ST2L Negatively Regulates Therapeutic TLR9 Activation in Asthma

by

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To my family with love

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Asthma is a major cause of morbidity worldwide and this disease continues to increase in severity and prevalence, especially in the developed world. Clinical studies involving CpG-ODN therapy in asthmatics have identified that CpG-ODN drives a Th1 response, but does not inhibit airway hyperresponsiveness (AHR) in clinical asthma. In a murine fungal model of asthma, TLR9 plays a critical role in antifungal responses and is necessary for effective CpG-ODN therapy of asthma. Our experimental investigations have demonstrated that CpG-ODN administration promotes elevations in IL-12 levels, but no decrease in AHR in fungal asthma. These findings have provided a model in which to explore the mechanisms that inhibit CpG-ODN therapy from having efficacy for asthma. The IL-1R family protein ST2L is the receptor for IL-33. ST2L is present on immune and non-immune cells particularly during Th2-mediated disease. Recently, ST2L was identified as a negative regulator of MyD88-mediated TLR signaling. We have observed through immunohistochemical staining that ST2L progressively increases in the lung in asthmatic C57BL/6 mice along with the levels of TLR9. Herein, we have investigated whether blockade of ST2L enhances the therapeutic properties of CpG-ODN in a chronic fungal model of asthma. In our asthma model, C57BL/6 mice are sensitized to soluble *A. fumigatus* antigens and challenged with live *A. fumigatus* conidia. We investigated our model of fungal asthma first in TLR9$^{+/+}$ and TLR9$^{-/-}$ mice followed by site directed therapy through either the intranasal (IN) or systemic intraperitoneal (IP)

route from days 14 to 28-post conidia challenge. Additionally, mice were treated with an antibody targeting ST2L, IP CpG, IgG control, or combinations of the antibodies with CpG from days 14 to 28-post conidia challenge. Airway responses were attenuated in mice treated with α-ST2L+CpG, but not α-ST2L alone or CpG alone. *In vitro* studies with bone marrow derived DCs showed that targeting ST2L with CpG therapy decreased pSTAT3 levels in these cells, while increasing IL-12p70 levels. Together these data demonstrate that systemic TLR9 activation can reverse all features of chronic fungal asthma when delivered concomitantly with an anti-ST2L antibody. These data suggest that ST2L blockade enhances CpG-mediated therapeutic effects in asthma.

CHAPTER 1

INTRODUCTION

Asthma

Asthma is a disease of the airways that manifests as a complex syndrome with many clinical phenotypes in both adults and children. Asthma is characterized by airflow obstruction, bronchial hyperreactivity, and airway inflammation. Asthmatic disease begins for many patients in infancy, and both genetic factors (atopy) (1, 2) and environmental factors (viruses, allergens, and occupational exposures) (3-5) contribute to the initiation and progression of disease. Inflammation is a core component of asthmatic disease and in order to comprehend the pathogenetic mechanisms underlying the many variants of asthma, it is essential to identify factors that initiate, intensify, and regulate the inflammatory response of the airway.

The association of asthma with allergies has been known for some time, however, until recently the mechanism of this association was unknown. Recent investigations into the mechanisms behind asthmatic disease have helped to define the pathways behind allergic airway inflammation in animal models and relate it to the clinical manifestations of asthma. These studies have allowed for a greater understanding of therapeutic targets in the treatment of asthma and the role of immune responses in the pathogenesis and progression of allergic airway disease (6).

Immunopathology of Asthma

Inflammation was first identified as a component of asthma at autopsy in patients with fatal asthma. Airways in these individuals showed neutrophil and eosinophil infiltration, mast cell degranulation, sub-basement-membrane thickening, loss of epithelial-cell integrity, and mucus occlusion of the bronchial lumen. Hyperplasia and hypertrophy of bronchial smooth muscle and hyperplasia of goblet cells were also observed. These findings were considered to be characteristic of fatal asthma, but not necessarily of other forms of the disease (6).

Substantial inflammation has also been observed in bronchial-biopsy specimens from patients with asthma. Patients with mild variants of the disease have also been observed to exhibit lung inflammation (7). These inflammatory changes can occur throughout the central and peripheral airways and often vary depending on disease severity (8-11). Shedding of the airway epithelium, collagen deposition below the basement membrane, mast-cell degranulation, and lymphocytes and eosinophil infiltrates in the airway are frequent characteristics observed in the inflamed lungs of asthmatics. Infiltrating cells in the airway are often activated, and are thus primed to release mediators of asthma such as cytokines, histamine, and leukotrienes into the airway, creating and exacerbating the symptoms of asthma (6).

The presence of cytokines and chemokines from inflammatory cells in the lung that mediate inflammation and cellular chemotaxis in bronchoalveolar-lavage (BAL) fluid or pulmonary secretions are characteristic of asthmatic disease (12). Some cytokines elicit inflammatory responses by activating transcription factors, proteins that bind to the promoter region of genes and initiate transcription of pro-inflammatory genes.

Transcription factors involved in asthmatic inflammation include nuclear factor-κB (NF-κB), activator protein-1 (AP-1), nuclear factor of activated T cells (NFAT), cyclic AMP response-element binding protein (CREB), and various members of the family of signal transduction-activated transcription (STAT) family of factors. These transcription factors act on genes that encode inflammatory cytokines, chemokines, adhesion molecules, and other proteins that induce and perpetuate inflammation. Corticosteroids which are frequently prescribed for use by asthma patients act by downregulating the action of transcription factors in order to reduce inflammation (13).

Asthma and Allergic Inflammation

Allergic inflammatory processes in asthma are initiated with the recognition of allergens by cells of the innate immune system such as dendritic cells. Epidemiologic and clinical observations have linked IgE antibodies to the severity of asthma (14) and the initial and sustained responses of the airway to allergens. In order to initiate the synthesis of IgE, inhaled allergens must encounter dendritic cells that line the airway. These dendritic cells then migrate to draining lymph nodes, where they present processed antigen to T and B cells (15). Interactions among these cells elicit responses that are influenced by cytokines and the presence or absence of costimulatory molecules. For a switch to the synthesis of IgE, the first signal is delivered by interleukin-4 (IL-4) or interleukin-13 (IL-13), cytokines that are associated with what are known as Th2 type responses, when these cytokines bind to receptors on B cells **(Fig. 1.1)**. The receptors for interleukin-4 and interleukin-13 share a common chain and use the same signal-transduction pathway involving the transcription factor STAT-6 (16). The second signal

is delivered when CD40 on B cells binds to its ligand on T cells. Additional interactions between other ligand and receptor pairs (between CD28 and B7 and between ICAM-1) can complement or up-regulate the T cell–dependent activation of B cells that follows the binding of CD40 to its ligand (17).

Once synthesized and released by B cells, IgE antibodies briefly circulate in the blood before binding to high-affinity IgE receptors (FcεRI) on the surface of mast cells in tissue or peripheral-blood basophils **(Fig. 1.1)**, and low-affinity IgE receptors (FcεRII, or CD23) on the surface of lymphocytes, eosinophils, platelets, and macrophages. Whether the binding of IgE to its low-affinity receptors activates cells and contributes to inflammation is not yet known. Soluble FcεRII receptors, however, appear to be important in regulating IgE synthesis. Molecular bridging of FcεRI receptors, which occurs when allergen interacts with receptor-bound IgE molecules, causes activation of the cell and the release of preformed and newly generated mediators. Basophils and mast cells that are important in allergic inflammation and asthma can also secrete the cytokines IL-4 and IL-13 and express CD40L, however, IgE cross-linking does not occur by allergen on these cells, and these cells most likely amplify rather than induce the synthesis of IgE (6, 17).

In allergic asthma, genetically susceptible atopic individuals mount a chronic Th2 cell-type of immune response to allergens like house-dust mites (HDMs), fungi, plant pollen, and animal dander. These responses are measured clinically by the presence of a serum IgE response or a positive skin-prick test. Bronchial biopsy studies in human asthmatics have revealed a predominant eosinophilic airway inflammation is accompanied by the accumulation of helper T cells at the airway wall. $CD4^+$ T cells in

human asthmatics as well as in mouse models of asthma secrete IL-4, IL-5, IL-13, and tumor-necrosis factor α (TNF-α), cytokines consistent with the presence of inflammatory T helper 2 (Th2) cells (18). Investigations in mouse models with blocking antibodies or transgenic modeling have shown that these cytokines individually can produce many of the major features of asthma such as IgE synthesis (IL-4 acting on B cells), airway eosinophilia (IL-5 acting on bone marrow progenitors), goblet cell metaplasia (IL-4 and IL-13 acting on airway epithelia), and airway hyperreactivity (AHR) (IL-13 acting on bronchial smooth muscle cells) (16). Differences between asthma in animal models and that in human patients has led to the identification of several other subsets of Th cells that could play a significant role in asthma pathogenesis (19). IL-9 has been observed in mouse asthma models and in humans and is strongly associated with mast cell accumulation in the airways, as well as AHR and mucus cell metaplasia. It still remains to be established whether IL-9 derives from a dedicated Th9 cell (20). Closely related to the Th9 cell subset, some patients with severe airway obstruction may also have a Th17 cell component to their asthma (21, 22). Regulatory cell populations such as allergen-specific Treg cells that can downregulate allergic inflammation through instruction by TGF-β and/or IL-10 have also been observed in a many asthmatic patients (23).

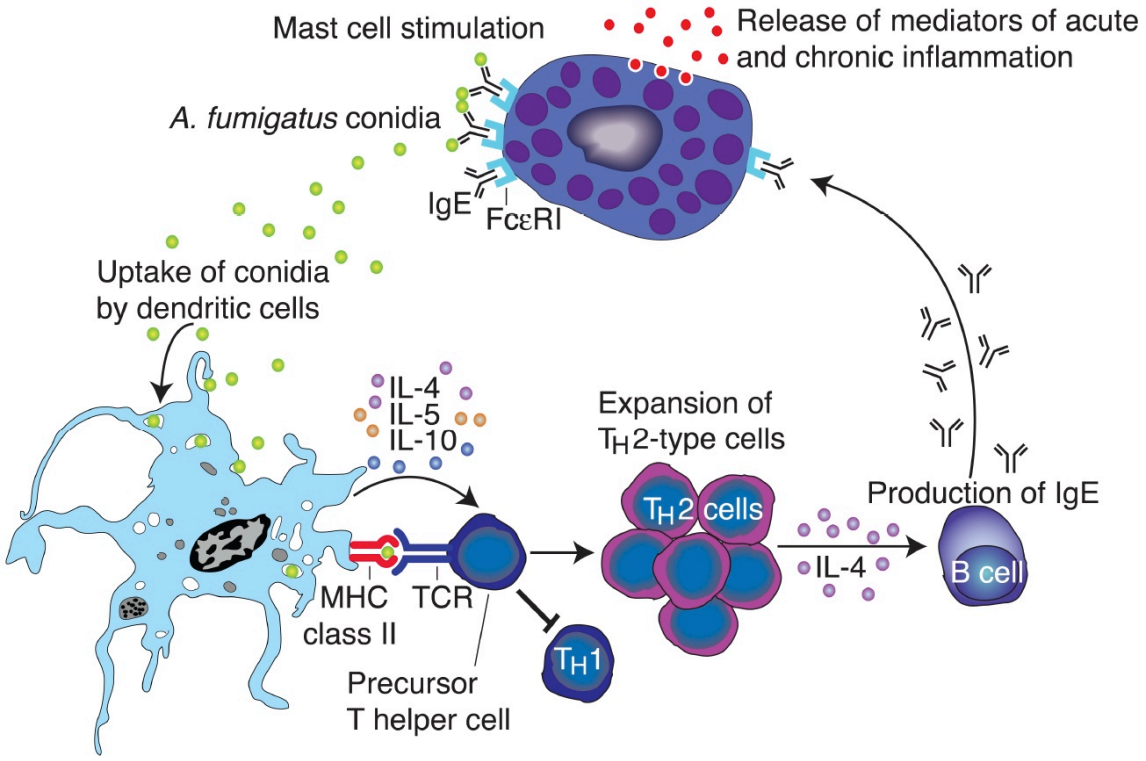

Figure 1.1. ***Cellular signaling associated with the pathogenesis of allergic asthma.*** Dendritic cell recognition of *A. fumigatus* conidia leads to dendritic cell activation and presentation of fungal components on MHC II receptors to T cells via the T cell receptor (TCR). Atopic individuals with a predisposition to develop asthma exhibit elevated CD4+ Th2 cells in circulation, whereas normal individuals exhibit predominantly Th1 cells that act in a protective manner against allergic airway disease. Th2 cells secrete IL-4, which promotes B cell production of IgE, an antibody elevated in the serum of asthmatics. IgE can promote activation of other cell types such as mast cells, which release mediators promoting acute and chronic inflammation upon cross linking of FcεRI receptors on their surface post IgE binding of allergen epitopes.

Dendritic Cells and Asthma

Dendritic cell initiated cytokine synthesis occurs in response to recognition of allergens by these cells. Recognition of factors such as respiratory virus infection, fungal conidia, exposure to airborne pollutants (tobacco smoke, diesel particles, and ozone), or physical stimuli (exercise and cold air) can modify or exacerbate asthmatic disease (24). Asthma is therefore more accurately described as a disorder of the adaptive immune response in response to pattern recognition by the resident or structural and innate immune cells such as bronchial epithelial cells, mast cells and basophils, natural killer (NK) cells, NKT cells, and dendritic cells. The lung network of dendritic cells (DCs) found throughout the conducting airways, lung interstitium, lung vasculature, pleura, and bronchial lymph nodes perform a sentinel function in the pulmonary immune response **(Fig. 1.2)** (25). DCs recognize inhaled antigens through expression of evolutionarily ancient pattern-recognition receptors such as Toll-like receptors (TLRs), NOD-like receptors (NLRs), and C-type lectin receptors that can recognize pathogen-associated molecular patterns (PAMPs) present on virtually any inhaled pathogen, allergen, or substance (22). Lung DCs also express receptors for inflammatory mediators (so-called "alarmin" type proteins such as HMGB1, and IL-33) that are released upon damage to the tissues by pathogens, trauma, vascular damage, or necrosis (26, 27). By expressing all these direct and indirect sensors for danger in the airways, and at the same time possessing the ability to migrate to the regional lung-draining lymph nodes and process antigens, DCs form a core component of innate and adaptive immunity in the lung (28).

Lung DCs were originally characterized in the mouse as a single cell population with a high degree of expression of CD11c and MHCII (29), but today these cells have

been differentiated into at least five different subsets with differing functions in the lungs (25). Lung dendritic cells originate from the bone marrow and traffic to the lungs as immature cells that are activated and mature in response to pathogen or allergen exposure in the lung **(Fig. 1.3)**. The mouse lung is grossly divided into large conducting airways and lung interstitium containing alveolar septa and capillaries where gas exchange is taking place (30). In steady-state conditions, the conducting airways are lined with an intraepithelial network of $MHCII^{hi}CD11c^{hi}$ cells that are mostly $CD11b^{-}$. In the mouse and rat these cells also express langerin and the mucosal integrin CD103 ($\alpha_E\beta_7$). This cell population has the ability to extend dendrites into the airway lumen through formation of tight junctions with bronchial epithelial cells (31-34). The lamina propria below the epithelium of the conducting airways contains $MHCII^{hi}CD11c^{hi}$ cells that highly express CD11b and are a rich source of proinflammatory chemokines (34, 35). The $CD11b^{+}CD103^{-}$ subset of these cells also express the SIRPα molecule, a binding partner to CD47 involved in DC migration (36). A similar broad division into $CD11b^{+}$ and $CD11b^{-}$ can also be applied to lung interstitial DCs obtained by enzymatic digestion of peripheral distal lung (31, 37). Both $CD11b^{+}$ and $CD11b^{-}$ subsets express high amounts of CD11c, and are known as conventional DCs (cDCs). Another population of cells that are $CD11c^{int}$ are the plasmacytoid DCs (pDCs) and these cells also express Siglec-H and BST1, among other markers (31, 38, 39). The alveolar spaces also contain $CD11c^{hi}MHCII^{hi}$ DCs and are easily collected through bronchoalveolar lavage (BAL) of the airways. Lung alveolar macrophages are another cell type that also expresses high amounts of CD11c, but lacks CD11b expression (28). Under inflammatory conditions, such as viral infection or fungal allergen challenge, there is recruitment of additional

subsets of $CD11b^+$ monocyte-derived DCs that rapidly upregulate CD11c and retain expression of Ly6C and are easily confused with resident lung $CD11b^+$ cDCs (31, 40, 41).

