led to an accumulation of activated CD4+ T cells, eosinophils and goblet cell hyperplasia [250]. Since the initial observation, several studies have confirmed a role for endogenous mDCs in the induction of Th2 pulmonary immune responses driving allergic asthma [251, 252].

pDCs can influence the decision between tolerance and immunity to inert inhaled antigens (ex. ova with no endotoxin) by modulating mDCs. A single intratracheal (i.t.) administration of OVA containing very low to no endotoxin does not result in Th2 cell-associated eosinophilic airway inflammation upon repeated challenge with OVA aerosol. However, tolerance can be broken if the antigen mixture contains an adjuvant such as (ex. endotoxin or lipopolysaccharide, LPS) [253]. Various mechanisms of how adjuvants are able to amplify an immune response have been proposed throughout the years, one of the central ones is
through activation of mDCs by engaging PRRs [254, 255]. Heer et al demonstrated that exposure to inhaled LPS-free OVA can result in sensitization and development of eosinophilic, Th2 rich airway-inflammation in mice depleted of pDCs [256]. Further they demonstrated that pDCs suppress the generation of effector T cells and that adoptive transfer of pDCs can prevent the development of asthma. Lewkowich et al showed that, C3H/HeJ mice, which are naturally resistant to the development of AHR, showed increase antigen uptake of HDM in pDCs rather than mDCs [249]. Neutralization of pDCs, with the anti-CD25 mAbs reversed their resistance suggesting that pDCs can suppress the development of allergic airway responses.

Recently, intraepithelial DCs (CD103+ DCs) have gained attention for their role in asthma pathogenesis. In a mouse study, using thrombomodulin, the ligand for CD141 (BDCA3) expressed on CD103+ DCs, Takagi et al showed that inhalation tolerance achieved by suppression of CD4+ T cell–dependent allergic airway inflammation requires CD103+ DCs. This report suggested that CD103+ DCs promote immunosuppression; furthermore other reports suggest that CD103+ mucosal DCs can confer protection by inducing anti-inflammatory Foxp3+ Tregs [257]. However, modulating CD103+ DCs directly in an OVA-LPS model, Nakano et al recently demonstrated that mice lacking CD103+ DCs displayed diminished Th2 priming and allergic airway inflammation [258].

The human equivalents of all three subsets of murine DCs (mDCs, pDCs and CD103+ DCs) have been identified. However, little is known about their specific functions, anatomical distributions and their role in asthma pathogenesis
1.4.3 Epithelial Cells

Lung airway epithelial cells lie at the interface between the host and the environment representing the first line of defense against exposure to pathogens, allergens and pollutants [261]. In the quiescent state, the lung epithelium forms a tightly regulated impermeable barrier. However, during chronic inflammatory conditions, such as in asthma, there is evidence of a loss of barrier function [262]. In addition, epithelial cells are reported to have an activated phenotype in asthmatics compared to controls. In asthmatics, epithelial cells display a stressed phenotype as compared to controls evidenced by enhanced expression of heat shock proteins and transcription factors such as NFkB and AP1 [263]. Moreover, there are studies suggesting that there is enhanced apoptosis of epithelial cells from asthmatic patients compared to healthy controls [264].

Epithelial cells, like DCs, express a wide range of PRRs that initiate allergen recognition leading to rapid release of anti-microbial peptides and inflammatory mediators. Recent studies have highlighted the role of PRRs on the airway epithelium in regulating the function and activation of DCs in the lung [219]. Utilizing chimeric mice lacking TLR4 on either radiosensitive hematopoietic or radio-resistant structural cells, Lambrecht and colleagues showed that TLR4 activation on stromal lung cells was essential for the recruitment, migration, and activation of DCs and subsequent development of AHR following exposure to HDM [261, 265]. As previous studies have reported that in a resting state, epithelial cells are hypo-responsive to endotoxin due to low expression of
cognate signaling molecules or intracellular localization of TLR4, exposure to allergen in susceptible individual might trigger other PRRs along with TLR4 on the epithelium resulting in initiation of allergic immune responses [261]. Therefore, there is a threshold of epithelial cell activation that is required to trigger DCs in initiating a maladaptive asthmatic immune response. In addition, epithelial cells can also condition the inflammatory response generated by DCs through secretion of cytokines such as CCL20, IL-8, GROα, Granulocyte Monocyte Colony stimulating factor (GMCSF), IL-33, IL-25, and TSLP. In particular, GM-CSF, IL-33, IL-25 and TSLP have been shown to promote DCs initiation of Th2 immune responses.

GM-CSF is classically characterized as a hematopoietic growth factor enhancing the proliferation of myeloid cells (DCs, macrophages) from bone-marrow progenitors, GMP [266]. Apart from its role in the development of myeloid cells, GM-CSF has been shown to modulate the function of mature immune cells, particularly DCs, including enhanced proliferation, phagocytosis, antigen presentation and antigen cross-presentation [266]. Several studies have reported enhanced GM-CSF from bronchial epithelial cells from asthmatics compared to controls [267]. Moreover, a polymorphism in the GMCSF gene has been described in a Swedish atopic asthmatic population [268]. Studies from murine models have suggested that GM-CSF promotes DCs to enhance Th2 immune response. In mice, inhalation tolerance to OVA can be broken if mice constitutively express GM-CSF prior to antigen challenge [269]. These GM-CSF transgenic mice produce more Th2 cytokines, IgE, eosinophilia, mucus hyper-
secretion and AHR upon repeated intra-tracheal administration of OVA than non-transgenic controls. The role of GM-CSF in allergic airway disease extends beyond facilitating allergic sensitization, as administration of GM-CSF during aeroallergen challenge in previously sensitized mice exacerbates airway eosinophilic inflammation [267, 270]. Consistently, GM-CSF blockade results in diminished Th2 responses and as well as AHR in a model of diesel-exhaust particle [271] and HDM-induced asthma [272]. Along with promoting Th2 immune response, GM-CSF can also potentiate Th17 immune responses as indicated by numerous studies [273]. GM-CSF induces IL-23, a critical Th17 maintenance factor, and therefore it has been reported to promote IL-23 driven Th17 immune response in autoimmune diseases [273]. As T cells do not express the GM-SCF receptor, GM-CSF signaling through DCs guides the differentiation of Th17 cells in vivo. Therefore, decreasing GM-CSF in models of allergen-induced asthma can affect the disease phenotype by downregulating either/or both Th2 and Th17 immune responses.

In addition to epithelial cells, GM-CSF can be also be secreted by T cells and eosinophils, however none of the studies discussed above address the source of GM-CSF that is relevant in their model of allergic asthma. Therefore, careful studies are needed to address the relevant source of GM-CSF. While during the sensitization phase, epithelial cells may be the major contributor of GM-CSF, during the challenge phase GM-CSF from other cellular sources might also affect the disease outcome.

TSLP is another epithelial-derived pro-Th2 mediator, which has been
reported to be elevated in bronchial biopsies and sputum of asthmatics. Moreover, polymorphisms in the TSLP gene are associated with increased risk for asthma [274, 275]. TSLP acts as maturation factor on DCs resulting in upregulation of co-stimulatory molecules (CD40, CD80, CD86, Ox40L) and production of cytokines that are part of the Th2 axis of inflammation [276]. Moreover, TSLP has been shown to facilitate the generation of Th2 immune responses by suppressing the development of a Th1 immune response by DCs [277]. Overexpression of TSLP in bronchial epithelium has been shown to induce AHR in a murine model utilizing OVA [278]. However, in murine asthma models that utilized native allergens (HDM, peanut), the neutralization of TSLP does not necessarily result in a reduction in the features of allergic asthma [279]. Apart from having a pro-inflammatory role, TSLP also promotes epithelial cell repair, therefore careful studies will be required before targeting TSLP in allergic asthma.

Ever since its discovery as a strong Th2 initiator, IL-25 (also known as IL-17E) has been investigated in the pathogenesis of asthma. IL-25 is produced by eosinophils, basophils and mast cells, as well as by epithelial cells. Several studies have linked IL-25 to airway inflammation, showing that overexpression of IL-25 by the airway epithelium induces Th2 responses. Moreover intranasal administration of rIL-25 alone induces AHR [280]. Conversely, IL-25 receptor (IL17RB)-deficient mice or mice treated with IL-25 neutralizing antibody have reduced Th2 cytokines, eosinophilia and AHR following allergen challenge [281-283]. Moreover, polymorphisms in the IL17RB gene have been associated with
susceptibility to asthma [284]. However IL-17B can also bind this receptor with lower affinity as compared to IL-25, and its role in asthma remains unknown.

Among all the cytokines secreted from the epithelium, IL-33 is unique as it is found in the nucleus of stromal cells (mainly endothelial and epithelial cell). In addition to acting as a traditional cytokine, IL-33 also has transcriptional regulatory properties [285]. The receptor for IL-33, ST2, is present in multiple isoforms, including a membrane-bound form (ST2L), which together with the interleukin-1 (IL-1) receptor accessory protein (IL-1RAcP) forms the transmembrane IL-33 receptor. A soluble form of the receptor (sST2) may act as a decoy receptor for IL-33 [286]. In addition to higher Il33 mRNA levels in lung biopsies, elevated levels of ST2 are reported in the sera of asthmatics with acute exacerbations [287, 288]. In a murine model, blockade of IL-33 (using neutralizing Ab) or its receptor ST2 (anti-ST2 antibodies or ST2-Ig fusion) decreases antigen-induced eosinophilia, serum IgE, goblet cell hyperplasia and AHR [289, 290]. IL-33 instillation has also been shown to induce AHR in the absence of allergen, T or B-lymphocytes [291]. Further, IL-33 transgenic mice develop spontaneous pulmonary allergic inflammation dominated by eosinophils, goblet cell metaplasia and Th2 cytokines [292]. In the past decade, numerous mechanisms through which IL-33 can promote Th2 immune responses have been proposed through studies in murine models. In addition to directly activating DCs to promote the polarization of naïve Th cell into a Th2 lineage [293], IL-33 can directly act on Th2 cells enhancing secretion of IL-5 and IL-13 [294]. Moreover, antigen-pulsed dendritic cells pretreated with IL-33 were better than
untreated antigen-pulsed DCs at driving a Th2 response following secondary antigen challenge [295]. Also, IL-33 is also a potent activator of mast cells, basophils, neutrophils and macrophages facilitating the survival, and production of pro-inflammatory mediators from these cells during pulmonary inflammation [285] [276].

Besides driving inflammatory response, signals from epithelial cells can also suppress inflammation. Epithelial cell derived mediators such as prostaglandin E2 (PGE2), lipoxin A4, resolvins and protectins are broadly anti-inflammatory in pulmonary immune response and the majority of these are reported to be reduced in asthmatic subjects [261].

In summary, asthma research has undergone tremendous changes in the past two decades and now the respiratory epithelium is considered the cornerstone of asthma pathogenesis and not just a mere barrier or means of gas exchange in the lungs. Airway epithelium takes an active part in innate immune responses and by controlling the activation and programming of T cells through APCs, epithelial cells also dictate adaptive immune responses. As DCs do not produce a “Th2-specific factor”, it is thought that epithelial cells via secretion of pro-Th2 cytokines (IL-33, IL-25, TSLP) instruct DCs to promote Th2 cell-mediated immune response in asthmatics. Hence, epithelial cells are thought to be a major locus of control of asthmatic immune responses.

1.4.4 Innate Lymphoid Cells (ILCs)

A previously unrecognized population of innate immune cells, innate
lymphoid cells (ILCs) have recently been described and found to be important regulators of innate immunity and inflammation at mucosal surfaces [296]. Three main features define ILCs: the absence of recombination activating gene (RAG)-dependent rearranged antigen receptors (TCR); a lack of myeloid cell and dendritic cell phenotypical markers; and their lymphoid morphology [297]. Although ILC’s share morphological, development and functional similarities with CD4+ T helper cells, they do not express cell-surface markers that are associated with these or other cell lineages like NK cell (NK1.1, CD49b), B cells (B220), DCs (CD11b, CD11c), and macrophages (CD11b, F4/80) [296]. However, ILCs are dependent on IL-7 and IL-2 signaling and hence express IL-7Ra (CD127) and IL-2Ra (CD25) for their development and maintenance. Like T cells, ILCs originate from CLP population in the bone marrow under the control of transcription factor repressor, inhibitor of DNA-binding 2, Id2 [298]. Moreover, ILCs produce many T cell associated cytokines and as they do not express a TCR, they are not under the guidance of antigen presentation by an APC [299]. ILCs can be further divided into three subsets – group 1 ILCs (ILC1s), group 2 ILCs (ILC2s) and group 3 ILCs (ILC3s), based on their ability to produce cytokines associated with Th1 (IFNγ), Th2 (IL-5, IL-13) and Th17 (IL-17A, IL-22) cells respectively [299].

ILC2s were first discovered in mice in 2010 by three independent groups which gave them different names including: natural helper cells (NHCs), as they promote B cells produce antibody; Nuocytes, because they produce IL-13 (nu = 13th letter of the Greek alphabet), and type 2 innate helper cells (Ih2) [300].
Multipotent progenitor population type 2 (MPP type 2) cells are another IL-25 responsive, type 2 cytokine producer of the ILC2 family, which is mostly found in gut-associated lymphoid tissue (GALT) [301]. The initial knowledge about ILC2s in guiding Th2 immunity came from helminth infection models in mice. As ILC2s are considered to be the major responders to IL-25 and IL-33, the cytokines pivotal in mounting protective CD4+ Th2 immune response, it was not surprising that ILC2s were also protective in helminth infections. Utilizing IL-13-eGFP reporter mice, Niel et al demonstrated that ILC2s expand following IL-25 and IL-33 administration and represent the major source of IL-13 post-infection with *N. brasiliensis* [302]. Further, adoptive transfer of cultured IL-13 sufficient but not IL-13 deficient ILC2s restored rapid worm expulsion in IL-25 receptor deficient mice. Even though type 2 ILC-derived cytokines are sufficient to resolve a helminth infection, the presence of Th2 cells (even if IL-4 and IL13 deficient) is still essential for effective helminth expulsion because Th2 cells are required to maintain type 2 ILCs numbers during an infection [303].

While our knowledge about the importance of ILC2s in health and disease in animal models has increased, it was not until 2011 that the presence of ILC2s was first reported in two independent studies in humans [296]. Monticelli et al reported the presence of Lin- cells expressing CD25 (IL-2 receptor), ST2 (IL-33 receptor) and CD127 (IL-7 receptor) in lung tissue and BAL from humans, resembling characteristic phenotype of mouse ILC2s [304]. Spits & colleagues provided a thorough characterization of human ILCs and showed that fetal and adult CD161+CRTH2+ ILCs isolated from the lung or intestines were responsive
to IL-25, IL-33, and IL-2 and produced IL-13, similar to the mouse ILC2s [305]. Detection of ILC2s in the lungs has led to the exploration of these cells in pulmonary responses. In a model of virus-induced acute asthma, Umetsu & colleagues showed that Rag2 KO mice, which lack lymphocytes, develop neutrophilic airway inflammation and AHR (methacholine) upon H3N1 infection [306]. They further showed that following H3N1 infection, IL-33 produced from alveolar macrophage is crucial in expansion of ILC2s (IL-5+ IL13+) and that mouse lacking either IL-33 receptor, ST2 or ILC2s (via antibody depletion) did not mount AHR. Moreover, adoptive transfer of IL-13 sufficient and not IL-13 deficient ILCs was shown to be sufficient to drive AHR in IL-13 deficient host. Therefore, IL-33 responsive, IL-13 secreting ILC2s are crucial in virus-induced AHR. Studies by other groups highlighted the importance of ILC2s in antigen-induced allergic asthma models. Utilizing IL-4 and IL-13 dual reporter mice, McKenzie and colleagues demonstrated following exposure to either IL-25, IL-33 or OVA antigen, IL-13+ ILCs accumulate in the lungs [307]. Furthermore, transfer of WT (Il13+/+) ILCs and not KO (Il13/-) ILCs into IL-13 deficient host resulted in AHR and eosinophilia following rIL-25 instillation. Importance of IL-13 secreting ILC2s in allergen-induced AHR, goblet cell hyperplasia and eosinophilia was also demonstrated in mouse models utilizing glycolipid and protease (papain) antigens [308, 309]. Similarly, Wolterink et al showed that ILC2s increase in lung and BAL fluid and constitute the major source of IL-5 and IL-13 after HDM or OVA induced allergic asthma [310].

Detection of IL-17A or IL-22 secreting ILC3s in adult human tonsils and
intestinal tissues was reported before detection of ILC2s [296]. Like their CD4+ Th17 counterparts, IL-17A producing ILC3s have been shown to be pathogenic in colitis in mice and humans [311, 312]. Conversely, in the context of fungal infections, ILC3s have been shown to be critical in acute response to *Candida albicans* as depletion of these cells rendered mice highly susceptible to oropharyngeal candidiasis [313, 314]. A recent study by *Umetsu and colleagues* in mice showed that IL-17A+ ILC3s facilitates obesity-induced AHR [315]. Mice fed a high fat diet displayed severe AHR to methacholine compared to those fed a standard diet and this was dependent on IL-17A as mice lacking IL-17A got obese but did not mount AHR. Further they showed that, IL-1β produced from macrophages caused AHR by inducing IL-17A production in obese mice. Moreover, the IL-1β–induced AHR response required ILC3 cells, as it was abolished by depletion of ILC3 cells in obese mice. Apart from this report, there has been no direct evidence supporting the role of ILC3s in allergic or non-allergic asthma. As IL-17A secreted from CD4+ Th17 cells can drive severe forms of asthma it would be of interest to determine whether IL-17A+ ILC3s also promote disease severity.

It has been more than a decade since we have known that IL-13 is necessary and sufficient to induce AHR [152]. However, until recently, the source of IL-13 which drives allergen-induced AHR was thought to be only the CD4+ Th2 cells. However, we now know that IL-13 produced from ILC2s can mediate AHR independent of Th2 cell immune responses. Following an encounter with an allergen, epithelial cells can secrete the cytokines crucial for ILC recruitment into
the lungs, namely IL-25 and IL-33. Therefore, epithelial cells via controlling ILC2s can modulate the course of asthma pathogenesis. In the field of asthma immunology it was thought that the purpose of innate immune responses was to instruct/shape adaptive T cell-mediated immune responses that govern the course of the disease. However, now it is clear that two innate compartments, epithelial cells and ILC2s, can interact leading to the initiation of asthmatic immune response independent of the adaptive immune response.

1.5 Pattern Recognition Receptor (PRR)

Inflammation is a protective response generated for effective clearance of detrimental stimuli, as well as a healing process for repairing damaged tissue [316]. However, an uncontrolled or aberrant immune response against invading pathogens can result in tissue injury and hence prove detrimental to the host. The inflammatory response to pathogens ensues upon recognition of specific conserved structures known as Pathogen Associated Molecular Patterns (PAMPs) by host’s germline-encoded PRRs. PAMPs acts as non-self signals for the presence of pathogens to immune cells. PAMPs are unique to classes of pathogens and because these motifs are crucial for pathogen survival, they are not altered, suppressed or easily hidden by pathogens [317]. In addition to exogenous PAMPs, PRRs have also been shown to recognize endogenous molecules secreted by damaged cells, Damage Associated Molecular Patterns (DAMPs) [316]. Once triggered by binding of PAMPs or DAMPs, PRRs induce downstream signaling pathways, involving nuclear factor-kappa B (NF-κB),
mitogen-activated protein kinase (MAPK) resulting in secretion of pro-inflammatory cytokines and chemokines [318].

PRRs are broadly classified in four families: Toll-Like Receptors (TLRs), C-type Lectin Receptors (CLRs), Nod-Like Receptors (NLRs) and RIG-I-Like Receptors (RLRs). RLRs and NLRs are strictly associated with sensing intracellular PAMP [316]. While CLRs can sense extracellular PAMP, TLRs can recognize both intra- and extracellular PAMPs [316]. Depending upon the antigen/pathogen in context, various PRRs can be engaged leading to initiation of an immune response. While our knowledge regarding the PRRs that mediate Th1 and Th17 immune response is ample, we still lack in understanding of the PRRs involved in initiating Th2 immune responses.

