I, Catherine M Buckingham, hereby submit this original work as part of the requirements for the degree of Master of Science in Immunobiology.

It is entitled:
The Effect of IL-17A on Dendritic Cells

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ABSTRACT

Asthma is a chronic inflammatory disorder of the airways classically thought to result from an excessive Th2-dominated immune response to inhaled environmental antigens such as house dust mite (HDM). Recently though, the observation that individuals with severe asthma express high levels of IL-17A and IL-17F suggests that Th17 cytokines may play a role in more severe disease. In support of this, we have found that A/J mice, which develop severe AHR, mount a mixed Th2/Th17 response following HDM inhalation, while C3H/HeJ mice, which develop mild AHR, mount an exclusively Th2-biased immune response. Supporting a role for IL-17A, blockade of IL-17A in A/J mice diminished AHR, while exposure of C3H/HeJ to HDM+IL-17A exacerbated disease. In preliminary studies that form the basis of this thesis, we found that IL-17A plays different roles at different phases of the allergic response. Specifically, if present at challenge, IL-17A enhances IL-13 driven signaling while the presence of IL-17A at allergen sensitization regulates the number of Tregs present in the lung following allergen exposure. However, the mechanism by which IL-17A induces negative effects on Tregs is unknown. As DCs are critical players in initiating the immune response at allergen sensitization by activating T cells and influencing their development and differentiation, we hypothesized that this impact on Tregs may be due to IL-17A mediated effects on DCs. Indeed, treatment with IL-17A after HDM exposure enhanced DC antigen uptake, MHCII and PD-L2 surface expression, and production of inflammatory cytokines known to antagonize Tregs (IL-6, TNFα). Additionally, IL-17A significantly reduced levels of IL-10, an important immunosuppressive cytokine recently associated with inhibition of the Th17-promoting cytokine, IL-23. These data suggest
that the presence of IL-17A during allergen sensitization may set up a positive feedback loop that further promotes Th17-driven immune responses but impairs Tregs leading to the development of severe asthma. Further understanding the mechanisms that initiate a mixed Th2/Th17 phenotype in response to allergens should inform the development of improved therapies for severe asthma.
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<td>AHR</td>
<td>Airway Hyperresponsiveness</td>
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<td>APC</td>
<td>Antigen Presenting Cells</td>
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<td>BAL</td>
<td>Bronchoalveolar Lavage</td>
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<td>CD</td>
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<tr>
<td>RORγt</td>
<td>Retinoic Acid-Related Orphan Receptor-gamma T</td>
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<tr>
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<td>Transforming Growth Factor-beta</td>
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<tr>
<td>Th</td>
<td>T helper</td>
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<tr>
<td>TNFα</td>
<td>Tumor Necrosis Factor-alpha</td>
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<td>Treg</td>
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Chapter One: Introduction
Definition of Asthma

Asthma is an ancient disease with evidence of treatment in Egyptian hieroglyphics, *The Papyrus Ebers*, (circa 1550 B.C.) [1, 2]. The word, “asthma” is believed to come from the Greek word “aazein,” meaning to exhale with open mouth, or to pant. The first written record of asthma was in Homer’s *The Iliad*, in one instance describing the death of a warrior who died with “asthma and perspiration” [2, 3]. Although Hippocrates first used “asthma” in the medical context (460-360 B.C.), it was Aretaeus (1st Century A.D) who gave such accurate clinical descriptions as including symptoms “heaviness of the chest” and “difficulty breathing when running or on a steep road” [2, 4].

Today, the World Health Organization (WHO) describes asthma as a “chronic disease characterized by recurrent attacks of breathlessness and wheezing, which vary in severity and frequency from person to person. During an asthma attack, the lining of the bronchial tubes swell, causing the airways to narrow and reducing the flow of air into and out of the lungs.”

Asthma: Prevalence and Economic Burden

Worldwide, it is estimated that 300 million people suffer from asthma and 250,000 deaths were attributed to asthma in 2005 [5]. Although asthma affects people of all ages, it typically develops during childhood. In fact, asthma is the most common chronic disease among children [6]. In the United States, over 34 million people [7], including 9 million children, have been diagnosed with asthma [8].
Aside from staggering incidence and mortality numbers, asthma imposes significant financial burden on the American economy. Considering that asthma accounts for about 500,000 hospitalizations, 13 million missed school days, and 10 million missed workdays per year [8], it is not surprising that the annual economic cost of asthma in the United States was estimated at about $20 billion in 2007 [7].

Asthma Immunopathology

Asthma is a chronic inflammatory disease of the lung. The symptoms of asthma referred to above arise as a result of airway hyperresponsiveness (AHR) and airway inflammation leading to variable airway obstruction. Pathologically, asthmatic airways are characterized by an infiltration with eosinophils and neutrophils, hypertrophy of the bronchial smooth muscle, and goblet cell hyperplasia [9]. The etiology of this response is not known, but is thought to be due to exposure to environmental triggers in genetically susceptible individuals. Some of the environmental triggers include aeroallergens (house-dust mite, pollen, pet dander), viruses, and pollutants.

Asthma is a heterogeneous disease. Some individuals are atopic, meaning they have elevated allergen-specific serum IgE levels, while others are not. Interestingly, the pathological picture is much the same in atopic and non-atopic asthma. Likewise, the severity of disease varies widely in that the majority of patients with asthma have mild to moderate disease that can be effectively controlled with regular treatments, including inhaled corticosteroids, long-acting beta-2-agonists, and, if necessary, supplementation by a short-acting bronchodilator for rescue medication [10, 11]. However, there is a small subset, less than 10%, of asthmatics who, even with high doses of medication, do not have controlled disease. This minority group is classified as severe asthmatics. In
addition to the infiltrating eosinophils in the airways of mild to moderate asthmatics, there is an influx of neutrophils in severe asthma. The neutrophilia associated with severe asthma is not reversed by steroid treatment and may explain the uncontrolled disease state of many severe asthmatics. Medical care of severe asthmatics account for a disproportionate amount of health care costs and morbidity associated with the disease [11, 12].

**CD4⁺ T Cells in Asthma**

As critical players in antigen-specific immune responses, T lymphocytes have been implicated in allergic disease. There are increased numbers of T lymphocytes in the bronchial mucosa of patients with allergic asthma when compared to non-atopic (non-allergic) controls [13, 14]. Additionally, there is also an increase in overall T cell activation in allergic individuals [14, 15], suggesting an inappropriate response to non-infectious particles. Studies show that CD4⁺ T cells are the dominant T cell population that drive disease in the airways, as depleting CD4⁺ T cells, either by antibody depletion [16] or gene targeting [17] in mouse models results in complete abrogation of allergen-induced AHR. However, similar targeting of CD8⁺ T cells has no effect on airway responses of allergen-challenged mice [18].

**CD4⁺ T Cell Subsets**

When allergens are processed and resulting peptides are presented by antigen presenting cells (APCs) to naïve CD4⁺ T cells (Th0) in the context of major histocompatibility complex (MHC) II and other co-stimulatory molecules, Th0 cells can
then differentiate into one of a number of functional subsets, Th1, Th2, T regulatory, or Th17, each with a particular phenotype. Subset differentiation is mostly determined by APC-derived signals, primarily cytokines.

Th1 cells are important mediators of the cellular immune response to pathogens such as bacteria, viruses, and fungi by releasing inflammatory cytokines such as TNFβ and IFNγ. Th1 cells develop in response to high levels of IL-12 in the surrounding environment. Also, IL-18 is important for Th1 effector responses as it synergizes with IL-12 to induce IFNγ synthesis [19].

Th2 cells are critical in protection against parasites and in the pathogenesis of allergic disease. Classical Th2 cells secrete IL-4, IL-5, and IL-13, and can mediate immune allergic responses including IgE production, eosinophilia, and mucus production. To date, differentiation factors critical for Th2 cell development are not well understood. Although IL-4 is necessary for in vitro Th2 differentiation, Th2 cells can still arise in IL-4/IL-4Rα-deficient mice (as reviewed in [20]). Recent evidence suggests that TSLP (thymic stromal lymphopoietin) may be necessary for Th2 cell development [21].

There has been recent evidence that in the presence of IL-4 and TGFβ, Th2 cells can be reprogrammed into Th9 cells [22]. Indeed, in the absence of IL-6, TGFβ can reduce expression of the Th2-associated transcription factor GATA-3 and subsequent Th2 cytokine production [23]. Th9 cells produce IL-9 and IL-10. Despite their production of anti-inflammatory cytokine IL-10, in mouse models Th9 cells do not provide protection from the development of autoimmune disease [22]. In fact, as IL-9 was originally identified as a growth factor for mast cells [24] and suggested to prime mast cells to respond to allergen challenge [25], Th9 cells have recently been implicated
in the development of allergic disease. However, so far, this subset has not been identified in humans.

T regulatory cells (Tregs) display immunosuppressive functions by preventing activation and function of effector T cells, partly due to production of immunoregulatory cytokines such as TGFβ and IL-10. The T regulatory cell subset is very important in maintaining self-tolerance and suppressing inappropriate responses to benign and foreign particles (such as allergens). The best characterized regulatory T cells (Tregs) can be defined as CD4+ T cells which express high levels of CD25 (IL-2 receptor) and the transcription factor Foxp3 (Forkhead box P3) [26]. In general, regulatory T cells can be divided into two categories: natural and inducible (nTregs and iTregs, respectively). While nTregs develop in the thymus [27], iTregs develop in the periphery [28]. TGFβ is a critical cytokine for Tregs due to its ability to maintain nTregs [29] as well as driving iTreg development in the periphery [30].

Recently, a new CD4+ Th subset was discovered and referred to as Th17 cells. Th17 cells produce potent inflammatory cytokines such as IL-17A and IL-17F. Th17 cells provide protection in certain infections but they are most strongly associated with neutrophil inflammation and autoimmune disease. TGFβ and IL-6 are the known Th17 differentiation factors [31-33]. With TGFβ as a known critical factor for regulatory T cell development, these observations were somewhat surprising. Bettelli et al. [31] speculate that the amount of TGFβ present in the environment may be the determinant of naive T cell polarization: high levels promoting Treg development, while low levels synergize with IL-6 to drive Th17 differentiation. Also, although not necessary for differentiation, IL-23 is a critical Th17 survival factor [34, 35]. Treatment with TGFβ, IL-
6, or IL-23 results in upregulation of the orphan nuclear receptor RORγt [36], which is known to bind to the IL-17A gene and induce transcription in naïve CD4+ T cells [36, 37].