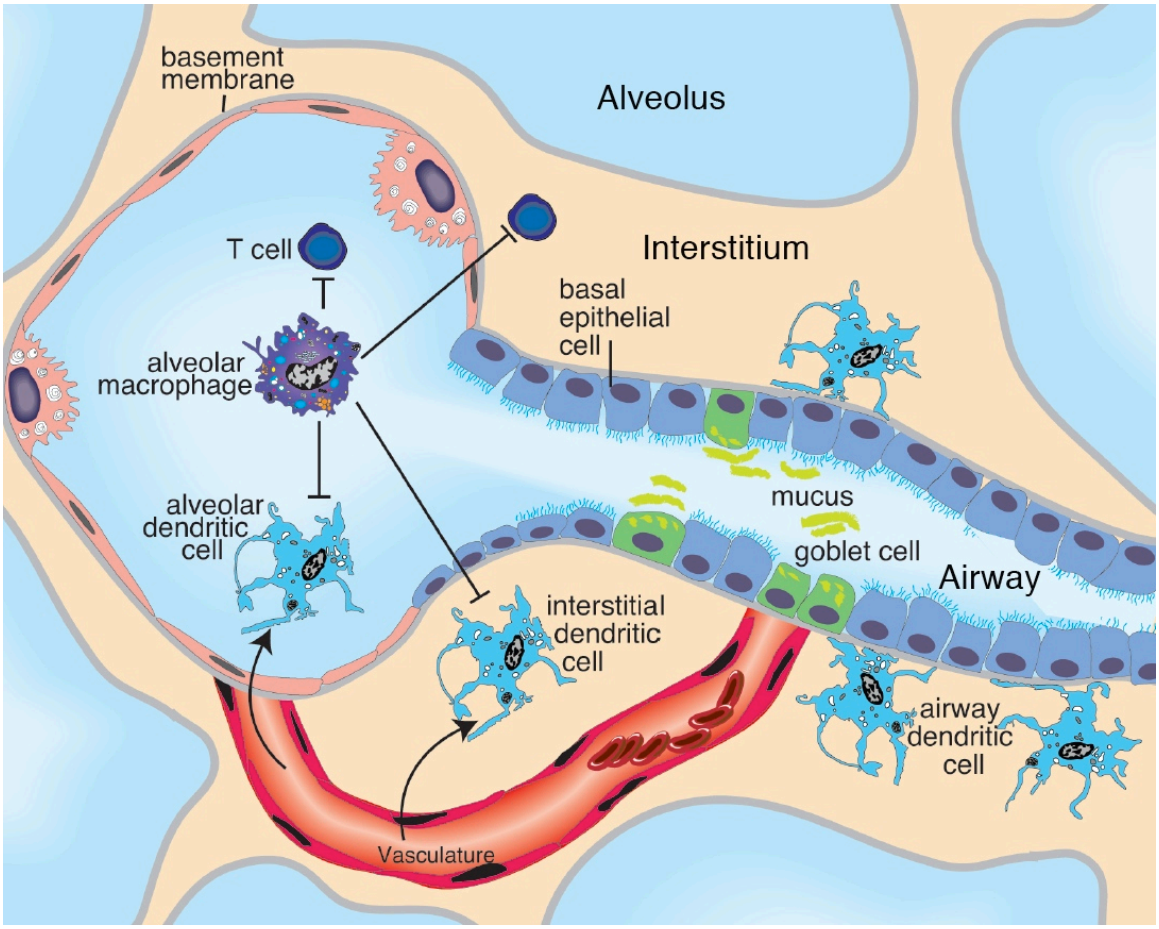

Figure 1.2. ***Dendritic cells in the lung.*** Dendritic cells (DC) within the lung environment include airway DC, interstitial DC, and alveolar DC. All of these cell types are able to recognize pathogens through the use of pathogen recognition receptors such as toll-like receptors and initiate adaptive immune responses to allergens upon activation of T cells in the lung draining lymph nodes.

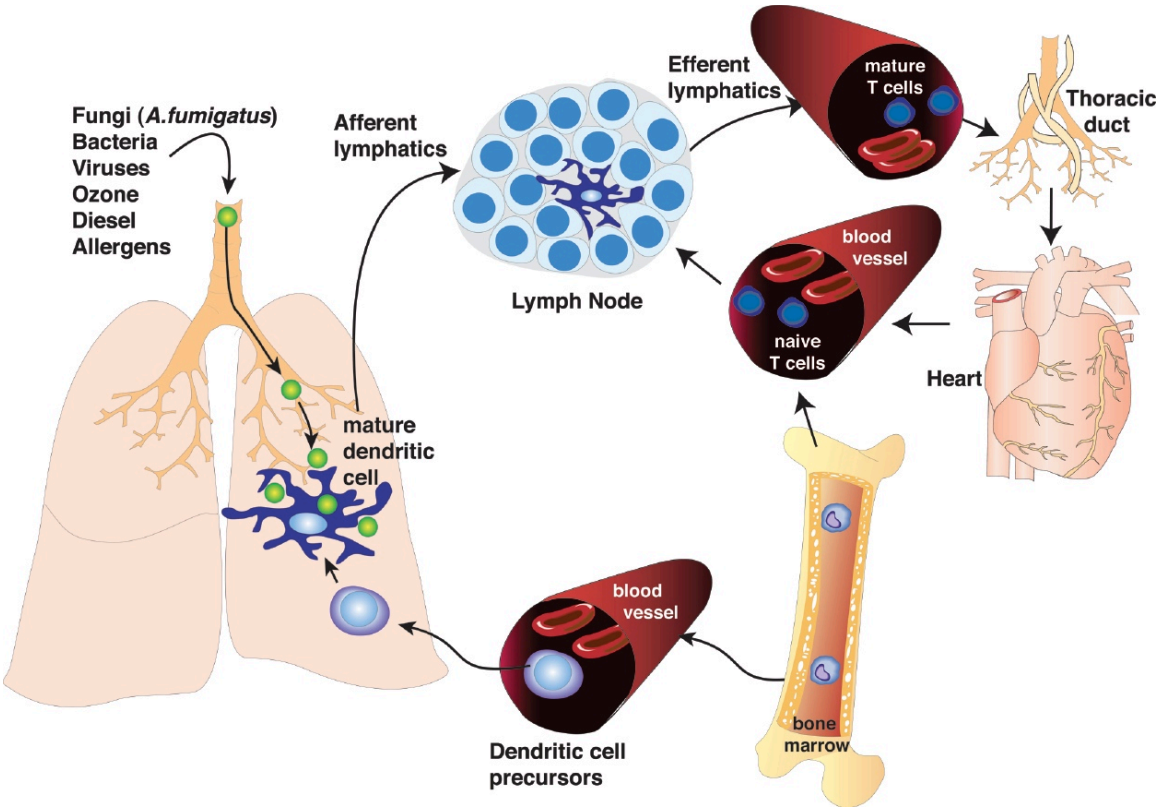

Figure 1.3. ***Origin of lung dendritic cells.*** Dendritic cells within the lung environment originate from the bone marrow, like all immune cells, and traffic to the lung as immature cells. Exposure to allergens such as *A. fumigatus* activates dendritic cells and allows them to traffic to the local lung draining lymph nodes where they can activate T cells based upon the signals they were exposed to in the lung.

Allergic Sensitization and the Epithelial-DC interaction

Most clinically relevant allergens have the potential to modify epithelial barrier function or activate airway epithelial cells or innate and adaptive immune cells, like DCs and basophils (42). For example, proteases from fungi such as *Aspergillus* have either proteolytic activity or enhance TLR responsiveness, explaining why *Aspergillus* conidia fungal proteases act as both allergens and a Th2 cell adjuvant (43, 44). Proteases increase the permeability of the bronchial epithelium by cleaving the tight junctional proteins claudin and occludin, thus gaining access to the DC network (45). Along the same line, TLR4 signaling is also involved in recognition of allergens such as house dust mites (HDM), which also express proteases such as Der p1 (46). It has also been shown that epithelial TLR4-driven responses activate Th2 cell immunity to HDM allergen recognition, through release of innate pro-Th2 cytokines, like GM-CSF, IL-33, TSLP, and IL-25 (42). The TLR-, C-type lectin-, or proteolytic-mediated activation of epithelial cells by *Aspergillus* or HDM can lead to release of these innate cytokines or other mediators that subsequently program DCs to become Th2 cell inducers (42, 47). GM-CSF promotes DC maturation and breaks inhalation tolerance, and previous studies have demonstrated that HDM-driven asthma is neutralized by blocking GM-CSF (48). IL-33 is made by epithelial cells, boosts Th2 cytokine production, and promotes goblet cell hyperplasia. Recently, IL-33 was shown to promote Th2 cell differentiation by programming the function of DCs (49).

Epithelial-DC interactions might also be necessary for preventing overt Th2 cell responses. It was recently shown in an *in vitro* study that monocyte-derived DCs that were differentiated in the presence of a bronchial epithelial cell line were instructed to

produce IL-12, IL-6, IL-10, and TNF-α, thus promoting Th1 cell responses, away from potentially harmful Th2 cell responses through epithelial-derived type I interferon production (49). These findings suggest that epithelial cells modulate local DC differentiation to optimize antimicrobial defenses in the airways and in the process downmodulate capacity for expression of potentially damaging Th2 cell mediated immunity. A defective innate immune response of epithelial cells that impacts DC differentiation or activation may therefore be at the core of allergic sensitization.

The Th1 and Th2 Cell Imbalance and the Pathogenesis of Asthma

The increasing prevalence of asthma in industrialized nations has led to the development of the "hygiene hypothesis" (50, 51). This hypothesis proposes that the immune system of the newborn infant is naturally skewed toward Th2 cells and relies on environmental signals to produce a balanced immune response. Factors that enhance Th1-mediated responses and that are associated with a reduced incidence of allergy, asthma, or both include infection with Mycobacterium tuberculosis, measles virus, and hepatitis A virus (52-54); increased exposure to infections through contact with older siblings (50); attendance at a day-care facility during the first six months of life (55); and a reduction in the production of interferon-γ as a result of decreased exposure to environmental endotoxin or to polymorphisms of the major endotoxin receptor (CD14) that diminish the response to endotoxin (56). Restoration of the balance between Th1 cells and Th2 cells may be hindered by alterations in gastrointestinal flora due to frequent administration of oral antibiotics (51). It has also been suggested that transplacental

transfer of allergens and cytokines may also play a role in the development of allergic asthma (57).

The relevance to asthma of allergic sensitization is supported by the evolution of the disease in later childhood. Many children with asthma have positive skin-prick tests to extracts of protein from house-dust mites, cockroaches, pets (especially cat dander), and the fungi conidia (4, 58-60). In 6-year-old children with asthma, sensitization to spores from the fungus *Alternaria* was associated with a significantly reduced frequency of remission of asthma by the age of 11 years (9% in those who were sensitized vs. 39% in those who were not sensitized) (4). Thus, it appears that the genetic background plays a role in the generation of a cytokine imbalance that promotes the formation of IgE and that allergens in the local environment determine the specificity of the antibody response (61). Sensitization to certain allergens, such as cockroach and fungal allergens, has also been associated with an increased risk of asthma-related morbidity (59), respiratory arrest during exacerbations of asthma (62), and the development of asthma (4, 60).

Therapy of Asthma

In the past decade, the treatment of asthma has emphasized long-term suppression of airway inflammation plus relief of symptoms with quick-acting bronchodilators such as beta-agonists. Inhaled corticosteroids are the most effective agents available for the symptomatic control of asthma and improvement in pulmonary function, but their potential side effects when used in higher doses have led to the use of adjunctive therapies (63-65). Concomitant treatment with long-acting beta-agonists, theophylline, and leukotriene antagonists have all been shown to help control asthma while minimizing the doses of inhaled corticosteroids that are needed (66, 67).

Corticosteroids whether used alone or in combination with other therapies do not consistently alleviate airway inflammation in patients with asthma (68). For this reason, other approaches that modulate IgE-associated immunologically mediated inflammatory responses are in use or are under development. DNA vaccines such as therapeutic CpG-ODN that target the Toll-like receptors and similar methods of down-regulating antigen-specific Th2-mediated responses are currently an active area of research (69, 70). The efficacy of these therapies highlights the important contribution of allergic inflammatory mechanisms in the pathophysiology of asthma in many patients and provides a basis for targeting these mechanisms therapeutically.

Aspergillus Fumigatus

The genus *Aspergillus* comprises a few hundred species of fungus sharing a common asexual spore forming structure, the aspergillum (71). *Aspergillus fumigatus* (*A. fumigatus*), one member of this genus, is a ubiquitously present saprophytic fungus in the environment. Innocuous in immunocompetent individuals, *A. fumigatus* is a known causative agent for a number of diseases, from allergic airway diseases such as allergic bronchopulmonary aspergillosis (ABPA) to invasive aspergillosis (IA), an often-fatal infection of the lung commonly observed in immunocompromised patients. *A. fumigatus* is only a small fraction of airborne fungus and most individuals inhale thousands of fungal conidia each day with no visible effect. *Aspergillus*, however, is responsible for the majority of clinical fungal complications (72, 73). For example, in a 1990 study of hospital air, it was found that *A. fumigatus* made up 0.3% of all fungal isolates in the air,

but was discovered in 44% of patients (74, 75). Thus, *A. fumigatus* is a significant pathogen and opportunistic mold that is in need of further clinical and basic research.

Reproduction and Growth

A. fumigatus is normally isolated in soil samples and plays a vital role in recycling carbon and nitrogen from decaying matter (76). *A. fumigatus* is an asexually reproducing haploid organism that begins its life cycle as a spore (conidia), which germinates to form hyphae that extend into a conidiophore structure (77). The conidiophore contains a single layer of cells, known as phialide, from which individual conidia bud following contraction of the phialide (78, 79). Thousands of conidia, 2-3μm in diameter, are released from every conidiophore during sporulation. Most individuals inhale thousands of such conidia daily from their environment with no visible effect. Some recent studies have provided evidence that *A. fumigatus* has the ability to reproduce sexually, however the main form of reproduction appears to be through the asexual route (77, 80). The recent decoding and analysis of the entire genome of *A. fumigatus* (81) has helped to provide further insights into the lifecycle of this fungus and its associated pathogenicity.

Temperature, stress, and nutrient availability regulate the transition of *A. fumigatus* conidia into its invasive hyphal form (73). *A. fumigatus* is a relatively thermotolerant fungus, adding to its pathogenic potential. Conidial growth can occur from below 22°C to 55°C, while conidia can survive temperatures as high as 70°C (78, 82). Environmental stress also contributes to *A. fumigatus* growth. When the resting conidia of *A. fumigatus* are placed in an appropriate milieu and given nutrients and water, they begin the process of germination (83). The conidia undergo a period of isotropic

growth before switching to a polar growth program and producing a germ tube (84, 85). At the beginning of the process of germination, conidia swell and produce increased β-glucan moieties on their surface. It is also during this process of transition from resting to swollen conidia that *A. fumigatus* switches from no oxygen consumption to a respiration process involving oxygen (86). Nutrient uptake and availability also contribute to *A. fumigatus* growth. The protein *rhbA* is for example plays a key role in nitrogen sensing and utilization and is implicated in *Aspergillus* virulence (87). Further understanding of the growth requirements of *A. fumigatus* may provide new insights into fungal survival and virulence, thus offering new treatment strategies for *A. fumigatus*-related diseases.