Allergens were initially thought to be allergenic because of their ability to bind IgE. However, no single structures were identified which categorized an allergen. With the discovery of the importance of PAMPS-PRR interactions it is being hypothesized that allergens contain PAMPS that are recognized by the innate immune system. Complex allergens such as HDM, which are known to contain multiple allergenic peptides with diverse structures and biological functions, may activate multiple PRR pathways. Perhaps its potency as an allergenic trigger is due to the fact that it is able to activate multiple innate immune pathways. Although the exact PRRs and their contribution to HDM-induced asthmatic responses are not completely understood, studies are underway to identify the crucial allergen containing PAMPs and PRRs by which they are recognized in the development of type 2 immunity.
1.5.1 TLRs

An allergic immune response is generated against generally innocuous allergens, because, allergens like pathogens contain PAMPs and can mimic a microbial stimulus against which the innate immune system may react adversely in susceptible individuals. Among the PRRs that can sense PAMPs, TLRs are one of the most well characterized PRRs to date. TLRs were named as such because they resembled the protein encoded by the *toll* gene in *Drosophila*. Since their discovery in 1988 in *Drosophila*, these evolutionary conserved receptors have been reported to play a crucial role in mammalian immune sensing [319]. Briefly, TLRs consist of a conserved cytoplasmic domain, a transmembrane Toll/IL-1R (TIR) domain and diverse extracellular leucine-rich repeat (LRR) domain [320]. The LRR domain is involved in PAMP recognition and because of its diverse nature, structurally unrelated ligands can be sensed by various TLRs. Ligand binding initiates conformational change, and dimerization of the receptor, leading to recruitment of downstream signaling molecules. So far, 11 TLRs (TLR1-TLR11) have been recognized in mammals and are reported to have differential cellular localization [321]. TLR1, TLR2 TLR4, TLR5 and TLR6 are located on the cell surface and can be recruited to phagosomes after activation [322]. However TLR3, TLR7, TLR8 and TLR9, all of which are involved in the recognition of nucleic-acid-like structures, are not expressed on the cell surface [322]. While TLR1-TLR9 are conserved between human and mouse, TLR10 is reported to be functional in humans and not in mouse [321]. Conversely, the *Tlr11* gene is transcribed in mice but has been reported to have
a premature stop codon in humans [321].

Gain of function SNPs in the TLR1, TLR6 and TLR10 gene have been reported to have protective effects on atopic asthma [320]. These SNPs were associated with enhanced Th1 cytokine (IFN-γ) and reduced Th2-cytokine (IL-4) production after ex vivo stimulation [320]. While TLR4 is the most widely studied TLR in asthma pathogenesis, numerous studies of the effects of TLR4 polymorphisms on allergic asthma have yielded mixed results [323]. Epidemiological studies in humans have often linked exposure to endotoxin or LPS, the TLR4 ligand in early life with protection against the development of allergic diseases [324]. However, contradictory results have also been reported in humans where exposure to LPS in asthmatic individuals exacerbates bronchial reactivity [325]. Studies in murine models have suggested that the outcome of allergic airway disease is highly dependent on the dose of LPS utilized. Bottomly & colleagues reported that while low level of inhaled LPS along with OVA (OVA-LPSlo) promotes Th2 response, high levels of LPS with OVA (OVA-LPShi) facilitate Th1 responses in a model of allergic sensitization and challenge [254]. Following this, another study by the same group utilizing bone-marrow chimeric mice demonstrated that TLR4 expression on the hematopoietic cells was necessary and sufficient in mediating Th1 sensitization to OVA-LPShi [326]. However, they depicted that TLR4 in both hematopoietic and non- hematopoietic compartments was required for Th2 sensitization to OVA-LPSlo [326]. Surprisingly, mice expressing TLR4 on only stromal cells generated a robust Th2 response instead of a Th1 response upon exposure to OVA-LPShi [326]. Further,
they showed that stromal TLR4 signaling leads to the maturation of Th2-inducing DCs. The author proposed a model in which stromal cells (likely epithelial cells) require a threshold level of TLR4 activation to independently drive Th2 sensitization and if this threshold is not achieved, LPS sensitivity is increased by TLR4-expressing cells of the hematopoietic compartment (DCs), thereby permitting Th2 sensitization to low levels of LPS [326]. However, studies by Lambrecht and colleagues showed that TLR4 expression on lung structural cells and not in the hematopoietic compartment is sufficient to promote DCs to induce a Th2 immune responses following exposure to HDM containing low levels of LPS [265]. The discrepancies between these two studies might be due to the differences in the nature and type of TLR4 agonists utilized. Unlike the OVA-LPS model wherein only LPS can induce TLR4 activation, HDM might contain additional endogenous TLR4 agonists which trigger the receptor in the stromal compartment thereby promoting a Th2-immune response [324]. In summary, TLR4 expression on lung structural cells and not hematopoietic cells seems to be necessary to promote DCs to induce a Th2 immune response.

In addition to microbial contaminants, allergens contain proteins that can mimic specific TLR signaling components and may drive the aberrant immune response seen in allergic asthmatics. Studies have demonstrated that one of the major allergenic peptides contained in HDM, Der p 2 shares structural similarity to MD2, the endotoxin-binding component of the TLR4 complex [327, 328]. Leveraging this finding, Karp & colleagues demonstrated that in addition to structural homology, Der p 2 also exhibits functional mimicry of MD2 through its
ability to activate TLR4 [329]. Specifically, they showed that mice sensitized and challenged with Der p 2 developed a Th2-rich allergic inflammatory response characterized by BAL eosinophilia, IgE synthesis and goblet cell metaplasia [329]. Moreover, mice lacking TLR4 (Tlr4 -/-) did not develop Th2 inflammation upon Der p 2 sensitization and challenge. In contrast, Der p 2 exposure induced features of allergic asthma in mice lacking MD2 (MD2-/-) suggesting that Der p 2 could reconstitute the TLR4 receptor complex in the absence of MD2. Therefore, it might be possible that the genetic propensity to develop a strong Th2 immune response is due to the fact that in susceptible individuals innocuous proteins are mistaken for microbes. Alternatively, immunosuppressive pathways, which normally inhibit the activation of these pathways, may be defective.

Like TLR4, TLR2 is also known to bind lipids. However, it can also form heterodimers with other TLRs to recognize different structures. For example, diacylated lipopeptides are sensed by TLR2 and TLR6, while triacylated lipopeptides, such as Pam₃CysSK₄, are recognized by the combined TLR2/1 complex [330]. Lipoproteins such as lipoprotein I (Pseudomonas aeruginosa) are recognized by TLR2/4 [330]. The role of TLR2 in asthma is controversial; studies have reported both protective and enhancing effects of TLR2 stimulation (using Pam₃CysSK₄) on Th2 immune responses and disease pathogenesis. For example, mice sensitized and challenged with OVA and Pam₃CysSK₄, as compared to those treated with OVA alone, had exaggerated AHR, increased numbers of BAL eosinophil and enhanced Th2 cytokines [331]. In contrast, Patel et al reported that Pam₃CysSK₄ can reverse established OVA-induced
airway inflammation. They showed that Pam$_3$CysSK$_4$ treated OVA-challenged mice had a profound reduction in the BAL total cell count, eosinophilia, AHR [332]. Based on the above-mentioned studies it seems that TLR2 stimulation can have varying effects on allergic asthma. While TLR2 triggering during the sensitization phase can enhance the development of allergic asthma, TLR2 stimulation can be beneficial in established disease. Like TLR4, the dual role of TLR2 in asthma can also be partly dependent on the dose of agonist utilized. Independent of its role on epithelial and APC compartment, recent studies indicate that TLR2 can be expressed by T cells and can act as a co-stimulatory molecule on activated T cells [333]. Interestingly, TLR2 triggering on Tregs is accompanied by a loss of their suppressive ability [334]. Therefore, enhanced Th2 polarization and low immunosuppressive capacity of T regulatory cells following TLR2 activation might underlie the development of allergic diseases [335].

As viral and bacterial infections can exacerbate allergic asthma, TLRs that can sense the ligands released from such infectious agents have gained attention in modulating the disease outcome. Among the intracellular TLRs that can recognize cytoplasmic nucleic acids during viral or bacterial infections, TLR9 and TLR3 have recently gained attention. Unmethylated CpG dinucleotides present in particular base contexts in bacterial, but not vertebrate DNA can cause activation of B cells, NK cells and the secretion of Th1 cytokines [336]. Utilizing such bacterial DNA mimics (oligodeoxynucleotides, ODN) Kline et al reported that airway eosinophilia, IgE production, and bronchial hyperreactivity were
prevented in mice administered with CpG ODN alongwith Ag [336]. While CpG ODN diminished the induction of IL-4 expression in the lungs, it promoted the release of lung IL-12 and IFN-γ, thereby shifting the balance from a Th2 to a Th1 immune response [336]. Consistent with the finding in a murine model, Beeh et al recently reported that repeated subcutaneous injections of QbG10, a TLR9 agonist results in considerable reduction or withdrawal of ICSs, while asthma symptoms remained stable or even improved in few asthmatics [337]. All studies focusing on TLR9 have reported a protective effect of TLR9 stimulation on allergic asthma and therefore this may serve as a good candidate for therapeutics in future.

Unlike TLR9, which has been implicated in modulating Th2 immunity, TLR3 has been reported to modulate allergen-induced Th2 and Th17 immune responses. TLR3 activation on bronchial epithelial cells can cause the release of pro-Th2 inflammatory cytokines like TSLP [338]. Tanaka et al reported that human CD11c+ DCs primed with the TLR3 agonist, poly I:C can induce IL-23 secretion. While TSLP alone did not, a combination of TSLP and poly I:C synergistically induced the production of IL-23 [338]. Further, DC primed with TSLP and poly I:C were better at differentiating naïve CD4+ T cells into Th17+ cells than DCs treated with poly I:C alone [338]. In vivo studies by Jeon et al showed that mice sensitized and challenged with OVA plus poly I:C (low and high dose) had higher total inflammatory cell counts in the BAL fluids compared to those sensitized and challenged either with PBS, OVA, or low or high doses of poly I:C alone [339]. In particular, macrophage and neutrophil counts and not
eosinophils were higher in the OVA plus poly I:C groups than in the other groups [339]. Further, neutrophilic rich inflammation and AHR following OVA+poly I:C sensitization and challenge was dependent on signaling through TLR3 as only WT and not Tlr3 -/- mice developed the phenotype [339]. Interestingly, the author reported that IL-13, IL-4 and STAT6 were required for the development of lung inflammation in the OVA+low-dose dsRNA (poly I:C) model [339]. However, IFNγ and T-bet were crucial for the development of AHR and non-eosinophilic lung inflammation as seen in their OVA+high-dose dsRNA model [339]. Therefore, dsRNA exacerbates asthma and while a high dose promotes Th1, a low dose of dsRNA favors a Th2-driven inflammation. This phenomenon recapitulates data seen with the dosage of LPS required to drive allergen responses towards either Th2 or Th1 immune responses.

In summary, the role of TLRs in asthma pathogenesis is complex. According to the hygiene hypothesis, the increased incidence of allergic diseases in developed nations is due to low exposure to bacterial insults during childhood. Activation of TLRs through bacterial and viral ligands in early life is considered to deviate the immune response from a Th2 to a Th1 phenotype, thus conferring protection from the development of allergic disease. However, there are reports suggesting that TLR triggering might confer risk for the development of allergic diseases. Polymorphisms in CD14, which associates with both TLR4 and TLR2, are associated with increased risk of allergic diseases [340]. Moreover, bacterial and viral infection often results in asthma exacerbations. Therefore, there is a lack of understanding about the role of TLR triggering through microbial exposure
in early life in impacting allergic diseases outcome.

1.5.2 NLRs

NLRs are intracellular PRRs involved in the recognition of cytosolic PAMPs or DAMPs. NLRs are expressed in a variety of cell types, including DCs, macrophages and epithelial cells [341]. In humans, 23 NLR family members exist and in mice at least 34 Nlr genes have been reported [341]. Structurally, NLRs are large multi-domain proteins consisting of three subunits [342].

NOD1, which responds to meso-diaminopimelic acid (meso-DAP) in bacterial cell wall peptidoglycan (PGN) and NOD2 that recognizes conserved muramyl-dipeptide (MDP) motif, were the first NLRs to be discovered [343]. Although both NOD1 and NOD2 are associated with the recognition of bacterial products, polymorphism in their genes or associated adaptor molecules is associated with increased risk of atopy and allergic asthma [344-347]. Studies in murine models have reported that Nod1 and Nod2 activation can result in antigen-specific Th2 responses when their ligands are given systemically [348]. However, intratracheal administration of Nod1 and Nod 2 agonist was reported to have varying effects on antigen-specific Th2 immune response [348]. Recently, Duan et al reported that intranasal exposure of the NOD2 agonist, MDP and not NOD1 agonist PGN results in suppression of inhalation tolerance against OVA antigen [348]. Further, lung DCs from mice exposed to MDP in comparison to control mice, have higher expression of TSLP and IL-25, both of which promoted IL-4 producing Th2 cells and suppressed FoxP3+ Tregs [348].
In addition to PAMPs, recognition of DAMPs by NLRs has been linked to the modulation of Th2 immunity. Allergens might trigger release of DAMPs, which are present intra-cellularly under homeostatic conditions, but are released extracellularly upon physical or metabolic stress [349]. Two such DAMPs, ATP and uric acid (UA) are released from dying or necrotic cells and can signal as alarmins for immune system activation [350, 351]. A crucial step in induction of Th2 immune response by UA and ATP is the cleavage of pro-IL-1β into bioactive form via caspase 1. Activation of caspase 1 in turn is dependent on activation of NLRP3, an NLR. Activation results in oligomerization of NLRP3 and recruitment of the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD) [352]. Following this, pro-caspase-1 is recruited by ASC resulting in a functional NLRP3 inflammasome and leading to caspase-1 activation [352].

Lambrecht & colleagues reported that following allergen exposure, ATP accumulates in the airways of asthmatics and in mice [353]. Further, enzymatic (apyrase) neutralization of extracellular ATP or blockade (using suramin) of ATP receptors (P2 receptors) prior to allergen challenge in mice resulted in diminished eosinophilic airway inflammation, Th2 cytokines and AHR. Moreover, mice exposed to ATP along with OVA had enhanced mDCs recruitment and activation in the lungs when compared to mice receiving OVA only.

Another study by Lambrecht & colleagues demonstrated that alum, a prototypical Th2 adjuvant, induces the release of uric acid (UA) that promotes Th2 immunity by activating DCs [349]. Moreover, UA is also released upon primary exposure of the lung to HDM, as well as upon allergen challenge in
sensitized human asthmatics and mice [349]. Although UA is a known stimulator of NLRP3 inflammasome, the adjuvant effect of alum on Th2 cell immunity is independent of the NLRP3 inflammasome activation [349]. Interestingly, triggering of Syk kinase, a prototypical kinase downstream of CLR activation, was shown to be crucial in DCs for mounting Th2 cell immunity to UA [349]. Therefore, in addition to PAMPs, activation of NLRs by endogenous DAMPs released during allergen sensitization might contribute to the induction of Th2 immune responses.

1.5.3 RLRs

RLRs belong to a family of RNA helicases involved in sensing of intracellular viral RNA and DNA [354]. As viral RNA usually contains a modified 5’ triphosphate end, which is not present in capped or processed host RNA, RLRs recognize such motifs to distinguish between the two types of RNA [355]. Certain viruses are able to escape immune sensing by post-transcriptionally modifying their terminal 5’ triphosphate end into forms that are not recognized by RLRs [356]. RLRs consist of three members; namely, RIG-I (retinoic acid-inducible gene I), which is the founding member and therefore best characterized of this family, MDA5 (melanoma differentiation associated factor 5), and LGP2 (laboratory of genetics and physiology 2 and a homolog of mouse D11lgp2) [354]. Unlike, the anti-viral TLRs (TLR3, TLR7, TLR8 and TLR9) that are located in endosomes, RLRs detect viral RNA in the cytoplasm [357]. Upon activation by viral RNA/DNA, RLRs signaling triggers NFκB mediated downstream signaling
involving MAP kinases and Interferon Regulatory factor (IRF), together culminating in the release of type I IFNs.

Given the pathogenic role of viral infection in asthma exacerbations, it is surprising that no polymorphisms in the gene for RLRs have been identified with increased risk for asthma or allergy in humans. Respiratory tract viruses, such as rhinovirus, respiratory syncytial virus (RSV), influenza viruses, coronaviruses, human metapneumoviruses, parainfluenza viruses, adenoviruses, and bocaviruses, have all been detected in subjects with asthma exacerbations [358]. Specifically, 85% of acute asthma exacerbations in infants and children are caused by viruses [355]. Moreover, nearly 40–85% of asthma exacerbations are associated with upper-respiratory-tract virus infections, of which 60% are caused by rhinoviruses [359]. A lack of animal models in which rhinoviruses can bind and replicate has hindered our understanding of the mechanism underlying rhinovirus-induced asthma exacerbation. Almost 90% of rhinovirus serotypes bind to human and not murine ICAM-1 expressed inter-cellularly in the respiratory tract [360]. The remaining 10% utilizes a member of the low-density lipoprotein receptor family and can bind to both human and mouse receptors [360]. Recently Bartlett et al reported the generation of a novel genetically modified mouse (expressing human ICAM-1) that can be successfully infected with rhinovirus. In this model, rhinovirus infection induced allergic airway inflammation with or without allergen (Ova) [360]. Hopefully, in the future, we will be able to dissect the role of virus sensing through RLRs in asthma pathogenesis utilizing these new mouse models.
1.5.4 C-type Lectin receptors (CLRs)

‘Lectin’ is a collective term used to define proteins that can recognize sugar moieties on the cell surface of prokaryotes and eukaryotes, facilitating the uptake and neutralization of pathogens [361]. The term “C-type lectin” was first coined to identify a group of Ca\(^{2+}\)-dependent carbohydrate-binding (lectin) proteins that were different from the rest of the animal lectins [362]. CLRs contain at least one carbohydrate recognition domain (CRD) that consists of a unique structural fold. The structural fold harbors conserved residues or motifs that determine the carbohydrate specificity of the CLR [363]. CLRs consists of both soluble and trans-membrane members. While soluble CLRs are found in plasma, trans-membrane CLRs are shown to be expressed on immune cells. Based on their molecular structure, trans-membrane CLRs were first distinguished into two broad types, Type 1 and Type II wherein the N termini is oriented extracellularly or into the cytoplasm of the cell, respectively [364]. Type I C-type lectins (Macrophage Mannose receptor (MMR) and DEC-205), also known as the mannose receptor family, contain an amino-terminal cysteine-rich repeat, a fibronectin repeat (FN) and multiple carbohydrate recognition domains (CRDs) [364]. However, Type II CLRs or asialoglycoprotein receptor family consisting of DC-SIGN, Langerin, CLEC10A and CLEC5A, contains only 1 CRD at their carboxy-terminal extracellular domain [364]. Type II CLRs can be further divided into the dectin-1 subfamily consisting of Dectin-1 (Clec7a), CLEC12A, CLEC2, CLEC9A, CLEC12B and the DCIR subfamily containing Dectin-2, CLEC4C,
Mincle and DCIR [363]. Based on the CRD domains, different CLRs can recognize specific carbohydrate moieties. Table 1 below gives a comprehensive list of CLRs, their expression by cell type, sugar moieties recognized and signaling motifs required for signaling.

Table 1. CLR Family Members and Their Signaling Motifs

<table>
<thead>
<tr>
<th>CLR</th>
<th>Cell types expressing the receptor</th>
<th>Ligand/carbohydrate moieties</th>
<th>Signaling Motifs or adaptor proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannose Receptor MR/ CD206</td>
<td>DCs and Macrophages</td>
<td>High mannose, fucose and sulphated sugars</td>
<td>ND</td>
</tr>
<tr>
<td>DEC205/CD205</td>
<td>DCs</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DC-SIGN/CD209</td>
<td>DCs</td>
<td>High Mannose and fucose</td>
<td>NONE</td>
</tr>
<tr>
<td>Langerin</td>
<td>Langerhan cells and dermal DCs</td>
<td>High mannose, fucose and N-acetylgalactosamine</td>
<td>Proline-rich domain</td>
</tr>
<tr>
<td>MGL/CLEC10A</td>
<td>DCs and Macrophages</td>
<td>Terminal N-acetylgalactosamine</td>
<td>None</td>
</tr>
<tr>
<td>CLEC5A</td>
<td>Monocyte and Macrophages</td>
<td>ND</td>
<td>DAP10 and DAP12</td>
</tr>
<tr>
<td>Dectin-1/CLEC7A</td>
<td>DCs, Macrophage, monocyte and B cells</td>
<td>β-1, 3-glucan</td>
<td>YxxL</td>
</tr>
<tr>
<td>MICL/CLEC12A</td>
<td>DCs, monocyte, macrophages and neutrophils</td>
<td>ND</td>
<td>ITIM</td>
</tr>
<tr>
<td>CLEC2</td>
<td>Platelets</td>
<td>ND</td>
<td>YxxL</td>
</tr>
<tr>
<td>DNGR1/CLEC9A</td>
<td>BDCA3+ DCs, monocytes and B cells</td>
<td>ND</td>
<td>YxxL</td>
</tr>
<tr>
<td>Cells</td>
<td>Macrophages</td>
<td>ND</td>
<td>ITIM</td>
</tr>
<tr>
<td>-------</td>
<td>-------------</td>
<td>----</td>
<td>------</td>
</tr>
<tr>
<td><strong>CLEC12B</strong></td>
<td>ND</td>
<td>ITIM</td>
<td></td>
</tr>
<tr>
<td><strong>Dectin-2/ CLEC6A</strong></td>
<td>mDCs, pDCs, B cells and Neutrophils, macrophages</td>
<td>High Mannose and α-mannans</td>
<td>FcRγ</td>
</tr>
<tr>
<td><strong>BDCA2/ CLEC4C</strong></td>
<td>pDCs, monocytes and macrophages</td>
<td>ND</td>
<td>FcRγ</td>
</tr>
<tr>
<td><strong>Mincle/ CLEC4E</strong></td>
<td>DCs, monocyte, macrophages</td>
<td>Alpha-mannose</td>
<td>FcRγ</td>
</tr>
<tr>
<td><strong>DCIR/ CLEC4A</strong></td>
<td>mDCs, pDCs, macrophages, B cells and neutrophils</td>
<td>ND</td>
<td>ITIM</td>
</tr>
</tbody>
</table>


Because of their ability to recognize major components of the fungal cell wall (mannose and β-glucans), CLRs are crucial in initiating and mediating antifungal Th17 immunity. Particularly four CLRs, i.e. Dectin-1, Dectin-2, the mannose receptor (MR), and DC-SIGN have been shown to mediate fungal binding, phagocytosis and induction of antifungal mediators and cytokines such as IL-8 and IL-17A [365]. The host and not just the invading pathogen or allergen can also express sugar moieties recognized by MR, DC-SIGN and Dectin-2. However, Dectin-1 is unique in this regard as it recognizes β-glucan moieties, which are not expressed in mammals and therefore serves as an excellent non-self PAMP.
1.6. Dectin-1

Dectin-1 is a type II membrane receptor consisting of ligand binding C-terminal C-type lectin domain, a short stalk region, a single transmembrane domain, and a short 40 amino acid amino-terminal intracellular tail [366]. In 2001, a year later than the discovery of dectin-1 in mice [367], dectin-1 was cloned from DCs in humans [368]. Dectin-1 is localized on human chromosome 12 (12p12.3-p13.2) in the region of Natural Killer gene complex (NKC) [368]. Human Dectin-1 displays 60% sequence identity and 71% homology to mouse Dectin-1 [368]. Alternate gene splicing in both murine and human dectin-1 homologues generates multiple splice variants although each has two major isoforms [369]. The major isoforms differ in either the presence or absence of the stalk, isoforms A and B, respectively, and despite the large number of mRNA transcripts (8) identified in humans, both humans and mice tend to preferentially express the B or the stalkless isoform of Dectin-1 [366].