In addition to environmental triggers (i.e. DC-derived cytokines), fully-differentiated Th effector cells, themselves, also determine the balance between the subsets, as there is inherent antagonism between the subsets. The Th1/Th2 counter-regulation is well-defined and multi-faceted. For example, while IL-4, a primary Th2 cytokine, can inhibit Th1 effector responses (IFNγ production), treatment with Th1-related agents, such as IL-12 and IFNγ can prevent downstream Th2-mediated responses. However, although Th1 and Th2 cells can mediate disruptions to each other’s development and function, they are still considered to be “terminal subsets.” Conversely, the more recently discovered T regulatory cells and Th17 cells are more plastic [38]. Both Treg and Th17 populations require TGFβ during development, but in the absence of IL-6, TGFβ can induce naïve T cells to express Foxp3 [39]. Foxp3 can inhibit Th17 differentiation by antagonizing RORγt activity, as well as reduce IL-17A, IL-17F, and IL-21 mRNA expression. However, if IL-6 is present in the microenvironment, it can overcome this suppressive action by Foxp3 and promote IL-17-producing cells [40]. Together, this evidence suggests that both the environmental signals and Th effectors and the cytokines they produce determine the balance between the subsets and may promote dominance of one subset over the others.

**Specific Roles of CD4+ T Cell Subsets in Asthma (Figure 1.1)**

There is substantial evidence that allergic asthma is driven by an aberrant Th2-skewed immune response. Numerous human studies of lung and blood associate
asthma with increased gene expression or secretion of IL-4 [41-44], IL-5 [41-43], and IL-13 [42, 44-46]. Besides high baseline levels in asthmatics, there is an increase in Th2 cytokines after allergen challenge [47, 48]. However, the primary Th1 effector cytokine IFNγ was found at significantly lower levels after allergen challenge, when comparing atopic asthmatics to non-asthmatic controls [49], indicating that allergic responses are specifically associated with the Th2 phenotype. The definitive pathogenic role of Th2 lymphocytes was demonstrated in an adoptive transfer study in mice in which the transfer of Th2 cells into the lungs of naïve mice induced allergic inflammation and AHR, while transfer of Th1 cells induced inflammation but not AHR [50]. Together, these studies suggest a polarization toward Th2 cells that drives the asthmatic phenotype.

Th2-derived cytokines IL-4, 5, and 13, play a critical role in the development of the asthmatic phenotype. IL-4 is thought to be important in Th2 differentiation [51] and IgE antibody synthesis from B cells [52]. In fact, attenuation of allergic inflammation is seen in IL-4 deficient mice [53]. However, studies suggest that although IL-4 may be needed for initial Th2 induction, they are not critical for the effector phase of the allergic response [54].

As IL-5 is a critical factor for eosinophils, and eosinophilia is a common feature of asthma, many studies have sought to neutralize IL-5 and prevent the development of eosinophilia and AHR. In animal studies, although IL-5 blockade consistently abolished eosinophilia [55-57], the effect on AHR is contradictory [56, 58-61]. However, mice deficient in IL-5 or IL-5Rα chain do not develop lung eosinophilia or AHR after allergen challenge (as reviewed in [62]). Together, this evidence suggests that although IL-5 is
important in allergic airway inflammation, treatment with neutralizing anti-IL-5 antibodies may not be sufficient to prevent the development of the asthmatic phenotype.

Unique among the Th2 cytokines, IL-13 is necessary and sufficient to induce the cardinal symptoms of allergic asthma [63]. IL-13 has a key role in the allergic response due to its actions on epithelial and smooth muscle cells [64, 65]. Treatment with IL-13 alone can drive eosinophilia, mucus hyperproduction, and AHR in mice [63, 66]. Even in the presence of IL-4 and IL-5 in the airways, IL-13 deficient mice fail to mount AHR and mucus hypersecretion in an OVA model [67].

**Impaired T Regulatory Cells in Asthma**

Although asthma may arise due to aberrant expansion of Th2 cells themselves, it is also possible that Th2 immune responses dominate as a result of impaired counter-regulation by T regulatory cells. In several *in vitro* studies, Tregs were able to inhibit proliferation and cytokine production of allergen-stimulated effector T cells [68, 69]. In animal studies, Tregs have been shown to play a protective role against allergen-induced AHR as transfer of antigen-specific Tregs prevented the development of AHR and airway inflammation [70] in an OVA model. Moreover, in a HDM model, depletion of Tregs enhanced AHR, IgE synthesis, Th2 cytokine production, and airway inflammation [71].

However, although regulatory T cells can inhibit Th2 responses and protect against AHR, when cells from allergic asthma patients were studied, both the numbers and suppressive abilities of regulatory T cells were diminished [68, 72]. Hartl et al. discovered that the numbers of CD4+CD25hi T cells (as a percentage of cells in BAL)
and Foxp3 expression were reduced in the blood of asthmatic children compared to controls [72]. Other studies reported reduced in vitro suppressive function of Tregs isolated from the blood of allergic asthmatics when compared to those of non-asthmatic controls [73]. Interestingly, depletion of Tregs in strains of mice that are highly susceptible to HDM-induced AHR, had no effect on airway responses after HDM challenge, while Treg depletion in resistant strains of mice enhanced AHR. This data suggests a present but impaired Treg population in susceptible mice that cannot provide protection against disease as they do in resistant mice [71]. Together, these studies suggest that whether due to abnormal recruitment, development or function, Tregs within asthmatics may be compromised and unable to provide protection against disease.

**Th17 Cells in Severe Asthma**

Knowing IL-17 is critical for neutrophil recruitment and expansion and that lung neutrophilia is associated with severe asthma, many researchers have begun to explore the role of Th17 cells in asthma. Several studies have reported increased IL-17A found in the sputum, peripheral blood, bronchoalveolar lavage (BAL) fluid, and lung tissue of severe asthmatics [74-77]. Additionally, in response to methacholine, there was a positive correlation between an increase in AHR and IL-17A levels in the sputum of asthmatics [74]. It is commonly known that steroids have little effect on patients with neutrophilic asthma. Recently, researchers reconstituted SCID mice with Th17 cells and found the subsequent neutrophil inflammation and AHR after OVA challenge to be resistant to dexamethasone [78], linking IL-17A to severe, steroid-resistant asthma.
The major gene targets of IL-17 include pro-inflammatory chemokines (KC, IL-8, MIG, MIP2, IP10, CCL20), hematopoietic cytokines (IL-6, TNFα, G-CSF, GM-CSF), anti-microbial substances (β-defensins, mucins), and acute phase response genes (serum amyloid A, Lipocalin 2/24p3, S100 proteins) [as reviewed in [79]]. By regulating both neutrophil chemoattractants (KC, MIP-2, and IL-8 in humans) and neutrophil-activating cytokines (G-CSF and GM-CSF), IL-17 has proven to be sufficient to induce neutrophilic inflammation [80, 81]. In relation to our studies, this is particularly interesting because neutrophils have been implicated in playing a role in asthma pathogenesis in severe cases. In fact, in a comparison of BAL and airway biopsies between severe and moderate asthmatics, there were significantly greater percentages of neutrophils found in the severe individuals [82]. Another study focusing on sputum inflammatory cell profiles found increased neutrophils, IL-8, and MPO in severe compared to moderate asthmatics [83].

In animal studies, there has been controversy as to the role of IL-17 in asthma, some reporting IL-17 as protective while others contend that IL-17 is pathogenic. There are a number of potential explanations for such divergent claims. First, the mode of allergen exposure varies greatly from group to group and can affect the results. Wilson et al. [84] found that whereas allergen exposure via the airways induced robust Th17 and mild Th2 responses, sensitization via the peritoneum induced only Th2 responses. Another common discrepancy between the studies is the strain of mouse that is used, a critical variable as there is differential expression of IL-17 between the strains [85]. In fact, many studies [84, 86, 87] use BALB/c mice, which have been shown to produce low levels of IL-17 and this may explain the variability in the results. Finally, IL-17 has a
dynamic role throughout the allergic response and therefore, based on the timing of a study the role of IL-17 may differ. One group, found that the presence of IL-17A has pathogenic effects at sensitization, but protective effects at challenge [88]. Collectively, although studies reveal a strong association between IL-17 and severe asthma, the exact role of IL-17 in asthma and the mechanisms by which it contributes to allergic responses remains unclear.
Figure 1.1 The role of CD4\(^+\) T cell subsets in asthma.
Dendritic Cells

As dendritic cells are known to instruct T cell differentiation through several signals they provide to Th cells, it is possible that altered dendritic cell function may lead to an imbalance in T cell subsets produced during asthma. Just as in other tissues that are exposed to many foreign bodies, such as the skin and gut, the lung has an elaborate network of dendritic cells (DCs), which can be found throughout the conducting airways and bronchial lymph nodes [89]. There is evidence that dendritic cells beneath the barrier epithelial cell layer can extend their dendrites into the airways, sampling and picking up particles [90-93]. Dendritic cells perform a “unique sentinel function” in pulmonary immunology in that they can recognize dangerous and/or foreign substances through expression of pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), C-type lectin receptors (CLRs), and NOD-like receptors (NLRs) [94]. DCs that reside in the periphery have an “immature” phenotype, specialized in recognition and uptake of antigens but not yet capable of stimulating naïve T cells due to insufficient co-stimulatory molecule expression. However, DCs undergo maturation either through direct interaction with an antigen or through indirect stimulation from the environment, usually by surrounding cells releasing inflammatory cytokines (IL-1β, TNFα, IL-6, GM-CSF). Previously, we have found that DC phenotype was differentiated in the presence of GM-CSF [95], enhancing DC activation and maturation state by increasing antigen-uptake and CD86 and MHCII expression. More recently, reports suggest that TSLP, an epithelial cell-dervied cytokine, can activate dendritic cells such that they drive Th2 effector responses [21]. Conversely, treatment with IL-10 or TGFβ have been shown to induce “tolerogenic” dendritic cells with an immature
phenotype (i.e. reduced antigen-uptake and CD86 expression) [96, 97]. Together, this evidence suggests that environmental stimuli can alter the DC maturation state and, therefore, influence the downstream T cell effector responses. Once matured, a DC displays reduced antigen uptake but will acquire a phenotype of “professional” APCs (antigen presenting cell) by expressing all the co-stimulatory molecules on its surface and releasing chemokines to attract and stimulate naïve T cells. Mature DCs then migrate to the T-cell areas of draining lymph nodes and present their antigen to naïve T cells. This DC-T cell interaction in the lymph node is critical to the immune response to that particular antigen. By secretion of a specific cytokine pattern, co-stimulatory molecule expression, antigen burden, and maturation status, DCs have an opportunity to influence T cell differentiation. Thus, altered DC signals may be the difference between tolerance and allergic inflammation.

**Dendritic Cells in Asthma**

There is experimental evidence that dendritic cells (DCs) play a pivotal role in the development of allergic disease. Researchers have shown that either endogenous lung DCs [95] or bone-marrow-derived DCs transferred into the airways [98] are sufficient to promote a Th2 response after allergen challenge. This response is due to the antigen presented by the DCs, themselves, and not the antigen alone, as transferred MHCII-deficient DCs into wild-type hosts fail to induce a Th2 phenotype [99]. However, using diphtheria toxin in CD11c-DTR transgenic mice [100] or a suicide-gene” approach [98], which both deplete CD11c⁺ DCs dendritic cells, resulted in reduced eosinophilia, goblet
cell hyperplasia, and AHR. However, the exact phenotype of DC that confer susceptibility to different forms of asthma are unknown.