Aspergillus Associated Diseases

A. fumigatus is responsible for a wide range of diseases that can be categorized as allergic, saprophytic, or invasive. Allergic diseases include *Aspergillus* sinusitis, allergic bronchopulmonary aspergillosis (ABPA), severe asthma with fungal sensitization (SAFS), and hypersensitivity pneumonias (88-90). ABPA is a disease of particular clinical interest as it features type I, II, and IV hypersensitivity reactions, and because it predominately affects asthmatic and cystic fibrosis patients, occurring in 2% and 35% of patients, respectively (91, 92). Saprophytic aspergillosis, characterized by the formation of an aspergilloma or "fungal ball", is usually observed in patients with a pre-existing lung conditions, such as sarcoidosis and tuberculosis, diseases that generate space for *A. fumigatus* to grow (82, 93, 94). Aspergillomas that form in the saprophytic form of *Aspergillus* infection are spherical masses of fungal material, consisting of hyphae

surrounded by reproductive fungal units. Disruption of aspergillomas in the lung can lead to blood in the mucus and internal bleeding as the blood vessel surrounding the aspergilloma are disrupted (95, 96). The third form of *Aspergillus* induced disease is invasive aspergillosis, which includes airway invasive aspergillosis and chronic necrotizing apergillosis (88). The common therapy for *Aspergillus* infection are antifungals such as amphotericin B and voriconizole, but in several instances individual drugs alone do not kill the fungus and combination therapies must be used to effectively treat infection (97). There are several strains of *Aspergillus* that are resistant to antifungal therapy, thus further research into *Aspergillus* infection is necessary to understand the evolving nature of *Aspergillus* fungi (98). The range and complexity of *Aspergillus* associated disease necessitates further study of the fungus in order to enhance therapeutic strategies for such disorders.

Immunity to Aspergillus fumigatus

The innate immune response in immunocompetent individuals is usually able to prevent growth and infection from inhaled *Aspergillus* conidia. The adaptive immune response to the fungus is characterized by a protective Th1-response, leading to the production of associated pro-inflammatory cytokines, which promote fungal clearance. The innate immune response to *A. fumigatus* occurs in three steps: exposure, recognition, and resolution. Different innate immune responses are required to recognize the fungus depending on whether it is in the resting, swollen, germinated, or hyphal stage of growth. Recognition of *A. fumigatus* is dependent on pattern recognition receptors (PRRs) such as soluble receptors, Toll-Like Receptors (TLRs), and surface receptors such as dectin-1

that recognize fungal β-glucans (99). Clearance of fungus and resolution of infection occurs by phagocytosis of fungal material and the production of antimicrobial products within cells.

Alveolar macrophages first recognize inhaled conidia, and are able to phagocytose and kill conidia using reactive oxygen species (100, 101). Conidia phagocytosed by macrophages swell as they germinate leading to the presentation of fungal β-glucans and activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase production in macrophages (99, 100, 102). NADPH converts oxygen into superoxide anion and downstream reactive oxidant intermediates (100). These reactive oxygen species can lead to the production of acids that can kill conidia in the phagolysosome following the fusion of the phagosome with the endosome (103). Corticosteroids commonly used as therapeutics in asthma patients inhibit macrophage killing of conidia, leading to rapid germination and hyphal growth (100, 101).

Myeloid dendritic cells (DCs) are another population of innate immune cells that produce strong, anti-fungal responses. DCs have been identified to clear *A. fumigatus* conidia and hyphae (104). DC maturation post recognition of conidia and hyphae leads to the production of pro-inflammatory cytokines such as TNF-α, IL-12, IL-6, and IL-10 (105-107). DC recognition of fungal components through the innate immune system leads to the activation of the adaptive immune response to further control and clear the fungus. One process through which this occurs is through DC activation of fungal-reactive, IFN-γ producing Th1-cells post conidia recognition (104).

Molecular Mechanisms of Recognition of Aspergillus fumigatus

Several pathogen recognition receptors, including Toll-Like Receptors (TLRs), are associated with fungal recognition and immune activation. TLRs 2, 4, and 9 have been shown to recognize conidia and hyphal components (108-111). TLR2 and TLR4 expression on human neutrophils, monocytes, and macrophages have been shown to produce antifungal responses. Studies involving mice lacking the TLR adaptor protein, MyD88 demonstrate reduced survival following conidia challenge. TLR2 deficient mice, however, have been shown to be the exception to this as they are not more susceptible than wild-type mice. TLR2 has been suggested to provide a protective effect against germinated conidia. $TLR2^{-/-}$ mice have also been shown to secrete significantly less TNF-α, IL-12, and MIP-2α during conidial challenge (112). TLR4 has been shown to play a critical role in antifungal immune responses. Cyclophosphamide immunosuppressed mice deficient in TLR4 signaling significantly decreased survival following conidial challenge when compared to wild-type mice (113). Recent investigations have also identified that hypomethylated CpG DNA is present in *Aspergillus* that can potentially activate TLR9 (111).

TLR-independent modes of *A. fumigatus* recognition also play significant roles in fungal recognition. The dectin-1 and the dendritic cell marker DC-SIGN both play important roles in fungal recognition (99, 114-116). Dectin-1 is a receptor known to bind β-(1,3) glucan, polysaccharide-derived moieties present on the surface of germinating conidia and hyphae, but not on resting conidia (114, 117). Activation of dectin-1 receptor leads to the production of pro-inflammatory cytokines, chemokines, and reactive oxygen intermediates (114, 118). Dectin-1 is a critical element in fungal recognition, as dectin-1-deficient mice have been found to have significantly higher mortality after

fungal challenge. This effect has been attributed to the substantial decrease in pro-inflammatory cytokine production during conidial challenge found in these mice when compared to wild-type mice (115). DC-SIGN, a type II C-type lectin found on macrophages and myeloid DCs, has been shown to bind conidia and mediate phagocytosis (116). Our initial set of studies observed a link between TLR9 signaling and dectin-1 recognition of *Aspergillus* conidia in the swollen state.

Toll-like Receptor 9 Biology

The key feature of innate immune cells that enables them to detect and categorize infection are pattern-recognition receptors (PRRs), which bind certain general types of molecules that are expressed across broad classes of pathogens, but which are absent or restricted in some way in vertebrates (119). Toll-like receptors (TLRs) are the best understood of the PRRs, of which 13 are known in humans (120). TLRs that are specific for molecules characteristic of extracellular pathogens, such as lipopolysaccharides or lipopeptides, are expressed at the cell surface, whereas TLRs that detect intracellular pathogens are expressed within innate immune cells and are specific for nucleic acids. For example, TLR9 detects unmethylated CpG dinucleotides, which are relatively common in the genomes of most bacteria and DNA viruses, but which are suppressed and methylated in vertebrate genomes. TLR9 is endosomally localized and this allows efficient detection of invading viral nucleic acids, while preventing inadvertent stimulation by CpG motifs within self DNA **(Fig. 1.4)** (121).

Different immune cells express distinct subsets of the TLRs, which likely enables the immune system to tailor its responses against different pathogen classes (120).

Among resting human immune cells TLR9 is expressed primarily or exclusively in B cells and plasmacytoid dendritic cells (pDC), a specialized type of dendritic cell that produces most of the type I interferons (IFN) that are made in response to viral and intracellular pathogens (122). Some studies have also reported functional TLR9 expression in activated but not in resting human neutrophils and pulmonary epithelial cells (123-125), but the biological significance of this TLR9 expression is unknown.

Targeted immune activation by CpG ODN

Most types of immune cells do not express TLR9, and so are not activated directly by CpG DNA. All of the cellular immune effects of CpG ODN in humans are thought to result directly and indirectly from activating TLR9-expressing pDC and B cells. Plasmacytoid DC activated through TLR9 secrete IFN-α, which drives the migration and clustering of pDC in the marginal zone and outer T-cell areas of the lymph node, where they are better able to stimulate adaptive immune responses (126). TLR9-stimulated B cells and pDC show increased expression of co-stimulatory molecules, resistance to apoptosis, upregulation of the chemokine (C-C motif) receptor CCR7, and secretion of T_H1-promoting chemokines and cytokines such as monocyte inflammatory protein-1 (MIP1), IFN-γ-inducible 10-kDa protein (IP10) and other IFN-inducible genes (127). Co-activation of naive, germinal centre or memory B cells through the B-cell-antigen receptor and TLR9 induces their differentiation into plasma cells (128); for memory B cells, activation through TLR9 alone is sufficient to drive differentiation to plasma cells (129, 130). The B-cell-stimulatory effect of TLR9 activation is so great that the efficiency of hybridoma generation from purified primary human memory B cells is

improved from 1–2% without CpG to 30–100% with the addition of a CpG ODN (131). Although CpG-induced plasma cell differentiation does not require T-cell help, its efficiency is enhanced by interactions with pDC, together with B-cell receptor crosslinking (132). The net effect of TLR9 activation is to induce T_H1-biased cellular and humoral effector functions of innate and adaptive immunity **(Fig. 1.5)**.

TLR9 is an intracellular protein and stimulation of immune cells by CpG ODN requires internalization (133). ODN internalization occurs spontaneously in culture without the need for uptake enhancers or transfection, is temperature- and energy-dependent, and appears to be relatively sequence-independent. The earliest steps in the CpG-induced signal transduction pathways can be blocked by inhibitors of endosomal acidification/maturation (134-137) or by inhibitors of phosphatidylinositol 3-kinase, which seems to have a role in ODN internalization (138). Following internalization into an endosomal compartment, CpG motifs are bound and recognized by TLR9, leading to the rapid recruitment and activation of the adaptor molecules MyD88, interleukin-1 receptor-associated kinase-1 (IRAK1), interferon regulatory factor-7 (IRF7), and tumor-necrosis factor-α receptor activated factor-6 (TRAF6) (135, 139-143). This process results in the rapid activation of several mitogen-activated protein kinases, including extracellular receptor kinase (ERK), p38, and Jun N-terminal kinase, as well as the IκB complex, and these pathways converge on the nucleus to alter gene transcription through transcription factors such as NF-κB and AP-1 **(Fig. 1.4)** (144-151).

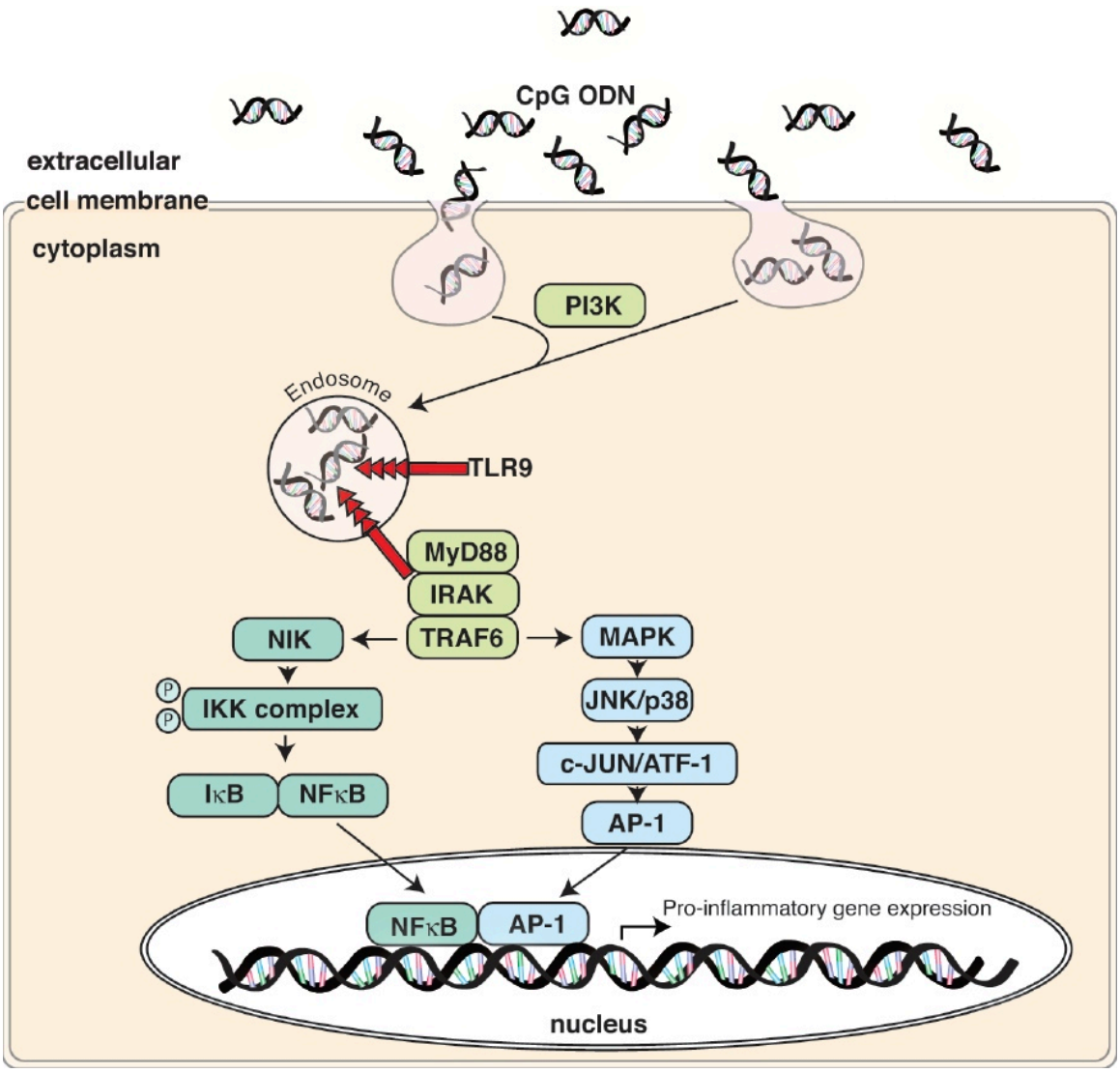

Figure 1.4. ***Toll-like Receptor 9 biology.*** Antigen Presenting Cells like dendritic cells express Toll-like Receptor 9 (TLR9) internally on endosomes. TLR9 signals through a MyD88-dependent pathway upon recognition of its ligand, hypomethylated DNA containing repeats of cytosine and guanine residues termed CpG repeats (CpG-ODN). TLR9 signaling occurs through TRAF6, the IKK complex and NF-κB, ultimately resulting in pro-inflammatory gene expression.

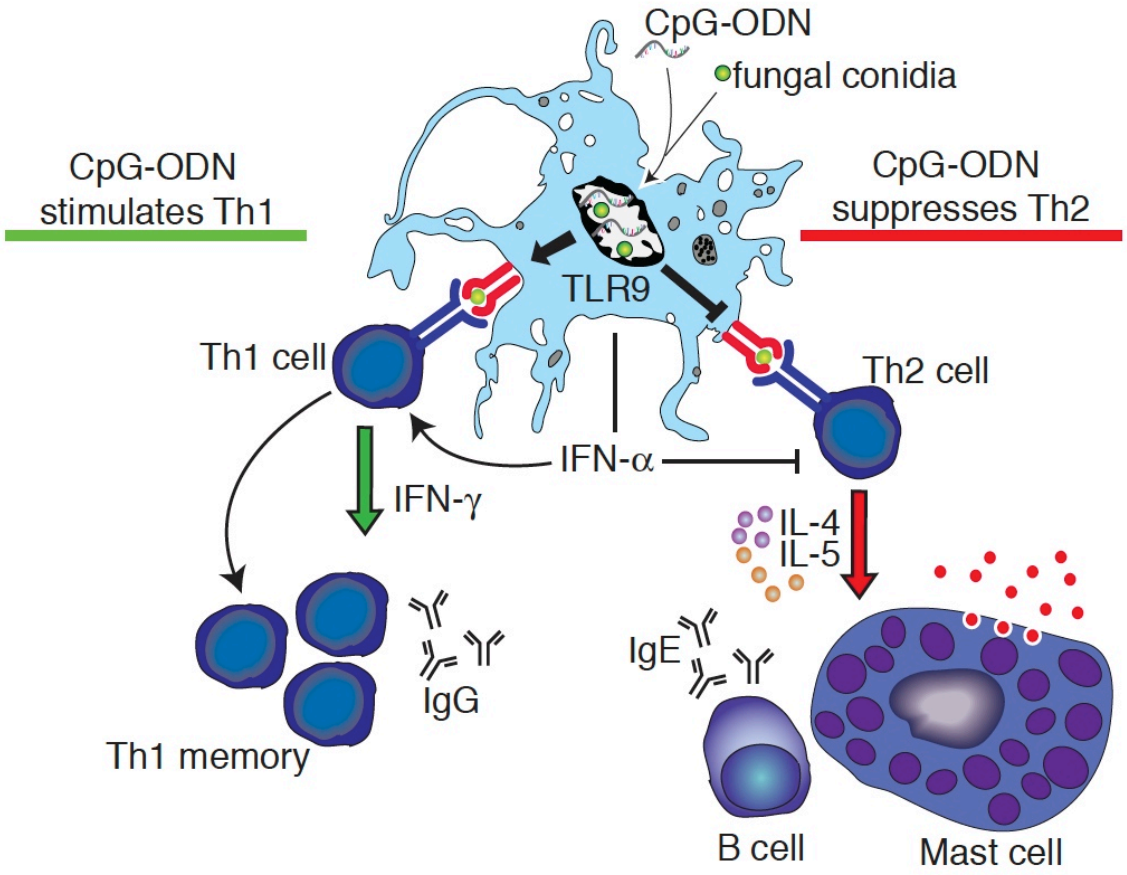

Figure 1.5. ***Intended effects of TLR9 activation on immune responses.*** Activation of TLR9 on APCs such as dendritic cells by CpG-ODN leads the production of factors such as IFN-γ that bias the T cell response toward Th1 immunity. The intended effect of therapy with CpG-ODN is to shift the immune response away from Th2 responses associated with the pathogenesis of asthma and toward Th1 responses that can promote resolution of inflammation.