1.6.1. Dectin-1: Ligand binding and intracellular signaling

Unlike many other CLRs, ligand recognition or binding by dectin-1 is not necessarily dependent on calcium [370]. Although Dectin-1’s putative ligand is β-glucan, β-glucans can also bind to the complement receptor 3 (CR3), lactosylcermaide and CD5 [371-373]. β-Glucans are naturally occurring polysaccharides of D-glucose monomers linked through β-glycosidic linkages [374]. β-glucans are conserved structures found abundantly in fungi, plants, however they are not found in mammals [375]. In fungi, β-glucans constitute the
major structural components of the cell wall and exists as $\beta-1 \rightarrow 3$ linked glucose polymers with $\beta-1 \rightarrow 6$ linked side chains of varying length and distribution [374]. In the cell walls and other structures of plants, they can also occur as $\beta-1 \rightarrow 3, 1\rightarrow 4$ linked polymers [374].

The binding affinity of Dectin-1 for $\beta$-glucan can vary from very low to very high (2.2 pM-2.6 mM) and this is dependent on the source, size and number of side branches on $\beta$-glucan [376]. Binding by high-molecular weight $\beta$-glucans isolated from cell wall fractions such as zymosan (from *Saccharomyces cerevisiae*) and curdlan (*Agrobacterium biobar*) can initiate dectin-1 signaling. However, low-molecular-weight $\beta$-glucans, such as laminarin (brown algae) are biologically inactive in inducing dectin-1 signaling [374]. Like TCR and BCR, the intracellular tail of Dectin-1 contains immunoreceptor tyrosine-based activation motifs (ITAMs) like motif [377]. However, unlike other ITAM bearing receptors, Dectin-1 has only one instead of two ITAM motifs, hence it is referred to as hemITAM. Following ligand binding, the phosphorylation of tyrosine residues in the intracellular tail of Dectin-1 facilitates the recruitment and docking of Src and Syk kinases. However ligand binding can trigger activation of both Syk kinase-dependent and independent pathways downstream of Dectin-1 (Refer. Diagram 1). Syk-dependent activation of NF-$\kappa$B can be further categorized into canonical and non-canonical routes [369]. In the canonical pathway, the IKK phosphorylates I$\kappa$B complex, releasing NF-$\kappa$B which can then translocate into the nucleus and regulate gene transcription [369]. Activation of the IKK complex, in turn, is dependent on Dectin-1 mediated phosphorylation of Syk kinase, which
further phosphorylates PLCy2. Activated phosphorylated PLCy2 then catalyzes the hydrolysis of PIP3 to membrane-anchored DAG and soluble IP3 [369]. IP3 causes the release of calcium from the ER, which together with DAG results in activation of PKC [369]. Active PKC then phosphorylates CARD-9, promoting the association of Bcl10 and Malt1, culminating in the formation of CARD-9 complex. The CARD9-Bcl10-Malt1 complex activate subunits of NF-κB through the IKK complex [369]. In the non-canonical pathway, Syk can activate NF-kB inducing the kinase (NIK) pathway, leading to activation of NF-κB [369]. In addition, Dectin-1 also activates a Syk-independent pathway via its tri-acidic [DED] motif involving Raf-1 activation by Ras [369]. Interestingly, the uptake of zymosan particles by both BMMC and peritoneal macrophages was shown to be independent of Syk phosphorylation but dependent on the DED motif [378].
Diagram 1: Signaling downstream of dectin-1


The nature of the ligand bound by Dectin-1 determines the routing of the receptor from the cell surface following ligand binding. Binding to particulate
ligand promotes lysosomal trafficking of the receptor, whereas binding to soluble ligands (laminarin) promotes recycling of the receptor [370]. An elegant study by Underhill & colleagues recently suggested that dectin-1, unlike many other PRRs (TLR) can distinguish between soluble and particulate ligands [379]. The authors compared whole glucan particles (WGP), a particulate β-glucan preparation from *Saccharomyces cerevisiae* preparation with various soluble *S. cerevisiae* β-glucans in regards to their capability to activate Dectin-1 signaling. They reported that only particulate, but not soluble ligand can activate dectin-1 signaling leading to induction of Syk phosphorylation, NFκB activation, TNFα production and phagocytosis in macrophages, dendritic cells and neutrophils [379]. Moreover, all the soluble β-glucans blocked Dectin-1-mediated particulate β-glucan responses in primary BMMC and BMDC [379]. Further they showed that the mode of presentation of the β-glucan is crucial in activating Dectin-1 signaling as, like the β-glucan particles, soluble β-glucans immobilized on tissue culture plates or polystyrene latex beads stimulated robust Dectin-1-dependent responses [379]. Dectin-1 receptors cluster at the contact site with either particulate β-glucan, live fungi or β-glucan coated plates, following which the inhibitory phosphatases, CD45 and CD148 could exit from the synapse, allowing the activation of Src and Syk kinases [379]. In contrast, upon binding of soluble β-glucans to Dectin-1, CD45 remained co-localized with Dectin-1 at the cell surface, inhibiting the activation of Dectin-1 signaling [379]. Therefore, the ability to distinguish between soluble and particulate ligand allows the receptor to become activated only in the context of an active fungal infection.
1.6.2 Dectin-1: Mediation of anti-fungal immune responses

Originally thought to be a DC-specific receptor, Dectin-1 is now known to be expressed by many other cell types such as monocytes, macrophages, neutrophils, T cells, B cells, eosinophils, mast cells and microglia [380]. Dectin-1 has been shown to be crucial in host defense against numerous fungal infections. Utilizing mice lacking Dectin-1 (Clec7a -/-) generated in their lab, Taylor et al reported that Clec7a -/- mice, compared to control mice (WT), displayed enhanced systemic dissemination, tissue specific fungal colonization and consequently had a poor survival rate upon live C. albicans infection [381]. Moreover, following i.p. infection with C. albicans, fewer monocytes, neutrophils and eosinophils were recruited to the peritoneal cavity of Dectin-1 -/- mice than WT mice. Defective recruitment was concurrent with low levels of IL-6, MCP-1, CCL-2, CCL-3, MIP1a, G-CSF and GM-CSF in the peritoneal lavage fluid of Dectin-1 -/- mice as compared to WT controls [381]. However, studies by Ohno & colleagues utilizing another Clec7a -/- mouse reported that Dectin-1 is required for clearing Pneumocystis carinii but not Candida albicans [382]. Further, they reported that Sparassis crispa glucan (SCG), a soluble, β-1, 6-branched β-1, 3-glucan purified from an edible mushroom can induce maturation (upregulation CD80 and CD40) of immature WT BMDC but not of Dectin-1 -/- DC [382]. However, BMCDs from WT and Dectin-1 -/- mice were similarly matured in response to LPS, suggesting that Dectin-1 is not required for the maturation of DCs. Moreover, antigen-presentation and T cell-mediated responses were comparable between WT and Dectin-1 KO DCs [382]. However, defective ROS-
production by macrophages in Dectin-1 KO mice rendered them more susceptible to *Pneumocystis carinii* infection [382]. The discrepancy in the two studies regarding the role of dectin-1 in *C. albicans* infection may be due in part to the differences in the mouse strain utilized. It is also possible that the content of β-glucan or its size or structure might be different between the two stains of *C. albicans*. Studies by Underhill & colleagues have shown that Dectin-1 mediated recognition of *C. albicans* by macrophages is dependent on fungal morphology [383]. During budding and cell separation, a permanent scar develops that exposes the shielded β-glucan component of the cell wall [383]. Exposed β-glucan sensing by dectin-1 triggers antimicrobial responses, including phagocytosis and activation of reactive oxygen production [383]. However, during filamentous growth, no cell separation or subsequent β-glucan exposure occurs, and the yeast fails to activate Dectin-1 on macrophages [383].

Concurrent with the significant role of Dectin-1 in clearance of *Candida* in mice, humans with a homozygous stop-codon mutation (Y238X) in the gene encoding Dectin-1 were reported to be more susceptible to muco-cutaneous candidiasis [384]. The mutation resulted in low expression, β-glucan binding, and defective cytokine (IL-17, IL-6) production after stimulation with β-glucan or *C. albicans* [384]. Interestingly, fungal phagocytosis and fungal killing were comparable between monocytes isolated from patients with this mutation and those from healthy controls [384].

In addition to *C. albicans*, Dectin-1 has been shown to be the primary driver of anti-fungal immunity to *Aspergillus fumigatus* infection. Dectin-1 KO
mice as compared to WT controls demonstrate impaired lung neutrophil recruitment and function (ROS production, fungal killing) and IL-17A production leading to uncontrolled *A. fumigatus* lung growth [385]. Moreover, in response to *A. fumigatus*, alveolar macrophages from WT mice did but Dectin-1 -/- mice failed to produce proinflammatory mediators, IL-1α, IL-1β, TNF-α [385]. Studies by other groups have suggested that low levels of the anti-inflammatory cytokine, IL-10 are the cause of enhanced lung pathology seen during *A. fumigatus* infection in Dectin-1 -/- mice [386].

*A. fumigatus* primarily affects patients undergoing solid organ transplants and those receiving immunosuppressive drugs, which can result in invasive pulmonary aspergillosis (IPA) [387]. Recent study reported that patients expressing Y238X polymorphism in dectin-1 gene, undergoing hematopoietic stem cell transplantation had increased susceptibility to aspergillosis [386]. Interestingly, the risk for aspergillosis was greatest when the polymorphism was present in both the donors and the recipients [386]. Utilizing murine models, the authors reported that while the infection was severe in Clec7a KO host receiving Clec7a KO bone marrow cells, and moderate in WT bone marrow into a KO host, there was no increased susceptibility in KO into WT pairings, suggesting that Dectin-1 expressed in the non-myeloid/non-hematopoietic compartment may be playing a protective role [386, 387].

The role of Dectin-1 in regulating immune responses has revolved around its effect on hematopoietic cells, especially myeloid cells. However, recently there have been a few reports demonstrating a role for Dectin-1 on cells other than
APCs. Lee et al reported that Dectin-1 expression is induced in the human alveolar epithelial cell (A549) line following *Mycobacterium tuberculosis* (Mtb) infection [388]. Moreover, selective knock-down of CLEC7A on A549 cells resulted in decreased Mtb internalization, ROS generation and production of pro-inflammatory cytokines (TNFa, IL-6, CXCL8) concurrent with enhanced intracellular Mtb replication [388]. Together, their data indicates that Dectin-1 on airway epithelial cells is protective against Mtb infection [388]. Utilizing another human bronchial epithelium cell line (16HBE), Sun et al. demonstrated that *A. fumigatus*-induced production of ROS and anti-microbial mediators is dependent upon the upregulation of Dectin-1 mRNA and protein [389]. An *in vivo* study by Rand et al. demonstrated that exposure to low-doses of curdlan induces dectin-1 expression on AMs, alveolar type II cells (ATII), and non-ciliated, respiratory bronchiolar epithelia as assessed by in situ hybridization (ISH) and immunohistochemistry (IHC) [390].

Apart from the *in vitro studies outlined above*, there have been no reports exploring the role of epithelial cell expression of Dectin-1 on the modulation of allergen-driven immune responses *in vivo*. In light of the knowledge gained from studies with TLRs, wherein the expression of PRR on the non-hematopoietic compartment can dominate and modulate the immune response through hematopoietic cells, and the growing recognition of the role of the epithelium in asthma, further studies are needed to explore the role of dectin-1 expression on the airway epithelium in asthma.
1.7 CLRs in allergic asthma pathogenesis

Although CLRs are best characterized for their role in modulating fungal-induced Th17 immune responses, recent evidence has emerged that suggests that carbohydrate moiety recognition through CLRs is important in Th2 immune responses as well as Th17-mediated immune responses. During Schistosoma mansoni parasitic infection, the host mounts a strong Th2 immune response, which is crucial for expulsion of the parasite. The anti-parasitic Th2 immune response was shown to be dependent on carbohydrate moiety sensing of schistosoma’s soluble egg antigen (SEA). Mice sensitized and challenged with normal SEA in comparison to those treated with de-glycosylated SEA had greater serum IgE and IL-4, IL-5 upon restimulation [391]. Specifically, Lacto-N-fucopentaose III (LNFPIII), a polylactosamine sugar, the predominant carbohydrate in SEA was shown to function as an adjuvant in inducing Th2 type response by DCs in vivo [392]. Immature human monocyte-derived DCs (moDCs) were shown to rapidly internalize schistosome soluble egg antigen (SEA) through various CLRs (DC-SIGN, MGL and MR) that synergize with the TLR4 pathway to induce Th2 immune response [324]. In conclusion, studies in helminth models have revealed a previously unrecognized role for carbohydrate moiety sensing on APCs in inducing Th2 immune responses.

1.7.1 Carbohydrate allergen recognition in asthma

While a Th2 immune response is beneficial for expulsion of parasites from the host, it can be detrimental in the context of Th2 driven allergic diseases.
Utilizing native peanut allergen (PNAg) and its’ deglycosylated form (dPNAg), Shreffler et al reported that glycoproteins on allergens acts as Th2 adjuvants. While native PNAg could, dPNAg did not activate moDCs (MHC-II) and subsequent T cell proliferation [393]. Further, they showed that binding of the major glycoallergen protein, Ara h1 through carbohydrate binding sites of DC-SIGN (CD209) on myeloid DCs is essential for induction of Th2 immune responses in vivo [393]. In humans, moDCs stimulated with DC-SIGN ligand compared to controls are better in driving naïve T cells into a Th2 lineage while suppressing Th1 differentiation [394]. Although genetic variations in DC-SIGN receptors have been shown to influence the susceptibility to several infectious agents such as Mycobacterium tuberculosis, human immunodeficiency virus-1 and dengue virus, no such study has been reported for asthma [395]. However, two CD209 polymorphisms, rs11465413 and rs8112555, were shown to be significantly associated with atopic sensitization in the KOALA birth cohort [395].

In the context of allergy and asthma, MR was one of the first CLR s to gain attention. Gene-mapping linkage analyses in both humans and mice have identified MR as a candidate gene for development of allergen-induced airway hyperresponsiveness (AHR) [396]. Interestingly, MR has been shown to recognize numerous clinically relevant allergens from HDM (Der p 1, Der p 2), cat dander (Fel d 1) and peanut (Ara h 1) [396]. Silencing of MR expression on monocyte derived-dendritic cells inhibits the Th2 cell bias, downstream of Der p 1 exposure [397]. Recognition of Fel d 1 through the mannose receptor determines its allergenicity, as mice lacking MR produce less total IgE and allergen specific
IgE as compared to WT mice following sensitization and challenge [398]. Therefore, MR promotes allergic Th2 immune response upon exposure to various allergens.

Recent studies have highlighted the role of other CLRs in modulating allergen-induced Th17 responses in addition to Th2 immune responses. For example, Barrett et al reported that extracts from the house dust mite species, *Dermatophagoides farinae* (Df) and *Dermatophagoides pteronyssinus* stimulates the production of cysteinyl leukotrienes (cys-LTs) from mouse BMDCs and pulmonary CD11c+ cells in a Dectin-2 dependent manner [399]. Adoptive transfer of Df-pulsed WT and not Dectin-2 deficient DCs was sufficient to induce pulmonary inflammation i.e, recruitment of eosinophils and induction of Th2 cytokines [400]. Sensitization with Df-pulsed BMDCs from either mice lacking the critical enzyme in cys-LT generation (LTC4 synthase -/-) or the type 1 cys-LT receptor (CysLT1R -/-) did not induce airway inflammation [400]. Therefore, Dectin-2 promotes dust-mite induced Th2 immune responses through generation of cysteiny1 leukotrienes. While the above mentioned studies dictate that dectin-2 sensing of dust-mite allergen is critical during the sensitization phase, later studies from the same group indicate that dectin-2 regulates the effector phase of allergen-induced pulmonary inflammation independently from its role in sensitization [401]. Further, Norimoto et al recently reported that in addition to Th2, dectin-2 also modulates house dust mite-induced Th17 cell differentiation promoting allergic airway inflammation in mice [402].
1.7.2 Role of Dectin-1 in Asthma Pathogenesis

Numerous studies have linked exposure to β-glucans with Th2 immune responses such as observed during allergic asthma. Mice pre-exposed to β-glucan had higher titers of OVA specific IgE than controls in a model of OVA sensitization and challenge [403]. In humans, administration of β-glucan alone has been shown to be sufficient in driving eosinophil infiltration in the lungs with a concomitant decrease in their numbers in the blood [404]. Several studies have measured the exposure to molds by estimating the content of (1→3)-β-D-glucan in indoor environments and correlated the levels with the presence of clinical symptoms or signs of airway inflammation [405]. All studies have linked high levels of β-glucan exposure to enhanced respiratory symptoms, and airway inflammation. In some of these studies high exposures were linked to atopy and a decrease in FEV1 [406-409]. However in the majority of these studies, the levels of endotoxin were either not accounted for or were reported to be high. Therefore, it is hard to dissect the role of each component (endotoxin vs. β-glucan) on airway inflammation. Nonetheless, these studies indicate that β-glucan exposure might be deleterious and might exacerbate allergic asthma. In contrast, other studies point towards a protective role for β-glucan in allergic inflammation. Subcutaneous administration of particulate β-1-3-glucan was shown to increase serum IL-10 levels in asthmatics [410]. Moreover, patients receiving β-glucan had less asthma symptoms at the end of the study [410]. In a birth cohort study, it was reported that exposure to high (1-3)-β-D-glucan concentration and not endotoxin was associated with lower chances of wheezing
wheezing and allergen sensitization [411]. In another study, orally ingested, superfine dispersed β-1, 3-glucan (SDG) in individuals with atopy led to reduction of increase in both allergen-specific and total IgE titers [412]. Therefore, studies in humans suggest that β-glucans may either promote or inhibit allergic asthma, perhaps depending on their exact structure and their solubility characteristics.

Murine studies have provided direct evidence of the role of β-glucan on allergic immune responses. Recently, Kawashima et al reported that i.p. administration of curdlan (a linear β-glucan) significantly inhibited antigen (Ag)-induced eosinophil recruitment and Th2 cytokine production in the airways [413]. These studies have all inferred the involvement of dectin-1 through association with the promiscuous ligand, β-glucan. However, studies showing direct linkage between the Dectin-1 receptor and Th2 immune responses are limited. A study by Mintz-Cole et al in a model of mold-induced asthma showed that while Dectin-1 promotes Th17 immune responses, it might inhibit the development of a Th2 immune response [414]. Using two different molds the authors reported that while mice exposed to C. cladosporioides spores induced AHR, eosinophilia, and a predominant Th2 response, A. versicolor exposed mice mounts a strong Th17 response and neutrophilic inflammation, but very mild AHR [414]. In their model, IL-17A was protective as its blockade in A. versicolor-exposed mice led to development of exacerbated AHR compared with animals receiving isotype Ab [414]. As Dectin-1 has been shown to mediate anti-fungal Th17 immune response, they next compared WT and Clec7a−/− mice in their model. Clec7a−/− and WT mice exposed to C. cladosporioides had equivalent AHR and
eosinophilia [414]. However, *Clec7a*−/− mice had fewer Th17 cells and increased Th2 cells in the lungs compared with wild-type mice following *A. versicolor* exposure [414]. In the absence of Dectin-1, *A. versicolor* induced robust AHR and eosinophilia, which the author proposes is due to a decrease in the protective Th17 immune response. However, it might be possible that the robust AHR could be due to an increase in Th2 immune response seen in *Clec7a*−/− mice. In an airway infection-model wherein a Th17 immune response is required to clear the infection, blocking IL-17A might prove detrimental to the host. Despite the suggestion that dectin-1 may differentially regulate fungally-induced Th17 and Th2 immune responses depending on the presence or absence of B-glucans, evaluation of the role of dectin-1 in recognition of other non-infectious respiratory allergens has not been conducted to date.