**Lung DC Subsets: Distinct Roles in Allergic Responses**

There are specialized DC subsets that have distinct functions in allergen responses. In the mouse lung, there are two major pulmonary DC subsets that have been identified: CD11c⁺CD11b<sup>bright</sup>Gr<sup>-</sup>myeloid dendritic cells (mDCs) [101] and CD11c<sup>low</sup>CD11b<sup>neg</sup>CD317<sup>+</sup>Gr<sup>+</sup>plasmacytoid dendritic cells (pDCs) [102].

Numerous studies have shed light on these subsets. After exposure to antigen, mDCs express high levels of MHCII and CD11c [93] and low levels of the classical co-stimulatory molecules CD80 and CD86 [102-105]. Also, the mDCs are able to capture antigen rapidly in the airways [90, 106, 107] and have high migratory capacity for trafficking antigen to the draining lymph node [90, 106, 107]. It’s not surprising, then, that mDCs are thought to have greater potential to drive a strong adaptive immune response. pDCs, on the other hand, are associated with tolerance. Compared to mDCs, pDCs have a relatively immature phenotype, expressing low levels of MHCII, but high levels of the inhibitory molecule PD-L1 [108].

These differences translate in asthma disease models. Adoptive transfer of mDCs prior to allergen challenge has been shown to induce Th2 responses in the airways [109-112]. Conversely, adoptive transfer of pDCs provided protection from eosinophilia, goblet cell hyperplasia, and increased Th2 cytokine production, while pDC depletion in a resistant host led to an enhanced asthmatic phenotype [108]. Also, although there seemed to be no impact of pDCs on T cell proliferation, there was a
greater number of regulatory T cells in pDC-sufficient mice [108], likely due to the high level of PD-L1 on the surface of the pDCs.

**DC-Derived Signals for T Cell Activation**

Given that naïve CD4⁺ T cells: 1) require MHC II-mediated presentation of processed antigen; 2) require co-stimulatory signals in order to elicit an effector response; and 3) can differentiate into a number of different subsets based on the cytokine milieu, dendritic cells are significant in determining the balance between an inflammatory and regulatory response to allergen in the airways. Although antigen presentation induces general T cell activation, the pattern of co-stimulatory molecules and cytokine production can greatly influence the type of Th effector response.

**DC Maturation State: Co-Stimulatory Molecule Expression**

Based on the pattern and magnitude of expression of co-stimulatory molecules, DCs establish the balance between positive signals and negative signals given to resting T cells. The CD80/CD86:CD28/CTLA-4 is the best characterized of the T cell activation pathways. This pathway is made up of two B7 family members, CD80 and CD86, both of which can bind to either the stimulatory receptor CD28 or the inhibitory receptor CTLA-4 [as reviewed in [113]]. Although CD28 is constitutively expressed, CTLA-4 is upregulated upon T cell activation and has higher affinity for both CD80 and CD86 [114].

Despite their shared receptors, CD80 and CD86 are neither equivalent nor redundant. In human studies, there are conflicting results concerning CD80 and CD86
in atopic asthma. When comparing allergic to non-allergic patients, CD80, but not CD86, expression was upregulated on alveolar macrophages [115]. However, when analyzing soluble forms (which have also been associated with CD28 ligation and subsequent T cell activation) [116], high levels of CD86, and not CD80, are linked to severe asthma and increased AHR [117]. Animal models support the theory that it is CD86, not CD80, expression that is required for the development of AHR. In an OVA model, Keane-Myers et al. [118] used anti-CD80 and anti-CD86 antibodies to delineate the contribution of each to disease. CD86 blockade abolished allergen-induced AHR, eosinophilia, and elevated serum IgE levels as well reduced Th2 cytokine (IL-4 and IL-5) levels in the BAL. However, although treatment with anti-CD80 antibody significantly reduced pulmonary eosinophilia, it had no affect on AHR, serum IgE levels, or Th2 cytokines [118].

Another important B7 family member is PD-1, named programmed-death 1 because it was initially discovered on T cells undergoing cell death [119]. The main function of PD-1 as an inhibitory receptor became quite clear as multiple autoimmune diseases spontaneously arise in PD-1 deficient mice [120]. There are two known PD-1 ligands, PD-L1 and PD-L2. PD-L1 is expressed on resting B and T cells, most non-hematopoietic cells, as well as macrophages and dendritic cells. Conversely, PD-L2 is transiently expressed on macrophages and DCs after stimulation. IL-4, anti-CD40, and GM-CSF are the main stimuli that induce PD-L2 up-regulation. [121]

Functional studies of PD-L1 and PD-L2 have yielded conflicting results. Initially, as known ligands for PD-1, PD-L1 and PD-L2 were considered to act solely as immunosuppressive molecules. Some investigators report that \textit{in vitro} addition of PD-1
ligands suppress T cell effector response [122, 123], while studies indicate that PD-L1 and PD-L2 can stimulate T cell proliferation [124, 125]. Sharpe et al. suggests in her review “B7 family revisited” [113] that perhaps these contradictory results may be due to a second, as yet unknown, receptor that promotes T cell activation. In fact, a study using mutant PD-L1 and PD-L2 molecules, which were unable to bind PD-1, maintained an ability to stimulate T cells [126].

In relation to asthma, E. Ofıazoglu et al. [127] found sustained up-regulation of both PD-1 and PD-L2 (mainly on DCs) in the lungs of asthmatic mice. Although in vitro addition of PD-L2-Fc led to decreased T cell proliferation and cytokine production, in vivo treatment in an OVA mouse model exacerbated disease-elevated serum IgE levels, enhanced eosinophilia and lymphocyte infiltration in the airways, and increased Th2 cytokines [127]. In our own studies (unpublished, Lewkowich), using A/J (susceptible to HDM-induced AHR) and C3H/HeJ (relatively resistant to HDM-induced AHR) mice, we discovered opposing functions of PD-L1 and PD-L2. Treating mice with neutralizing antibodies against PD-1, PD-L1, or PD-L2, we found that while blocking PD-1 and PD-L1 exacerbated disease, the blockade against PD-L2 provided protection against AHR. These results indicate that while PD-1 and PD-L1 typically are immunoregulatory, PD-L2 is pathogenic and enhances disease (perhaps by engagement with an unknown stimulatory receptor). Interestingly, PD-L1 was not protective in the susceptible A/J mice suggest that there may be a defect in the PD-L1/PD-1 pathway. Finally, knowing that regulatory T cells are protective against the development of allergic asthma, it is important to note that studies suggest PD-L1 can both enhance Treg Foxp3 expression
and suppression capabilities [128]. This Treg supporting role of PD-L1 may be a way in which it provides protection in allergic asthma.

Collectively, previous studies suggest that aside from DC co-stimulation providing a necessary signal for T cell activation, the specific pattern of co-stimulatory molecules expressed on the surface can influence the type of T cell response.

**DC-Derived Cytokines Influence T Cell Differentiation**

One of the most important signals by which DCs influence T cell differentiation is through their ability to secrete specific cytokine patterns that drive the specific Th cell responses to antigen stimulation (Figure 1.2). By releasing previously mentioned differentiation factors, DCs can promote Th differentiation: Th1 (IL-12, IL-18), Th2 (TSLP), Th9 (TGFβ), Tregs (TGFβ, IL-10), or Th17 cells (TGFβ, IL-6, IL-23). Also, DC-derived cytokines can tip the balance between subsets by promoting one over others or inhibiting one, which allows others to dominate.

The balance between Th17 and T regulatory cells is strongly affected by environmental cytokines. Besides the plasticity during differentiation, the maintenance of Tregs and Th17 cells can also be fragile, mostly based on the IL-10/IL-23 axis. IL-10, an immunoregulatory cytokine released by dendritic cells or active T regulatory cells, can inhibit production of IL-23 [31]. As IL-23 is a necessary survival factor for Th17 cells, IL-10 could potentially lead to a Th17 population contraction. However, if IL-23 is present in the environment, it can, in concert with IL-6, genetically reprogram fully differentiated Tregs toward the Th17 lineage [40, 85]. Together, this data suggest that
DC-derived cytokines can influence both T cell differentiation and maintenance of Th subsets.

Figure 1.2 The role of DC-derived cytokines in determining naïve T cell polarization.
**Potential Role of IL-17A in Driving Severe Asthma: Modulation of DC Function**

In an effort to elucidate the role of IL-17A in severe asthma, Lajoie et al. [85] used a well-characterized mouse model in which the strains have differences in susceptibility to severe disease. Using mice that have moderate (C3H/HeJ) and severe (A/J) susceptibility to HDM-induced AHR showed that the susceptible (A/J) mice produced both IL-17A and Th2 cytokines, while the less susceptible (C3H/HeJ) mice produced only Th2 cytokines and little to no IL-17A in response to HDM challenge. Blocking IL-17A in the A/J mice throughout allergen exposure significantly reduced AHR; whereas, treating the C3H mice with recombinant IL-17A exacerbated AHR [85]. From these studies [85], it was concluded that in contrast to the Th2 phenotype in C3H mice, A/J mice have mixed Th2/Th17 phenotype that associates with severe disease. This study identifies that IL-17A plays a pathogenic role in the development of the asthmatic phenotype. However, with previous studies suggesting that IL-17A acts differently at sensitization vs. challenge [88], we decided to further explore this possibility by blocking IL-17A at different times throughout allergen responses. Whether IL-17A was blocked at sensitization or challenge with an anti-IL-17A mAb, both groups showed a decrease in AHR, compared to the isotype-control (unpublished data, Lajoie et al.), but had distinct phenotypes. Compared to control and the challenge-blockade, only the mice treated with anti-IL-17A mAb at sensitization had an increase in T regulatory cells. Together with our previous findings that indicated an impaired T regulatory cell population in susceptible mice [71], the evidence suggests that IL-17A drives Th subset imbalance in susceptible A/J mice during sensitization, skewing towards Th17 cells and away from T regulatory cells.
It is possible that IL-17A may regulate T cell differentiation by mediating effects on DCs, important regulators of T cell development and differentiation. In fact, mDCs isolated from A/J mice, previously shown to make IL-17A after HDM challenge, were found to have an increased ability to take up antigen, express maturation markers as well as preferentially produce Th17-skewing cytokines (IL-6, IL-23) [95]. Knowing that dendritic cells play a critical role during sensitization to determine the asthmatic response (not only do dendritic cells present antigen to naïve T cells and thus drive the adaptive immune response, but they also instruct the response by unique cytokine profile production), we hypothesize that IL-17A produced early during the immune response contributes to the initiation of the asthmatic response through its effect upon dendritic cells, altering the phenotype of dendritic cells such that they promote an environment that shifts the balance of Th cell differentiation towards Th17 and away from T regulatory cells, thereby enhancing its own production and driving a severe asthma phenotype. To test this hypothesis we propose the following specific aims:
Specific Aims and Hypotheses

1. To determine the effects of IL-17A on dendritic cell phenotype *in vitro*.

   We hypothesize that IL-17A treatment *in vitro* will induce increased antigen uptake, a co-stimulatory molecule expression pattern that’s associated with allergic responses (upregulated MHC II, CD86, PD-L2 expression and/or down-regulated PD-L1), and a cytokine profile that promotes Th17 cells buts inhibits Tregs (high levels of IL-6, TNFα, IL-1B, IL-23; decreased levels of TGFβ and IL-10).