IL-33 and ST2 Biology

ST2 (also called IL1RL1, DER4, T1 and FIT-1) is a member of the Toll-like/IL-1-receptor superfamily. Members of this superfamily are defined by a common intracellular domain, called the Toll/Interleukin-1 receptor (TIR) domain. This domain is approximately 160 amino acids in length and is composed of a central five-stranded β-sheet surrounded by five α-helices located on the cytosolic end of the protein (152). The Toll-like/IL-1-receptor superfamily can be divided into three subfamilies based on their extracellular domains: the IL-1 receptor-like subfamily, the Toll receptor subfamily and a family comprised of their adaptor proteins. The IL-1 receptor-like subfamily is characterized by three linked immunoglobulin motifs on the extracellular domain of the protein. Members of this family include the type I and II IL-1Rs (IL-1R1 and IL-1R2), the IL-18R, their accessory proteins IL-1RAcP and IL-18RAcP, and ST2, among others. The Toll receptor subfamily is characterized by extracellular leucine-rich repeat motifs and is represented by the TLRs, one of which is TLR9 (153, 154). Thus, there is some structural similarity between ST2 and TLR9.

A family of five adaptor molecules comprised of myeloid differentiation factor 88 (MyD88), MyD88-adaptor-like protein (MAL, also known as TIRAP), TIR-domain-containing adaptor protein inducing IFN-β (TRIF, also known as TICAM1), TRIF-related adaptor molecule (TRAM, also known as TICAM2) and sterile α- and armadillo-motif containing protein (SARM) has also been identified. These proteins act as intracellular signaling molecules for proteins in this family, and the interaction between these

intracellular proteins, the TLRs (specifically TLR9) and ST2 represents a major theme behind the work presented here.

Isoforms

The gene for ST2 is approximately 40 kb on human chromosome 2q12, and is part of the larger human IL-1 gene cluster of approximately 200 kb (Genbank accession number AC007248). ST2 is conserved across species, with homologues in the genomes of mouse (*Mus musculus* chromosome 1), rat (*Rattus norvegicus* chromosome 9) and fruitfly (homologues to the *Drosophila melanogaster* Toll protein). ST2 was first discovered in 1989 by two independent laboratories working with growth-stimulated mouse 3T3 fibroblasts who described a 2.7 kb transcript encoding a ~37 kD unglycosylated secreted protein corresponding to a 60–70 kD glycosylated product, which today is known as the soluble form of ST2, sST2 (155-159). A 5 kb transcript related to the earlier identified protein was identified in 1993 with a putative transmembrane motif. The protein product of this transcript is today known as transmembrane receptor ST2L (160). The soluble sST2 and the transmembrane ST2L forms arise from a dual promoter system that drives differential mRNA expression (161-163). sST2 lacks the transmembrane and cytoplasmic domains contained within the structure of ST2L and includes a unique nine amino-acid C-terminal sequence (164). ST2L, the membrane bound form of ST2, is the focus of the third and most recent component of our studies in how it can regulate TLR9 signaling.

Two additional isoforms of ST2 have thus far been identified: ST2V and ST2LV. The ST2 proteins are similar to the structure of the type I IL-1 receptors which are

comprised of an extracellular domain of three linked immunoglobulin-like motifs, a transmembrane segment and a TIR cytoplasmic domain. ST2V and ST2LV are two splice variants of ST2. Loss of the third immunoglobulin motif and alternative splicing in the C-terminal portion of ST2, resulting in a unique hydrophobic tail, produces ST2V (165), whereas alternative splicing, leading to deletion of the transmembrane domain of ST2L, produces ST2LV (166).

Expression and tissue localization

The earliest expression of ST2 in mice is in fetal liver tissue with restricted expression in haematopoietic organs in the adult. More detailed investigation revealed that ST2L is restricted to the surface of Th2 and mast cells, but that it is not expressed by Th1 or other immune cells (167-169). ST2L may therefore serve as a marker for effector Th2 cells (170).

sST2 expression is inducible and appears to be restricted to integument (including fibroblasts), retinal, mammary and osteogenic tissues (162, 171). Mouse dermal tissue can be stimulated to express sST2 after exposure to ultraviolet radiation or the proinflammatory cytokines tumor necrosis factor (TNF), IL-1α and IL-1β. Constitutive sST2 expression has been suggested to be more ubiquitous in humans (172).

Experiments with human tissue have revealed that the splice variant ST2V is expressed predominantly in gastrointestinal organs including stomach, large and small intestine, and spleen. Its expression is notably absent from liver and cardiac tissues, and confocal microscopy in cells transfected with ST2V suggests restricted localization at the plasma membrane (173).

ST2LV was recently discovered in a search for a chick (*Gallus gallus*) ST2 homologue. This isoform seems to be expressed during the latter half of embryogenesis in cerebral, ocular, cardiac and pulmonary tissue, and its expression declines relative to the robust expression in ocular tissue in the adult (166). The cellular localization, as well as the expression and tissue localization, of ST2LV in other species is not yet known.

IL-33, the ligand for ST2L, was only recently identified. A β-trefoil fold protein sequence derived by superposition of IL-1 and fibroblast growth factor (FGF) protein structures was used to mine the public genomic database, which lead to the discovery of a novel member of the IL-1 family from a dog cDNA library. *In vitro* translation of this protein and treatment with caspase-1 yielded a processed protein of 18 kD that activated the ST2 receptor (174). This protein was named IL-33 and now has been classified as a member of the IL-1 interleukin family, whose members are characterized by an array of 12 β-strands (the IL-1/FGF β-trefoil fold) and the absence of a classical secretory N-terminus peptide sequence.

The IL-33 protein had previously been isolated in a search for the ST2 ligand, in quiescent 3T3 cells that were precipitated by an ST2-Fc fusion protein. These molecules were approximately 32 kD and 18 kD in mass, which probably represented the uncleaved and mature IL-33 proteins, respectively (175). In addition, in 2003, as part of a search for transcripts unique to high endothelial venules, a gene was identified with a sequence that localized to human chromosome 9p24.1. Antibodies raised against this peptide sequence precipitated a 30 kD protein (176). The sequence of this protein was later confirmed to be identical to that identified by computational methods (177).

IL-33 contains a putative DNA-binding domain (176) and is localized in the nucleus, most notably within heterochromatin subdomains and mitotic chromosomes. The N-terminus of IL-33 contains an evolutionarily conserved homeodomain-like helix–turn–helix (HTH) DNA binding domain, which is necessary and sufficient for nuclear targeting and is involved in the repression of transcription that has been ascribed to IL-33 (178). Precursor IL-33 may require caspase cleavage to yield a mature protein capable of nuclear targeting (179). The existence of a cytokine as an extracellular ligand as well as an intranuclear effector is not unprecedented; a similar function has previously been noted for IL-1α and HMBG1 (180). Thus, IL-33 might have intracellular functions that are independent of binding to the ST2 receptor.

IL-33/ ST2 signaling

The mode by which IL-33 exerts its effect has not been fully established but it probably acts similarly to other members of the IL-1 family, specifically IL-1β and IL-18. It has been proposed that upon synthesis, precursor IL-33 enters specialized secretory lysosomes. Caspase-1-dependent cleavage of pro-IL-33, lysosomal navigation and fusion with the cell plasma membrane may result in the release of IL-33 into the interstitium as an active cytokine (174, 181). IL-33 could then interact with its receptor on a target cell membrane to affect downstream signaling and/or be transported to the target cell nucleus, where it could act as a DNA binding factor. However, although *in vitro* evidence of caspase-1 cleavage has been published (174, 179), this has yet to be definitively established *in vivo* (177). A caspase-1 cleavage sequence within the primary IL-33 protein structure is not conserved across all species (177) and definitive localization of

pro-IL-33 within lysosomal structures has not yet been reported. The heterochromatin binding of IL-33 resembles the biology of IL-1α more closely than other members of the IL-1 family and, as has recently been suggested for pro-IL-1α, it is possible that caspase-1 acts as a secretory targeting factor for pro-IL-33. IL-33 might also act in an autocrine fashion as well as a secreted paracrine or endocrine effector, but active secretion of IL-33 from cells has also not yet been documented.

Features of the Toll-like receptor/IL-1-receptor system that are specific to IL-33 signaling have recently been clarified. IL-33 appears to bind a receptor complex composed of ST2L and IL-1RAcP. The affinity of IL-33 for ST2L is enhanced in the presence of IL-1RAcP, and mast cells from IL-1RAcP-deficient mice failed to release IL-6 upon IL-33 exposure (182). In agreement with this finding, IL-1RAcP-null mice exposed to IL-33 failed to mount the typical cellular hyperplasia and inflammatory-cell infiltrate that is seen in wild type counterparts (183). Additionally, the inability of D6/76 E4 cells to respond to IL-33, can be rescued by IL-1RAcP transfection, which leads to downstream NF-κB activation (184).

Events downstream of IL-33 stimulation may include phosphorylation of extracellular signal-regulated kinase (ERK) 1/2, p38 MAPK, JNKs and activation of NF-κB (174). TRAF6 appears to be required for IL-33-mediated NF-κB activation and downstream induction of Th2 cytokines, however, it has been suggested that IL-33-mediated ERK activation might be TRAF6 independent (185). In multiple models, activation of ST2L results in AP-1 activation independent of NF-κB (186), and activation of NF-κB in HEK293 cells can be inhibited by prior ST2L transfection. Thus, ST2L has been identified to exert an anti-NF-κB effect by sequestering MyD88 and MAL **(Fig. 1.6)**

(187). In basophils, cytokine release upon exposure to IL-33 requires ST2L and MyD88 (188). These observations have indicated that IL-33 may function as a negative modulator of NF-κB and TLR/IL-1-receptor signaling.

Role of IL-33/ST2 in disease

IL-33 was originally described as a modulator of inflammation, tipping the balance towards CD4+ Th2 mediated immune responses. IL-33 may serve as a chemotactic factor for Th2 cells (189) and induce the production of the Th2-associated interleukins IL-4, IL-5 and IL-13 (190). Mice lacking the gene for IL-4, the prototypical Th2 cytokine, still express ST2L on the surface of mature Th2 cells (191), but the ability to mount a Th2-cytokine response to an antigen is reduced in these cells compared with wild-type controls (192). Mice with targeted deletion of ST2 develop normally and exhibit normal Th2-cell maturation, but display altered Th2-mediated responses in an antigen-specific fashion. They are able to mount an IL-4 and IgE response to infection with the helminthic parasite *Nippostrongylus brasiliensis* that is equivalent to their wild-type counterparts but do not form pulmonary granulomas in response to intravenous injection of *Schistosoma mansoni* eggs (193, 194). Taken together, these data suggest that ST2L and IL-4 production could serve as markers for distinct subpopulations of Th2 cells or as parallel complimentary systems, each sufficient but not necessary for the initiation of Th2 immune responses to antigen (195).

The observation that ST2L is a cell surface marker as well as an effector molecule in the regulation of Th2 cell function is consistent with subsequent studies demonstrating

a role for IL-33/ST2 in diseases associated with a Th2 response such as asthma, rheumatoid arthritis, collagen vascular diseases and pleural malignancy.

It has long been held that T-cells have a central role in the pulmonary response to allergen (195). Specifically, evidence points to a primary contribution from Th2 cells (196). Mice lacking both IL-4 and IL-5 exhibit airway hyper-responsiveness following exposure to aerosolized ovalbumin, implicating an IL-4/IL-5 independent process (197). This process may involve the IL-33/ST2 system as most, though not all, studies examining ST2 suggest that it is required for antigen-induced airway inflammation (193, 198-200).

ST2-null mice deficient in functional ST2 have been generated and studied (193). At 20 weeks of age, the mice are both healthy and fertile. Both mast cells and splenic CD4+ cells in these mice are skewed towards the Th2 lineage by *in vitro* exposure to IL-4 and anti-INFγ antibody. The resulting Th2-developing cells produced equivalent amounts of IL-4 as cells from their wild-type mice. Pre-immunized ST2-null and wild-type animals exposed to aerosolized ovalbumin displayed a quantitatively equivalent IgE and eosinophil response. Pulmonary histology from ST2-null mice display widespread inflammation. By contrast, using similar models of pulmonary inflammation, several studies have suggested a critical role of sST2 in Th2 cell function. Intravenous gene transfer of murine sST2 (201) or administration of an immunoglobulin against IL-33 have been observed to result in significant attenuation of airway inflammation in response to aerosolized ovalbumin challenge (202). The reason for the discrepancies between studies utilizing antibody-based IL-33 inhibition versus genetic deletion of ST2 is unclear. Genetic deletion of ST2 should result in the loss of all ST2 isoforms unlike selective loss

of sST2 function with an antibody-based approach. It is possible that genetic deletion of ST2 resulted in compensatory induction of other Th2 activating pathways. Furthermore, the use of an antibody against IL-33 may exert non-specific effects that might not have been initially appreciated. However, the totality of data suggests that the IL-33/ST2 system is involved in Th2-mediated immune responses in the lung.

There is evidence that IL-33 plays a role in mast cell activation. Exposure of human or murine mast cells to IL-33 results in the secretion of various interleukins and chemokines (203, 204). Exposure of mice to exogenous IL-33 also results in airway hyperresponsiveness and airway goblet-cell hyperplasia in a lymphocyte-independent manner (205). Direct exposure to IL-33 results in epithelial hypertrophy and mucus accumulation in bronchial structures (174). In keeping with the proposed decoy function ascribed to sST2, pre-exposure to sST2 results in reduced production of Th2 cytokines in a mouse model of allergen-induced pulmonary inflammation (198). Serum levels of sST2 are elevated in patients who suffer from an acute exacerbation of bronchial asthma compared with healthy controls (206). Serum sST2 levels are also increased in both serum and BAL samples from patients with acute eosinophilic pneumonia (207). Our third set of studies investigate the role of the IL-33-ST2 axis in a fungal model of asthma and the implications this pathway has on TLR9 signaling.

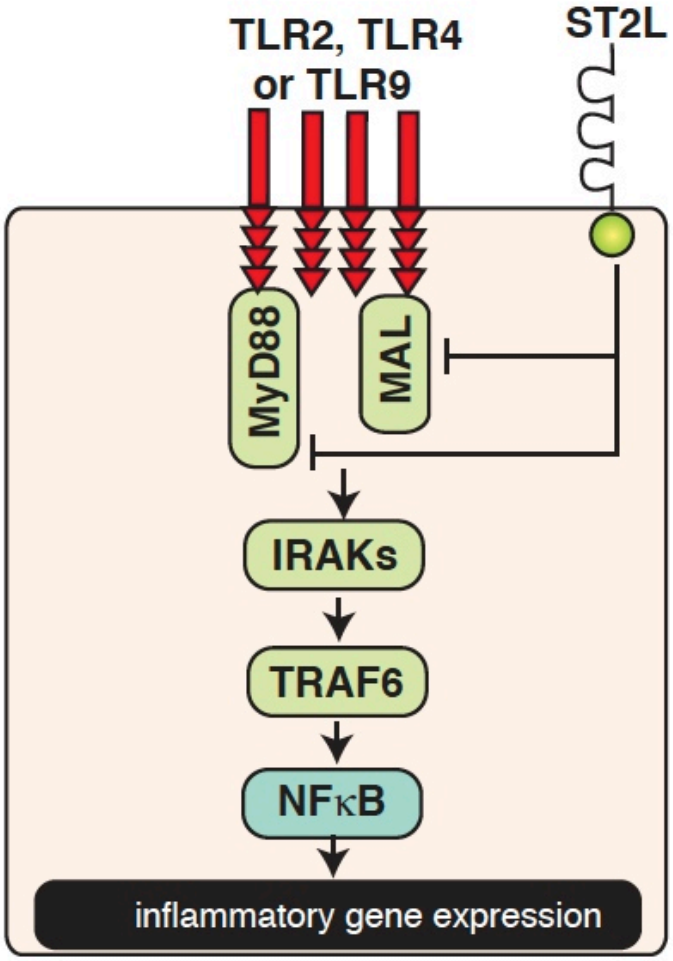

Figure 1.6. ***ST2L inhibition of TLR9 through MyD88 sequestration.*** ST2L inhibition of TLR9 signaling occurs through the sequestration of MyD88 upon IL-33 binding and activation of ST2L, preventing MyD88 interaction with TLR9. This inhibitory action of ST2L acts to prevent TLR9 activation of NF-κB and pro-inflammatory gene expression.