Recent studies from our lab have suggested that β-glucan contained in HDM has a biological effect on lung epithelial cells. Specifically, we demonstrated that β-glucan might be crucial in dust-mite sensing on the epithelium [415]. Specifically, Nathan et al demonstrated that HDM-induced rapid secretion of CCL20 from 16HBEs. Inherent protease activity or TLR stimulation by HDM was not responsible for CCL20 secretion [415]. However, pretreatment of 16HBE cells with β-glucans competitively inhibited HDM-induced CCL20 production [415]. Further, enzymatic digestion of β-glucan in HDM by β-glucanase treatment dramatically reduced epithelial CCL20 secretion [415]. Therefore, β-glucan moieties in HDM can induce rapid secretion of CCL20 from epithelial cells *in vitro*. As dectin-1’s putative ligand is β-glucan, it can be
speculated that dust-mite might be sensed by the receptor leading to induction of CCL20 in vivo. However, as β-glucan can be sensed by receptors other than Dectin-1, care must be taken when interpreting these results. Nonetheless, as CCL20 is known to recruit immature DCs to the lungs following allergen-exposure, dectin-1 might promote allergic immune responses. Thus careful in vivo studies are required to dissect the role of recognition of HDM containing PAMPs through dectin-1 in regulating allergic immune responses.

Thus the goal of the work contained in this thesis is to determine the contribution of dectin-1 in mediating type 2 immune responses to the common allergen, house dust mite. As sensitization to HDM is a major risk factor for the development of asthma, we will focus our studies on the role of dectin-1 in regulating the development of asthma to this common allergen. Based on the current evidence, we hypothesize that dectin-1 recognition of ligands in HDM promotes the development of the asthmatic phenotype.

The specific aims of this project are the following:

1.8. Specific Aims

1) To determine the role of dectin-1 in dust-mite mediated asthma pathogenesis

2) To determine the mechanism(s) through which dectin-1 regulates asthma pathogenesis

3) To begin to explore the importance of dectin-1 in human asthma
Chapter 2: Materials and Methods

In this chapter, we will describe the general methods to be utilized throughout the thesis.

2.1 Mice

*Clec7a*-/- mice were originally generated on the 129/SvEv background and backcrossed for 10 generations to the C57BL/6 background, and bred at Taconic farms. We obtained them from Taconic farms and bred them to WT C57BL/6 mice purchased from Taconic in order to generate *Clec7a*+/+ Het mice. All the experiments were performed using littermate control WT (*Clec7a*+/+) and KO (*Clec7a*-/-) mice generated from *Clec7a*+/+ Het * Clec7a*-/+ Het breeding in JHSPH animal facility. Mice were housed in a specific pathogen free animal facility in micro-isolator cages. Mice were provided autoclaved food (Lab diet 5010) and water ad libitum. All procedures were approved by the Animal Care and Use Committee of Johns Hopkins University.

2.2 Allergen sensitization and challenge

Mice were sensitized to HDM by intraperitoneal (i.p.) injection with PBS (100ul, Mediatech, Manassas, VA) or HDM (10ug/100uL PBS, Greer Laboratories, Lenoir, NC) on days 0 and 7. Following this, mice were challenged intratracheally with PBS (40ul) or HDM (100ug/40ul) on days 14 and 21. Seventy-two hrs. after the last HDM challenge (Day 24), the allergic phenotype was assessed (described below).
2.3 Airway responsiveness measurement

Airway reactivity measurements were done seventy-two hrs. after the last I.t. HDM challenge. Briefly, mice were anesthetized by i.p. administration of ketamine (2mg/mouse) and xylazine (0.3mg/mouse) and then tracheotomized before insertion of an 18 gauge cannula into the trachea. Mice were then paralyzed with suxamethonium chloride (3mg/kg), intubated and respirated at a rate of 120 breaths per minute with a constant tidal volume (0.2 ml). After a stable baseline was achieved, mice were exposed to nebulized methacholine (30mg/ml). After 10 seconds, dynamic airway pressure (cm H$_2$O × s) was recorded for 5 min. Following airway reactivity measurements, serum BAL fluid, lung tissue (RNA/cell culture) were collected, and processed as discussed below.

2.4 Bronchoalveolar lavage

Mice were lavaged using a 1 ml syringe containing 1 ml of cold (kept on ice) Hank's Balanced Salt Solution (HBSS, Gibco, NY) through the cannula and collected in a 1.5ml microcentrifuge tube (USA scientific). Lavage fluids were then spun at 500xg for 5 min followed by 1 min pulse at 5000xg. Supernatants were removed and transferred to a new 1.5 ml microcentrifuge tube and stored at -80°C. The pellet was resuspended in 200ul of Ack buffer for 3 min at room temperature to lyse the red blood cells. One ml of 10% FBS (in PBS) was then added to each tube and the lavage was spun again at 500xg for 5 min. Supernatants were then discarded and the pellet was resuspended in 200ul of 10% FBS (in PBS) for cell counts using trypan blue. Cells were then processed
(cytospin) for staining with Diff-Quik (Siemens, Newark, DE) to identify eosinophils, neutrophils, macrophages and lymphocytes.

2.5 Serum collection and IgE antibody measurement

After airway reactivity measurements and BAL collection, the abdomen was disinfected with alcohol and the peritoneal cavity was exposed and the inferior vena cava was severed to collect blood into a separator tube (BD microtainer, Franklin lakes, NJ). Separator tubes were then centrifuged at 10,000 rpm for 7 mins, and the serum was collected from the top layer using a pipette. Serum was stored in 96 well culture supernatant plates at -80°C. Serum IgE ELISAs were done using BD OptEIA kit (BD Biosciences, San Jose, CA).

2.6 Lung cell harvest and restimulation

Following BAL and serum collection, whole lungs were excised and placed on a cell strainer (70µ) in a 6-well tissue culture plate. Tissue was minced and incubated for 45 min at 37°C in 6 ml of serum-free RPMI medium containing Liberase Cl (0.5 mg/ml, Roche, Madison, WI) and DNase I (0.5 mg/ml, Sigma, St. Louis, MO). Cell strainer containing lung was placed on a sterile 50 ml tube and then lung was homogenized through the filter using a plunger from a 3 ml syringe to obtain single cell suspension. The cell strainer was then rinsed with 6ml of media from the 6-well plate and then with 10ml of complete RPMI. Cells were then centrifuged at 1600 RPM for 6 minutes and the cell pellets were resuspended in 3 ml of ACK lysing buffer and incubated for 3 minutes. Ten mls of
complete culture medium was then added to each tube and the cells were centrifuged again at 1600 RPM for 6 minutes. Cells were resuspended in 1 ml of RPMI and counted using trypan blue. Lung cells (125,000 cells/well) were cultured in 96 well flat bottom culture plates with PBS or HDM (100ug/ml) for 72 hrs. Afterwards the supernatants were harvested and stored in-80C for analysis by ELISAs.

2.7 Cytokine ELISAs
IL-33 (BAL fluid), IL-10, IL-13, IL-17A, IL-5 and IFN-g (lung cell restimulation) levels were measured using ELISA duosets from R&D Systems (Minneapolis, MN).

2.8 Quantitative real-time RT-PCR
Gene expression was measured by real-time PCR using specific primer pairs. For mouse samples, target gene expression was normalized to expression of the S14 gene. For human samples, target gene expression was normalized to S13 gene.

2.9 Histology
To determine the degree of inflammation and mucus metaplasia, formalin-fixed lung sections were embedded in paraffin wax, cut into 5µ sections and stained with hematoxylin & eosin (H&E) and periodic-acid Schiff (PAS) for mucus. The slides were examined with a 20x objective and a minimum of three airway sections per animal were examined. Examiners were blinded
2.10 Bone marrow dendritic cell (BMDC) culture

As described [190], bone marrow cells from WT and dectin-1 deficient mice were cultured in complete RPMI (cRPMI containing 10% FBS) supplemented with GM-CSF (10 ng/ml, Peprotech) on days 0, 3 and 6. BMDCs were harvested on day 7 using enzyme-free cell dissociation buffer (Gibco, Grand Island, NY). For stimulations with allergen or other stimulants, cells were counted and plated in cRPMI at 250,000 cells/well in a flat-bottom 96 well plate for twenty-four hrs before stimulants were added. For HDM uptake studies, at the end of Day 7 GMCSF-supplemented BMDC cultures, cells were harvested and plated at a density of 100,000 cells/well in a round-bottom 96 well plate in cRPMI. The following day, cells were serum starved (in RPMI containing 0.1% FBS) overnight. Cells were then stimulated with either PBS or Ax-405 labeled HDM (at various doses) at 37°C for 3 hrs. Cells were then stained with the following fluorescently-labeled antibodies, CD11c, CD11b, MHC-II and CD86 for further analysis.

2.11 Ex vivo flow cytometry analysis: Lung single cell suspension was plated in a round-bottom 96 well flat-bottom plate for staining. Cell staining was performed at 4°C following incubation with mouse FcBlock (BD) for 30 min. Data were acquired with an LSRII flow cytometer (BD Biosciences, San Jose, CA) equipped with lasers tuned to 488nm, 633nm, and 405nm. Spectral overlap was compensated and analyzed using the FACSDiVa software (BD Biosciences).
2.12 **Dectin-1 expression**

Lung cells \((1 \times 10^6)\) were plated for analysis of various cell populations (hematopoietic and non-hematopoietic) for Dectin-1 expression at baseline and after dust-mite exposure utilizing following antibodies. Anti-CD45 FITC (30-F11), anti-EpCAM PECy-7 (G8-8), anti-CD11c Ax700 (N418), anti-CD11b efluor 450 (M1/70) and anti-Dectin-1 PE (RH1)

2.13 **Lung inflammatory cell profile:**

Myeloid DCs (CD11b+CD11c+Gr1- MHCII+), plasmacytoid DCs (CD11b-CD11c+Gr1+CD317+), CD103+DCs (CD11b-CD11c+CD103+), neutrophils (CD11b+Gr1+), monocytes (CD11b+ Gr1-) and alveolar macrophages (CD11b-CD11c+Gr1-CD317-) were quantified at the end of in-vivo experiment using following antibodies. Lung cells \((1 \times 10^6)\) were stained with anti-CD11c-Ax700 (N418), anti-CD11b-PE-Cy7 (M1/70), anti-Gr1-APC-Cy7 (RB6-8C5), CD317 APC (BST2), MHC-II PE (M5/114. 15. 2) and CD103 PercpCy5.5 (M290).

2.14 **T cell flow**

Lung cells \((0.5 \times 10^6)\) were stimulated with PMA (100 ng/ml) and ionomycin (1 µg/ml) for ~16h, then Brefeldin A and monensin (eBioscience) were added for 4 hr. Cells were fixed, permeabilized and stained with anti-CD4-PE-Cy7 (RM4–5), anti-IL-13e660 (eBio13A), anti-IL-17A BV421 (TC-11-18H10) anti-IFNg Ax700 (MG1.2) and FoxP3 PE (FJK-16S).
2.15 ILC flow

Lung-single cell suspensions were plated at a density of $5 \times 10^6$ cells in cRPMI in a 6 well-flat bottom culture plate. Cells were re-stimulated with PMA (100 ng/ml) and ionomycin (1 µg/ml) for ~16h, then Brefeldin A and monensin (eBioscience) were added for 4 hr. ILC2s (Lineage-, CD45+, ST-2+, IL-13+) were identified using following antibodies. Cells were first gated on FSC-singlets and then SSC-singlets in order to analyze single cell events. Live cells identified using fixable viability dye (efluor-660) were then gated on lineage markers. Lineage (Lin) cocktail (PerCPCy5.5) contains anti-CD3ε (145-2C11), anti-TCRβ (H57-597), anti-CD11b (M1/70), anti-CD11c (N418), anti-Gr1 (RB6-8C5), anti-B220 (RA3-6B2), and anti-NK1.1 (PK136). Lin- cells were then gated using anti-CD45 (30-F11), anti-ST-2 FITC (T1/ST2) and anti-IL-13 PE (ebio13A).

2.16 Dectin-1 overexpression (16HBE)

16HBE cells (10,000) were plated in a 96 well flat-bottom culture plate in cDMEM overnight. Cells were then transfected with hDectin-1 or control vector (pCDNA 3.1) using FuGENE HD (Promega) for 24hr. Following day the cells were starved (DMEM containing 0.1% FBS) and 16hrs later the cells were stimulated with media or HDM (100µg/ml).

2.17 Dectin-1 ligand depletion from HDM

To deplete HDM of dectin-1 binding proteins, we pulled out dectin-1 ligands using dectin-1 Fc. To this end, HDM was incubated with recombinant protein (Dectin-1
Fc, CD40 Fc) overnight at 4C on a rocker. Twenty-four hrs later, protein G magnetic beads were added and incubated for 4hrs at 4C on rocker. At end of 4hr, removed the Ligand-Dectin-1 Fc-ProteinG or CD40 Fc-Protein G complex by magnetic separation.

2.18 Bone-marrow chimera

Seven to eight week old WT or Dectin-1 KO mice were irradiated at a dose of 10 Gray(Gy). Fresh bone-marrow (2 × 10^6 cells/100 ul cRPMI) isolated from non-irradiated WT and Dectin-1 KO mice was then supplement back (retro-orbital injection) to irradiated mice. In order to avoid infections, irradiated mice were then kept on drinking water supplemented with Neomycin (1.1mg/ml) for 2 weeks post-radiation. At the end of 2 weeks, mice were switched back to neomycin-free water.

The degree of chimerism was evaluated based on the differential expression of CD45 isoforms. As our experimental mice (WT and Dectin-1 KO) express CD45.2 isoform, we utilized B6.SJL-Ptprc^a Pepc^b/BoyJ (Jackson labs) mice which is a congeneric C57BL/6 mice harboring the CD45.1 isoform to evaluate chimerism. B6.SJL-Ptprc^a Pepc^b/BoyJ mice are Dectin-1 sufficient and hence used as WT controls. We transferred either CD45.1 WT BM (BoyJ) into irradiated Dectin-1b KO host (expressing CD45.2). Another group of irradiated WT (BoyJ, CD45.1) mice received dectin-1 KO BM (CD45.2). At the end of 5 months the expression of CD45.1 and CD45.2 isoforms in the lungs was evaluated.
2.19  *In vivo* anti-ST2 antibody blockade

Mice were sensitized and challenged with HDM or PBS as described before (2.2). Twenty-four hrs prior to each PBS/HDM sensitization and challenge, mice were given either isotype control antibody (Rat IgG2a, BioXcell) or anti-ST2 antibody (Amgen) at a concentration of 250µg in 100ul PBS i.p.

2.20  **Statistical analysis.**

Analysis of variance (ANOVA) followed by the Newman-Keuls test was used for analysis of differences among multiple groups. Student's t-test was used for comparisons between two groups. P values of less than 0.05 were considered significant.
Chapter 3: Role of Dectin-1 in dust-mite mediated allergic asthma

3.1 Introduction:

Asthma is a chronic inflammatory disease that has reached epidemic proportions. Although the exact etiology of asthma is unknown, the disease is thought to result from maladaptive immune responses to ubiquitous, otherwise innocuous environmental antigens, referred to as allergens. Allergens, by definition, are proteins that have the ability to elicit powerful T helper 2 responses, culminating in IgE antibody production in susceptible individuals. Why specific proteins cause aberrant T and B cell responses is a fundamental question that has remained largely unanswered.

Given the diverse nature, structure and biological functions of various allergens, it does not seem likely that a single characteristic confers allergenicity. Indeed it has been shown that no one structural characteristic explains the ability of allergens to induce allergic inflammation. Recently it has been proposed that allergens are linked by their ability to activate the innate immune system at mucosal surfaces. It is thought that allergen might contain, structures that resemble Pathogen Associated Molecular Patterns, PAMPs, recognition of such entities by innate immune cells such as APCs, epithelial cells could initiate maladaptive immune responses. Such motifs or PAMPs can activate specific pattern recognition receptors on immune effector cells such as APC leading to naïve T cell differentiation into effector subsets mediating asthma pathogenesis. Alternatively, PRRs expressed on airway epithelial cells might mediate the
release of factors (IL-25, IL-33, TSLP), which have been shown to be important in mediating type 2 immune responses. These cytokines, in turn, can then initiate immune responses through interactions with either classical APCs or innate immune cells such as mast cells, basophils, and ILCs.

Of the allergens, house-dust mite is the most common aero-allergen to which atopic asthmatics are sensitized [416]. Sensitization to HDM has been shown to be a major risk factor for the development of asthma [416]. Among the various species of dust-mites, *Dermatophagoides pteronyssinus* and *Dermatophagoides farinea* are the most common ones against which individuals are sensitized against. The allergens from dust-mite are classified into various groups depending on their biological functions. Twenty-four distinct classes of allergenic peptides have been identified in dust-mite [417]. Broadly, the HDM-contained allergens are grouped into four classes; those displaying protease activity, binding affinity for lipids, non-proteolytic enzymes, and non-enzymatic entities [417]. Category 1 contains allergens belonging to group 1, 3, 6 and 9 that display cysteine proteinase, trypsin-like, chymotrypsin-like, and collagenolytic-like serine proteinase activity respectively [417]. Category 2 includes those allergens belonging to groups 2, 5, 7, 13, 14, and 21, displaying affinities to bind fatty acid and lipids. Category 3 allergens with non-proteolytic enzymatic activities such as amylases, glutathione-S-transferases, and arginine kinases, fall in groups 4, 8 and 20 allergens respectively. Various allergens contained in groups 12, 15, and 18 display homologies with chitinases, hydrolases that digest chitin. Category 4 contains dust-mite muscle derived proteins tropomyosin (group 10) and
paramyosin (group 11). Allergenicity to dust-mite can occur as a result of sensitization to allergenic components contained in the dust-mite itself (discussed above) and/or to those microbial-contaminants contained in fecal pellets [418]. Indeed, several microbial PAMPs like chitin, LPS and β-glucan are present in the mite fecal pellet and hence inhalation of these can alter sensitization in allergic individuals [418]. While the source of chitin maybe the mite itself, symbiotic fungal species in the gut of dust-mite might contribute to the β-glucans found in dust-mite fecal pellets. Therefore, sensing of numerous PAMPs or allergenic moieties in complex allergens can dictate one’s propensity to mount an asthmatic phenotype.

Among the PRRs being investigated in the asthma field, TLRs have been the most studied. Exposure to endotoxin (LPS), the TLR4 ligand has been reported to promote or inhibit ones’ likelihood to develop allergic asthma in humans [324]. Studies in mouse models indicate that depending on the dosage of LPS, mice can either develop a Th2 rich (Ova+ low LPS) or a Th1 high (Ova+ high dose LPS) immune response. In a model of dust-mite driven allergic lung inflammation, Trompette et al reported that Der p 2 is a functional mimic of MD-2, a component of the TLR4 signaling complex. Der p 2 bound to LPS can activate TLR4 signaling thereby driving Th2-mediated allergic inflammation in the absence of MD-2 [329]. Therefore, components of allergen extract can act as mimics activating PRRs on immune effector cells.

In addition to TLR ligands, allergens have also been shown to contain carbohydrates that activate a class of carbohydrate moiety sensing receptors,
referred to as the C-type lectin receptors (CLRs). Indeed the association of allergenic proteins such as Der p 2, Bermuda grass allergen and BG60 with specific glycans has been shown to contribute to their allergenicity through activation of CLRs [419]. Recent studies in various helminth and allergen models have highlighted the role of carbohydrate moiety recognition (in pathogen) in driving strong Th2 immune responses through CLRs like the mannose receptor (CD206), Dectin-2, and DC-SIGN [391, 393, 397, 406].

Among the CLRs, dectin-1 is unique, as it primarily recognizes β-glucan moieties, which are not expressed in mammals. Since it’s discovery, the CLR Dectin-1 has been shown to initiate and amplify Th17 responses, and to confer protection against fungal infections [374]. However, the role of β-glucan exposures in modulating asthma pathogenesis is conflicting, as β-glucans have been shown to have either a pro- or anti-allergic role [403, 405, 410]. β-glucan is a promiscuous ligand as it can be sensed by receptors other then dectin-1, such as CR3, lactosylceramide and CD5 [371-373]. Nevertheless, in the current literature, it has been assumed that β-glucan-driven biological effects are equivalent to those driven by Dectin-1. Because β-glucan can be sensed by more than one receptor, it is therefore not necessarily correct to assume that all of β-glucan’s effects are mediated through dectin-1. Unfortunately most of the studies of the effects of β-glucan on allergic asthma have perhaps been incorrectly attributed to dectin-1 activation. Utilizing an indirect approach, Kawashima et al have reported that i.p. administration of the fungal antigen curdlan (a β-glucan) significantly inhibited Ag-induced Th2 responses [413]. As β-glucan can be
sensed by multiple receptors, it is not clear if β-glucan sensing through dectin-1 is responsible for the inhibition of allergic inflammation in their study. However, recent studies by Mintz-cole in a model of mold-induced allergic inflammation have indicated that while dectin-1 promotes Th17 immune responses to fungal antigens, it also inhibits Th2-mediated immune responses [414].