2. To determine the effects of IL-17A on dendritic cell phenotype *in vivo*.

   We hypothesize that IL-17A will mediate effects on pulmonary dendritic cells similar to those of IL-17A-treated BMDCs discussed in Aim1. In addition, we hypothesize that there will be an increased mDC population compared to pDCs found in the airways after IL-17A treatment.
Animals

5-6-week old C3H/HeJ mice were purchased from Jackson Laboratories and housed in an environmentally controlled, specific pathogen-free facility at Cincinnati Children’s Hospital Medical Center. Cincinnati Children’s Hospital Medical Institutional Animal Care and Use Committee approved all animal studies.

in vivo Mouse Treatment

6-week-old C3H/HeJ mice were treated with 40µl PBS, 100µg HDM extract (Greer Laboratories) or 100µg HDM extract with 15µg recombinant mouse IL-17A (eBioscience) intratracheally (I.T.) on day 0. Where indicated, the HDM used was labeled with AlexaFluor405 (Invitrogen) according to manufacturer’s standards. Mice were sacrificed 24 hours after HDM exposure and lungs removed for further analysis.

Bone Marrow Derived Dendritic Cell Cultures

Bone marrow cells were cultured (3.0 x10^5 cells/ml) in complete RPMI 1640 media plus 10% (vol/vol) FBS supplemented on days 0 and 3 with 10ng/ml GM-CSF (Peprotech) in 50mls in a T175 flask. On day 6, cells were harvested, by pipetting out suspended cells and using cell disassociation buffer (5ml/T175 flask; Gibco) to remove adherent cells. After washing cells with complete RPMI (10% FBS), viable cells were counted by trypan blue exclusion. Cells were then at a concentration of 6.0 x 10^5 cells/well with media alone, 30µg Alexa Fluor 405-conjugated HDM (AF405-HDM), or AF405-HDM and 100ng/ml recombinant IL-17A (R&D Systems), in a total volume of 250µl for 24 hours.
**Lung Cell Isolation**

Lungs were removed and, after mincing, were placed in 6 ml RPMI 1640 media containing 0.5mg/ml DNase I (Sigma Aldrich) and Liberase DL (Roche Diagnostics) and incubated at 37°C for 45 minutes. After digestion, the remaining tissue was forced through a 70mm cell strainer to make a single-cell suspension. Cells were pelleted and red blood cells lysed with 5ml of ACK lysis buffer. Remaining cells were washed with complete RPMI (10% FBS) and live cells are counted using trypan blue exclusion. Cells are then cultured at 250,000 cells/well of a flat-bottom 96-well dish in 250µl/well. Cells were stimulated with culture medium alone, concanavalin A (conA) at 5µg/ml or HDM at 30µg/ml. After 72 hours, supernatants were harvested and frozen at -80°C until cytokine analysis.

**Real-Time PCR**

Whole lung tissue samples were lysed with TRIzol (Invitrogen) for RNA extraction. Expression of *Rps14* (ribosomal protein S14), *Pdcd1lg2*, *Il10*, *Il12a*, *Il23a*, TGFβ1, and *Foxp3* was measured in RNA samples isolated from whole lung samples from C3H/HeJ mice treated with PBS, HDM or HDM+rIL-17A as well as from media or IL-17A-treated C3H/HeJ BMDCs using real-time PCR as previously described [141]. Primers were obtained from mouseprimerdepot.nci.nih.gov and were designed to span at least one intron to avoid co-amplification of genomic DNA. PCR values were normalized to expression of *Rps14*. Primers were: *Rps14* sense TGG TGT CTG CCA CAT CTT TGF ATC; *Rps14* anti-sense ACT CAC TCG GCA GAT GGT TTC CTT; *Pdcd1lg2* TTG CCT GGT CAT CTG; *Pdcd1lg2* anti-sense AGG ACA CTG CTG CTA; *Il10* sense TGC TAA
CCG ACT CCT TAA TGC; \textit{Il10} anti-sense CTG CTC CAC TGC CTT GC; \textit{Il12a} sense GCT TCT CCC ACA GGA GGT TT; \textit{Il12a} anti-sense CTA GAC AAG GGC ATG CTG GT; \textit{Il23a} sense GAC CCA AAG CCA GTC AAG GA; \textit{Il23a} anti-sense CAT GGG GCT ATC AGG GAG TA; \textit{TGFb1} sense AAT TCC TGG CGT TAC CTT GG; \textit{TGFb1} anti-sense GGC TGA TCC CGT TGA TTT CC; \textit{Foxp3} sense TTG GAA GAC AGT CAC ATC; \textit{Foxp3} anti-sense CGT TCA AGG AAG AAG AGG.

\textit{Flow Cytometry}

Staining reactions were performed at 4°C after incubation of cells with Fc Block (mAb 2.4G2; derived from cell line HB-197; American Type Culture Collection) for 30 minutes. mDCs (CD11c\(^+\), CD11b\(^+\), Gr1\(^{-}\)neg, CD317\(^{-}\)neg), pDCs (CD11c\(^{\text{low}}\), CD11b\(^{-}\)neg, Gr1\(^+\), CD317\(^+\)), alveolar macrophages (CD11c\(^+\), CD11b\(^{-}\)neg, Gr1\(^{-}\)neg, CD317\(^{-}\)neg), neutrophils (CD11c\(^+\), CD11b\(^{\text{bright}}\), Gr1\(^{\text{bright}}\)) were quantified using anti-CD11c-APC (HL3), anti-CD11b-PE-Cy7 (M1/70), anti-CD317-Alexa Fluor 488 (927) and anti-Gr1-APC-Cy7 (RB6-8C5). Co-stimulatory molecule expression was examined using PE-conjugated mAbs to MHC Class II (14-4-4s), CD80 (16-10A1), CD86 (GL1), B7-H1 (MIH5) and B7-DC (TY25). Dead cells were excluded using the viability dye 7-AAD. All antibodies were purchased from BD Biosciences or eBioscience.

For quantification of lung, regulatory T cells 1 x 10\(^6\) lung cells were fixed for 30 minutes in Fix/Perm buffer (eBioscience), and non-specific Ab binding was blocked using Fc Block (2.4G2) in Permeabilization Buffer (eBioscience) for 30 minutes. Cells were stained with anti-CD4-PE Cy7 (RM4-5; 25-0042) and anti-Foxp3-APC (FJK-16s) antibodies for 30 minutes and subsequently washed twice before re-suspension in flow
buffer. All steps described for regulatory T cell staining were performed using Permeabilization Buffer (eBioscience).

For quantification of lung, IL-17A producing T cells, $1 \times 10^6$ lung cells were stimulated with phorbol 12-myristate 13-acetate (50ng/ml) and ionomycin (100ng/ml). After 18 hours, brefeldin A and monensin (eBioscience) were added for 4 hours. Then, cells were fixed with Fix/Perm buffer (eBioscience), and non-specific Ab binding was blocked using Fc Block (2.4G2) in Permeabilization Buffer (eBioscience) for 30 minutes. Cells were stained with anti-CD4-PE Cy7 (RM4-5; 25-0042) and anti-IL-17A-Alexa Fluor 647 (17b7; 51-7177; eBioscience). After washing the cells twice with Permeabilization Buffer, they were re-suspended in flow buffer.

**Determination of Cytokine Concentration**

Cytokine levels in lung cell culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) using matched antibody pairs purchased from R&D Systems (IL-6, TNFα, IL-23, IL-10, IL-12p40, and IL-13). The assays were conducted according to manufacturers’ recommendations. Optical density (OD) readings of samples were converted to picograms per milliliter using values obtained from standard curves generated with serial dilutions for each recombinant cytokine (0-5000 pg/ml).

**Statistical Analysis**

Student’s t-test was used for comparisons. P values of less than 0.05 were considered significant.
Introduction

Although asthma has been classically defined as a Th2-dominated disease, recent evidence suggests that it is a more heterogeneous and complex disease than can be explained within the Th2-paradigm. Most notably, severe asthmatics have increased IL-17A levels in the airways as well as neutrophilia [75]. This pattern is also associated with steroid resistance, which may explain the correlation of neutrophil inflammation with sudden-onset fatal asthma in humans [78]. In animal models, Th17 cells can induce neutrophil inflammation in the airways and, in concert with Th2 cells, drive exacerbated airway hyperresponsiveness.

In our model of HDM-induced AHR, there is a positive correlation between expression of lung IL-17A and severity of disease. In A/J mice, which are susceptible to allergen-induced AHR, blockade of IL-17A throughout HDM exposure significantly reduced airway responses [85]. To delineate the effects of IL-17A presence early versus late during HDM treatment, IL-17A was blocked only during HDM sensitization versus only during HDM challenge. Interestingly, both protocols yielded similar decreases in AHR, but distinct cellular profiles emerged. Unique to the blockade at sensitization, T regulatory cell numbers were increased compared to the isotype-control and the blockade at challenge (unpublished, Lajoie). This evidence implies that IL-17A has a negative impact on regulatory T cells, potentially affecting recruitment, development, and/or function. This is of particular interest as Lewkowich et al. [71] have shown that depletion of naturally-occurring T regulatory cells (nTregs) at sensitization in C3H/HeJ mice increased AHR to levels similar to that of A/J mice. Conversely, depletion of nTregs in A/J mice had no effect on airway responses. Based
on this, we hypothesized that due to elevated levels of IL-17A in A/J mice after HDM-exposure [85], Tregs are negatively affected and this impairment leads to exacerbation of the asthmatic phenotype.

In another study exploring the differential AHR phenotypes between the strains, Lewkowich et al. [95], found an inherent difference in the dendritic cells. Pulmonary DCs from A/J mice produced more *Il23a* mRNA as well as other Th17-skewing cytokines, potentially promoting a Th17 effector response. Together, this implies that in the susceptible A/J strain, there is a more permissive Th17 environment that may be mediated by dendritic cell-derived signals.