STAT3 Biology

The STAT protein family consists of seven members, which are encoded by distinct genes (208-210). One distinguishing feature of the proteins encoded by these genes is that they both transduce signals through the cytoplasm and function as transcription factors in the nucleus (211-213). STATs were originally discovered through their capacity to mediate signaling from IFN and IL-6 receptors following engagement with their respective cytokines (209, 211-218). Cytokine receptors are usually activated by receptor-associated tyrosine kinases, most prominently the Janus kinase (JAK) family kinases (JAK1, JAK2, JAK3 and TYK2) (208, 209, 211, 212, 219). Following phosphorylation of specific tyrosine residues in STAT proteins, they form stable homodimers or heterodimers with other STAT proteins through reciprocal phosphotyrosine–SRC homology 2 (SH2) domain interactions. Each STAT family protein responds to a defined set of cytokines, and each also regulates, with other transcription factors and/or cofactors, a group of specific genes. Importantly, many of the downstream target genes of STATs encode cytokines and growth factors, the receptors of which signal through the same STATs, thereby providing a mechanism for autocrine and paracrine STAT activation. In the case of STAT3, IL-6 signaling through GP130 (also known as IL6ST)–JAK, STAT3 is phosphorylated. This is followed by homodimerization and translocation to the nucleus where it binds to STAT-binding elements in the promoters of various IL-10-inducible genes, including suppressor of cytokine signaling 3 (SOCS3) and Bcl3 (220, 221).

STAT3 deletion is embryogenically lethal, and myeloid cell-specific STAT3-deficient mice develop severe enterocolitis (221, 222). STAT3 deletion in bone marrow

cells leads to overly activated innate immune responses and interferes with the adaptive immune system by inhibiting the induction of antigen-specific T-cell tolerance (223, 224). STAT3 gene inactivation also leads to an aggressive and fatal form of enterocolitis mediated by IL-12 (225). In the nucleus STAT3–STAT3 homodimers modulate the expression of genes encoding T_H1-skewing cytokines such as IL-12 and other mediators crucial for the classic physiological acute phase response and cancer-promoting inflammatory conditions **(Fig. 1.7)** (214, 226, 227). Our third set of studies investigates the role of STAT3 in regulating inflammation through its action inhibiting NF-κB binding to the IL-12 promoter. We investigate the role the protein plays in the regulation of inflammation during therapeutic blockade of ST2L preventing binding of IL-33 in dendritic cells.

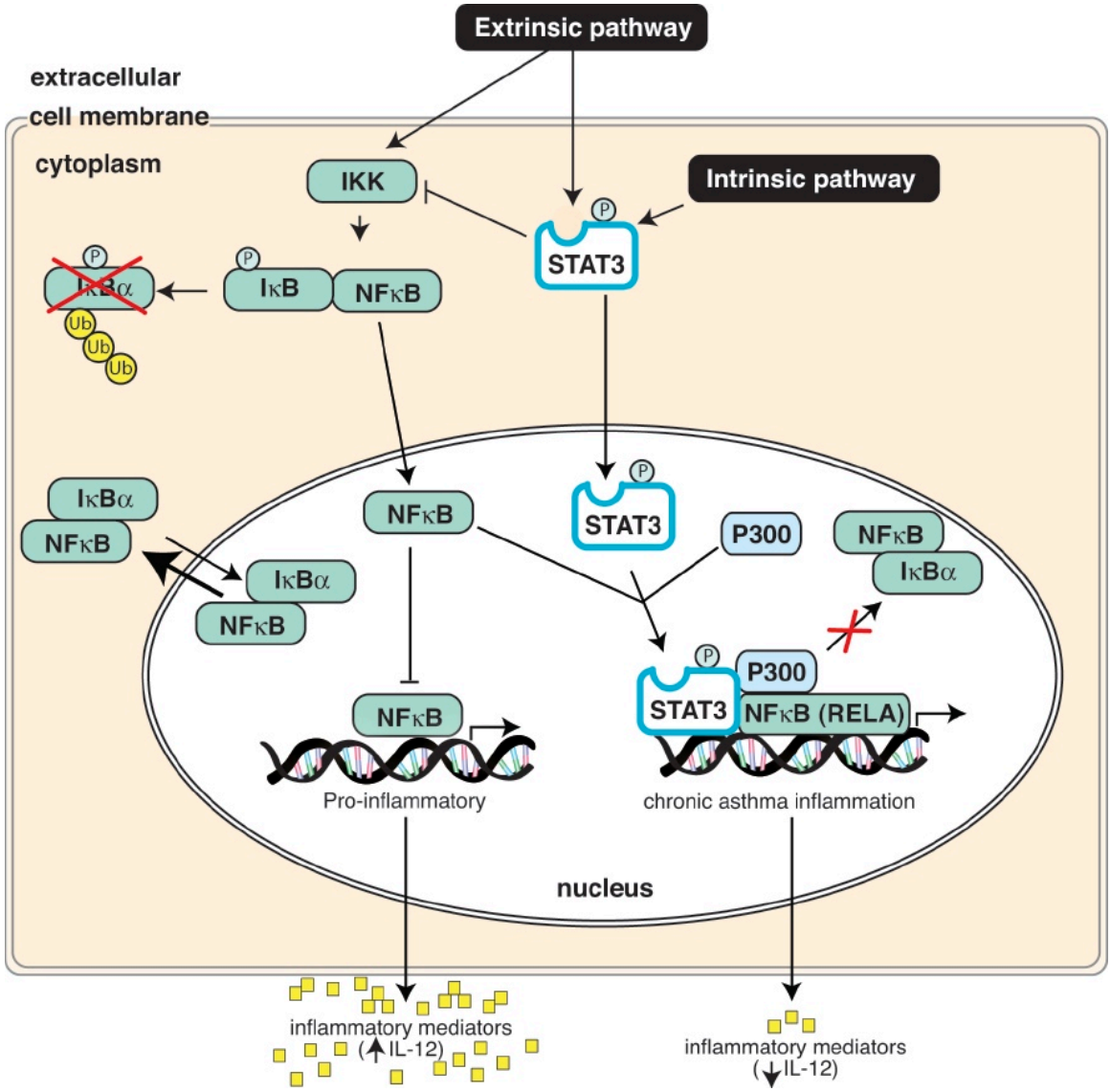

Figure 1.7. ***pSTAT3 inhibition of NF-κB.*** STAT3 is phosphorylated at Tyr705 by Jak2 kinase allowing it to dimerize through SH2 domain interactions and enter the nucleus to effect transcriptional activation. Binding of phosphorylated STAT3 to the transcription factor NF-κB inhibits the action of NF-κB mediated pro-inflammatory gene expression. In the absence of significant pSTAT3, NF-κB is able to bind to the promoter regions of pro-inflammatory genes and activate transcription of genes for cytokines such as IL-12.

Summary and Chapter Outline

Asthma is a chronic disease that affects more than 23 million Americans and many millions more around the world. Several therapies against asthma such as bronchodilators and corticosteroids are currently in use, however, side effects of long-term therapy and the inability for such therapeutics to effectively control the symptoms of asthma in the long term have led to the search for more effective immunotherapeutics. Over the past decade, therapies targeting Toll-like receptors such as TLR9 have provided promising results in animal models, but have yet to bear such results in human trials. To examine the reason why TLR9 targeted therapies in humans have failed, we have investigated the role of TLR9 in a well established fungal model of asthma (Chapter 3) (228, 229) and examined the ability for CpG-ODN therapies to provide effective therapy in site directed, but not systemic therapy (Chapter 4) (230). Most recently, we have studied the effects of targeting a negative regulator of TLR9 signaling, ST2L, using an antibody blocking the binding of its ligand, IL-33, to attempt to understand why intranasal, but not systemic CpG-ODN therapy is effective against the airway hyperresponsiveness characteristic of asthmatic disease (Chapter 5). In summary, CpG-ODN therapy against asthma has been extensively studied and tested in both humans and animal models, but thus far has proven effective only in the latter mode of study. My studies have focused on identifying and understanding the factors and mechanisms involved in the inability of TLR9 targeted therapies to work in human trials. The work presented here suggests a significant role for ST2L in the regulation of TLR9 signaling in the context of asthma and provides impetus for further work to understand this protein as it pertains to the therapy of allergic airway disease.

CHAPTER 2

MATERIALS AND METHODS

Mice

Homozygous, female TLR9 gene deficient ($TLR9^{-/-}$) mice on a Balb/c background were bred at the University of Michigan. Female, wild type Balb/c ($TLR9^{+/+}$) mice at 6-8 weeks of age were purchased from The Jackson Laboratory (Bar Harbor, ME). Female, wild type C57BL/6 mice at 6-8 weeks of age were purchased from Taconic Farms (Germantown, NY). Both groups of mice were maintained in a specific pathogen free (SPF) facility for the duration of the study. Prior approval for mouse usage was obtained from the University Committee on Use and Care of Animals (UCUCA) at the University of Michigan.

Aspergillus fumigatus growth and isolation

Lyophilized *Aspergillus fumigatus* strain 13073 conidia (American Type Culture Collection, Manassas, VA) stored in PBS with 0.1% BSA and glycerol were aseptically inoculated on Sabouraud Dextrose Agar plates (Teknova, Hollister, CA) and were cultured for 10 days at 37˚C until mature conidia cultures were visually apparent (i.e. dark green coloration). Conidia were washed from culture plates using 50 ml sterile PBS with 0.1% Tween 80, strained through sterile gauze to remove hyphal contamination,

centrifuged, and finally reconstituted to a concentration of 1.7 x 10^8 conidia/ml. Conidia were then either used immediately (designated throughout as resting conidia) for intratracheal (i.t.) injections or incubated for 6 hours in sterile PBS with 0.1% Tween 80 at 37˚C to allow for germination (designated as swollen conidia) prior to i.t. injection. All visible conidia were swollen after 6 hours of incubation at 37˚C, hyphal growth was not detectable until at least 8-12 hours incubation at 37˚C and was minimal at this timepoint. Conidia were collected in a laminar flow hood using sterile techniques and N95 respirator masks to prevent endotoxin contamination.

Invasive aspergillosis model

A well-described model of invasive aspergillosis was used in this study (231). To induce neutropenia, mice were injected with α-GR-1 antibody (100μg in PBS) 24 h prior to i.t. challenge. Briefly, mice were anesthetized with a mixture of ketamine and xylazine, the trachea was exposed by blunt dissection of adjacent muscle tissue, and 5.0 x10^6 *A. fumigatus* resting or swollen conidia suspended in 30 μl of PBS with 0.1% Tween 80 were administered by i.t. injection. For survival studies, mice were monitored every 12-24 h after the *A. fumigatus* conidia challenge and euthanized if they appeared moribund. Whole lung samples were processed for proteomic or histological analysis (see below) at either day 2 or 4 after conidia injection. In our studies, the extent of neutropenia was approximately 90% and rebound of neutrophil numbers began to occur 24-48 hours after injection of anti-GR-1 antibody. Full reconstitution of neutrophil numbers occurred by 3-5 days post anti-GR-1 injection.

Chronic fungal asthma model

TLR9$^{+/+}$ and TLR9$^{-/-}$ mice were sensitized with single 100 µL intraperitonial (i.p.) and subcutaneous (s.c.) injections of *Aspergillus* antigen (500 µg of antigen diluted in 5 mL of Incomplete Freund's Adjuvant and 5 mL of sterile saline). These were followed one week later by three intranasal (i.n.) treatments (20 µL of a 1 µg/µL solution in sterile saline) with *Aspergillus* antigen, each spaced one-week apart. A commercially available preparation of soluble *A. fumigatus* antigens as has been previously described in detail was used (232). Seven days after a third intranasal challenge, each mouse received 5 x 10^6 resting or swollen conidia suspended in 30 µl of PBS tween 80 (0.1%; vol/vol) via i.t. administration. Immediately prior to and at days 7, 14, and 28 after an intratracheal *A. fumigatus* resting or swollen conidia challenge, bronchial hyper-responsiveness was assessed in a Buxco^{TM} plethysmograph (Buxco, Troy, NY). Briefly, sodium pentobarbital (Butler Co., Columbus, OH; 0.04 mg/g of mouse body weight) was used to anesthetize mice prior to their intubation and ventilation with a Harvard pump ventilator (Harvard Apparatus, Reno, NV). Once baseline airway resistance was established, 50 µg/ml of methacholine was nebulized, and airway hyperresponsiveness was monitored for approximately 5 min. The peak increase in airway resistance was then recorded. Once airway responsiveness had returned to baseline, each mouse was challenged again with a higher dose of nebulized methacholine (80 µg/ml). Again, airway hyperresponsiveness was monitored for approximately 5 min and peak resistance was recorded. After the assessment of airway hyperresponsiveness, blood was removed for Ig analysis (see below) and whole lungs were dissected from each mouse and snap frozen in liquid nitrogen for genomic and proteomic analysis, or fixed in 10% formalin for

histological analysis **(Fig. 2.1)** (see below). Leg bones were also dissected for the retrieval of bone marrow and the culture of bone marrow-derived dendritic cells (DCs).

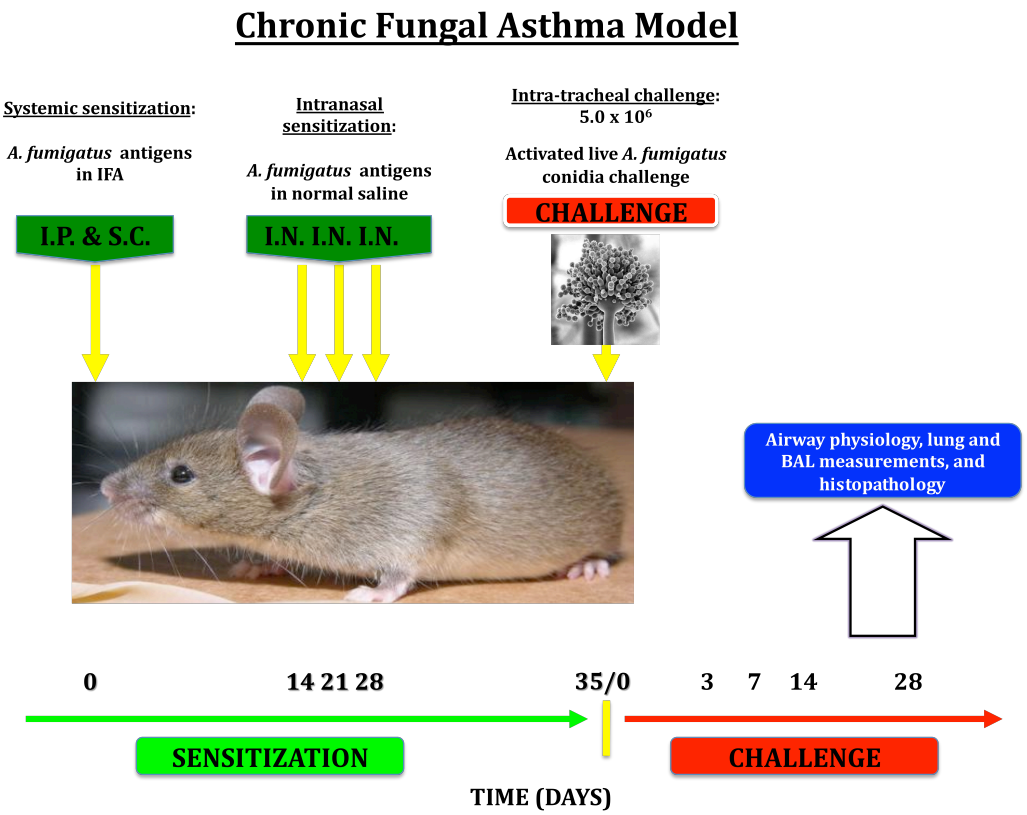

Figure 2.1. Aspergillus fumigatus *chronic fungal asthma model.* Chronic model of asthma involving sensitization to *Aspergillus fumigatus* proteins and challenge with live conidia. Sensitization phase involves exposure to fungal antigens via intraperitoneal, subcutaneous, and intranasal routes. Fungal challenge is with live fungal spores via the intratracheal route. Data is collected and analyzed during the challenge phase.