Although dectin-1’s role in fungal antigen mediated immune responses is well characterized, studies of it’s role in HDM-mediated immune responses are lacking. Initial studies from our lab indicated that β-glucans in dust-mite extracts induce CCL20 secretion from a human bronchial epithelial cell line [415]. This was the first report suggesting that HDM contained β-glucans could activate host epithelial cells. As CCL20 is crucial in recruiting immature dendritic cells into the lung, and mice lacking the cognate receptor for CCL20, CCR6 (CCR6−/−) were shown to have significantly reduced allergen-induced allergic asthmatic immune responses as compared to receptor sufficient WT mice, we hypothesized that β-glucans may induce allergic inflammation [420]. However, in these studies the role of dectin-1 in mediating the epithelial cell response to HDM contained β-glucans, was not directly examined. As the role of dectin-1 in type 2 immune responses to allergens remains unclear, the goal of the current study was to directly determine the role of dectin-1 in the development of immune responses to the common allergen, house dust mite. Based on our earlier published findings, we hypothesized that the β glucan: dectin-1 pathway will be crucial in mediating the development of type 2 cytokine-mediated asthmatic immune responses to the common allergen, HDM. To test this hypothesis, we evaluated
the asthmatic phenotype in dectin-1 deficient and C57BL/6 wildtype littermate controls following sensitization and challenge with house dust mite.

3.2 Results:

3.2.1: In vivo expression profile of Dectin-1

To determine the role of dectin-1 in dust-mite mediated allergic asthma, we first investigated whether dectin-1 was expressed in the lung and whether it was regulated by allergen exposure. To this end, we compared dectin-1 mRNA levels in the lungs of mice challenged with either PBS or HDM. Dectin-1 expression was detected in the lungs of PBS-sensitized and exposed mice (Fig. 1A), however it was significantly suppressed after allergen exposure. Interestingly, dectin-2, a CLR that has been shown to promote Th2-rich allergic asthma in a model of dust-mite induced allergic asthma, is upregulated following allergen exposure (Fig.1B) [399]. Therefore, our data suggests that dust-mite exposure promotes the expression of PRRs (ex: Dectin-2) that exacerbate allergic asthma. Based on our data that dust-mite exposure represses the expression of dectin-1 (Fig.1A), it can be inferred that dectin-1 might protect against the development of the allergic response.

3.2.2 Dectin-1 inhibits dust-mite mediated allergic asthma

To determine whether dectin-1 mediates or inhibits dust-mite-induced immune responses, we compared the asthmatic phenotype in mice sufficient (Clec7a+/+, wildtype, WT), and either deficient in one (Clec7a+-/−, heterozygous,
het) or both (Clec7a-/-, KO) copies of the dectin-1 gene. The airway responses to cholinergic agonist (methacholine) stimulation were comparable between PBS-challenged WT, het and KO mice (Fig. 2A). Following HDM challenge, WT, het and Clec7a-/- mice had significantly higher AHR than their PBS-challenged controls. Surprisingly, mice deficient in both copies of the dectin-1 gene (Clec7a-/-) had significantly higher airway hyperresponsiveness compared to WT and het mice (Fig. 2A). Thus, loss of both copies of the dectin-1 gene is necessary to render mice more susceptible to dust-mite induced AHR. These data demonstrate that the loss of dectin-1 enhances dust-mite driven AHR, or that dectin-1 normally serves to protect against the development of dust-mite-induced AHR.

In order to determine whether the alterations observed in AHR in dectin-1 mice were associated with inflammatory cell changes in the lung, we evaluated the cellular composition of the BAL fluids. Although the total number of cells in the BAL was not different between WT and KO mice exposed to HDM (Fig. 2C), the composition of the cells was different (Fig. 2D). The increase in AHR seen in dectin-1 KO mice was associated with a higher percentage of eosinophils in the BAL of dectin-1 KO mice as compared to WT mice following allergen exposure (Fig. 2D). Consistent with the literature [421] demonstrating that dectin-1 regulates neutrophil recruitment, we observed that the loss of dectin-1 resulted in a significantly lower recovery of neutrophils in the BAL of HDM-challenged mice (Fig. 2D)[422]. Histological assessment of the airways showed that while both WT and dectin-1 KO mice exposed to HDM had significant increases in the
degree of mucus staining in the airways as compared to PBS-challenged mice, dectin-1 KO mice had severe mucus plugging of the airway lumen, which was not present in WT mice challenged with HDM (Fig. 2E).

Despite the effects of dectin-1 deficiency on the traditional parameters of a type 2 immune response (AHR, eosinophilia, and mucus production) we did not see any significant differences in the magnitude of IgE production, as total serum IgE levels were comparable between WT and KO mice (Fig. 2B). The data is suggestive that dectin-1 does not play a role in allergen sensitization but it regulates the magnitude of dust-mite driven asthma exacerbation.

3.2.3 Dectin-1 inhibits dust-mite induced Th2 cytokines while promoting Th17 cytokines

As dectin-1 KO mice have exaggerated responses indicative of a type 2 immune response (AHR, eosinophilia, mucus hypersecretion), we compared T cell cytokine patterns in the lungs. Exposure to HDM induced the prototypical Th2 cytokines i.e. IL-13, IL-4 and IL-5, as well as the Th17 cytokine, IL-17A (Fig. 3A, 3B, 3C, 3D) in the lungs of both WT and dectin-1 KO mice. However, compared to HDM-challenged WT mice, Il13 mRNA was expressed at higher levels in the lungs of dectin-1 KO mice (Fig. 3A). Although not significant, Il15 levels were also higher in dectin-1-deficient mice, which could explain the increase in BAL eosinophils in dectin-1 KO mice (Fig. 3B). Given the known pro-Th17 role of Dectin-1, it is not surprising that dectin-1 KO mice had significantly lower levels of dust-mite induced Il17a, as compared to WT mice (Fig. 3D).
Although studies from our lab and others have shown that IL-17A can exacerbate allergic asthma [190], others have shown that it can inhibit type 2 immune responses at the initiation of T cell immune response [194]. Taken together these results suggest that dectin-1 likely suppresses type 2 immune responses, while driving Th17 immune responses following dust-mite exposure. The low levels of IL-17A in dectin-1 KO mice (compared to WT mice) did not correlate with asthma phenotype suggesting that dectin-1 regulation of Th17 responses directly does not modulate disease exacerbation. However, the increases in Th2 cytokine expression (IL-13, IL-5) seen in the lungs of dectin-1 deficient mice tracks with enhanced AHR, mucus production and eosinophilia.

3.2.3 Dectin-1 inhibits mDCs recruitment in the lungs.

As APCs such as DCs are known to widely express dectin-1, we next sought to determine if the receptor modulates DCs function following dust-mite exposure. DCs are pivotal in differentiation of naïve CD4+ T cells into effector T cell subsets. As our data suggests that dectin-1 might be inhibiting Th2 responses, we sought to determine whether the dectin-1 deficiency alters the types of DCs [i.e. mDCs (pro-inflammatory) and pDCs (anti-inflammatory)] present in the lungs following HDM exposure. To this end, single cell lung suspensions from mice sensitized and challenged with PBS or HDM were stained with various fluorescently labeled antibodies to identify various DC and other inflammatory cell populations in the lungs. FACS analysis revealed that, PBS-challenged mice had a significant greater number of mDCs
(CD11b+CD11c+Ly6G-MHC-II+) in the lungs of dectin-1 KO mice as compared to WT mice (Fig.4A). Similarly, following dust-mite exposure, mDCs are present in higher numbers in the lungs of dectin-1 KO mice as compared to WT mice (Fig.4A, 4B). Therefore, dectin-1 seems to be controlling the number (development/ recruitment) of mDCs in the lungs (Fig. 4A & 4B). We did not see differences in the recruitment of protective pDCs (CD11b-CD11c+Ly6C+CD317+) or inflammatory monocytes (CD11b+Ly6C+Ly6G-) (Fig.4C, 4D, 4E, and 4F) in the lungs of WT or dectin-1 KO mice following PBS or allergen exposure. However, PBS-challenged dectin-1 KO mice had significantly lower neutrophils (CD11b+CD11c+Ly6C-Ly6G+) (Fig. 4G and 4H) as compared to WT controls. Compared to PBS challenge, HDM exposure leads to the reduction in the numbers of neutrophils in the whole lungs of both WT and dectin-1 KO mice. As these mice were lavaged prior to isolation of lung cells, we believe that the PMNs may have exited the lungs into the BAL fluids. Thus, the flow data is not likely an accurate reflection of PMN numbers in the lung. Therefore, in the absence of dectin-1, a greater number of DCs are recruited into the lungs, which might drive a higher CD4+Th2 immune response, thereby explaining the exaggerated asthma phenotype in the receptor deficient mice as compared to receptor sufficient mice.

3.2.4 Dectin-1 does not modulate the phagocytic or activation potential of DCs following dust-mite exposure
As our studies suggested that DC were increased in dectin-1 deficient lungs, and dectin-1 has been shown to be a bonafide phagocytic receptor, we asked whether dectin-1 expression alters the phenotype of DCs following dust-mite exposure. To test this hypothesis, we exposed BMDCs derived from WT and dectin-1 KO mice to fluorescently labeled HDM and examined HDM uptake and costimulatory molecule expression (MHC-II, CD86) by flow cytometric analyses.

Upon examining the CD11c+CD11b populations, we report that HDM content per cell (MFI) (Fig.5A) is not impacted by dectin-1 deficiency and neither is MHC-II (Fig.5B) or CD86 (Fig.5C) expression. Despite dectin-1’s known role as a phagocytic receptor for β-glucan rich fungal particles, it does not seem to be important in regulating HDM phagocytosis by DCs. Consistent with the hypothesis, earlier studies have shown that while dectin-1 deficiency affected DCs phagocytic and activation potential to fungal antigen, the responses were similar upon exposure to LPS [382].

3.2.5 Dectin-1 does not modulate adaptive CD4+ T cell immune responses

As DCs are known to be crucial in driving generation of CD4+ T cell immune responses, we wanted to determine if loss of dectin-1 impacts the generation of adaptive T cell immune responses following dust-mite exposure. To quantify the magnitude and type of adaptive T cell immune response, we analyzed the prototypical Th2 cytokines from lung-cell cultures of WT and dectin-1 KO mice previously sensitized and challenged with PBS or HDM upon
secondary exposure to the allergen. Surprisingly, in re-stimulated cultures of lung cells from HDM-exposed WT and Clec7a−/− mice, dust-mite recall induced similar levels of Th2 cytokines, IL-13 and IL-5 (Fig. 6A and 6B).

As lung cell restimulation is most likely recalling HDM-specific CD4+ T cell, we next, compared the levels of IL-13+ Th2 cells (CD3+CD4+) in the lungs of dectin-1 KO and WT mice, sensitized and challenged with PBS or HDM by flow cytometry. We observed a robust HDM-induced increase in lung IL-13+ T cells; however, both WT and KO had equivalent frequencies of IL-13+ T cells (Fig. 6C and 6D). Because Il13 message was higher in dectin-1 KO mice (Fig. 3A), while T cell-derived IL-13 or IL-13+ T cells were not different between WT and dectin-1 KO mice, our data suggests that dectin-1 likely controls IL-13 production from a non-CD4+ T cell source.

In contrast to IL-13+ cells, we did not observe increases in the numbers of HDM-induced Th17 cells (CD4+CD3+IL-17+) in the lungs of WT or dectin-1 KO mice exposed to HDM (Fig. 6E, 6F). However PBS-challenged, dectin-1 KO mice had a lower frequency of Th17 cells as compared to PBS-challenged WT mice (Fig. 6E and 6F). Similarly, Th1 (CD3+CD4+IFNγ+) cells were significantly lower following PBS challenge in the KO mice as compared to controls (Fig. 6G and 6H). These data are consistent with the reports of others showing that dectin-1 drives Th1 and Th17 responses [423]. Further, as studies in both humans and mouse have shown a protective/anti-inflammatory role of regulatory T cells in asthma, we next sought to determine whether dectin-1 exerts its protective role through promoting Tregs [211, 213-215]. Our data shows that dectin-1 does not
affect the frequency of HDM-induced lung Tregs (CD3+CD4+FoxP3+) (Fig. 6I and 6J). Tregs are known to suppress immune responses through production of the cytokine, IL-10. **However, there are no differences in the levels of IL-10 produced from lung-T cell restimulation cultures of WT and dectin-1 KO mice (data not shown).**

### 3.3 Discussion

Herein we report that dectin-1 controls against the magnitude of type 2 immune responses to the common allergen, house dust mite. Specifically, in the absence of the receptor, mice develop exaggerated AHR (Fig. 2A), eosinophilia (Fig. 2D) and mucus plugging (Fig. 2E) of the airway lumen following HDM sensitization and challenge. Therefore, dectin-1 normally serves to control the magnitude of the asthmatic immune response. Consistent with its inhibitory role, the development of a productive allergic response in wildtype mice is associated with suppression of the expression of this inhibitory receptor (Fig. 1A). These results suggest that loss of the balance between dectin-1 mediated suppression and other pro-allergic signals, might dictate one’s propensity to mount deleterious immune responses to ubiquitous airborne allergens.

The functional airway changes observed in dectin-1 deficient mice (compared to WT mice) are associated with an increase in lung Type 2 cytokine expression levels (i.e. IL-13 and IL-5) following dust-mite exposure. Despite dectin-1’s effect on the functional features of the disease, it did not have any effect on the serum levels of IgE in HDM-challenged mice, suggesting that it
does not regulate sensitization to HDM. The lack of effect of dectin-1 on IgE is not necessarily surprising given that IL-4 mRNA levels were not different between WT and dectin-1 KO mice. Unlike in humans, in mice IL-4 is the primary regulator of IgE synthesis by B cells. Therefore, although dectin-1 regulates the magnitude of some pathophysiological manifestations of a type 2 immune responses, it does not seem to modulate the propensity for sensitization to environmental allergens.

The discrepancy between the production of IL-13/IL-5 and IL-4 begs the question as to why dectin-1 specifically alters certain CD4+Th2 cytokines and not others. Likely, dectin-1 controls the production of IL-13 and IL-5 from non CD4+ Th2 cell sources. These cytokine patterns are reminiscent of ILC2s which are known to produce IL-13 and IL-5 but not IL-4. The possibility that dectin-1 suppresses the production of type 2 cytokines from non-CD4+ Th2 cells is supported by our studies in both HDM restimulated lung-cell cultures and lung CD4+ T cell populations (assessed by flow cytometry) wherein no differences in IL-13/IL-5 production or CD4+CD3+IL-13+ Th2 cell numbers were found between receptor sufficient or deficient mice.

Although we saw small increases in the recruitment of mDCs in the lungs of dectin-1 KO mice at 72 hrs after the last allergen challenge, dectin-1 expression on DCs does not appear to regulate their phenotype (i.e. allergen uptake) or subsequent activation on a per cell basis. This is concurrent with published findings that loss of dectin-1 does not alter DC phagocytic and activation potential upon exposure to LPS [382]. The lack of differences in DC
activation is consistent with the fact that the recruitment and activation of CD4+ Th2 cells did not differ between WT and dectin-1 KO mice. It is possible that the approaches we utilized to assess the in vivo CD4+Th2 cell phenotype may not have allowed us to capture changes occurring in the lungs early after allergen challenge, however, as we are able to detect differences in IL-13 and IL-5 gene expression at this time point, it is most likely that the protective effects of dectin-1 are independent of its effects on DC-CD4+Th2 cell interactions. Again, these results support, but do not prove, the hypothesis that IL-13 and IL-5 are regulated by dectin-1 through it’s effects on innate immune cell sources of these cytokines.

Consistent with the well-described role of dectin-1 in mediating Type 17 immune responses against fungal antigens, we report that dectin-1 KO mice had significantly reduced IL-17A and neutrophils in the lungs following dust-mite exposure [381]. The source of reduced IL-17A in the lungs of dectin-1 KO mice do not appear to be CD4+Th17 cells, as dust-mite did not induce significant elevations in lung Th17 cells over that observed in PBS-control mice. Therefore it is likely that dectin-1 promotes IL-17A secretion from non-CD4+ T cell sources, such as NK cells, neutrophils, ILC3s or γδ T cells. Interestingly studies indicate that dectin-1 agonists can drive IL-17A production from neutrophils, ILC3s or γδ T cells [314].

The fact that IL-17A levels were reduced in dectin-1 KO mice despite an increase in the asthmatic phenotype suggested that IL-17A and PMNs did not contribute to the heightened allergic disease phenotype in receptor deficient mice. The role of IL-17A in asthma pathogenesis has been complex with studies
showing both its ability to inhibit [194] and to augment [190] IL-13-induced allergic phenotype.

Although our studies clearly demonstrate that dectin-1 is protective, the identity/nature of the PAMP contained in dust-mite remains to be defined. Based on the studies by Underhill & colleague, we can speculate that the dectin-1 ligand in allergen extracts is in the non-soluble form as soluble ligands are not able to activate the receptor [379]. As dust-mite contains the fungal derived β-glucan, it is possible that these moieties might be playing a role in dectin-1 mediated biological functions in our model. Interestingly, studies by Mintz-Cole et al reported that based on the presence or absence of exposed β-glucan moieties on their surface, different molds did or did not mount a dectin-1 dependent Th17 immune response, respectively [414]. While the mold, C. cladosporioides that do not display β-glucan moieties on their surface induced a robust Type 2 mediated inflammatory response (CD4+IL-13+Th2 cells, eosinophils), it did not mount a Type 17 immune response. In contrast, the mold A. versicolor, decorated with exposed β-glucan moieties on their surface induced a mixed Th17/Th2. Taken together the results suggest that sensing of allergen-contained β-glucans is potentially necessary for induction of Th17, but not Th2 immune responses. In other words, non β-glucan carbohydrate moieties are able to induce Th2 immune responses. Based on these studies, it is possible that recognition of fungal-derived β-glucans contained in dust-mite through dectin-1 might be promoting the Type 17 immune response, which may in turn, inhibit the generation of Type 2
immune responses. This is consistent with previous reports of the ability of T cell subset-derived cytokines to inhibit each other.

Another possible explanation is that dectin-1 might modulate Type 2 and Type 17 immune response independently of each other through direct interaction with two different ligands contained in dust-mite. As dust-mite is a complex mixture of various allergenic moieties further studies will be required to identify the ligand for dectin-1 receptor.