In the development of the asthmatic phenotype, dendritic cells are critical players during allergen sensitization. Dendritic cells are primary antigen presenting cells and provide necessary signals for downstream T cell activation. Besides MHCII-mediated antigen presentation, dendritic cells express co-stimulatory molecules and cytokines, which can influence the type of Th effector response. In terms of co-stimulation, while CD86 and OX40L are associated with the development of Th2 responses, PD-L1/PD-1 are associated with tolerogenic immune responses. Likewise, the DC cytokine profile during antigen presentation can determine Th subset development. For this particular study concerning Th17 cells and Tregs, we will focus specifically on TGFβ, IL-6, IL-23, IL-10, and TNFα, classifying TGFβ, IL-6, and IL-23, as Th17-promoting cytokines and TGFβ and IL-10 as Treg-promoting cytokines. We will also look at TNFα as, along with IL-6, it is an inflammatory cytokine known to inhibit Treg development and function.

The imbalance between Th17 cells and T regulatory cells in A/J mice after sensitization to HDM suggest an aberrant Th17 subset development. Knowing that
dendritic cells play a critical role during sensitization to determine the asthmatic response, partly due to their influencing the Th effector response (by antigen presentation, co-stimulatory pattern, and unique cytokine profile), we hypothesize that IL-17A drives the initiation of the asthmatic response by its effect upon dendritic cells, altering the phenotype of dendritic cells such that they promote an environment that shifts the balance toward Th17 and away from T regulatory cells.

Methods

After allergen challenge, A/J mice are predisposed to promote mixed Th2/Th17 responses leading to severe airway responses, while C3H/HeJ mice produce Th2 cytokines but little to no IL-17A and develop moderate AHR [95]. However, after reconstitution with IL-17A, C3H/HeJ mice mount a severe disease phenotype similar to that of A/J mice [85]. Knowing that C3H/HeJ BMDCs express receptors for IL-17A (data not shown), we chose to treat C3H/HeJ BMDCs with IL-17A to assess how this modifies DC function as compared to HDM alone.

Gating strategy for mDC isolation by flow cytometry

After staining bone marrow-derived DCs (generation and staining described in Materials and Methods), mDCs were isolated based on size, viability, and CD11c expression (Figure 3.1A). mDCs were then analyzed for antigen uptake and co-stimulatory molecule expression.
Results

To determine the direct effect of IL-17A on dendritic cells in the context of HDM challenge, bone marrow-derived DCs (BMDCs) from C3H/HeJ were generated by exposing bone marrow cells to GM-CSF for 6 days, a protocol shown to induce primarily mDCs. BMDCs were then pulsed with PBS, AF405-HDM alone, or AF405-HDM+IL-17A for 24 hours, and then assayed for antigen-uptake, co-stimulatory molecule expression, and cytokine production. Only live (7-AAD\textsuperscript{neg}), CD11c\textsuperscript{+} cells were analyzed for HDM uptake and co-stimulatory molecule expression (Figure 3.1A). Cytokines were measured from supernatants of BMDC cultures.

*IL-17A treatment enhances HDM uptake*

Compared to media-treated BMDCs, there was a significant (~5-fold) increase in fluorescence after treatment with AF405-HDM, indicating that the C3H/HeJ BMDCs are functional and able to phagocytose the fluorescently-labeled HDM. With additional IL-17A treatment, the amount of HDM taken up per cell (as indicated by the mean fluorescent intensity (MFI)) was moderately, but significantly increased (Figure 3.1B). Also, the percent of HDM-positive CD11c\textsuperscript{+} cells were also affected by IL-17A treatment (Figure 3.1C), increasing from 34% (HDM alone) to 40% (HDM and IL-17A). The ability of IL-17A to enhance antigen uptake affects both individual dendritic cells (increased antigen burden) as well as the CD11c\textsuperscript{+} population (more cells taking up HDM), as shown in Figure 3.1C. As antigen-uptake and processing is important for DC maturation and subsequent activation of naïve T cells, the increase of dendritic cells
taking up antigen in the presence of IL-17A may indicate one way in which IL-17A can enhance allergic response.

*IL-17A treatment upregulates PD-L2 expression*

As expression of molecules involved in antigen presentation and co-stimulation are required for activation of naïve T cells and subsequent induction of the adaptive immune response, we analyzed expression of MHCII, CD80, CD86, PD-L1, and PD-L2 after HDM treatment with or without IL-17A. Although MHCII, CD80, CD86, and PD-L1 were unaffected, PD-L2 expression was enhanced following IL-17A treatment. Consistent with previous studies in which DCs express PD-L2 after antigen stimulation, more dendritic cells expressed PD-L2 after HDM exposure (compare 35% of media-treated CD11c+ cells with 49% of HDM-treated CD11c+ cells). The percentage of PD-L2+, CD11c+ cells was increased with additional IL-17A treatment, reaching 53% of the population (Figure 3.2A). Also, Pdcd1lg2 message levels were increased with IL-17A when compared to HDM treatment alone or media-control (Figure 3.2B). When only CD11c+, HDM+ cells were examined, there was an increase in the percentage of PD-L2+ cells with added IL-17A stimulation (Figure 3.2C), indicating a positive correlation between IL-17A and DC maturation after antigen-uptake. However, although HDM treatment did upregulate PD-L2 surface expression on individual DCs (increased MFI), IL-17A did not affect PD-L2 expression on a per cell basis (Figure 3.2D). These data indicate that while IL-17A induces more CD11c+ cells to express PD-L2, it does not increase surface expression of PD-L2 on individual DCs (Figure 3.2E).
IL-17A treatment induces inflammatory cytokine release while inhibiting IL-10

By enhancing antigen uptake as well as Pdcd1lg2 (PD-L2) mRNA, IL-17A may promote DC maturation and down-stream T cell activation. However, dendritic cells can influence naïve T cell differentiation by releasing cytokines that can affect differentiation, survival, and/or expansion of the different Th subsets, determining the type of T cell effector response. In relation to our studies concerning Th17 and Tregs, we looked specifically at TGFβ, IL-6, IL-23 as Th17-promoting cytokines, TGFβ and IL-10 as Treg-promoting cytokines, and IL-6 and TNFα as Treg-inhibiting cytokines.

To determine the cytokines released by the C3H/HeJ BMDCs after HDM exposure with or without IL-17A, culture supernatants were harvested and/or RNA extracted for analysis. In terms of cytokines involved in Th17 and Treg differentiation, both TGFβ (shown as mRNA expression) and IL-6 were induced by HDM, although increases in TGFβ levels were not statistically significant (Figure 3.3A). IL-23, a critical factor for Th17 survival, was also induced upon HDM stimulation (Figure 3.3A). With additional IL-17A treatment, IL-6 and IL-23 production trends to increase but it is not statistically significant. These data suggest that IL-17A may induce DCs to produce Th17-promoting cytokines.

Upon stimulation with HDM treatment, alone, DCs produced significantly more IL-10, shown by both increased protein and message levels (Figure 3.3B). In this case, the effect of IL-17A on IL-10 production was evident, reducing levels by 30% or more. As IL-10 is an important Treg-promoting cytokine, the ability of IL-17A to inhibit DCs from producing IL-10 may be one way that IL-17A regulates the Treg population.
As previously stated, both IL-6 and TNFα are associated with impaired T regulatory cell development and/or function. Similar to IL-6, there was significant induction of TNFα after HDM treatment (Figure 3.3C). In the presence of IL-17A, TNFα production was greatly enhanced. In fact, of all the cytokines analyzed, TNFα had the highest protein levels.

From this data, we conclude that IL-17A can alter the cytokine profile of stimulated dendritic cells that may promote an environment conducive for Th17 cells. But, even more striking, our evidence suggests that IL-17A can induce a cytokine expression pattern (reduced IL-10, enhanced TNFα) that specifically antagonizes Tregs.

Conclusion

Collectively, these data support our hypothesis that treatment with IL-17A can alter DC phenotype, enhancing activation and maturation status as well as inducing a cytokine profile that may promote Th17 cells while antagonizing Tregs. These effects may be due to direct action of IL-17A or a synergy between IL-17A and the media-supplemented GM-CSF, a synergy that has been previously shown [129]. These IL-17A-mediated effects on DCs are also replicated in vivo, as will be discussed in the following chapter.
Figure 3.1 IL-17A enhances HDM-uptake. C3H BMDCs were cultured with PBS, AF405-HDM, or AF40-HDM+IL-17A in vitro as described in Materials and Methods. DCs were harvested 24 hours after treatment and analyzed by flow, gating on live, CD11c+ cells (A). PBS (black bars), HDM alone (green bars), and HDM+IL-17A (red bars)-treated DCs were analyzed for allergen content, or HDM MFI (B) and proportion of HDM+CD11c+ cells (C). Histogram compares HDM-content and % of HDM+ cells between media (gray histogram), HDM (green histogram) and HDM+IL-17A (red histogram)-treated CD11c+ cells. Mean+ SEM shown (n= 1; representative of three independent experiments). *, **, and *** indicate significant differences of p<0.05, p<0.01, and p<0.001, respectively.
Figure 3.2 IL-17A enhances PD-L2 expression. C3H BMDCs were cultured with PBS, AF405-HDM, or AF40-HDM+IL-17A in vitro as described in Materials and Methods. DCs were harvested 24 hours after treatment. CD11c+ cells treated with media (black bars), HDM alone (green bars), or HDM+IL-17A (red bars) were compared for proportion of PD-L2+ cells (A), PD-L2 mRNA expression (B), proportion of HDM+PD-L2+ cells (C), and PD-L2 MFI (D). Histogram compares PD-L2 surface expression and % of PD-L2+ cells between media (gray histogram), HDM (green histogram) and HDM+IL-17A (red histogram)- treated CD11c+ cells. Mean+ SEM shown (n= 1; representative of three independent experiments). *, **, and *** indicate significant differences of p<0.05, p<0.01, and p<0.001, respectively.
Figure 3.3 IL-17A increases inflammatory cytokine release but reduces IL-10 levels. C3H BMDCs were cultured with PBS, AF405-HDM, or AF40-HDM+IL-17A in vitro as described in Materials and Methods. DC culture supernatants were harvested 24 hours after treatment. CD11c+ cells treated with media (black bars), HDM alone (green bars), or HDM+IL-17A (red bars) were compared for TGFβ mRNA expression and release of cytokines IL-6, IL-23 (A). IL-10 protein and message levels were compared between the groups (B), as well as TNFα production. Mean± SEM shown (n= 1; representative of three independent experiments). *, **, and *** indicate significant differences of p<0.05, p<0.01, and p<0.001, respectively.
Chapter Four: *In Vivo Effects of IL-17A on DC Phenotype*
Introduction

From our previous *in vitro* studies (chapter 3), we have found that IL-17A can directly enhance dendritic cell antigen uptake and PD-L2 expression as well as induce a DC cytokine profile that is associated with Treg inhibition. However, as there are many factors in the *in vivo* environment which may influence the effects of IL-17A on DC phenotype and function such as the fact that multiple types of DC exists which may respond differently to IL-17A and that other cell types that can also be affected by IL-17A treatment and may alter DC phenotype, it is necessary to confirm our *in vitro* results *in vivo*.