Whole lung RNA isolation and TAQMAN analysis

Total RNA was isolated from homogenized mouse lungs using Trizol reagent (Invitrogen/ Life Technologies, Carlsbad, CA). Purified RNA was treated with DNAse and reverse transcribed into cDNA using TAQMAN reverse Transcription Reagents (Applied Biosystems, Foster City, CA). For quantitative TAQMAN analysis, a total of 0.2 μg of total RNA was reverse transcribed to yield cDNA, and pre-developed TAQMAN Gene expression Assays were used to quantify IL-10, CXCL10, CCL5, CCL2, CCL3, dectin-1, IL-23p19, IL-12p35, IL-12p40, IL-25, and IL-13 as per manufacturer's (Applied Biosystems, Foster City, CA) instructions. GAPDH was analyzed as an internal control and gene expression was normalized to GAPDH before the fold change in gene expression was calculated. The fold changes in transcript expression were calculated via the comparison of gene expression in naïve whole lung samples using the formula $2^{-(ddCt)}$, which were assigned a value of 1 to that in whole lung samples from mice with chronic fungal asthma.

Whole lung proteomic analysis

Murine IL-4, IL-5, IL-10, IL-13, IFN-γ, TGF-β, TNFα, IL-12, CXCL10, CCL11, CXCL9, CCL21, CCL6, CCL2, CCL3, CCL5, CCL22, and CCL17 were determined in 50-μl samples from whole lung homogenates using a standardized sandwich ELISA technique previously described in detail or through a bead based multiple target sandwich ELISA system (BIO-PLEX®, Biorad Laboratories, Hercules, CA). Recombinant murine cytokines and chemokines (R&D systems, Rochester, MN) were used to generate the standard curves from which the sample concentrations were derived. The limit of ELISA

detection for each cytokine was consistently above 50 pg/ml for sandwich ELISA and 1 pg/ml for BIO-PLEX®. The cytokine and chemokine levels in each sample were normalized to total protein levels measured using the Bradford assay.

Whole lung histological analysis

Whole lungs from *A. fumigatus*-sensitized $TLR9^{+/+}$ and $TLR9^{-/-}$ mice prior to and at various times after *A. fumigatus* conidia challenge were fully inflated with 10% formalin, dissected, and placed in fresh 10% formalin for 24 h. Routine histological techniques were used to paraffin-embed the entire lung, and 5 μm sections of whole lung were stained with hematoxylin/eosin, Gomori methenamine silver (GMS) to detect *Aspergillus* conidia, Periodic Acid Schiff, or Masson-Trichrome.

CpG treatments in a chronic fungal asthma model

$TLR9^{+/+}$ and $TLR9^{-/-}$ mice were sensitized with a commercially available preparation of soluble *A. fumigatus* antigens as has been previously described in detail (228). Seven days after a third intranasal challenge, each mouse received 5 x 10^6 swollen conidia suspended in 30 μl of PBS tween 80 (0.1%; vol/vol) via i.t. administration as previously described (229). Beginning at day 14 after conidia challenge, both groups of TLR9 received either 5 μg of a mouse variant of CpG (HyCult Biotechnology, Uden, The Netherlands) similar to CpG-B DNA containing mouse optimal sequence GACGTT (optimal human sequence is GTCGTT) dissolved in 20 μl of distilled water for intranasal (IN) instillation or 200 μl of distilled water for intraperitoneal (IP) injection every other day until day 28 after conidia. The CpG used herein is a 20-mer synthetic

oligodeoxynucleotide (ODN) that has the following sequence: 5'-tccatgacgttcctgatgct-3' and contains a CpG-DNA motif, which mimics the immunostimulatory effects of bacterial DNA.

At day 28 after intratracheal *A. fumigatus* swollen conidia challenge, bronchial hyper-responsiveness was assessed in a Buxco^{TM} plethysmograph (Buxco, Troy, NY) (228). Briefly, sodium pentobarbital (Butler Co., Columbus, OH; 0.04 mg/g of mouse body weight) was used to anesthetize mice prior to their intubation and ventilation with a Harvard pump ventilator (Harvard Apparatus, Reno, NV). Once baseline airway resistance was established, 420 mg/kg of methacholine was administered intravenously through the tail vein, and airway hyperresponsiveness was monitored for approximately 2 min. The peak increase in airway resistance was then recorded. After the assessment of airway hyperresponsiveness, blood was removed for Ig analysis (see below) and whole lungs were dissected from each mouse and snap frozen in liquid nitrogen or fixed in 10% formalin for genomic, proteomic, and histological analysis (see below).

Serum IgE and IgG2a analysis

Serum levels of IgE and IgG2a at day 28 after conidia in TLR9$^{+/+}$ and TLR9$^{-/-}$ groups were analyzed using complementary capture and detection antibody pairs for IgE (PharMingen, San Diego, CA). Immunoglobulin ELISAs were performed according to the manufacturer's directions. Duplicate sera samples were diluted to 1:20 for IgE determination. Immunoglobulin levels were then calculated from optical density readings at 492 nm, and immunoglobulin concentrations were calculated from a standard

curve generated using recombinant IgE or IgG2a (the standard curves ranged from 0-1000 ng/ml).

Whole lung RNA isolation and TAQMAN analysis

Total RNA was isolated from homogenized mouse lungs using Trizol reagent (Invitrogen/ Life Technologies, Carlsbad, CA). Purified RNA was treated with DNAse and reverse transcribed into cDNA using TAQMAN reverse Transcription Reagents (Foster City, CA). For quantitative TAQMAN analysis, a total of 0.2 μg of total RNA was reverse transcribed to yield cDNA, and pre-developed TAQMAN Gene expression Assays were used to quantify IFN-α, IFN-β, CXCL10, MARCO, SRAI/II, TLR2, TLR3, and TLR9 as per manufacturer's (Applied Biosystems) instructions. GAPDH was analyzed as an internal control. Gene expression was normalized to GAPDH before the fold change in gene expression was calculated. The fold changes in transcript expression were calculated via the comparison of gene expression in naïve whole lung samples, which were assigned a value of 1 to that in whole lung samples from mice with chronic fungal asthma.

Whole lung proteomic analysis

Murine IL-4, IL-5, IL-10, IL-13, IFN-γ, TGF-β, TNFα, IL-12p70, CXCL10, CCL11, CXCL9, CCL21, CCL6, CCL2, CCL3, CCL5, CCL22, CCL17, MARCO, and SR-A levels were determined in 50-μl samples from whole lung homogenates using a standardized sandwich ELISA technique previously described in detail or through a bead based multiple target sandwich ELISA system (BIO-PLEX®, Biorad Laboratories,

Hercules, CA). Recombinant murine cytokines and chemokines (R&D systems, Rochester, MN) were used to generate the standard curves from which the sample concentrations were derived. The limit of ELISA detection for each cytokine was consistently above 50 pg/ml for sandwich ELISA and 1 pg/ml for BIO-PLEX®. The cytokine and chemokine levels in each sample were normalized to total protein levels measured using the Bradford assay.

CpG and α-ST2L treatments in a chronic fungal asthma model

C57BL/6 mice were sensitized with a commercially available preparation of soluble *A. fumigatus* antigens as has been previously described in detail (228). Seven days after a third intranasal challenge, each mouse received 5 x 10^6 swollen conidia suspended in 30 μl of PBS tween 80 (0.1%; vol/vol) via i.t. administration as previously described (229). Beginning at day 14 after conidia challenge, mice received 5 μg of CpG (HyCult Biotechnology, Uden, The Netherlands), 5 μg of scrambled CpG (HyCult Biotechnology, Uden, The Netherlands), control IgG, α-ST2L (Centocor, Radnor, PA), or a combination of either α-ST2L or control IgG with CpG. α-ST2L is a chimeric mAb with a mouse IgG1 Fc and its IgG isotype control is an anti-KLH mIgG1. Each treatment was dissolved in 200 μl of distilled water for intraperitoneal (IP) injection every other day until day 28 after conidia. The CpG used herein is a 20-mer synthetic oligodeoxynucleotide (ODN) that has the following sequence: 5'-tccatgacgttcctgatgct-3' and contains a CpG-DNA motif, which mimics the immunostimulatory effects of bacterial DNA.

At day 28 after intratracheal *A. fumigatus* swollen conidia challenge, bronchial hyper-responsiveness was assessed in a Buxco^{TM} plethysmograph (Buxco, Troy, NY) (228). Briefly, sodium pentobarbital (Butler Co., Columbus, OH; 0.04 mg/g of mouse body weight) was used to anesthetize mice prior to their intubation and ventilation with a Harvard pump ventilator (Harvard Apparatus, Reno, NV). Once baseline airway resistance was established, 420 mg/kg of methacholine was administered intravenously through the tail vein, and airway hyperresponsiveness was monitored for approximately 2 min. The peak increase in resistance was then recorded. After the assessment of airway hyperresponsiveness, blood was removed for Ig analysis (see below) and whole lungs were dissected from each mouse and snap frozen in liquid nitrogen or fixed in 10% formalin for genomic, proteomic, and histological analysis (see below).

Bone marrow-derived dendritic cell culture and isolation

Myeloid DCs were isolated and prepared by culture of bone marrow obtained from allergic TLR9$^{+/+}$ and TLR9$^{-/-}$ mice at various times after conidia challenge and cultured for 6 days with murine granulocyte-macrophage colony-stimulating factor (GM-CSF, 20 ng/ml, R&D systems, Minneapolis, MN) followed by selection sorting for CD11c+ cells through Magnetic Activated Cell Sorting (MACS) purification (Miltenyi Biotech, Bergisch Gladbach, Germany). Cells were cultured for 6 or 24 h for RNA or protein determination, respectively, with medium only, CpG (2 nM), Pam3Cys (2.5 μg/mL), or Poly I:C (50 μg/mL) and analysis was performed according to the TAQMAN and Proteomic Analysis protocol described earlier.

Bone Marrow-Derived Dendritic Cell and Whole lung RNA isolation and TAQMAN analysis

Total RNA was isolated from dendritic cells or homogenized mouse lungs using Trizol reagent (Invitrogen/ Life Technologies, Carlsbad, CA). Purified RNA was treated with DNAse and reverse transcribed into cDNA using TAQMAN reverse Transcription Reagents (Foster City, CA). For quantitative TAQMAN analysis, a total of 0.2 μg of total RNA was reverse transcribed to yield cDNA, and pre-developed TAQMAN Gene expression Assays were used to quantify CXCL10, CCL3, ST2, and IL-33 as per manufacturer's (Applied Biosystems) instructions. GAPDH was analyzed as an internal control. Gene expression was normalized to GAPDH before the fold change in gene expression was calculated. The fold changes in transcript expression were calculated via the comparison of gene expression in naïve whole lung samples, which were assigned a value of 1 to that in whole lung samples from mice with chronic fungal asthma.

Bone Marrow-derived Dendritic Cell and Whole lung proteomic analysis

Murine IL-4, IL-10, IL-12p70, CXCL10, CCL3, ST2, and IL-33 levels were determined in 50-μl samples from dendritic cell samples or whole lung homogenates using a standardized sandwich ELISA technique previously described in detail or through a bead based multiple target sandwich ELISA system (BIO-PLEX®, Biorad Laboratories, Hercules, CA). Recombinant murine cytokines and chemokines (R&D systems, Rochester, MN) were used to generate the standard curves from which the sample concentrations were derived. The limit of ELISA detection for each cytokine was consistently above 50 pg/ml for sandwich ELISA and 1 pg/mL for BIO-PLEX®. The

cytokine and chemokine levels in each sample were normalized to total protein levels measured using the Bradford assay.

Splenic Dendritic Cell Culture and Flow Cytometric Analysis

Whole spleen samples were minced using surgical scissors and incubated in RPMI 1640 supplemented with 5% FCS, type IV collagenase (Sigma-Aldrich), and DNase for 45 min. Cells were flushed through a nylon mesh filter and washed with FACS buffer, consisting of Ca^{2+}- and Mg^{2+}-free PBS with 0.1% azide, 1% BSA, and 5 mM EDTA as described. Cells were sorted for CD11c+ cells through magnetic activated cell sorting purification (Miltenyi Biotech, Bergisch Gladbach, Germany). Cells were then cultured for 12 h with medium only or CpG (2 nM). Before surface staining with labeled Abs for CD11b and MHCII from eBioscience, nonspecific binding was blocked by incubating cells with purified rat anti-mouse CD16/CD32 (FcγIII/II receptor) mAb from eBioscience. Flow cytometry analysis was performed with a BD LSR II cytometer, and data were analyzed using FlowJo 8.2 software.

Immunohistochemical staining of Paraffin-embedded tissues

Paraffin-embedded whole lung samples were analyzed using routine immunohistochemical techniques for the presence of MARCO, SR-A, ST2L or TLR9. Goat anti-mouse MARCO and SR-A polyclonal antibodies were obtained from R & D Systems (Minneapolis, MN). Rabbit anti-mouse IL-33, ST2L and TLR9 polyclonal antibodies were obtained from Alexis Biochemicals (San Diego, CA), Abcam (Cambridge, MA), and Imgenex (San Diego, CA), respectively. Briefly, 5-micron

histological sections were dewaxed with xylene, rehydrated in graded concentrations of ethanol, and stained with the mouse HRP-DAB cell and tissue staining kit according to the manufacturer's instructions (R & D systems). Other histological samples were immunostained with control antibodies (IgG isotype controls and HRP substrate).

STAT3 immunoblotting

BMDCs were lysed in 1% Nonidet P-40-containing lysis buffer. Analysis of cell lysates by SDS/PAGE and blotting were performed as described by Kawakami et. al. (233) using anti-Stat3, anti-phospho-Stat3(Tyr-705), and anti-SOCS3 antibodies from Cell Signaling Biotechnology (Beverly, MA). Naïve BMDCs were treated with custom siRNA constructs (Thermo Scientific Dharmacon) according to the sequences used by Kortylewski et. al. (234). Bands were quantitated using ImageJ64 software from the NIH.

Statistical Analysis

All results are expressed as mean ± standard error of the mean (SEM). A Student's T test or Analysis of Variance (ANOVA) and a Student-Newman-Keuls Multiple Comparison test were used to determine statistical significance on a normal distribution between $TLR9^{+/+}$ and $TLR9^{-/-}$ mice and mice in each treatment group at day 28 after the conidia challenge; $P<0.05$ was considered statistically significant.

CHAPTER 3

TLR9 MODULATES IMMUNE RESPONSES TO *ASPERGILLUS FUMIGATUS* CONIDIA IN IMMUNODEFICIENT AND ALLERGIC MICE.

Summary

The role of toll like receptor-9 (TLR9) in anti-fungal responses in the immunodeficient and allergic host is presently unclear. Herein, we investigated the role of TLR9 in murine models of invasive aspergillosis and fungal asthma. Neutrophil-depleted TLR9 wildtype ($TLR9^{+/+}$) and TLR9 deficient ($TLR9^{-/-}$) mice were challenged with resting or swollen *A. fumigatus* conidia and monitored for survival and lung inflammatory responses. The absence of TLR9 delayed, but did not prevent, mortality in immunodeficient mice challenged with resting or swollen conidia compared with $TLR9^{+/+}$ mice. In a fungal asthma model, $TLR9^{+/+}$ and $TLR9^{-/-}$ mice were sensitized to soluble *A. fumigatus* antigens and challenged with resting or swollen *A. fumigatus* conidia, and both groups of mice were analyzed prior to and at days 7, 14, and 28 following the conidia challenge. When challenged with resting conidia $TLR9^{-/-}$ mice exhibited significantly lower airway hyperresponsiveness compared with the $TLR9^{+/+}$ groups. In contrast, *A. fumigatus*-sensitized $TLR9^{-/-}$ mice exhibited pulmonary fungal growth at days 14 and 28 after challenge with swollen conidia, a finding never observed in their allergic wildtype counterparts. Increased fungal growth in allergic $TLR9^{-/-}$ mice correlated with markedly decreased dectin-1 expression in whole lung samples and isolated dendritic cell populations. Further, whole lung levels of IL-17 were lower in

allergic TLR9$^{-/-}$ mice compared with similar TLR9$^{+/+}$ mice. Together, these data suggest that TLR9 modulates pulmonary antifungal immune responses to swollen conidia, possibly through the regulation of dectin-1 expression.