In summary, we have identified a novel role for the dectin-1 receptor in regulating the major hallmarks of asthma including AHR, eosinophilia and mucus production. HDM contains multiple allergenic PAMPs that can be sensed by various PRRs modulating the course of asthma pathogenesis. It can be hypothesized that dust-mite through down-regulation of anti-allergic PRRs such as dectin-1 and upregulation of pro-allergic PRRs such as TLR4, Dectin-2 (Fig.1B) might lead to initiation of asthma in susceptible individuals. Concurrently, while dust-mite exposure significantly downregulates dectin-1 expression (Fig.1A), it upregulates dectin-2 in whole lungs (Fig.1B). Perhaps these pathways, pro-allergic and anti-allergic regulate each other. The regulation can occur at various levels i.e. inhibition of receptor expression and/or downstream signaling pathways. Taken together these results suggest that dysregulation of dectin-1-mediated suppression of type 2 immune responses may be an important determinant of susceptibility to the development of asthma.
3.4: Figures for Chapter 3

Figure 1: Dust mite exposure downregulates dectin-1 mRNA expression in the whole lungs of mice: 7 weeks old A/J mice from Jackson farms were exposed to either PBS (40µl) or HDM (100µg/40µl) intra-tracheally. Seventy-two hours following the treatment mice were sacrificed and small lung lobe was collected. Following RNA extraction and cDNA synthesis, dectin-1 gene expression was quantified (Chapter 2.8). Data represent mean dectin-1 gene expression (relative to S14) +/- SEM (n = 7 mice/group). ** and *** indicates P < 0.01, P<0.001, respectively.
Fig. 1: Dust mite exposure downregulates dectin-1 mRNA expression in the whole lungs of mice.
Figure 2: Dectin-1 suppresses the magnitude of dust-mite mediated allergic asthma: Seven weeks old WT or Clec7a−/− mice (Method 2.1) were treated with PBS or HDM as described (Chapter 2.2). Seventy-two hours following the last PBS/HDM challenge, airway function (APTI) (A), serum IgE levels (B) BAL cellularity (C,D) was determined (Chapter 2.3, 2.5, 2.4). Mucus production (E) was assessed following staining as described earlier (Chapter 2.9). Data represent means +/- SEM (n = 8 mice/group). In panel 2(A) # or * indicates, P < 0.05 compared to WT PBS and Het. (Clec7a+/-) PBS respectively, while ** indicates P < 0.01 vs. WT HDM. In panel 2D, * and # represents p <0.05 vs. WT HDM.
Fig. 2: Dectin-1 suppresses the magnitude of dust-mite mediated allergic asthma

(A) APTI (cm H2O x sec)

(B) IgE (ng/mL)

(C) Total Cells/mL BAL* 10^5

(D) Cell counts (% BAL)

(E) WT Clec7a-/-

PBS HDM
Figure 3: Dectin-1 negatively controls dust-mite induced IL-13 production:

Seven weeks old WT or Clec7a-/− mice (Chapter 2.1) were treated with PBS or HDM as described in methods (2.2). Seventy-two hours following the last PBS/HDM challenge, lung lobes were isolated for extraction RNA and quantitative PCR assessment of gene expression as described in Chapter. 2.8. Data represent means +/- SEM (n = 8 mice/group). * indicates, P < 0.05 vs. WT HDM.
Fig. 3: Dectin-1 negatively controls dust-mite induced IL-13 production:

(A) IL-13 mRNA expression

(B) IL-4 mRNA expression

(C) IL-17A mRNA expression

(D) IL-5 mRNA expression
Fig. 4: Dectin-1 promotes mDCs recruitment into the lungs: Seven weeks old WT or Clec7a-/- mice (Chapter 2.1) were treated with PBS or HDM as described (Chapter 2.2). Seventy-two hours following the last PBS/HDM challenge single lung cell suspensions (Chapter 2.6) were generated, plated and stained for flow cytometric analysis of various cell populations as described in Chapter 2.12, (lung inflammatory cell profile). Data represent means +/- SEM (n = 6 mice/group). *, **, *** indicate P < 0.05, 0.01, 0.001, respectively.
Fig. 4: Dectin-1 promotes mDCs recruitment into the lungs
Fig. 5: Loss of Dectin-1 does not alter DCs phagocytic capacity. Seven weeks old naïve WT and dectin-1 KO mice were sacrificed and bone marrow cells were isolated and cultured according to the methods described in Chapter 2.10. At the end of day 7 of culture in the presence of GM-CSF, BMDCs were harvested and plated at a concentration of $0.1 \times 10^6$ cells/250ul cRPMI in 96-well round plate. BMDCs were serum starved overnight, followed by stimulation with fluorescently labeled (Ax-405) HDM for 3hrs. At the end of 3hrs, cells were stained for flow analysis (Chapter 2.11). In panel (A), (B) and (C), data is representative of MFIs from HDM+ CD11c+CD11b+ cells.
Fig. 5 Loss of Dectin-1 does not alter DCs phagocytic capacity

(A)

HDM Content (MFI)

(B)

MHC-II MFI

(C)

CD86 MFI
Figure 6: Dectin-1 does not modulate dust-mite induced CD4+ T cell immune responses. At the end of the in-vivo experiment (Chapter 2.2), single lung cell suspensions (Method. 2.6) from WT and Clec7a-/ mice were plated for lung cell-restimulation cultures (2.6) and cell supernatants were analyzed for cytokine by ELISA (2.7). Simultaneously, cells were plated and stained for various CD4+ T cell markers and analyzed by flow cytometric analyses as described in Chapter 2.12 (T cell flow). Data represent means +/- SEM (n = 8 mice/group). * indicates P < 0.05.
Fig. 6: Dectin-1 does not modulate dust-mite induced CD4+T cell immune responses
Chapter 4: Mechanism of dectin-1 mediated protection against house dust-mite induced allergic inflammation

4.1 Introduction:

Our results (shown in Chapter 3) indicate that dectin-1 protects against dust-mite driven allergic asthma through inhibition of Type 2 immune responses. Despite, the fact that dectin-1 regulates the lung expression levels of IL-13 and IL-5 in the context of HDM exposure, on our in vitro and in vivo measurements of CD4+ T cells did not demonstrate a role for dectin-1 in regulating their recruitment or their type 2 cytokine productive capacity. Consistent with these findings, our studies showed that while dectin-1 enhanced the recruitment of mDCs into the lung, on a per cell basis, it did not alter their ability to take up antigen and their subsequent activation status. Taken together these results suggest that dectin-1 does not alter the asthmatic phenotype or type 2 cytokine production via regulation of the DC-CD4+ T cell axis. However, given the potential caveats of measuring DCs and CD4+ T cells responses in vivo, we cannot entirely rule out a role for dectin-1 in regulating type 2 immune responses through classical APC/CD4+ T cell interactions.

As numerous studies have recently shown that innate immune lymphoid cells (ILC2s) are potent sources of both IL-13, and IL-5, but not IL-4, and that they play a role in type 2 immune responses, it is possible that dectin-1 may be regulating the recruitment and/or activation of these cells in the lungs following HDM exposure.
Although the mechanisms by which dectin-1 might regulates type 2 cytokine production from non-T cell sources is not known, it has been shown that signals derived from epithelial cells, such as IL-33 and IL-25 can both recruit these cells into the tissues and stimulate type 2 cytokine production. Recent studies with HDM have shown that unlike IL-25, IL-33 is required for dust-mite mediated allergic sensitization [424]. In addition to stimulating type 2 cytokine production from ILC2s, IL-33 can also induce IL-13/IL-5 secretion from other innate immune cells such as mast cells and basophils. Likewise, IL-33 can also bind its receptor (ST2) on DCs and drive adaptive type 2 immune-mediated responses, independently of ILC2s.

IL-33 is a nuclear cytokine that is present in cells of both hematopoietic and non-hematopoietic origin. Two independent studies utilizing IL-33 reporter mice have shown that in the naïve lung the majority of the IL-33+ lung cells are non-hematopoietic (CD45-) [425]. Similarly, following allergen exposure, the majority of IL-33+ cells in the lungs express epithelial cell markers (CD45-EpCAM+) [425]. These studies suggest that under both homeostatic conditions and following allergen exposure, epithelial cells are likely the major source of IL-33. Importantly, IL-33 levels have been shown to be significantly higher in the bronchial epithelium of asthmatics as compared to the tissues of healthy controls [288].

Thus the goal of this study was to determine the cell type(s) through which dectin-1 suppresses allergen-driven type 2 cytokine synthesis. We hypothesize that dectin-1 activation by HDM suppresses IL-33 production by cells of the non-
hematopoietic compartments (i.e. airway epithelium), thereby suppressing ILC2 recruitment and/or cytokine production, which in turn, suppress the magnitude of the asthmatic response.

4.2 Results

4.2.1 Dectin-1 in vivo expression: flow cytometry

Our data suggest that dectin-1 may play multiple roles in the response to allergen challenge (i.e. Th2, Th17), through its expression on multiple cells types, In order to gain a better understanding of the cell types expressing dectin-1 and whether receptor expression in the lung was modulated after allergen exposure, we used flow cytometry to identify dectin-1+ cells from lung single cell suspensions of mice challenged with either PBS (Fig. 7A) or HDM (Fig. 7B). Consistent with the literature, in lung samples from untreated mice Dectin-1 was expressed on hematopoietic (CD45+) cells including CD11c+CD11b+ myeloid DCs, CD11c+CD11b- alveolar macrophages and CD11c-CD11b+ (likely pDCs) cells (Fig.7A). Interestingly, a small population of epithelial cells (CD45-EpCAM+), but not all epithelial cells expressed Dectin-1 (Fig. 7A). Following allergen challenge, the percentage of cells in the whole lung that were positive for dectin-1 increased for every cell type except for CD11b+ CD11c- cells (Fig. 7B). However the amount of dectin-1 expressed per cell (mean fluorescence intensity, MFI) differs depending on the specific cell types examined (Fig. 7B). While dectin-1 MFI increased significantly (2921→5229) on CD11c+CD11b+ myeloid DCs, it is significantly decreased (25,609→15, 664) on epithelial cells
following dust-mite exposure (Fig. 7B). Dectin-1 MFI was not drastically modulated after allergen exposure in CD11c+CD11b- (2615→3519) and CD11c-CD11b+ (1022→1351) cells (Fig. 7A & 7B). Therefore, allergen exposure differentially regulates the expression of dectin-1 on the hematopoietic (mDCs) and non-hematopoietic cell (epithelial cells) compartments.

4.2.3 Dectin-1 on lung structural cells and not hematopoietic cells confers protection against dust-mite driven AHR.

In order to directly dissect the contribution of hematopoietic (DCs) and non-hematopoietic (epithelial cells) cell compartments to disease pathogenesis, we conducted bone-marrow chimera studies with WT and dectin-1 KO mice. Specifically, WT and dectin-1 KO mice were irradiated and reconstituted with BM from either WT or dectin-1 KO mice (as described in Chapter 2.18). Therefore, at the end of successful BM reconstitution, expression of Dectin-1 should be as shown in Table 3.
### TABLE 3. Chimerism of WT and dectin-1 deficient Mice

<table>
<thead>
<tr>
<th>DONOR</th>
<th>RECIPIENT</th>
<th>Dectin-1 expression on CD45+ hematopoietic cells</th>
<th>Dectin-1 expression on CD45- cells non-hematopoietic cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>WT</td>
<td>+</td>
<td>+</td>
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<tr>
<td>WT</td>
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After 5 months, the degree of chimerism was evaluated. Our results indicate that approximately 97% chimerism was achieved upon the transfer of either WT BM into a KO host or a KO BM into a WT host (Fig. 8). Therefore, the loss of dectin-1 in either donor bone-marrow hematopoietic stem cells or host stromal cells does not affect the degree of chimerism. Based on these results we started HDM sensitization and challenge of the mice according to the protocol described in Chapter 2.2.

Similar to our previous findings (Fig. 2A) wherein KO mice have higher AHR than WT mice, KO mice reconstituted with KO BM had higher AHR than WT mice given WT BM following HDM exposure (Fig. 9A). **Surprisingly, KO mice that received WT BM had exacerbated AHR as compared to WT mice**
receiving WT BM. Moreover, they (KO mice receiving WT BM) had significantly higher AHR than KO mice receiving WT BM (Fig. 9A). These results suggest that the loss of Dectin-1 on non-hematopoietic lung structural cells is sufficient to lose protection against dust-mite driven AHR. Therefore, Dectin-1 on lung-structural cells is protective against dust-mite driven asthmatic immune responses. In contrast, the data is suggestive that Dectin-1 expressed on hematopoietic cells (presumably DCs) promotes allergen-driven AHR as WT mice receiving Dectin-1 deficient BM had lower AHR compared to WT mice receiving Dectin-1 sufficient (WT) BM (Fig. 9A).

As seen before (Fig. 2C), loss of Dectin-1 mediated protection against AHR does not correlate with total BAL cell counts (Fig. 9B). While mice in all experimental groups had similar BAL cell (macrophage and epithelial cell) composition at baseline (PBS, Fig. 9C), there were differences after dust-mite exposure (Fig. 9D & Fig. 9E). Specifically, KO mice receiving KO BM and KO mice receiving WT BM had significantly higher proportion of eosinophils in the BAL. WT mice receiving KO BM did not have significantly higher eosinophils as compared to WT mice receiving WT BM. Therefore our data indicate that Dectin-1 on lung-structural cells and not hematopoietic cells inhibits dust-mite induced eosinophilia (Fig. 9D & Fig. 9D).

As far as neutrophils are concerned, our data show that the loss of Dectin-1 on both compartments in the lungs (i.e. CD45+ and CD45-) leads to reduced neutrophilia (Fig. 9E). However, Dectin-1 on either compartment is sufficient to promote neutrophils as WT mice given WT BM has equivalent percentages of
neutrophils in BAL as WT mice receiving KO or KO mice receiving WT BM (Fig. 9E). The data are suggestive that dectin-1 on both lung compartments, hematopoietic and non-hematopoietic, promotes allergen-induced neutrophilia.

Further, analysis of histological sections reveals that severe HDM-induced mucus production and plugging of the airway lumen is seen only in the KO mice receiving KO or WT BM (Fig. 9F). Interestingly, PAS staining is reduced upon transfer of dectin-1 deficient BM into a dectin-1 sufficient host as compared to all other groups (Fig. 9F). This suggests that dectin-1 signaling on hematopoietic compartment/cells promotes mucus production following dust-mite exposure. Taken together these results suggest that the loss of dectin-1 on lung structural cells and not hematopoietic cells results in eosinophilia (Fig. 9D), severe obstruction of the airways via mucus plugging (Fig. 9F) and exaggerated AHR (Fig. 9A).

To understand the contribution of various cell types expressing dectin-1 on adaptive CD4+ T cell immune responses, we investigated dust-mite induced Th2 cell recruitment and phenotype in chimeric mice. As before (Fig. 6C and 6D), HDM-induced Th2 cells were equivalent in WT mice given WT BM and KO mice given KO BM (Fig. 9G). Interestingly, dectin-1 expression on hematopoietic cells (WT BM given to KO host) increases the numbers of lung CD4+ T cells, although not significantly. These data suggest the loss of dectin-1 may promote the recruitment of Th2 cells (WT BM given to KO host vs. WT BM given to WT host, Fig. 9G). However, dectin-1 expressed on lung-structural cells might be opposing Th2 cells differentiation as Th2 cells are low when dectin-1 is only
expressed on epithelial cells [WT BM into KO host vs. KO BM into WT host, (Fig. 9G)].

In summary, protection against dust-mite driven AHR, mucus production, and lung eosinophilia is mediated through lung structural cells expressing dectin-1. In contrast, dectin-1 expression on hematopoietic cells seems to be promoting dust-mite driven Th2 inflammation. However, when dectin-1 is expressed on both compartments, dectin-1 mediated protection against allergic asthma dominates over the contributions from the non-hematopoietic compartment. However, as the number of mice surviving in these experiments is low (KO BM: KO host), this experiment will need repeating in future.

4.2.4 Dectin-1 is a critical regulator of dust-mite driven IL-33 production and ILC2 recruitment into the lungs.

As our data suggest that dectin-1 expression by lung structural cells and not hematopoietic cells is essential to confer protection against dust-mite driven type-2 immune responses (Fig.9), we focused our attention on defining the potential mechanisms by which dectin-1 on cells of the non-hematopoietic compartment, presumably epithelial cells may regulate type 2 immune responses in the lung.

To study the effect of dectin-1 on dust-mite induced IL-33 responses, we utilized 16HBE, a human bronchial epithelial cell line (Fig.10A). Cells were transfected with Dectin-1 or control plasmid before stimulation with HDM (see Chapter 2.16). In this cell line, HDM induces IL-33 production, however as
compared to cells transfected with the vector control, cells overexpressing Dectin-1 had significantly lower IL-33 production (Fig.10A). Therefore, Dectin-1 strongly inhibits dust-mite induced IL-33 production in epithelial cells. Moreover, dectin-1 specifically inhibits dust-mite driven IL-33, as IL-6 production was not modulated by overexpression of the receptor (Fig.10B) in epithelial cells.

Based on our in vitro studies, we investigated whether dust-mite driven IL-33 is also inhibited in vivo by dectin-1. As we don’t detect significant increases in the levels of secreted IL-33 (in BAL) or mRNA expression over PBS challenged mice at the end of the in vivo experiment (data not shown), we investigated dust-mite modulation of these cytokines at earlier time-points. Four hours following a single i.t. instillation of HDM, WT and dectin-1 KO mice were lavaged and IL-33 was measured in the BAL fluid by ELISA (Fig.10C). WT C57BL/6 mice do not mount a strong IL-33 response following dust-mite exposure as depicted by comparable IL-33 levels in PBS- and HDM-challenged WT mice (Fig.10C). However, upon loss of Dectin-1, there is a significant elevation of IL-33 in the BAL fluids (Fig.10C). Likewise, only upon loss of dectin-1 a surge in dust-mite driven IL-13 is seen in the BAL (Fig. 10D)

IL-33 is best known to recruit ILC2s and thus amplifying Type 2 immune responses in the lungs. Based on our in vitro (Fig. 10A) and in vivo data (Fig.10C & 10D), we hypothesized that Dectin-1 deficient mice will have a greater number of ILC2s following dust-mite exposure as compared to WT mice. Indeed, our ILC2 data (Fig.10E) mirrors the pattern of IL-33 (Fig. 10C) in vivo. WT mice do not mount a strong ILC2 (Lin-CD45+ST-2+IL-13+) response following
single exposure to dust-mite. However, upon loss of the protective dectin-1 receptor, significantly higher numbers of ILCs are recruited into the lungs following dust-mite exposure. These results strongly suggest that Dectin-1 is a critical negative regulator of IL-33 production from epithelial cells. C57BL/6 mice, which have a functional dectin-1 receptor, do not mount a strong ILC2 response upon dust-mite exposure. However, upon loosing dectin-1, IL-33 production, ILC2 recruitment and IL-13 production in the lungs is de-repressed and these are detectable in C57BL/6 mice. Taken together these results suggest that dectin-1 is a potent repressor of type 2 immunity through providing tonic suppression of epithelial production of IL-33 and IL-13-producing ILC2 recruitment into the lungs.

4.2.5 Dust-mite driven AHR exacerbation in Dectin-1 deficient mice is IL-33 dependent

In light of our in vitro (Fig. 10A) and in vivo data (Fig.10C and 10D) wherein dectin-1 strongly inhibits dust-mite induced IL-33, we next sought to determine whether blocking IL-33 signaling by inhibition of the IL-33R (ST-2), utilizing an antibody to ST2, would prevent the enhancement of the asthmatic phenotype observed in the dectin-1 receptor-deficient mice. Indeed, blocking the IL-33R (ST-2) in HDM-challenged dectin-1 KO mice prevented the enhancement of dust-mite AHR observed in dectin-1 KO treated with an isotype control matched antibody (Fig.11A). Interestingly, ST-2 blockade had no effect on allergen-driven AHR in C57BL/6 WT mice (Fig. 10A). This finding is
not surprising given the fact that we have not detected significant increases in dust-mite induced IL-33 levels over their PBS-challenged controls in WT mice (Fig. 10C). Only in the absence of dectin-1, are the C57BL/6 mice able to mount a strong dust-mite driven IL-33 response (Fig. 10C). The data is supportive of the hypothesis that Dectin-1 is a critical negative regulator of IL-33 driven AHR exacerbations following dust-mite exposure.

As we had noted before, no differences were seen in the total BAL cell counts (Fig. 11C) or total IgE production (Fig. 11B) after blockade with anti-ST2 in WT and Dectin-1 KO mice. Although neutrophils were lower in Dectin-1 KO mice than WT mice in HDM + Isotype Ab group, we did not see differences in eosinophil counts (Fig. 10D). In regards to eosinophils, we did not observe differences in eosinophil counts between WT and dectin-1 KO mice in either isotype treated or ST-2 blockade groups (Fig. 10D). Compared to our previous experiments (Fig. 2D), wherein following dust-mite exposure, we observe around 55% eosinophils in the WT and around 75% in the dectin-1 KO, in this particular experiment we see marked increases in their numbers (WT: around 80%). Therefore, it is likely that eosinophil responses are maxed out due to which we cannot see differences in between the groups. Experiments would have to be repeated again in future with more mice in each group. Nonetheless, our data suggests that dectin-1 by virtue of controlling IL-33 from epithelial cells protects against dust-mite driven asthma exacerbations.
4.3 Discussion

As we previously shown (Chapter 3) that dectin-1 regulated the asthmatic phenotype independently of classical APC-T cell interactions, we sought to understand through which cells dectin-1 mediates its protective effects. To this end, we evaluated the receptor expression in hematopoietic and non-hematopoietic compartments of the lungs. In support of the hypothesis, we observed that consistent with the differential regulation of the receptor on both lung compartments, we demonstrate that dectin-1's protective role is predominantly mediated through its expression on lung structural cells. Moreover, dectin-1 on cells of hematopoietic compartments likely promotes allergic disease phenotype. In support of the pivotal role for dectin-1 on non-hematopoietic cells, we show that dectin-1 inhibits the production of pro-Type 2 cytokine, IL-33 from epithelial cells. In vivo IL-33R blockade experiments supported the notion that dectin-1 mediates it protective effects through inhibition of lung IL-33 production. In turn, our data suggest that IL-33 then recruits ILC2s that produce type 2 cytokines and regulate the exaggerated response to HDM in dectin-1 KO mice.

In dectin-1 KO bone marrow chimera studies, we identified two distinct roles for the receptor in the regulation of allergic inflammation. On the one hand, the enhanced allergic response was only observed when dectin-1 was absent from the structural compartment or both the hematopoietic and non-hematopoietic compartments. These results suggest that the dominant effect of dectin-1 in controlling responses to HDM is due to its expression on lung resident
cells. Conversely, when dectin-1 is absent only on the hematopoietic cells the magnitude of the allergic phenotype is reduced. Therefore, depending on which cell-type dectin-1 is expressed on, it seems to differentially regulate the propensity of mounting a Type 2 driven asthmatic immune response.

Our earlier studies (Chapter 3) suggested that dectin-1 does not mediate its protective effect though modulation of the adaptive CD4+Th2 cells response. However our new findings with the bone-marrow chimera study have highlighted a role of dectin-1 in regulating the adaptive CD4+Th2 cell response. Based on the cell type it is expressed on, dectin-1 can either inhibit or promote a Th2 immune response. While dectin-1 expression on the non-hematopoietic compartment inhibits, its expression on the hematopoietic compartment likely amplifies dust-mite Th2 cell recruitment/differentiation/function. Upon loss of dectin-1 on both compartments, these opposing effects of dectin-1 cancel each other out and hence there are no differences in CD4+ Th2 cells in the lung (Fig.9G). Nonetheless, even when there are no increases in CD4+Th2 cells in the KO/KO mice as compared to WT/WT mice, we still observed exacerbated asthmatic phenotype in KO/KO mice mice. This is consistent with our previous findings (Fig.6) in which we depicted that the exacerbated Type 2 immune responses in dectin-1 KO mice following dust-mite exposure were not likely explained by changes in CD4+Th2 cell population. Nevertheless, dectin-1 deficient mice as compared to receptor sufficient mice display the hallmark symptoms associated with excessive Type 2 cytokine (IL-13, IL-5) responses, AHR, mucus production and eosinophilia. Given the adaptive T cell immune
responses are not different between WT and dectin-1 KO mice, we speculated that dectin-1 controls an innate source of IL-13.