There are a number of dendritic cell subsets that have been identified. For our purposes, we will focus on two prevalent pulmonary DC subsets identified in mice: myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs). mDCs and pDCs have distinct roles in the immune response to antigen. After antigen exposure, mDCs rapidly capture antigen [90, 106, 107] and display a greater maturation state [93, 102-105], as shown by increased MHCII, CD80, and CD86 expression. pDCs, on the other hand, maintain an immature phenotype even after antigen exposure, with increased expression of the inhibitory molecule PD-L1 [108]. In terms of asthma, mDCs are associated with the development of Th2 responses after allergen challenge [109-112], whereas, pDCs are associated with activating Tregs and subsequent protection from the development of the asthmatic phenotype [108, 130]. As our *in vitro* studies were conducted with DCs derived from the bone marrow, which are predominantly myeloid, studies to examine the impact of IL-17A on other lung resident DC subsets are required.
In contrast to *in vitro* studies, the lung is a complex environment with numerous cells types that, either directly or indirectly, interact with DCs, affecting their phenotype. Epithelial cells are in close proximity to dendritic cells and can provide signals that lead to DC activation. After allergen exposure, epithelial cells produce several chemokines and cytokines. For example, there is evidence that in response to HDM, epithelial cells can release TSLP, which induces DC activation and OX40L expression [21], and CCL20, which is a chemoattractant for immature DCs [131]. However, DCs can also receive immunoregulatory signals from surrounding cells. Tregs can also act as a regulator of DC activation. Tregs can suppress DC maturation (reduce co-stimulatory molecule expression) directly by PD-1/PD-L1 ligation or indirectly by IL-10 [71, 96]. Also, there is evidence that T regulatory cells can suppress dendritic cell (DC) activation by forming large aggregates around the DCs and inhibiting their maturation [68]. To complicate matters, adding IL-17A to this environment may alter DCs by IL-17A-mediated effects on other cell types. IL-17A is known to induce epithelial cells and structural cells to produce many pro-inflammatory chemokines and hematopoietic cytokines such as IL-6, TNFα, IL-8, CCL20, G-CSF, and GM-CSF [79]. A number of these are known to affect DCs. For instance, GM-CSF is an important DC survival factor and CCL20, as previously mentioned, recruits immature DCs.

Therefore, to determine the effects of IL-17A on dendritic cell phenotype in a way that is biologically significant, there are a number of factors that can only be addressed *in vivo*. As our previous finding that *in vivo* IL-17A blockade at sensitization resulted in increased Treg recruitment, we expect that IL-17A will alter the phenotype of DCs *in vivo*. 
in such a way that will promote a Th17-permissive environment and antagonize Treg differentiation, recruitment, or activation.

**Methods**

Consistent with *in vitro* bone-marrow experiments in chapter 3, C3H/HeJ mice were used for the following *in vivo* experiments. As previously discussed, C3H/HeJ mice have lower production of IL-17A following allergen treatment. For this reason, we chose to reconstitute IL-17A in this strain, and assess how this modifies DC function as compared to HDM alone.

**Gating Strategy for Isolation of Lung DC Subsets**

After staining lung cells (isolation and staining described in Materials and Methods), cells of interest were isolated based on viability (*Figure 4.1A*) and CD11c expression (*Figure 4.1B*). CD11c\(^+\) cells were then classified based on CD11b and CD317 expression (*Figure 4.1C*) with alveolar macrophages classified as CD11b\(^{\text{neg}}\)CD317\(^{\text{neg}}\) and pDCs classified as CD11b\(^{\text{neg}}\)CD317\(^{+}\). CD11b\(^{\text{bright}}\) cells were further divided based on Gr1 expression with mDCs classified as Gr1\(^{\text{neg}}\) and neutrophils (PMNs) as Gr1\(^{+}\). mDCs and pDCs were then analyzed for antigen uptake and co-stimulatory molecule expression.
Results

**IL-17A treatment increased neutrophil inflammation in the lungs after HDM-exposure**

As IL-17A is known to induce neutrophil recruitment to the tissues, we compared the frequency of neutrophils (defined as 7AAD$^{neg}$CD11c$^{+}$CD11b$^{bright}$Gr1$^{+}$ as shown in Figure 4.1D) in the absence or presence of IL-17A as a control for IL-17A bioactivity. In control, PBS-treated mice, neutrophils make up about 6% of the lung (gated only on live cells). This frequency reflects resident neutrophils and recently-recruited neutrophils due to PBS intratracheal treatment. After HDM exposure, neutrophil frequency is modestly, but not significantly increased (~7%). With the addition of IL-17A, there are significantly more neutrophils present in the lung, with an increased frequency of ~11% of live cells (Figure 4.2A). This evidence confirms that the IL-17A given is active and capable of inducing neutrophilia in the airways.

**IL-17A treatment increased mDC frequency; pDC frequency was unaffected**

To determine the effect of IL-17A on lung dendritic cells in context of allergen exposure, C3H/HeJ mice were given PBS, AF405-HDM, or AF405-HDM and IL-17A intratracheally. Lung cells were harvested from the mice 24 hours after treatment and compared between the groups for differences in lung DC subsets by flow cytometry. To assess the effect of IL-17A on lung DC subsets, we compared mDC and pDC frequencies between the treatments (Figure 4.2B). We classified mDCs as 7AAD$^{neg}$CD11c$^{+}$CD11b$^{bright}$Gr1$^{neg}$ cells and pDCs as 7AAD$^{neg}$CD11c$^{+}$CD11b$^{neg}$CD317$^{+}$ (Figure 4.1). At baseline, both mDC and pDC populations were present in the airways, where mDCs exceeded pDCs by 10-fold. In mice exposed to HDM, there were significant
increases in the frequencies of both mDC and pDC subsets, which indicated appropriate recruitment for subsequent antigen uptake. With additional IL-17A treatment, there was a significant increase in the frequency of mDCs present in the airways and this increase was not reflected in the pDC population. While the mechanisms for the IL-17A-mediated increase in mDC frequency are unclear, we speculate that IL-17A enhances mDC recruitment/survival in the lung due to its ability to induce CCL20, a chemokine for immature DCs, and GM-CSF, a DC survival factor, from epithelial and other structural cells [131]. As mDCs are associated with the development of antigen-specific Th2 cells and pDCs with the development of antigen-specific regulatory cells, the ability of IL-17A to induce greater mDC infiltration but not affect pDCs suggests that IL-17A may enhance downstream Th2 effector responses.

**IL-17A-treatment enhanced mDC HDM-uptake**

Gating on mDCs or pDCs from AF405HDM-treated mice revealed that both subsets had taken up antigen (Figure 4.3A). When analyzing allergen burden (as measured by AF405) and frequency of HDM+ cells (as a percent of the subset), this trend of antigen uptake is repeated (Figure 4.3B and 4.3C, respectively). However, differences in the magnitude of uptake between the two subsets suggest that mDCs were the primary population taking up antigen in the HDM treated groups. With additional IL-17A treatment, there is an increase in allergen burden in both subsets (Figure 4.3B). While IL-17A had a striking effect on mDCs, increasing both allergen burden and the frequency of HDM+ cells, the pDCs were modestly affected with a slight increase in HDM MFI and no change in the frequency of HDM+ cells (Figure 4.3B and
4.3C). These data indicate that IL-17A drives an enhanced mDC activation state and could, subsequently induce more robust T cell activation and effector responses.

Co-Stimulatory Molecule Expression

As previously described, DCs provide a number of necessary signals for downstream T cell activation, including those conveyed through co-stimulatory signals. Gated on mDCs derived from HDM-treated mice, we see a significant increase in the frequency of both MHCII⁺ and PD-L2⁺ cells, when compared to PBS-treated mice (Figure 4.4A). After IL-17A treatment, there is further increase (32% to 38%) in the frequency of MHCII⁺ cells but no significant difference in the frequency of PD-L2⁺ cells. This pattern is repeated upon co-stimulatory molecule expression analysis of only HDM⁺ mDCs, with an increase in MHCII⁺ cells, but not PD-L2⁺ cells, in the presence of HDM and IL-17A compared to HDM alone (Figure 4.4B). However, the number of MHCII and PD-L2 molecules (as measured by MFI) on the surface of mDCs was increased with HDM exposure and further increased with additional IL-17A treatment (Figure 4.4C). Together, these data suggest that IL-17A can induce mDC activation and greater mDC maturation state.

Cytokine Production

As cytokine milieu influences naïve T cell differentiation as well as survival and expansion of fully differentiated T cell subsets, we re-stimulated whole lung cell cultures with media, HDM, or concanavalin A (conA) for 72 hours and then analyzed cytokine production by ELISA. As mice were only given a single exposure of HDM, re-
stimulation with media or HDM yielded subtle responses. Re-stimulation with conA magnified responses and therefore, the following discussion details the conA re-stimulation results. For cytokines that are difficult to measure by ELISA, mRNA (extracted from whole lung cells before re-stimulation) expression levels were measured for comparison between the groups.

As expected, the critical Th2 cytokine IL-13 was induced with HDM treatment. However, levels of IL-13 were unaffected by IL-17A (Figure 4.5A). Il4 and Il5 message levels followed a similar pattern as IL-13 (not shown). These data indicate that at an early time-point, IL-17A neither inhibits nor exacerbates HDM-induced Th2 effector responses.

When the cytokines that are specifically involved in Treg and Th17 subset differentiation (TGFβ and IL-6) are analyzed, we see very different effects. The message levels of TGFβ1, a shared differentiation factor for Tregs and Th17 cells, was unaffected by HDM exposure with or without IL-17A (Figure 4.5B). IL-6 production, however, was induced by HDM and increased in the presence of IL-17A (Figure 4.5C). An environment with consistent levels of TGFβ and increased IL-6 is thought to be a Th17-promoting environment.

To determine the effects of IL-17A on IL-23, we compared p40, p35, and p19 between the groups, as p40 is a component of both IL-12 (when paired with p35) and IL-23 (when paired with p19). After HDM exposure, although neither p40 nor p35 were affected, message levels of p19 were increased (Figure 4.5D). Interestingly, with additional IL-17A treatment, p40 remained unchanged but the effects on p35 and p19 were opposing, with a decrease in p35 expression and an increase in p19 expression.
This pattern suggests that IL-17A induces IL-23 while inhibiting IL-12; however, a later time-point after IL-17A treatment may reveal definitive increases in IL-23. As yet, our current data is inconclusive.

Besides cytokines that are involved in Th17 and Treg differentiation and survival, we were also interested in Treg promoting and inhibiting cytokines as our previous study suggests that IL-17A negatively impacts T regulatory cells. IL-10, a Treg-promoting cytokine is unaffected by HDM exposure but, in the presence of IL-17A, IL-10 is significantly reduced (shown by both protein and mRNA levels, Figure 4.5E). In contrast, cytokines that antagonize Treg function and development, such as TNFα and IL-6, are both increased with IL-17A treatment, compared to HDM alone (Figure 4.5F).