Introduction

Aspergillus fumigatus is a ubiquitous but generally benign mold in immunocompetent individuals (90, 235, 236). Immunocompromised patients experiencing solid organ or hematopoietic stem cell transplant are at extreme risk for the development of invasive aspergillosis, and the lung is a major target organ due to its constant exposure to airborne *A. fumigatus* spores or conidia (237, 238). Further, a developing body of evidence suggests that *A. fumigatus* spores or conidia play a role in the exacerbation of allergic and asthmatic disease (239, 240). Though unknown, current evidence suggests the underlying mechanisms behind this phenomenon might lie in the pulmonary host immune response induced by this pathogen in individuals with underlying allergic airway disease (90). Experimental and clinical data show that the pulmonary immune response to *A. fumigatus* conidia in the immunocompromised and the allergic host is skewed towards Th2-type immunity instead of a protective Th1-type immune response (241). Therefore, therapeutic redirection of the immune response towards a protective Th1-type immunity is an attractive option for containment of invasive fungal growth in the immunodeficient host with fungal exacerbation of allergic airway disease and asthma.

The relatively frequent incidence of IPA in immunocompromised and neutropenic individuals combined with the dearth of knowledge about its immunopathogenesis has made IPA an important target of study. ABPA is a disease in which the characteristic Th2 immune activation that occurs produces asthma-like pathology complicated by fungal growth (235). Investigations in the area of innate immune receptors and soluble factors that inhibit or enhance immune activation during IPA or ABPA have led to

considerable progress in understanding its underlying mechanisms. In particular, inflammatory cytokines and chemokines such as TNF-α, (242-244) CCL2 (245) and CXCL10 (246) play critical roles in the activation and recruitment of immune cells such as dendritic cells and macrophages that further act to regulate inflammation at the site of *Aspergillus* infection. More recently, IL-17 has also been demonstrated to promote inflammation and infection during *Aspergillus* infection (247). Specifically, IL-17 neutralization has been demonstrated to increase fungal clearance, reduce inflammation, and restore protective Th1 antifungal responses (248).

Studies of the innate immune mechanisms, which recognize and eliminate fungi such as *A. fumigatus* have demonstrated the importance of the toll like receptors (TLRs) in innate immune signaling. In particular, it has been demonstrated that TLR2 and TLR4 provide critical anti-fungal immune signals in macrophages, neutrophils, and dendritic cells (113, 249), due in part to their collaboration with the essential anti-fungal β-glucan receptor dectin-1 (117, 250-252). Dectin-1 signals through a Syk and CARD-9 dependent pathway that leads to the induction of Th cells producing IL-17 (253). Dectin-1 is highly expressed on dendritic cells and macrophages and functions to recognize fungal β-glucans. Three forms of *Aspergillus* conidia are recognized and they include the resting form, the swollen form, and the hyphal form. Each of these forms is antigenically distinct. β-glucans are strongly expressed by swollen conidia (99, 254). TLR9, a MyD88 adapter protein-dependent receptor, has also been shown to contribute to the inflammatory pathology of the lung and early pulmonary responses during *Aspergillus* infection (249, 255), but the manner in which it regulates immune responses during aspergillosis have not been previously investigated. TLR9 detects *A. fumigatus* DNA,

resulting in the secretion of proinflammatory cytokines, which might contribute to the immune response to the pathogen (111). Also, recent studies of TLR9 polymorphisms have suggested that a single nucleotide polymorphism in TLR9 coincides with increased risk for clinical allergic bronchopulmonary aspergillosis (ABPA) (256), an allergic hypersensitivity to bronchial colonization by *Aspergillus*. Previous studies have demonstrated that fungal clearance of *Cryptococcus neoformans* is decreased in $TLR9^{-/-}$ mice (257). We have previously observed that the therapeutic targeting of CCR1 promoted an increase in TLR9 expression in a chronic fungal asthma model thus establishing a role for CCR1 in fungal asthma (258).

In the present study, we addressed the role of TLR9 in the innate immune responses elicited by *A. fumigatus* in murine models of invasive pulmonary aspergillosis (IPA) and chronic fungal asthma. We observed that TLR9 was induced in the lung following the introduction of either resting or swollen conidia into immunocompromised and allergic mice, and that it was particularly important for appropriate protective immune responses against swollen *A. fumigatus* conidia in the respiratory tract of both. Surprisingly, we observed that the absence of TLR9 also adversely affected the expression of dectin-1 and IL-17 in the lung in both the immunocompromised and allergic host. Thus, TLR9 appears to modulate the innate immune response directed against swollen *A. fumigatus* conidia, and this modulation appears to be specific for dectin-1 expression and the generation of IL-17.

Results

Neutrophil-depleted TLR9$^{-/-}$ mice were modestly protected from invasive aspergillosis compared with TLR9$^{+/+}$ mice.

It has been previously established that MyD88 dependent innate immune mechanisms, particularly involving TLR2, TLR4, and TLR9, are critical in the recognition of fungal components during *Aspergillus* infection (249). For example, neutrophil-mediated anti-fungal responses rely on these TLR pathways (113, 249). In a previous study, C57BL/6J TLR9$^{-/-}$ mice exhibited enhanced fungal clearance following exposure to resting conidia (249). Survival responses in neutrophil-depleted TLR9$^{-/-}$ and TLR9$^{+/+}$ mice on a Balb/c background following resting conidia or swollen conidia challenge are shown in **Figure 3.1 A & B, respectively**. Overall a significant difference in survival between TLR9$^{+/+}$ and TLR9$^{-/-}$ mice was observed in a model of invasive aspergillosis initiated by the administration of resting conidia into neutrophil depleted mice **(Fig. 3.1A)**. Intratracheal challenge of TLR9$^{+/+}$ and TLR9$^{-/-}$ mice with swollen conidia led to faster mortality in the TLR9$^{+/+}$ group but the overall survival in both groups was similar at day 4 **(Fig. 3.1B)**. Thus, these data suggested that TLR9 delayed but did not prevent mortality due to invasive aspergillosis in neutrophil-depleted mice.

Neutrophil-depleted TLR9$^{-/-}$ mice exhibited slower fungal growth compared with TLR9$^{+/+}$ mice following resting or swollen conidia challenge.

GMS-stained lung sections from TLR9$^{+/+}$ and TLR9$^{-/-}$ mice at day 2-post intratracheal challenge with resting conidia revealed increased *Aspergillus* growth in the TLR9$^{+/+}$ **(Fig. 3.2A)** versus the TLR9$^{-/-}$ **(Fig. 3.2B)** group. However, increased fungal

growth was observed in TLR9$^{-/-}$ mice at day 4 after swollen conidia challenge relative to TLR9$^{+/+}$ mice **(Figs. 3.2E & F)**. Together, these data indicate the lack of TLR9 allowed neutrophil depleted mice to slow, but not eliminate the growth of resting *Aspergillus* conidia in the lung, but TLR9 deficient mice exhibit increased fungal growth with swollen conidia challenge in neutrophil depleted mice.

Whole lung cytokine and chemokine changes were apparent in TLR9$^{+/+}$ and TLR9$^{-/-}$ mice during invasive aspergillosis induced by resting and swollen conidia.

Next, we examined whole lung cytokine and chemokine levels to determine significant mediators of the immune response to resting versus swollen *Aspergillus* conidia. All mediators were assessed via ELISA and BIO-PLEX® analysis of whole lung homogenates, which were taken from TLR9$^{+/+}$ and TLR9$^{-/-}$ mice two days post resting conidia challenge. Representative examples of these analyses are shown in **Figure 3.3**. A decrease in levels of TNF-α **(Fig. 3.3A)** and a significant decrease in CCL2 **(Fig. 3.3B)**, and CXCL10 **(Fig. 3.3C)** were observed in the knockout group compared with the wildtype group. Similar analysis of TLR9$^{+/+}$ and TLR9$^{-/-}$ mice four days post swollen conidia challenge demonstrated significant increases in levels of TNF-α **(Fig. 3.3D)**, CCL2 **(Fig. 3.3E)**, and CXCL10 **(Fig. 3.3F)** in TLR9$^{-/-}$ mice compared with TLR9$^{+/+}$ mice. In fact, whole lung homogenates from TLR9$^{+/+}$ mice appeared to express little, if any, of these proteins. Thus, these data show that the presence of TLR9 markedly altered the whole lung cytokine and chemokine response to *A. fumigatus* in the immunocompromised host; this receptor appeared to be required for the full initiation of immune activation to resting conidia but its presence appeared not to be required for

expression of TNF-α **(Fig. 3.3D)**, CCL2 **(Fig. 3.3E)**, and CXCL10 **(Fig. 3.3F)** in response to swollen conidia.

Sustained whole lung TLR9 transcript expression was observed in A. fumigatus-sensitized mice that received swollen conidia.

Because of the marked differences in the cytokine and chemokine responses to resting versus swollen conidia in neutrophil-depleted TLR9$^{-/-}$ mice, we next assessed the role of this TLR in a model of chronic fungal asthma. We have extensively characterized a model of chronic fungal asthma in which defective clearance of resting *Aspergillus* conidia introduced by intratracheal injection into *A. fumigatus* sensitized mice occurs (228, 259). We found that this defect is due to the inhibitory effect of allergic Th2-type immune response on the anti-fungal innate immune response (228, 259). The effect of resting and swollen conidia on whole lung TLR9 transcript expression in *A. fumigatus*-sensitized TLR9$^{+/+}$ mice is shown in **Figure 3.4**. At day 14, TLR9 transcript expression was increased approximately 2-fold in both groups of TLR9$^{+/+}$ mice. At day 28, only the group of TLR9$^{+/+}$ mice that received swollen conidia showed a persistent 2-fold increase in TLR9 transcript expression. These data indicate that whole lung TLR9 levels were maintained in wildtype mice, which were challenged with swollen conidia suggesting a role for TLR9 during allergic airway responses to this form of conidia.

Swollen conidia grow in A. fumigatus-sensitized TLR9$^{-/-}$ mice.

Previous studies have not explored the role of TLR9 during chronic fungal asthma responses. In the present study, we observed that fungal material was present upon GMS

staining of whole lung samples from *A. fumigatus* sensitized TLR9$^{+/+}$ and TLR9$^{-/-}$ mice challenged with resting conidia, and no differences were observed between these groups at any time after conidia challenge (not shown). However, marked differences were observed when *A. fumigatus* sensitized TLR9$^{+/+}$ and TLR9$^{-/-}$ mice were challenged with swollen conidia **(Fig. 3.5)**. *A. fumigatus* sensitized TLR9$^{+/+}$ mice challenged with swollen conidia exhibited small amounts of fungal material at day 14 **(Fig. 3.5A)**, which was gone by day 28 **(Fig. 3.5C)**. Surprisingly and unexpectedly, *A. fumigatus* sensitized TLR9$^{-/-}$ mice challenged with swollen conidia exhibited fungal growth at both times after conidia challenge **(Figs. 3.5B and 3.5D)**. At day 28, large masses of fungus were observed in the lungs of TLR9$^{-/-}$ mice **(Fig. 3.5D)**. Together, these histological analyses suggested that *A. fumigatus* sensitized TLR9$^{-/-}$ mice were unable to contain the growth of swollen conidia.

Conidia significantly augmented circulating total IgE levels in A. fumigatus-sensitized TLR9$^{-/-}$ mice regardless of germination status, while TLR9$^{-/-}$ mice showed significantly lower airway hyperresponsiveness following resting conidia challenge.

Elevated serum IgE levels characterize atopy and asthma in individuals (260, 261). Sensitized TLR9$^{-/-}$ mice that received an intratracheal challenge with resting conidia showed a significant increase in serum IgE levels at day 28 post conidia compared to TLR9$^{+/+}$ mice **(Fig. 3.6A)**. Likewise, *A. fumigatus* sensitized mice that received swollen conidia showed a significant 6-fold increase in serum IgE levels relative to TLR9$^{+/+}$ mice **(Fig. 3.6B)**. Importantly, there was a 100-fold difference in serum IgE levels between groups of mice receiving resting versus swollen conidia. These data

indicated that TLR9 regulated the IgE response in *A. fumigatus* sensitized mice and that swollen conidia provoked a more robust IgE response in *A. fumigatus* sensitized mice.

Allergic responses to *A. fumigatus* are characterized by airway hyperresponsiveness following a methacholine stimulus (228). This response differed significantly between *A. fumigatus* TLR9$^{+/+}$ and TLR9$^{-/-}$ mice given resting conidia **(Fig. 3.6C)**. At day 28 after resting conidia challenge, TLR9$^{-/-}$ mice showed significantly less airway hyperresponsiveness when compared with TLR9$^{+/+}$ mice. In contrast, airway hyperresponsiveness was similar in both groups of sensitized mice challenged with swollen conidia **(Fig. 3.6D)**. Thus, these data indicated that the absence of TLR9 dampened the airway allergic response to resting but not swollen conidia.

Divergent effects on whole lung cytokine and chemokine levels were observed in A. fumigatus-sensitized TLR9$^{-/-}$ mice following resting or swollen conidia challenge.

Next, we examined whole lung cytokine and chemokine levels to assess the nature of the cytokine/chemokine response elicited in allergic TLR9$^{+/+}$ and TLR9$^{-/-}$ mice following resting or swollen conidia challenge. ELISA and BIO-PLEX® analysis of whole lung homogenates taken from *Aspergillus* sensitized TLR9$^{+/+}$ and TLR9$^{-/-}$ mice challenged with either resting or swollen conidia revealed divergent effects on whole lung cytokine and chemokine levels. Many of the cytokines and chemokines measured in whole lung samples did not differ between sensitized TLR9$^{+/+}$ and TLR9$^{-/-}$ mice at day 14 and day 28 after resting conidia challenge **(Table 1)**. Significant differences between the two groups were noted, though, in IL-13 (at day 14) and CXCL9 (at both times after conidia) levels following resting conidia challenge. Following swollen conidia challenge,

significant differences in numerous whole lung cytokines and chemokine levels were observed at both times after conidia between sensitized $TLR9^{+/+}$ and $TLR9^{-/-}$ mice, including IL-5, IL-13, CXCL10, CCL6, CCL11, CXCL9, and CCL21 (**Table 1**). Most cytokine and chemokines were significantly lower in the $TLR9^{-/-}$ compared with the $TLR9^{+/+}$ group. However, both IL-13 and CCL6 were significantly higher in the $TLR9^{-/-}$ mice compared with the $TLR9^{+/+}$ group at day 14 after swollen conidia. Other examples of whole lung cytokine and chemokine levels in these groups relevant to fungal asthma are shown in **Figure 3.7**. Whole lung CCL2 levels were significantly decreased at day 28 in $TLR9^{-/-}$ mice challenged with resting conidia **(Fig. 3.7A)**, while in $TLR9^{-/-}$ mice, which received swollen conidia **(Fig. 3.7B)**, whole lung CCL2 levels were significantly increased. Whole lung CXCL10 levels were significantly increased at day 14 post resting conidia challenge in $TLR9^{-/-}$ mice, but significantly decreased at day 28 in this group **(Fig. 3.7C)**. Conversely, whole lung CXCL10 levels were significantly lower in sensitized $TLR9^{-/-}$ mice at both times after swollen conidia challenge compared with the $TLR9^{+/+}$ groups **(Fig. 3.7D)**. Finally, whole lung IL-17 levels were not significantly different between $TLR9^{+/+}$ and $TLR9^{-/-}$ mice at days 14 and 28 post conidia challenge with either resting or swollen conidia, but levels of this cytokine were consistently lower in the $TLR9^{-/-}$ group compared with the $TLR9^{+/+}$ group **(Fig. 3.7 E & F)**. These data showed that the cytokine responses evoked by swollen conidia in particular, were markedly different in the absence of TLR9, suggesting that this receptor had an important role in the regulation of immune responses to swollen *A. fumigatus* conidia.