The BM chimera experiments suggested that the protective effects of dectin-1 are indeed conferred through its effects on non-hematopoietic cells. As IL-33, which is released from epithelial cells is a pivotal inducer of type-2 immune responses, we focused on defining the role of dectin-1 on this axis. Interestingly, our data also indicates that C57BL/6 mice do not mount a strong IL-33-ILC2s-IL-13 (non-CD4+ Th2 cells) response following dust mite exposure. Only upon losing the inhibition conferred through dectin-1, a surge in IL-33 production occurs and hence IL-13+ ILC2s are observed in C57BL/6 mice. Concurrently, dampening the exacerbated IL-33 biological activities through IL-33R blockade was effective in inhibiting the AHR exacerbations in only dectin-1 KO mice and not WT mice.

Our data that dust-mite did not induce IL-33 in C57BL/6 mice is in agreement with few studies published recently [426]. However, in our hands (data not shown), BALB/c mice mount a significant IL-33 response following dust-mite exposure. Interestingly, genetically different strains of mice have been reported to have differential susceptibility to various fungal infectious models depending on the isoform of Dectin-1 expressed on immune cells. In humans and mice, alternative gene splicing results in multiple isoforms of Dectin-1. Among all of the isoforms (8), only the full-length isoform A and the isoform B (short-stalk) have functional ligand-recognition domains. Studies suggest that compared to C57BL/6 mice that primarily express isoform B, DBA/2 and BALB/c mice that
primarily express the full-length isoform (A) of Dectin-1 mounts a strong macrophage mediated anti-fungal Th17 immune response [427] [428]. The susceptibility is not attributed to the differences in the recognition of the ligand as studies have reported that there are no differences in the capacity of the two isoforms to bind the ligand, β-glucan. **Taken together these results suggest that compared to the isoform B, isoform A seems to be a stronger driver of Th1 and Th17 immune responses and hence BALB/c mice are able to while C57BL/6 mice are not able to mount a strong Dectin-1 mediated Type 17 response.** However, the role of different isoforms of the receptor is not evaluated on Type 2 immune responses. Interestingly, the dectin-1 overexpression vector used in our *in vitro* study (Fig. 10A) corresponds to the isoform B indicating that isoform B inhibits IL-33 production from epithelial cells. Based on our findings it can be speculated that isoform B (in C57BL/6) inhibits dust-mite driven IL-33 and hence an ILC2-driven type 2 cytokine response. Compared to C57BL/6 mice, BALB/c mice mount a much stronger dust-mite driven asthmatic immune response. Hence it is possible that differential isoform expression of Dectin-1 controls the susceptibility to mount a type 2-mediated asthmatic immune response in genetically divergent strains and humans.

Overall, our data suggests that dust-mite allergen is able to mount an exacerbated Type-2 driven asthmatic immune responses by circumventing the protective effects of dectin-1. Through selective repression of dectin-1 receptor on lung-structural cells, HDM moieties might be able to induce epithelial cell secretion of pro-Type 2 cytokine IL-33. In turn, IL-33 may drive an ILC2 mediated
maladaptive immune responses culminating in exacerbated disease. While certain components of allergens may promote sensitization via activation of selective PRRs, other PAMP-PRRs interaction downstream of allergen exposure may in turn repress the asthmatic immune response. These opposing pathways might maintain a fine balance and hence homeostasis in healthy individuals. However, dys-regulation of dectin-1 mediated protective effects might result in a propensity to mount an aberrant asthmatic immune response in susceptible individual following exposure to the allergen.
4.4: Figures for Chapter 4

**Fig. 7A: Dectin-1 is expressed on both hematopoietic cells and non-hematopoietic cells at baseline:** Seven weeks old C57BL/6 mice (n=3) breed in JHSPH facility were sensitized and challenged with PBS (as described, Chapter 2.2). At the end of the *in vivo* experiment (Chapter 2.2), single lung cell suspensions (Chapter 2.6) from mice were plated and stained for dectin-1 expression on various cell populations as described in (Chapter 2.12, dectin-1 expression). Data is represented as cells expressing dectin-1 as a percentage of parent (%P), total cells (%T) and dectin-1 MFI.

**Fig. 7B: Dust-mite exposure differentially modulates dectin-1 surface expression on cells of hematopoietic (CD45+) vs. non-hematopoietic origin (CD45-):** 7-8 week old C57BL/6 mice (n=3) breed in JHSPH facility were sensitized and challenged with HDM (as described, Chapter 2.2). At the end of the *in-vivo* experiment (method 2.2), single lung cell suspension (Chapter 2.6) from mice were plated and stained for dectin-1 expression on various cell populations as described in (Chapter 2.12, dectin-1 expression). Data is represented as cells expressing dectin-1 as a percentage of parent (%P), total cells (%T) and dectin-1 MFI.
Fig. 7A Dectin-1 is expressed on both hematopoietic cells and non-hematopoietic cells at baseline.
Fig. 7B: Dust-mite exposure differentially modulates dectin-1 surface expression on cells of hematopoietic vs. non-hematopoietic origin

Dectin-1 expression: HDM

<table>
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**Fig. 8: Dectin-1 deficiencies in either the donor or recipient do not modulate HSC capacity to engraft successfully:** B6.SJL-Ptprc\(^a\) Pepc\(^b\)/BoyJ (WT, CD45.1) or dectin-1 KO mice (CD45.2) were irradiated and supplemented with fresh bone-marrow (2 × 10\(^6\) cells/100 ul cRPMI) isolated from non-irradiated B6.SJL-Ptprc\(^a\) Pepc\(^b\)/BoyJ (CD45.1) or dectin-1 KO mice (CD45.2), as described (Chapter 2.18). At the end of 5 months the expression of CD45.1 and CD45.2 isoforms in the lungs was evaluated following staining with fluorescently labeled antibodies for these isoforms. Data is representative of n=2 mice each group.
Fig. 8 Dectin-1 deficiencies in either the donor or recipient do not modulate HSC capacity to engraft successfully
Fig. 9: Dectin-1 on lung structural cells and not hematopoietic cells is sufficient to confer protection against dust-mite induced allergic asthma:

Five weeks old WT or dectin-1 KO mice were irradiated at a dose of 10 Gray(Gy) followed by supplementation with fresh bone-marrow cells (2 \times 10^6 cells/100 ul cRPMI) isolated from non-irradiated WT and Dectin-1 KO (Chapter 2.18). Five months following the BM transfer, mice were sensitized and challenged with either PBS or HDM (Chapter: 2.2). Seventy-two hours following the last PBS/HDM challenge, airway function (APTI) (A), serum IgE levels (B) and BAL cellularity (Fig.9C, PBS challenged mice, Fig.9D & 9E) was determined (Method 2.3, 2.5, 2.4). Mucus production (F) was assessed following staining as described earlier (Method 2.9). Single-lung cell suspension (Chapter 2.6) from these mice were plated and stained with antibodies to determine to evaluate Th2 cells (Chapter 2.14). Data represent means +/- SEM (n = 4 mice/group). * indicates, P < 0.05
Fig. 9: Dectin-1 on lung structural cells and not hematopoietic cells is sufficient to confer protection against dust-mite induced allergic asthma.
Fig. 10 Dectin-1 is a critical negative regulator of dust-mite driven IL-33 production and ILC2s recruitment in the lungs:

Panel 10(A) & Panel 10(B): 16HBE cells were transfected with either control (pcDNA3.1) or dectin-1 expression vector (Chapter 2.16). Following day cells were serum starved overnight, followed by stimulation with either media or HDM (100ug/ml). Twenty-four hrs. later culture supernatants were harvested followed by quantification of cytokines, IL-33 and IL-6 (Chapter 2.17). Data represent means +/- SEM (n = 4). * indicates, P < 0.001 vs. Media pCDNA3.1, # indicates P < 0.01 vs. HDM pCDNA 3.1, ** indicates P < 0.01 vs. pCDNA 3.1 Media.

Panel 10(C) & 10(D): Eight weeks old WT and dectin-1 KO mice were exposed to either single challenge of PBS (40ul) or HDM (100ug/40ul). Four hrs. following the challenge, mice were lavaged (Chapter 2.4) and BAL cytokines (IL-33. IL-13) were measured by ELISA (Chapter 2.7). Data represent means +/- SEM (n = 6 mice/group). * indicates P < 0.001, # indicates P < 0.01

Panel 10(E): Eight weeks old WT and dectin-1 KO mice were exposed to either single challenge of PBS (40ul) or HDM (100ug/40ul). Twenty four following single challenge with PBS (40ul) or HDM (100ug/40ul) mice were sacrificed and single lung cell suspensions were stained with fluorescently labeled antibodies for detection of ILC2s (Chapter 2.15). Data represent means +/- SEM (n = 5 mice/group). & indicates, P < 0.05
Fig. 10 Dectin-1 is a critical negative regulator of dust-mite driven IL-33 production and ILC2s recruitment in the lungs
Fig. 11: Blockade of IL-33 receptor, ST-2 is sufficient to reduce dust-mite driven asthma exacerbation in Dectin-1 receptor deficient mice: Seven weeks old mice were sensitized and challenged with either PBS or HDM (Chapter 2.2). Prior to each sensitization and challenge WT and dectin-1 KO mice were treated with either isotype control Ab. or anti-ST2 Ab. (250ug/ml) as described(Chapter 2.19). Seventy-two hours following the last PBS/HDM challenge, airway function (APTI) (A), serum IgE levels (B) and BAL cellularity (C,D) was determined (Method 2.3, 2.4, 2.5). Data represent means +/- SEM (n = 4 mice/group). * indicates, P < 0.05 vs. WT (HDM+Isotype)
Fig. 11: Blockade of IL-33 receptor, ST-2 is sufficient to reduce dust-mite driven asthma exacerbation in Dectin-1 receptor deficient mice
Chapter 5: Major conclusions, discussion and future research

5.1 Introductory remarks:

Asthma is a debilitating inflammatory disease of the lungs affecting approximately 300 million people of all ages, gender and ethnicities worldwide [429]. The disease is a major public health problem, posing a significant burden on the healthcare infrastructure [430]. It is estimated that in 2008, around $18 billion in medical expenditures were attributed to asthma in the US [430]. Clinically, corticosteroids (broad immunosuppressant) and β-agonists (bronchodilators) are used in combination to control exacerbations in mild to moderate asthmatics [431]. However, severe asthmatics exhibit hypo-responsiveness to inhaled corticosteroids and as such they have a high morbidity and mortality rate [432]. The costs of care for these patients, constitutes the majority of the direct medical costs of asthma. As the current asthma therapies only offer temporary control of disease symptoms /exacerbations, there is a great need for a better understanding of mechanisms of disease initiation (genetics and environmental) in order to design therapies aimed at disease modification [433]. However, our knowledge of the sequence of events leading to the initiation of disease is limited.

5.2 Dectin-1 on lung structural cells is a critical negative regulator of dust-mite induced allergic asthma
In the current work, we sought to determine the role of dectin-1, a CLR in dust-mite mediated allergic asthma pathogenesis. We demonstrate that activation of the CLR, dectin-1, normally serves to dampen the development of asthmatic symptoms (i.e. AHR, eosinophilia, goblet cell hyperplasia) in response to the common allergen dust-mite (Chapter 3, Fig.2). In dissecting the underlying mechanisms of dectin-1’s protective effects, we report that dectin-1 on lung structural cells and not hematopoietic cells is sufficient to mediate protection against dust-mite driven asthma exacerbation (Chapter 4, Fig.10). Moreover, we demonstrate for the first time that dectin-1 regulates the initiation of allergen-driven immune responses via inhibition of IL-33 from the epithelium (Chapter 4, Fig 10), not through its effects on traditional APC-CD4+ T cell interactions (Chapter3, Fig.5 & 6). Further we report that dectin-1 by controlling dust-mite driven IL-33 production from epithelial cells inhibits the recruitment of ILC2s into the lungs (Chapter 4, Fig.10E). ILC2s, are a potent source of IL-13 and IL-5, which have been shown to be sufficient in inducing AHR, mucus production and eosinophilia in the absence of T cells [139]. Hence, dectin-1 on epithelial cells by virtue of controlling dust-mite driven ILC2s recruitment into the lungs likely inhibits IL-13 and IL-5 driven asthma pathogenesis (Chapter 4, Fig.13 & Fig.14). Taken together these results suggest that dectin-1 is a crucial PRR, mediating protection against dust-mite driven allergic asthma.

Although our work has provided substantial new insights into the mechanisms regulating type 2 immune responses, several questions remain regarding the role of dectin-1 in allergic asthma including: 1) Possible
mechanisms of dectin-1 inhibition of the IL-33-ILC2 axis; 2) identification of HDM-contained ligand(s) for dectin-1; and 3) the role of dectin-1 in human disease.

5.3 Possible mechanisms of dectin-1 inhibition of the IL-33-ILC2 axis

IL-33 is an archetypical alarmin that is secreted by stressed cells. Specific allergens (ragweed, *Alternaria alternata*) or agonists (ATP) are reported to induce IL-33 secretion from cells. Studies by Herbert & colleagues recently showed that Trefoil factor family peptides 2 (Tff2), a critical molecule of barrier function and repair function, drives IL-33 production that is pivotal in mediating Type 2 immunity to clear hookworm infection [434]. Dectin-1 likely regulates IL-33 secretion independent of Tff2 as in the whole lungs of WT and receptor deficient mice Tff2 expression seems to be comparable (data not shown). Nonetheless, allergen exposure can also result in apoptosis of airway epithelial cells causing the release of alarmins such as IL-33. Dust-mite has various protease allergens that have been reported to increase epithelial cell-permeability and cell death [435]. Recent studies have linked defective apoptotic epithelial cell clearance by adjacent healthy epithelial cells in inducing the secretion of the alarmin, IL-33 following allergen exposure. Elegant studies by *Juncadella et al* reported that following dust-mite exposure efficient clearance of apoptotic epithelial cells (AC) by healthy epithelial cells results in Rac 1 (a Rho GTPase) dependent secretion of anti-inflammatory mediators (IL-10, TGFβ, PGE2) [426]. However, specific depletion of Rac1 in airway epithelial cells leads to defective AC phagocytosis and clearance concomitant with profound IL-33 release and Type 2 rich allergic
inflammation. Surprisingly, in their model dust-mite exposure does not significantly induce IL-33 in BAL. Only upon the loss of Rac1 in the airway epithelial cells alone, is an increase in IL-33 is observed. This is reminiscent of the IL-33 (BAL) data in our model (Fig. 10C) wherein only upon the loss of dectin-1 are significant amounts of IL-33 detectable following dust-mite exposure. Interestingly dectin-1 has been reported to utilize the Rac1 GTPase for effective phagocytosis of zymosan in macrophages [436]. It can be hypothesized that following dust-mite exposure, dectin-1 utilization of Rac1 GTPase might be essential in effective clearance of apoptotic airway epithelial cells. Based on our data and that from the literature, it can be hypothesized that in the absence of dectin-1, the apoptotic cell clearance is defective leading to the release of IL-33 that initiates ILC2s driven allergic asthma. In the future, we can test this hypothesis by comparing the uptake of labeled apoptotic cells by epithelial cell lines (16HBE, BEAS2B, MLE-12) and IL-33 release (ELISA) in cells in which we overexpress dectin-1 and treat with or without the Rac1 inhibitor (commercially available). If the hypothesis is correct then, cells overexpressing dectin-1 should have heightened apoptotic cell uptake concurrent with low IL-33 response as compared to controls. However, in the presence of Rac1 inhibitor the apoptotic cell clearance and IL-33 inhibition due to dectin-1 overexpression should diminish.

Although IL-33 is known to be important in allergic asthma, little is known regarding IL-33 gene regulation, specifically in epithelial cells. A recent study by Vogel & colleagues indicates that various TLR agonists, poly I: C (TLR3), LPS
(TLR4) can induce IL-33 transcription in peritoneal macrophages, while P3C (TLR2) and CpGDNA (TLR9) do not [437]. Dissecting the molecular pathways, they reported that Il33 mRNA induction downstream to both TLR and non-TLR agonists is dependent on two transcription factors, IFN regulatory factor 3 (IRF3) and CREB. Studies in the past have indicated that dectin-1 synergizes with various TLR agonists (TLR2, TLR4) in the induction of TNFa from human-monocyte derived macrophages [438]. However Dalpke et al recently reported that dectin-1 activation leads to suppression of signaling downstream of TLRs [439]. Activation of dectin-1 following zymosan exposure leads to induction of suppressor of cytokine signaling (SOCS) protein 1 in macrophages. The authors reported that although activated SOCS1 does not act as a negative regulator for dectin-1 signaling, it inhibits TLR driven cytokine (IL-12p40) secretion. Studies from our lab (unpublished, not shown) indicate that dust-mite driven IL-33 secretion from epithelial cells (cell line) is dependent on TLR-4. Based on our data (Chapter 4, Fig.10), that dectin-1 strongly inhibits dust-mite driven IL-33 production, it will be interesting to investigate whether dectin-1 dampens TLR4 driven IL-33 production. The inhibition by dectin-1 can happen at the receptor level (down-regulation of TLR4) or downstream to receptor activation i.e. inhibition of signaling (through SOCS1). In order to investigate the potential role of TLR4-dectin-1 pathway in induction of IL-33 downstream of dust mite exposure, we can utilize overexpression studies in vitro. Cell lines that do not express these receptors (ex: Chinese hamster ovary cell, CHO) can be transfected with TLR4 and Dectin-1 overexpression vector alone or in
combination, and the effects on the IL-33 promoter (luciferase assay) can be evaluated. Further we can test the contribution of the TLR4 pathway in dectin-1 mediated IL-33 responses *in vivo* by blocking the TLR4 pathway selectively (utilizing R-LPS) in WT and dectin-1 KO mice prior to HDM challenge. If TLR4 pathway activation downstream of dust-mite is un-checked in dectin-1 KO mice, TLR4 inhibition will reduce IL-33 exacerbations in receptor-deficient mice.

To prove that heightened Type 2 immune responses in dectin-1 KO mice (compared to WT) following dust-mite exposure is directly driven by ILC2s specifically and not adaptive CD4+Th2 cells, we can breed dectin-1 KO mice to Rag1-/- mice (lack B cells). We would then treat these double knock out mice (dectin-1/-/rag1-/-, double knock out, DKO) with either isotype or an ILC2-depleting antibody (anti-CD90.2) and evaluate their asthmatic phenotype. We will expect to see heightened type 2 immune responses in DKO mice (compared to control mice), however ILC2 depletion should inhibit these responses (compared to isotype control). If this is not observed then most likely dectin-1 controls other innate source of IL-13 (NK, mast cells) that might be mediating exacerbated disease as seen in receptor deficient mice compared to controls.

5.4 Potential ligand(s) for dectin-1 in dust-mite extract

As sensing of PAMPs through PRRs is crucial in activating them, we hypothesized that there are specific carbohydrate ligand(s) for dectin-1 in the dust-mite allergen extract. Moreover, recent studies have reported that dectin-1 has an endogenous ligand, vimentin (an intermediate filament) [440]. The
authors reported that Vimentin is exposed at sites of arterial wall inflammation and promotes super-oxide anion production through dectin-1 activation on macrophages, contributing to the oxidation of lipids and accumulation of cholesterol in atherosclerotic lesions [440]. Hence, to rule out any role of endogenous ligands or any unforeseen developmental issues with receptor–deficient mice, we generated dectin-1 ligand-depleted HDM and tested its effect on the asthmatic phenotype in receptor sufficient WT mice. We hypothesized that removal of the ligand from dust-mite extract should exacerbate allergen-driven AHR in receptor-sufficient mice.