Together, these data suggest that IL-17A can induce an environment that antagonizes T regulatory cells, by skewing differentiation factors toward Th17 cells and away from Tregs as well as inducing inflammatory cytokines that inhibit Treg function and development.

IL-17A treatment inhibits Foxp3 expression

To confirm our previous findings that IL-17A negatively impacts Tregs, reducing this population, we analyzed Tregs after IL-17A treatment. Using flow cytometry, we compared the effects of IL-17A on the frequency of Foxp3+ cells, as a percent of CD4+ cells. Compared to PBS-treated mice, there was a significant increase in the frequency of Foxp3+ cells after HDM exposure (Figure 4.6A). However, in the presence of IL-17A, this increase in the frequency of Foxp3+ cells is completely abrogated, suggesting that IL-17A counteracts the HDM-mediated increase of Foxp3+ cells found in the lung.
Message analysis revealed a more striking pattern, where Foxp3 message levels significantly increased after HDM exposure and significantly decreased with additional IL-17A treatment (Figure 4.6B). These data indicate that IL-17A, likely through indirect mechanisms, negatively impacts Tregs.

Conclusion

Our data suggest that IL-17A amplifies HDM-induced pulmonary mDC antigen uptake and co-stimulatory molecule expression. IL-17A enhanced HDM-induced pro-inflammatory cytokines (IL-6, TNFα), but decreased levels of the immunosuppressive cytokine IL-10. The shift in balance of these cytokines mediated by IL-17A may not only promote overall lung inflammation and Th17 differentiation, but we have shown that it antagonizes Tregs. This suggests that IL-17A may exacerbate AHR by increasing DC function and activation while impairing Tregs.
Figure 4.1 Gating strategy for DC analysis. C3H/HeJ mice were sensitized to PBS, HDM, or HDM and mouse rIL-17A as described in Materials and Methods. Mice were sacrificed 24 hours later and lungs were removed. Live (A) CD11c+ (B) cells were further classified as pDCs, alveolar macrophages, (C) neutrophils, or mDCs (D) by flow cytometry.
Figure 4.2 IL-17A treatment increases the frequencies of neutrophils and mDCs in the airways. C3H/HeJ mice were sensitized to PBS, HDM, or HDM and mouse rIL-17A as described in Materials and Methods. Mice were sacrificed 24 hours later and lungs were removed. PMNs, mDCs and pDCs were identified by flow cytometry. The frequency of PMNs (A) mDCs and pDCs (B) in the lungs of PBS (black bars), HDM (green bars), or HDM and IL-17A (red bars) treated mice was determined. Mean+SEM shown. n=4 mice for PBS and HDM groups, n=6 mice for HDM and IL-17A group. *, **, and *** indicate significant differences of p<0.05, p<0.01, and p<0.001, respectively.
Figure 4.3 IL-17A enhances mDC antigen-uptake. C3H/HeJ mice were sensitized to PBS, AF405-HDM, or AF405-HDM and mouse rIL-17A as described in Materials and Methods. Mice were sacrificed 24 hours later and lungs were removed. Antigen uptake of mDCs or pDCs isolated from PBS (gray histograms), AF405-HDM (green histograms), and AF405-HDM and IL-17A (red histograms) treated mice was determined by flow cytometry (A). Antigen burden (B) and frequency of HDM+ cells (C) of gated mDCs (left panel) and pDCs (right panel) were compared between PBS (black bars), HDM (green bars) and HDM and IL-17A (red bars) treated mice. Mean+SEM shown. n=4 mice for PBS and HDM groups, n=6 mice for HDM and IL-17A group. *, **, and *** indicate significant differences of p<0.05, p<0.01, and p<0.001, respectively.
Figure 4.4 IL-17A upregulates PD-L2 and MCHII expression on mDCs. C3H/HeJ mice were sensitized to PBS, AF405-HDM, or AF405-HDM and mouse rIL-17A as described in Materials and Methods. Mice were sacrificed 24 hours later and lungs were removed. Co-stimulatory molecule expression of MHCII and PD-L2 on mDCs isolated from PBS (black bars), HDM (green bars), and HDM and IL-17A (red bars) treated mice were analyzed. The frequency of MHCII+ and PD-L2+ cells (A), as well as the frequency of HDM+MHCII+ and HDM+, PD-L2+ cells (B), and the number of MHCII and PD-L2 molecules expressed (C) were determined on gated mDC. Mean+SEM shown. n=4 mice for PBS and HDM groups, n=6 mice for HDM and IL-17A group. *, **, and *** indicate significant differences of p<0.05, p<0.01, and p<0.001, respectively.
Figure 4.5 While IL-17A induces inflammatory cytokine production, it inhibits IL-10.
C3H/HeJ mice were sensitized to PBS, HDM, or HDM and mouse rIL-17A as described in Materials and Methods. Mice were sacrificed 24 hours later and lungs were removed. RNA was isolated from whole lung ex vivo and message levels measured by real-time PCR. Some samples of whole lung were cultured and re-stimulated in vitro with concanavalin A. 72 hours later, supernatants from re-stimulated cultures were harvested for cytokine analysis by ELISA. IL-13 protein (A), TGFβ1 message (B), IL-6 protein (C), IL-12p40 protein, IL-12p35 message, IL-23p19 message (D), IL-10 message and protein (E), and TNFα protein (F) levels were compared between PBS (black bars), HDM (green bars), and HDM and IL-17A (red bars) treated mice. Mean+SEM shown. n=4 mice for PBS and HDM groups, n=6 mice for HDM and IL-17A group. *, **, and *** indicate significant differences of p<0.05, p<0.01, and p<0.001, respectively.
Figure 4.6 IL-17A inhibits Foxp3 expression. C3H/HeJ mice were sensitized to PBS, HDM, or HDM and mouse rIL-17A as described in Materials and Methods. Mice were sacrificed 24 hours later and lungs were removed. The frequency of Foxp3+ cells, as a percent of CD4+ cells (A) was determined by flow cytometry. Foxp3 message levels (B) were determined by real-time PCR. Comparisons were made between the groups: PBS (black bars), HDM (green bars), and HDM and IL-17A (red bars) treated mice. Mean + SEM shown. n=4 mice for PBS and HDM groups, n=6 mice for HDM and IL-17A group. *, **, and *** indicate significant differences of p<0.05, p<0.01, and p<0.001, respectively.
Although asthma is classically characterized as a Th2-dominant disease, there is evidence that increased pulmonary expression of Th17-related cytokines is associated with airway hyperresponsiveness and severe asthma in humans [74, 77]. In animal models, there has been controversy as to the role of IL-17 in asthma, some researchers claiming that IL-17 is pathogenic [84, 86, 87] while others argue that it is protective [88].

In our lab, using a mouse model displaying moderate or severe asthmatic phenotype that mirrors human disease, Lajoie et al. [85] recently reported that in A/J mice, which are susceptible to the development of severe HDM-induced AHR, there was an association with a mixed Th2/Th17 phenotype. Conversely, in C3H/HeJ mice, which develop moderate AHR in response to HDM, there was abundant production of Th2 cytokines but little to no Th17 cytokines. This study, revealing that there is differential endogenous production of IL-17 after allergen exposure, indicates that some of the controversy over the role of IL-17 in asthma may be due to experimental design such as the strain of mouse used.

To explore the mechanisms for differential IL-17A production between the strains, we examined the cytokines produced by pulmonary DCs, as they are known to play a role in T cell subset differentiation. Pulmonary DCs isolated from A/J mice displayed enhanced antigen uptake, co-stimulatory molecule expression, and production of Th17-skewing cytokines, which are relative to DCs isolated from C3H/HeJ mice [95]. Collectively, these data indicate that after allergen exposure, DCs from A/J mice preferentially promote Th17 responses and therefore, enhance IL-17A production and subsequent severe AHR. However, despite evidence that IL-17A plays a pathogenic role in the asthmatic phenotype, little is known about the mechanisms by which IL-17A contributes to disease. Our preliminary studies indicate that although IL-
17A is consistently pathogenic throughout the allergic response, the mechanisms by which IL-17A contributes to disease vary at different times. Specifically, if present at challenge, IL-17A enhances IL-13 driven signaling [85] while the presence of IL-17A at allergen sensitization is associated with a reduction in Treg numbers (unpublished, Lajoie), potentially removing important Treg-mediated suppression of downstream immune responses. In this current study, in order to explore potential ways that IL-17A may impact T cell differentiation, we examined the effects of IL-17A on dendritic cells, important players in T cell development and differentiation. We have shown that IL-17A regulates the dendritic cell phenotype in such a way that drives a Th17 permissive environment while antagonizing T regulatory cells, setting up a positive feedback loop that further promotes Th17-driven immune responses.

In studies to determine whether IL-17A altered DC phenotype, we found that, indeed, IL-17A treatment enhanced DC activation, indicated by increased DC antigen uptake and MHC II expression, suggesting that it may enhance allergen-driven sensitization. As MHCII-mediated antigen presentation is considered the “first” signal for T cell activation, the increased antigen uptake and MHCII expression of IL-17A-treated DCs suggests that IL-17A may provide more opportunities for DCs to present antigen and therefore, induce robust downstream T cell responses. Interestingly, the upregulation of MHCII expression was only observed on pulmonary DCs isolated after in vivo treatment with HDM+IL-17A, but not on bone marrow-derived DCs exposed to HDM + IL-17A in vitro, indicating that increased MHCII expression on DCs was not due to direct interaction with IL-17A. In fact, IL-17A induced a cytokine environment both in vitro and in vivo with abundant TNFα but suppressed IL-10 levels, which have both...
been associated with MHC II induction [132, 133]. GM-CSF, a cytokine both added to BMDC \textit{in vitro} cultures and induced by IL-17A from epithelial cells, is also known to upregulate MHC II expression on dendritic cells [134]. However, because this cytokine environment was present in both \textit{in vitro} and \textit{in vivo} experiments it does not explain why MHC II was only affected \textit{in vivo}. As IFN\(_\gamma\) is most highly associated with inducing MHC II expression on multiple cell types [133, 135], perhaps, although IL-17A does not affect pulmonary IFN\(_\gamma\) production in this study, the absence of IFN\(_\gamma\) \textit{in vitro} was sufficient to limit TNF\(\alpha\)/GM-CSF-mediated MHC II upregulation.

In contrast to MHC II, increased PD-L2 expression after IL-17A treatment was observed in both \textit{in vitro} and \textit{in vivo} conditions. IL-17A may directly induce PD-L2 expression or indirectly by inducing a cytokine that upregulates PD-L2 expression. As GM-CSF was likely present in both the \textit{in vitro} and \textit{in vivo} studies, as described above, and is known to upregulate PD-L2 [125], IL-17A may enhance GM-CSF upregulation of PD-L2. Currently the role of PD-L2 in asthma is not well understood with some reports suggesting a pathogenic role [127] while others contend that it is protective [136, 137].