In order to deplete the potential ligand from the dust-mite extract, we incubated recombinant dectin-1 receptor with the allergen and pulled out the receptor-ligand complex [as described in the Chapter (2.17)]. We utilized the remaining extract referred to as dectin-1 ligand depleted HDM (dHDM), to investigate the dust-mite induced allergic asthma phenotype in WT C57BL/6 mice. Interestingly, similar to the responses in dectin-1 deficient mice (Chapter 3, Fig. 2), mice sensitized and challenged with dHDM had significantly greater airway responses to cholinergic stimulation compared to those treated with control HDM (Fig. 12A). As seen before (Fig. 2B) with receptor–deficient mice, sensing of the dectin-1 ligand in dust-mite does not effect total IgE production (Fig. 12B). Although the number of total cells in the BAL was not different in the two types of HDM-treatment groups (Fig. 12C), there were differences in the cellular composition of the BAL. Eosinophils were significantly higher and neutrophils although not significantly different (p=0.08) were lower in the BAL of
mice treated with dHDM than mice exposed to control HDM (Fig. 12D). Analysis of lung sections revealed that control HDM induces mucus production, however it is significantly more pronounced in the dHDM-treated group (Fig.12E). Thus loss of the dectin-1 ligand in dust-mite leads to excessive mucus production and hence clogging of the airway lumen (Fig. 12E). In summary, depletion of the dust-mite ligand for dectin-1 leads to exaggerated AHR (Fig. 12A), BAL eosinophilia (Fig. 12D) and mucus metaplasia (Fig. 12E). Consistent with our previous finding that dectin-1 inhibits IL-33 production (Fig. 10), we show that WT C57BL/6 mice treated with dectin-1 ligand-depleted HDM had significantly more IL-33 in the BAL as compared to those treated with control HDM (Fig. 12F). Once again, the level of IL-33 after control-dust mite exposure was not significantly higher than that in the PBS controls (Fig. 10C, Fig.12F). Hence, a component of HDM is a specific ligand for dectin-1, recognition of which through the receptor confers protection against allergic asthma. Through multiple approaches (Fig.2 & Fig.12) we report that dectin-1 serves as a critical negative regulator of dust-mite induced IL-33 response, loss of which in receptor deficient mice leads to exacerbated asthmatic immune response as compared to controls. However, the specific identify of the dectin-1 ligand remains to be determined. In order to assess the functionality of the ligand, we will have to elute it off the dectin-1:Fc complex used to deplete the HDM. This can be achieved by using a competitive inhibitor for dectin-1 receptor, for ex. laminarin (biotinylated). At an ample concentration of the inhibitor, the ligand of interest might dissociate from the receptor. Following which, biotinylated laminarin can be removed using
streptavidin coated magnetic beads. The remaining extract will likely contain the potential ligand, whose functionality can be tested in in vitro cell cultures (inhibition of IL-33 response) or in-vivo (allergic phenotype).

Although we do not know the identity of the dectin-1 ligand in HDM, based on the literature there are several potential candidates. Dust-mite extract contains multiple allergenic proteins, the major ones being Der p 1, Der p 2 etc. against which the majority of allergic individuals are sensitized. Glycosylation patterns of both Der p 1 and Der p 2 have been reported in mediating their uptake through recognition of CLRs like mannose-receptor and DC-SIGN on dendritic cells [417]. Interestingly, while the native form of Der p 1 could induce TSLP secretion from the epithelial cell line (BEAS-2Bs), its deglycosylated form could not [417]. Therefore, sensing of glycosylation moieties on Der p 1 is crucial for inducing pro-Type 2 cytokine (TSLP) from epithelial cells. While in our hands, HDM (containing Der p 1) does not seem to modulate TSLP or IL-25 (in vitro & in vivo), recognition of Der p 1 through a CLR, might regulate IL-33 production from epithelial cells. Given, our data that dectin-1 is a critical negative regulator of IL-33 immune responses, it will be interesting to investigate whether sensing of Der p 1 (contained in dust-mite) through dectin-1 could modulate IL-33 secretion. To begin, we can determine whether Der p 1 by itself can induce IL-33 secretion and if it is modulated by overexpression or knock-down of dectin-1 in epithelial cell lines.

In addition to the allergens (Der p 1, Der p 2) contributed by mite itself, symbiotic microflora derived moieties like β-glucan, LPS, chitin (can also be
contributed by mite exoskeleton) present in mite fecal pellets can also modulate asthmatic immune responses in sensitized individuals. Studies in the past have shown that dectin-1 has a high binding specificity for (1→3)-β-D-glucans [376].

Given that the prototypical ligand for dectin-1 is a β-glucan one would anticipate that dectin-1 mediated protection is through sensing of β-glucan in dust-mite extract. However, we have data suggesting that β-glucan might exacerbate AHR (Fig.13). Mice sensitized and challenged with Ova+β-glucan (curdlan, a (1→3)-β-D-glucans) had higher AHR than those receiving only Ova. Our data (Fig.13) is in contradiction with a reported study wherein curdlan was shown to inhibit Ova (Alum adjuvant) induced allergic airway inflammation [413]. The differences between the published data and ours might be due to several factors. First, we do not utilize Alum (adjuvant) in our model. Moreover, while we utilize C57BL/6 mice, others have utilized BALB/c mice. Mice from varied genetic backgrounds express different isoforms of dectin-1. Specifically, C57BL/6 mice preferentially express the B isoform (major, short-stalk), while BALB/c mice express the A isoform (minor, full-length). It is possible that curdlan might activate one isoform preferentially over the other as has been previously shown for other β-glucans that can bind to dectin-1 [441]. Nonetheless based on our data it seems unlikely that sensing of (1→3)-β-D-glucans in dust-mite through dectin-1 will dampen AHR.

Alternatively, several studies suggest that dectin-1 might recognize chitin and mediate downstream biological effects. Chitin is a β-(1-4)-poly-N-acetyl d-glucosamine polysaccharide, abundantly found in cell walls (bacteria and fungi)
and exoskeleton of crustaceans (shrimp) and insect (dust-mite) [442]. A recent study suggests that fungal (C. albicans) recognition (cytokine secretion) by human PBMCs and mouse macrophages through dectin-1 can be blocked by chitin [443]. Moreover, Elias & colleagues reported that chitin induces IL-10 production from macrophages in a dectin-1 dependent manner. Therefore, there is a precedent for dectin-1 recognition of chitin moieties. In regards to allergic asthma, studies have shown that chitin can either promote or dampen the disease. However, recently Lockseley & colleagues reported that chitin (utilizing chitin beads, 70um) promotes TSLP, IL-25 and IL-33 secretion thereby enhancing ILC2s recruitment that drives eosinophilic airway inflammation [444]. In light of these data, our report that dectin-1 inhibits dust-mite induced ILC2s recruitment in the lungs suggest that chitin may not be the ligand for dectin-1 contained in dust-mite extract. However, chitin mediated biological effects are highly dependent on the size of chitin particles in question. While the large size chitin polymers are thought to be biologically inert, intermediate size (40-70um) are proinflammatory and the small size (less than 40um) chitin can dampen inflammation. Indeed, dependent on the size, chitin preferentially binds to different receptors, while small fragments (<40um) preferentially bind to dectin-1, larger (40-70um) moieties prefers TLR2 [445]. Hence, it might be possible that dectin-1 sensing of small size chitin moieties in dust-mite extracts may inhibit ILC2 driven allergic asthma. In the future, to test if dectin-1’s potential ligand is a chitin, we can use chitin-binding domain (CBD) proteins that specifically bind to chitin in order to analyze if they bind to chitin components from control HDM and
dHDM. If dectin-1 binds to chitin then CBD should only bind in the control HDM, but not in depleted HDM.

5.5: Dectin-1: Implications for human allergic disease

Dectin-1 (CLEC7A) is located in the NK-cell complex cluster on Chromosome 12 (12p13.2-12p12.3) in humans and on Chromosome 6 in mice. Dectin-1 is a transmembrane receptor containing an extracellular carbohydrate-ligand binding domain (CBD), transmembrane stalk and intracellular signaling tail. Alternate gene splicing generates multiple splice variants (8) although each has two major isoforms [369]. The major isoforms differ in either the presence or absence of the stalk, isoforms A and B, respectively, and despite the large number of mRNA transcripts (8) identified in humans, both humans and mice tend to preferentially express the B or the stalkless isoform of Dectin-1 [366]. In addition, only the isoform A and B can bind ligands because of a functional, extracellular carbohydrate recognition domain (CRD). Gene splicing results in deletion of CRD domain in isoform C and D. While in isoform E transmembrane region is deleted, isoform F has no stalk. Isoforms G and H have small insertions between exon 4 (both) and in stalk region (H). Frameshift mutations in all isoforms (except for A, B and E) result in premature stop codons for these and hence truncated proteins [446].

Based on our findings that dectin-1, expressed on epithelial cells is crucial in protecting against asthma exacerbation, we sought to determine whether the receptor expression is inhibited in bronchial epithelial cells of asthmatics as
compared to controls. To this end, we obtained bronchial epithelial cells from normal, mild, moderate and severe asthmatics. Severe asthmatics were actively being treated with corticosteroids to control asthma exacerbations. The first set of primers that we use to evaluate dectin-1 expression are designed in the CRD domain (exon 5 & 6) and therefore will likely amplify isoform A, B, E, F, G and H. The next set of primers were designed for the region of the stalk and therefore will amplify isoform A, C and G. Given the fact that only isoform A and B have a functional domain and biological activity, we will refer to the results from the first primer pair as total (A+B) and the second primer as the isoform A. We quantified either total or isoform A mRNA expression of CLEC7A mRNA expression relative to the A13 (house-keeping gene) from unstimulated bronchial epithelial cell samples.

Our preliminary findings suggest that total dectin-1 mRNA levels are downregulated in all asthmatic bronchial epithelium as compared to those from non-asthmatics (Fig. 14A). This is consistent with the notion that dectin-1 may protect against disease pathogenesis as our previous findings (in mice) suggests that allergen exposure (HDM) down-regulates total dectin-1 expression in the lungs (Fig.1). Interestingly, dectin-1 expression seems to be inversely correlated with asthma severity as compared to healthy controls (white bars) mild to moderate asthmatics (grey bar, Fig.14A) have lower Dectin-1 expression but it is higher than that in severe asthmatics (black bar, Fig. 14A). Specifically, isoform A (minor, full-length) expression seems to be variable, and because of high variation in the data it is not clear if isoform A tracks with asthma severity (Fig.
To determine the role of each mRNA specifically, RNA-Seq can be utilized to selective look at expression of each splice variant in samples from controls and asthmatics. Although additional studies with a larger cohort of samples is needed to confirm these data, taken together with our data from mouse studies, it can be inferred that loss of dectin-1 on bronchial epithelial cells results in increased susceptibility to allergic asthma.

While the mechanisms underlying dectin-1 gene suppression are not clear, multiple potential mechanisms are possible. First, polymorphisms in the regulatory/promoter regions that may result in lower dectin-1 expression in asthmatics is possible. As in the past, an early-stop-codon mutation in CLEC7A was reported to be associated with increased susceptibility to vulvo-vaginal candidiasis and aspergillosis, it will be interesting to evaluate if such SNPs associate with increased risk for allergic asthma [384]. Alternatively, is possible that the CLEC7A locus might be epigenetically silenced/repressed in asthmatics compared to controls as a result of allergen exposure. Alternatively, transcription factors, micro-RNA (miRNA) directly affecting CLEC7A expression might be modulated in asthmatics. All the mechanisms stated above (except for SNPs) can be upstream or downstream (direct or indirect) of allergen exposure in asthmatics leading to downregulation of dectin-1 on bronchial epithelium as compared to healthy individuals.

While there have been a plethora of studies dissecting the epigenetic modifications in CD4+ T cell subsets that might impact asthma pathogenesis, only one study to date has focused on airway epithelial cells. In comparing
differential DNA methylation patterns in airway epithelial cells, the authors reported that 13 CpG sites in healthy controls, 8 in atopics, and 6 in asthmatics (including atopic and non-atopic asthmatics) were unique to the disease phenotype [447]. Noteworthy, the authors did not found any differences in DNA methylation patterns in PBMCs from healthy, atopic and asthmatics. The authors reported that higher DNA methylation for STAT5A results in significantly lower STAT5A gene expression in asthmatics as compared to controls. In regards to allergic asthma, activation of STAT5A downstream to various cytokines (IL-2, IL-4 IL-7, IL-9, IL-15, IL-21 & TSLP) has been shown to promote the differentiation of Th2 cells and hence asthma pathogenesis. In Th2 cells, activated STATS 5A (downstream of IL-2, IL-7 and TSLP) synergize with IL-33 in induction and maintenance of GATA3, the master transcription factor for Th2 cell lineage. Therefore, while STAT5A activation in CD4 T cells promotes a Th2 phenotype, it might or might not function similarly through bronchial epithelial cells. Further, Stefanowicz et al also reported that Runx3, which has been reported to be protective against allergic asthma, is hyper-methylated and expressed at low levels in epithelial cells of asthmatic as compared to controls [447]. Interestingly, published Chip-Seq. databases have shown that both STAT5A and RunX3 can bind to the dectin-1 gene. Therefore, taking our data that dectin-1 expression is decreased in asthmatic epithelial cells with that provided by Stefanowicz et al, it is possible that STAT5A and/or RunX3 downregulation in asthmatic airway epithelial cells leads to repression of dectin-1 transcription.
Direct analysis of the dectin-1 promoter region has revealed that there are potential binding sites for transcription factors like PU.1, AP-1 and NF-kB [448]. NF-kB and AP-1 are activated downstream of various immune receptors and are therefore involved in many biological processes in variety of cell types [449]. PU.1 is primarily expressed on cells of hematopoietic origin, while nothing is known regarding its role on cells of non-hematopoietic origin in the lungs [450, 451]. PU.1 has been reported to drive dectin-1 expression in alveolar macrophages during Pneumocystis pneumonia infection [448]. Therefore, while PU.1 might modulate dectin-1 expression in cells of hematopoietic origin, it may or may not be active in lung epithelial cells. Another transcription factor, peroxisome proliferator-activated receptor gamma (PPARγ) was reported to control dectin-1 expression in macrophages during Candida albicans infection [452]. The authors reported that stimulation of peritoneal macrophages with PPARγ agonists (natural and synthetic) or IL-13 up-regulates Dectin-1 expression on the cell surface. Further they showed that IL-13 dependent upregulation of dectin-1 is PPARγ-dependent [452]. However, studies by Brossart et al showed that stimulation by the PPARγ ligand inhibits dectin-1 mediated DC activation by interfering with signaling pathways (CARD9, MAPK and NF-kB) downstream of dectin-1 [453]. Therefore, there are discrepancies in regards to role of PPARγ role in mediating dectin-1 expression and function. Nonetheless, PPARγ has been shown to be protective in mouse models of allergic asthma [454]. Utilizing a model of Ova sensitization and challenge, Honda et al showed that mice nebulized with ciglitazone (a PPARγ agonist) prior
to allergen challenge had significantly reduced AHR, mucus production, eosinophilia and airway remodeling as compared to controls [455]. Interestingly, the authors reported that PPARγ was mainly expressed in airway epithelial cells following allergen-sensitization. Further, SNPs in PPARγ have been linked with an increased risk for developing asthma [456]. PPARγ functions as a strong suppressor of immune response as in downstream of PRRs as in the presence of a PPARγ agonist, activation of signaling cascades downstream of TLR2, TLR3, TLR4 and TLR7 ligands are inhibited [453]. In summary, PPARγ has been widely reported as protective against the development of asthma and is primarily expressed in airway epithelial cells. Data from the literature (discussed above) suggests that PPARγ might regulate dectin-1 expression and/or function. Based on our findings that dectin-1 expression is down-regulated on bronchial epithelial cells from asthmatics as compared to controls, it will be interesting to investigate if PPARγ can modulate dectin-1 expression or function on airway epithelial cells. To test this hypothesis, dectin-1 expression patterns in epithelial cells can be evaluated following dust-mite exposure in presence of PPARγ receptor antagonists (commercially available).

5.6 Closing statements

In sensitized asthmatic individuals, allergenic triggers are able to initiate a maladaptive Type 2 immune response. Genetic predisposition and environmental modifiers are thought to strongly influence one’s propensity to mount an asthmatic Type 2 immune response. However, it is not clear as to why in a
subset of individuals an allergen is able to trigger a destructive asthmatic immune response, while not in the majority of individuals. It is thought that the host immune system mistakenly recognizes allergens as harmful entities leading to initiation of aberrant immune responses in sensitized individuals. Previous studies have shown that allergens in dust-mite extract can mimic microbial patterns or PAMPs that can be sensed by PRRs on host immune cells initiating allergic inflammation. However, since the dawn of PRR-PAMP era, PRR expression (dectin-2, DC-SIGN, MR) on APCs (DCs and macrophages) has been shown to predominate the course of allergic immune responses.

Herein we report that, Dectin-1 or Clec7a, a non-TLR PRR belonging to the class of CLRs, protects against the magnitude of dust-mite driven allergic asthma. Dectin-1 via strong inhibition of dust-mite driven IL-33 production and ILC2 recruitment protects against exacerbated allergen-driven AHR, eosinophilia and mucus production. Our studies are consistent with those published earlier wherein PRR (TLR4) expression on lung structural cells was shown to determine the course of allergic airway inflammation. TLR4 likely promotes dust-mite driven amplification of Th2 immune responses via conditioning of dendritic cells [265]. However, we report that dectin-1 likely mediates protection independent of its role through dendritic cells and adaptive T cell immune responses. Our work strengthens the idea that lung epithelial cells are the corner stone of asthma pathogenesis. Moreover, our work highlights the importance of dectin-1 in mediating the interaction between two innate immunity components (epithelial
cells-ILC2s), independent of adaptive T cell responses to protect against asthma pathogenesis.

While the importance of IL-33 in asthma pathogenesis is undebatable, knowledge of the mechanisms regulating IL-33 secretion following allergen exposure is limited. Our work contributes to the field by showing that sensing of dust-mite through dectin-1 on lung epithelial cell negatively controls the release of the alarmin, IL-33. This results in control of the recruitment of potent innate sources of IL-13 i.e. ILC2s into the lungs. Further, to the best of our knowledge this is the first time that triggering of a specific PRR on lung epithelial cells has been linked to ILC2 recruitment downstream of exposure to an aero-allergen. Our data in a mouse model is further strengthened by our preliminary findings in humans wherein we report that total dectin-1 expression is suppressed on airway epithelial cells in asthmatics as compared to controls.

Overall, our work provides substantial insights into the immunological mechanisms through which an aberrant asthmatic response might be prevented/suppressed in the host following exposure to ubiquitous allergens. Dysregulation of this suppressive pathway either due to genetic or epigenetic mechanisms might confer risk for the development of asthma in susceptible individuals. In light of our data, further research to define the ligand for dectin-1 might prove beneficial. Our data suggest that administration of ligand-mimics or agonists that selectively target dectin-1 on the lung epithelium might either control disease development or disease exacerbations.
5.7: Figures for chapter 5

**Figure 12. Sensing of a particular ligand in dust-mite extract through dectin-1 confers protection against severity of allergic asthma:**

**Panel 12(A) through Panel 12(E)** 7 weeks old C57BL/6 mice purchased from Taconic farms were sensitized and challenged with either PBS, control HDM or dectin-1 ligand depleted HDM (100ug/40ul) (Method: 2.17) as described (Method 2.2). Seventy-two hours following the last exposure, airway function (APTI) (A), serum IgE levels (B) BAL cellularity (C,D) was determined (Method 2.3, 2.4, 2.5). Mucus production (E) was assessed following PAS staining (Method 2.9). Data represent means +/- SEM (n = 4 mice/group). In panel 12(A), ***indicates P < 0.05 vs. PBS, * P< 0.05 vs. control HDM. In panel 12(D), * indicates P < 0.05 vs. control HDM

**Panel 12 (F):** Seven weeks old WT and dectin-1 KO mice were exposed to one time with either PBS, control HDM or dectin-1 ligand depleted HDM (100ug/40ul) (Method: 2.17) as described (Method 2.2). Four hrs. following the challenge, mice were lavaged (Chapter 2.4) and BAL cytokines (IL-33) were measured by ELISA (Chapter 2.7). Data represent means +/- SEM (n = 6 mice/group). *** indicates P < 0.001
Fig: 12 Sensing of a specific ligand in dust-mite extract through dectin-1 confers protection against severity of allergic asthma.
Fig. 13: (1→3)-β-D-glucan co-administration exacerbates allergen-driven AHR: 7 weeks old C57BL/6 mice were sensitized at Day 0 and Day 7 with either PBS (200ul) or Ova (20ug/200ul). On day 14 and day 21 mice were challenged (i.t.) with curdlan (100ug), Ova (750ug) or Ova (750ug)+curdlan (100ug). Seventy-two hours following the last (i.t.) challenge, APTI was assessed (Chapter:). Data represent means +/- SEM (n=5 mice).
Fig. 13: (1→3)-β-d-glucan co-administration exacerbates allergen-driven AHR

![Bar graph showing APTI (cm H20 x sec) for different treatments: PBS, β-glucan, Ova, Ova + β-glucan.]
Fig. 14: Dectin-1 expression on human bronchial epithelial cells inversely correlates with asthma severity: RNA was obtained from unstimulated bronchial epithelial cells from non-asthmatics (n=4), asthmatics (n=3) and severe asthmatics (n=3). Following cDNA preparation, mRNA expression of total dectin-1 expression or isoform A was evaluated. Data represent means +/- SEM.
(A) Total

CLEC7A mRNA expression

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<td>Severe asthmatics</td>
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(B) Isoform A

CLEC7A mRNA expression

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<tr>
<td>Severe asthmatics</td>
<td>0.001</td>
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REFERENCES

63. Huber, H.K., Pathology of bronchial asthma. Archives of Internal medicine, 1922: p. 689-760.


Kugathasan, K., et al., CD11c+ antigen presenting cells from the alveolar space, lung parenchyma and spleen differ in their phenotype and capabilities to activate naive and antigen-primed T cells. BMC Immunol, 2008. 9: p. 48.