In our previous studies (Lewkowich, unpublished data) using antibody blockade of PD-L2 during antigen exposure, we showed that PD-L2 blockade resulted in reduced AHR, suggesting that PD-L2 plays a pathogenic role. We have evidence that PD-L2 inhibits DC production of the Th1-promoting cytokine IL-12 and thereby enhances the Th2 effector response. Interestingly, Tomita et al. have shown that monocytes isolated from severe asthmatics produce significantly less IL-12 after re-stimulation in comparison to those isolated from moderate asthmatics or non-asthmatics [138]. Furthermore, IL-12 was shown to antagonize IL-13-inducible genes (Lewkowich, unpublished data),
suggesting that IL-12 can inhibit Th2 effector responses. Perhaps, by promoting PD-L2 expression, IL-17A indirectly suppresses IL-12, allowing Th2 effector responses in severe asthma.

Aside from enhancing DC antigen-uptake and co-stimulatory molecule expression, IL-17A induced DCs to produce Treg antagonizing cytokines, IL-6 and TNFα. Although TGFβ levels remained unaffected, the additional IL-6 induced by IL-17A suggests a shift from a Treg-promoting environment to a Treg-inhibitory one. There have been a number of studies that revealed IL-6 as a DC-derived cytokine that can block Treg suppression of T effector cells [139, 140] but they did not identify a mechanism. Recently, though, studies focused on ascertaining the plasticity of Tregs and Th17 cells have shown that IL-6 can inhibit Foxp3 expression in both nTregs and iTregs and induce them to produce IL-17 [40]. Perhaps, IL-17A impairs the Treg population during sensitization by inducing IL-6, which effectively reprograms Tregs into Th17 cells. In addition to IL-6, IL-17A treatment also induced abundant production of TNFα in DCs. The role of TNFα as a cytokine that directly inhibits Treg function by down-modulating Foxp3 expression has been reported in both rheumatoid arthritis [141, 142] and asthma models [73]. By its ability to induce IL-6 and TNFα from multiple cell types in the lung environment [80, 143], including dendritic cells as we have shown here, IL-17A may be able to regulate Treg development and function.

In addition to antagonizing Treg development, the ability of IL-17A exposed DCs to produce elevated levels of IL-6, and diminished levels of IL-10 also directly promote the development of Th17 cells. This can occur through multiple mechanisms. In concert with TGFβ, IL-6 induces ROR, a transcription factor sufficient to drive IL-17
expression [36]. Additionally, IL-6 drives upregulation of IL-23R surface expression [144], promoting Th17 cells, as IL-23 is a critical Th17 maintenance and survival factor [36, 145, 146]. Interestingly, IL-10, a cytokine recently shown to inhibit IL-23 [85], was reduced after IL-17A treatment. Perhaps with IL-17A reducing IL-10 production, IL-10 inhibition of IL-23 may be removed. Our current data collected 24 hours after HDM exposure does not indicate that IL-17A treatment significantly impacts IL-23 production. Therefore, it is possible that only IL-10 is affected and that reduction of IL-10 is sufficient to shift the T cell responses. Alternatively, IL-17A treatment may lead to increased production of IL-23 but this effect may only be observable at a later time point.

Additionally, the design of this experiment, sacrifice only 24 hours after allergen exposure, was not conducive for analysis of Th subset differentiation and recruitment to the airways, which we would expect to occur later. We predict that in mice treated with HDM+IL-17A, there would be an increase in the number of Th17 cells in the airways as our data indicate that IL-17A induces a lung environment that promotes Th17 cells.

The unique DC cytokine pattern profile induced by IL-17A treatment seen in our study is consistent with results from human studies that demonstrate an association between increased IL-17A, IL-6, and TNFα and reduced IL-10 levels and severe asthma [138, 147, 148]. Indeed, there are many groups exploring anti-TNFα therapies as a way to treat severe disease [149, 150]. As anti-TNFα therapy has been shown restore function to nTregs isolated from allergic asthmatics, this makes the potential role of IL-17A as an initiator of abundant TNFα production all the more significant. Our data indicate that although allergen exposure alone can drive IL-6 and TNFα production, this effect is greatly enhanced by additional exposure to IL-17A. Conversely, IL-17A
treatment abrogated HDM-induced IL-10 production. Collectively, this evidence suggests that IL-17A may be present early in the airways after allergen exposure and initiate the cytokine profile associated with severe disease in humans.

Our current data suggests that the initial differences we saw between A/J and C3H/HeJ DCs [95] may arise from differences in early production of IL-17A in the lung of A/J mice after HDM exposure. Our result that, in the presence of IL-17A, dendritic cells develop a “pro-asthmatic” phenotype is consistent with the description of DCs from IL-17A-producing A/J mice [95]. As DCs isolated from A/J mice preferentially produced Th17-skewing cytokines, the effects of early IL-17A on DCs indicates that IL-17A drives a positive feedback loop, perpetuating its own activity and driving severe disease.

Although our evidence clearly indicates that IL-17A can regulate the dendritic cell phenotype (Figure 5.1), further studies are required to determine if IL-17A-mediated effects on DCs contribute significantly to disease. To determine the role of IL-17A-treated DCs in the development of allergic responses, we could create a mouse on the A/J background in which there is selective deletion of Il17ra in CD11c cells (“floxed” Il17ra). We would then measure allergen-induced AHR. Using this method, we would be able to determine the contribution of IL-17A signaling on DCs and its role in the development of severe disease. We predict that in susceptible A/J mice, which display a mixed Th2/Th17 response after allergen challenge, the loss of DC responses to IL-17A would significantly reduce AHR and Th17 responses.

If we find that IL-17A-treated DCs are critical for the development of severe disease, additional studies to determine which IL-17A-mediated effects on DCs are sufficient to drive disease would greatly add to our understanding of the pathogenic role
of IL-17A at allergen sensitization. There are multiple IL-17A-induced, DC-derived factors (PD-L2, IL-6, TNFα) that could contribute to enhanced AHR. A systematic approach using neutralizing antibodies against PD-L2, IL-6, or TNFα during allergen sensitization (in mice that typically produce IL-17A in response to allergen) could be used to address this question.

Traditionally, IL-17A has been classified as an effector molecule involved in the induction of robust inflammatory responses. However, our studies suggest that there is an early source of IL-17A that is critical for the development of severe disease. Recent evidence suggests that early IL-17A production may be regulated by anaphylatoxins. Lajoie et al. reported that excessive C3a activation promotes DC production of IL-23, leading to the expansion of IL-17A-producing Th17 cells driving severe AHR [85]. However, providing a counter-regulatory balance, the complement component C5a can suppress production of IL-23, preventing expansion of IL-17A-producing Th17 cells and thereby, limiting the development of severe disease [85]. This reciprocal regulation of IL-23 responses is dependent upon IL-10, an inhibitor of IL-23, as C3a inhibits and C5a promotes production of this cytokine [85]. Interestingly, asthmatic triggers such as allergens, cigarette smoke, ozone, and infection have been shown to induce IL-17A production [87, 95, 151, 152] and these same triggers have been shown to exacerbate AHR in a manner dependent on the complement component C3a [153-157]. Therefore, after exposure to a variety of stimuli, C3a can become activated and induce Th17 responses leading to the development of severe asthmatic phenotype. Collectively, this suggests that an individual's ability to mount a Th17 response following exposure to environmental stimuli may be due to the balance of C3a/C5a. Further studies should be
done to determine if the early release of IL-17A is dependent on C3. Comparing early IL-17A production in the lungs of C3-sufficient and C3-deficient mice after allergen exposure would help elucidate the relationship between C3 activation and early IL-17A release.

In support of this hypothesis, there is evidence from human studies that individuals with severe asthma may have a genetic predisposition that shifts the balance towards C3a and away from C5a. Polymorphisms in C3 and C3a1 are associated with the development of asthma in humans [158, 159], whereas, polymorphisms in C5 are associated with protection from asthma in humans [159]. Therefore, in severe asthmatics, there may be preferential production/and or activation of C3 over C5 after exposure to environmental stimuli, shifting the IL-23/IL-10 axis toward IL-23 and predisposing towards enhanced early IL-17A production. Once produced, IL-17A can then induce pro-inflammatory cytokine production from the lung environment that may condition DCs to produce a cytokine profile promoting further IL-17 cytokine production and suppression of Treg differentiation and/or activation. Additionally, as IL-17A has been shown to directly induce production of C3 from pulmonary epithelial cells, its presence can in turn enhance local C3 levels, supporting IL-23 synthesis and further expanding Th17 cells, thereby perpetuating its own production [85]. Collectively, these studies suggest that early release of IL-17A in susceptible individuals may be due to exposure to various environmental triggers and a shift in the balance between C3a and C5a production.

Although our data suggests that there is an early source of IL-17A in the airways after allergen exposure in susceptible mice, the actual source is unknown. Aside from
Th17 cells, a number of cell types that are “early responders” have been identified to produce IL-17A such as \( \gamma \delta \) T cells, NKT cells, alveolar macrophages, neutrophils, or even naturally-occurring Th17 cells [160-164]. There is evidence that NKT and \( \gamma \delta \) T cells produce IL-17 in an IL-23 dependent manner [165, 166]. Knowing that C3a and C5a differentially regulate the IL-10/IL-23 axis, it is possible that the balance between C3a and C5a may determine the early release of IL-17A after exposure to previously listed stimuli. As our current studies suggest that there is an early source of IL-17A that may be important in promoting robust Th17 and Th2 responses, identification of the cellular source of IL-17A may be helpful in the development of treatments for severe asthma. The development of IL-17A reporter mice that express GFP under the IL-17A promoter would be a useful tool to explore early IL-17A sources in the airways after allergen challenge. Using flow cytometry, IL-17A producing cells could be isolated and then identified by back-gating.

In summary, we conclude that, in susceptible individuals, there is an early source of IL-17A produced after allergen sensitization that alters dendritic cell phenotype and results in cytokine release that inhibits regulatory T cells, promotes lung inflammation, and creates a Th17-favorable milieu, which have been shown to be correlated with disease severity. Expanding the traditional view of IL-17A as an effector molecule, our studies indicate that IL-17A acts as an initial factor that can regulate the nature and intensity of the downstream immune response by modulating dendritic cells. Excessive production of IL-17A at disease onset may be due to dysregulated complement pathways or other additional genetic and environmental factors. Our findings that IL-17A can initiate a positive feedback loop involving C3 that changes the balance
between regulatory and inflammatory responses may underlie the pathogenic role of IL-17A. Better understanding of the genetic and environmental triggers of C3 and early IL-17A production as well as the mechanisms that perpetuate their activity may inform future therapies in chronic inflammatory disease in humans.
Figure 5.1 The effects of IL-17A on dendritic cells.
References