Non-sensitized and A. fumigatus-sensitized TLR9$^{-/-}$ mice exhibit lower whole lung dectin-1 transcript expression relative to TLR9$^{+/+}$ mice.

Fungal β-glucans expressed on the surface of *Aspergillus* conidia are recognized through the innate immune receptor dectin-1 and this binding results in the generation of a Th17 response (99). Our TAQMAN analysis of whole lung samples from the IPA model suggested that dectin-1 expression was lower in the TLR9$^{-/-}$ group compared with the TLR9$^{+/+}$ group (data not shown). These data prompted us to analyze dectin-1 levels in this asthma model. The data indicated that lung dectin-1 expression was significantly decreased at day 28-post conidia challenge in TLR9$^{-/-}$ mice receiving resting conidia **(Fig. 3.8A)**. In TLR9$^{-/-}$ mice receiving swollen conidia, decreased expression of dectin-1 was observed at both day 14 and 28 after conidia **(Fig. 3.8B)**. These data indicated that TLR9 was involved in the regulation of dectin-1 expression in the lung, particularly following swollen conidia challenge.

Bone marrow dendritic cells exhibit significantly lower dectin-1 expression at day 14 after swollen conidia challenge in TLR9$^{-/-}$ mice compared with TLR9$^{+/+}$ mice.

To further explore the role of TLR9 on dectin-1 expression, we next assessed how this β-glucan receptor was regulated in dendritic cells from *A. fumigatus* sensitized TLR9$^{+/+}$ and TLR9$^{-/-}$ mice at days 14 and 28 after conidia challenge. TAQMAN quantitative PCR analysis of bone marrow derived dendritic cells stimulated with CpG revealed that dectin-1 expression was significantly lower at day 14 post conidia challenge in sensitized TLR9$^{-/-}$ mice compared with TLR9$^{+/+}$ mice but levels of this scavenging receptor were similar to wildtype levels at day 28 **(Fig. 3.9)**. In TLR9$^{+/+}$ sensitized mice,

dectin-1 levels increased in dendritic cells compared to unsensitized wild type mice and were maintained at increased levels at day 28. Thus, these data revealed that TLR9 is an important factor for dectin-1 transcript expression.

Discussion

In the present study, we characterized the altered immune responses in neutrophil-depleted and *A. fumigatus* sensitized TLR9$^{-/-}$ mice following their exposure to either resting or swollen conidia. While the absence of TLR9 had a minor albeit significant protective effect in invasive aspergillosis (i.e. survival) and allergic asthma (i.e. airway hyperresponsiveness) following challenge of neutrophil-depleted and *A. fumigatus* sensitized mice, respectively, with resting conidia, it was apparent that TLR9$^{-/-}$ mice were susceptible to *A. fumigatus* growth when given swollen conidia. This effect was most profound when TLR9$^{-/-}$ mice were sensitized to this fungus and challenged with swollen conidia. Interestingly, we observed that unsensitized and *A. fumigatus*-sensitized TLR9$^{-/-}$ mice expressed significantly lower dectin-1 in whole lung and isolated DCs, and we speculate that the lower expression of dectin-1 contributed to the impaired ability of these mice to respond to swollen conidia. Another indication that dectin-1 expression was lower in TLR9$^{-/-}$ mice was revealed by the lower IL-17 levels present in these mice. Thus, these data demonstrated that the absence of TLR9 markedly impaired the immune responses required to contain the growth of swollen conidia in allergic mice.

Much of the pathology associated with invasive aspergillosis is due to disregulated inflammatory processes (262). Inflammation is a necessary component of an effective immune response against fungus during invasive pulmonary aspergillosis. Properly regulated, inflammation can effectively contain *Aspergillus* conidia in the lung and promote their clearance, while inadequate or excessive inflammation can lead to IPA and/or damage of the lung tissues (263). In this study we found significant changes in the levels of TNF-α, CXCL10, and CCL2, all of which are significant mediators of

inflammation during IPA infection. TNF-α plays an important role in the recruitment of neutrophils, CXCL10 promotes dendritic cell and macrophage recruitment, and CCL2 acts as a chemoattractant for macrophages (246, 264). In our studies, we found that all three of these factors were decreased in TLR9$^{-/-}$ mice at day 2 post resting conidia challenge, but increased in TLR9$^{-/-}$ mice at day 4 post swollen conidia challenge. Previous reports involving conidia challenge in neutrophil-depleted mice have demonstrated an early elevation in inflammatory mediators such as TNF-α by day 2-post conidia challenge and normalization of mediator levels by day 4-post challenge (243). Our studies indicate the induction of TNF-α, CXCL10, and CCL2 is greatest at day 4 post swollen conidia challenge in TLR9$^{-/-}$ mice. We interpret these data to indicate that temporal changes in cytokines and chemokines are TLR9 dependent during IPA. TLR9 dependent effects on cytokine and chemokine generation appeared to provide significant protection during immune responses to resting conidia, but not during immune responses to swollen conidia. Further, these results indicate a role for TLR9 in reigning in the immune response to swollen conidia as TLR9$^{-/-}$ mice exhibit elevated levels of inflammatory cytokines at day 4 post conidia challenge.

Appropriate fungal recognition leads to a balanced immune response against *Aspergillus* conidia during pulmonary responses to *Aspergillus*. For example, TLR9 recognizes fungal DNA (111), while dectin-1 receptors have been demonstrated to recognize β-glucan components from the swollen conidia cell wall (114, 265). In addition to TLR9, the innate immune receptors TLR2, TLR4, and the adaptor protein MyD88 are also required for efficient conidial phagocytosis and immune responses to *Aspergillus* (117, 266, 267). MyD88-dependent signaling on dendritic cells is crucial for

priming antifungal Th1 responses (113). Polymorphisms in the TLR2 gene have been identified as important factors for susceptibility to development of allergic diseases and/or IPA (256, 268-270). TLR4 deficient mice possess neutrophils that are deficient in conocidal activity but phagocytose conidia normally (113, 249). Since β-glucans are abundant on swollen *Aspergillus* conidia, the dectin-1 pathway appears to be fundamentally important for the recognition of metabolically active conidia and the control of fungal infection (99, 271). The functional equivalence of dectin-1 in human cells and in murine models has been well characterized (272). Further, it has been established that fungal components such as chitin can stimulate IL-17A and induce acute inflammation through TLR 2 and a MyD88-dependent pathway (273). During IPA, IL-17A and IL-23 are rapidly produced at sites of infection (247, 263). IL-17 and IL-23 control the immune response to fungal infection and excessive IL-17 acts to enhance inflammation through PMNs. The Th17 pathway appears to directly contribute to defective pathogen clearance and the failure to resolve inflammation during *Aspergillus* infection (248). Thus, the TLRs and dectin-1 appear to cooperate in the recognition and elimination of *Aspergillus fumigatus* from the respiratory system.

Our studies indicate that TLR9 activation contributed to the susceptibility of neutropenic mice to resting or swollen conidia challenge. Two key observations in this IPA model included the following: 1) Fungal growth was slower in $TLR9^{-/-}$ mice challenged with either resting or swollen conidia; 2) The inflammatory response appeared to develop slower in the neutropenic $TLR9^{-/-}$ mouse following conidia challenge. Our data agree, in part, with those of Bellocchio et. al. (113), who showed that $TLR9^{-/-}$ mice on a C57BL/6 background were significantly protected from a multiple conidia

challenge. In that study it was not clear as to the activation state of the conidia, but if the conidia were in a resting state our present data and theirs coincide. Our data also suggest this protective effect could be due in part to lower tissue inflammation. However, neutropenic TLR9$^{-/-}$ mice were not protected from invasive aspergillosis due to swollen conidia. One explanation for this susceptibility might lie in the importance of dectin-1 during the response of the lung to swollen *A. fumigatus* (114). Indeed, targeting dectin-1 appears to render mice more susceptible to pulmonary invasive aspergillosis (99), whereas activating dectin-1 via dectin-1-Fc fusion protein demonstrated increased mouse survival in a model of IPA (274). Examination of whole lung samples from TLR9$^{+/+}$ and TLR9$^{-/-}$ mice revealed that dectin-1 transcript expression was lower in the knockout group compared with the wildtype group. Corresponding with lower dectin-1 expression were lower IL-17 levels. Together these data suggest that the lack of TLR9 is associated with lower dectin-1 expression, which might permit swollen conidia to grow in neutropenic TLR9$^{-/-}$ mice.

Allergic asthma is a Th2 cytokine dominated disease (258). During ABPA and fungal asthma, persistence of conidia drives airway hyperresponsiveness and airway remodeling. Our observations of these phenomena have led us to pose two major questions: 1. Why do immune cells hold onto conidia and yet are unable to kill them? 2. What factor(s) drive the clearance of fungus from the lungs of asthmatic mice? Several novel observations arose from the present study, which suggest that TLR9 has a major role in controlling fungal growth in the allergic lung. We observed that the asthmatic response to resting conidia was largely similar between TLR9$^{+/+}$ and TLR9$^{-/-}$ mice, although the latter group exhibited decreased airway hyperresponsiveness compared with

the former group. This decrease in the TLR9$^{-/-}$ group was coincident with decreased whole lung IL-13 levels. IL-13 acts to improve B cell production of IgE, one observation we did note with resting conidia at day 28. Further, IL-13 acts with TNF-α and histamines to increase mucus production and promote bronchoconstriction (228, 275-277). CCL2 neutralization in the lung at day 14 and 28 timepoints post conidia challenge decreases airway inflammation and airway hyperresponsiveness in a model of fungal asthma (245). Our observation that CCL2 levels significantly decreased in TLR9$^{-/-}$ mice challenged with resting conidia, could explain the decrease airway hyperresponsiveness we observed with resting conidia challenge. However, we observed that airway hyperresponsiveness in the TLR9$^{+/+}$ and TLR9$^{-/-}$ groups challenged with swollen conidia were similar, which was unexpected as the TLR9$^{-/-}$ mice contained markedly greater amounts of fungus/fungal material. Two potential explanations might reconcile this observation. First, Th2 factors that drive airway hyperresponsiveness, namely IL-5, IL-13, CCL11, and CCL21 were significantly lower in the TLR9$^{-/-}$ group compared with the TLR9$^{+/+}$ group. Second, containment of the fungus in the lungs of the TLR9$^{-/-}$ mice might have prevented disseminated infection and thus limited fungal antigen exposure in the lung. We noted a disconnect between serum IgE levels and the airway response in TLR9$^{-/-}$ mice that was in keeping with other previous finding regarding IgE and airway hyperresponsiveness (278). Serum IgE alone is not an accurate indicator of airway hyperresponsiveness in mice. The containment of fungus in the lungs of TLR9$^{-/-}$ mice was remarkable and similar to what is described clinically as fungal granulomas (279). The factors precipitating the marked fibrotic response in TLR9$^{-/-}$ mice are the subject of ongoing studies in the laboratory but we postulate that soluble factors such as CCL2 and

CCL5 might contribute to this phenotype. Both chemokines have well described pro-fibrotic roles in the lung. Thus, the lack of TLR9 resulted in a pulmonary environment characterized by less allergic airway disease due to resting conidia but increased remodeling due to the swollen conidia challenge.

Our data suggest that sustained dectin-1 levels mediated through TLR9 are important in antifungal responses against swollen conidia in the immunocompetent host. The immune response to resting conidia is mediated by MyD88 dependent TLR activation, including signaling through TLR2, TLR4, and TLR9 (113, 249). The lack of TLR9 is compensated by other TLRs in the case of resting conidia. However, TLR9 is required for full dectin-1 expression and its absence renders mice susceptible to swollen conidia. IL-17 has been demonstrated to play a role in inflammation during allergic asthma as it negatively regulates allergic asthma disease (280). From the present study it was apparent that TLR9$^{-/-}$ mice had lower dectin-1 expression, at various times during the course of IPA (data not shown) and chronic fungal asthma. One consequence of the decreased dectin-1 expression appeared to be a concomitant trend toward decreased IL-17 levels in asthmatic TLR9$^{-/-}$ mice exposed to swollen conidia. As mentioned above, the absence of IL-17 has been shown previously to augment features of allergic airway inflammation and our present data appeared to confirm these previous studies. Thus, the presence of TLR9 appears to be required for appropriate dectin-1 expression during fungal asthma responses evoked by swollen conidia.

Dendritic cells mediate the immune response against *Aspergillus* (104). During IPA infection, dendritic cell recruitment and activation are key to survival. Dendritic cell recruitment occurs initially through recognition of *Aspergillus* conidia by alveolar

macrophages in the lung and secretion of chemokine and cytokine signals (281). Further, dendritic cells themselves recognize and engulf *Aspergillus* conidia and aid in development of Th and neutrophil responses to the fungus (104, 107). Dectin-1 receptor expression on dendritic cells mediates inflammatory responses to *A. fumigatus* conidia. Silencing of dectin-1 results in reduced expression of proinflammatory cytokines (TNF-α and IL-12) (282). Further, dectin-1 Fc targeting of *Aspergillus* β-glucans has proven to be an effective means of improving the immune response to *Aspergillus* conidia (274). In the present study we observed a transient decrease in dectin-1 transcript expression in $TLR9^{-/-}$ DCs. It is possible that other cell types, including macrophages, also show impaired dectin-1 expression and these studies are ongoing.

In summary, TLR9 is relevant for pulmonary fungal responses against swollen conidia and is important for regulating inflammation that aids in the containment and clearance of *Aspergillus* **(Figure 3.10)**. This role appears to be related to altered dectin-1 expression. Dectin-1 plays an important role in the recognition of swollen *Aspergillus* conidia during IPA and fungal asthma and levels of dectin-1 are decreased in the absence of TLR9. Further studies are warranted to elucidate the direct role of dectin-1 in the fungal asthma response to swollen *A. fumigatus* conidia.

Table 3.1. Whole lung cytokine and chemokine levels at day 14 and 28 after resting or swollen conidia challenge in *A. fumigatus*-sensitized TLR9$^{+/+}$ and TLR9$^{-/-}$ mice.

Table 1	Resting Conidia				Swollen Conidia			
	Day 14		Day 28		Day 14		Day 28	
Cytokine/ Chemokine	TLR9$^{+/+}$	TLR9$^{-/-}$	TLR9$^{+/+}$	TLR9$^{-/-}$	TLR9$^{+/+}$	TLR9$^{-/-}$	TLR9$^{+/+}$	TLR9$^{-/-}$
IL-4	ND	ND	ND	ND	0.01±0.00	0.01±0.00	0.01±0.00	0.00±0.00*
IL-5	ND	ND	ND	ND	0.16±0.01	0.13±0.01*	0.11±0.01	0.04±0.01*
IL-10	ND	ND	ND	ND	0.31±0.01	0.25±0.02*	0.21±0.04	0.09±0.00*
IL-12	ND	ND	ND	ND	0.07±0.00	0.06±0.01	0.04±0.01	0.02±0.00
IL-13	0.04±0.01	0.02±0.00*	0.02±0.01	0.02±0.00	0.00±0.02	0.10±0.01**	0.08±0.01	0.04±0.00*
INFγ	ND	ND	ND	ND	0.18±0.01	0.15±0.02	0.12±0.02	0.06±0.01*
TGFβ	ND	ND	ND	ND	0.08±0.01	0.05±0.00*	0.04±0.00	0.03±0.00*
CCL11	ND	ND	ND	ND	0.26±0.02	0.18±0.03*	0.15±0.02	0.11±0.01*
CXCL9	0.07±0.01	0.04±0.00*	0.06±0.01	0.02±0.01*	0.08±0.01	0.06±0.01	0.06±0.00	0.03±0.00*
CCL21	ND	ND	ND	ND	1.12±0.07	1.49±0.11*	0.91±0.06	0.53±0.04*
CCL6	ND	ND	ND	ND	0.25±0.06	0.49±0.08**	0.17±0.03	0.23±0.05
CCL3	ND	ND	ND	ND	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00
CCL5	ND	ND	ND	ND	0.04±0.01	0.04±0.01	0.02±0.00	0.04±0.00*
CCL22	ND	ND	ND	ND	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00
CCL17	0.03±0.00	0.03±0.01	0.02±0.00	0.02±0.00	0.04±0.00	0.03±0.00*	0.03±0.00	0.03±0.00

*P≤0.05, **P≤0.01, ***P≤0.001, compared with TLR9$^{+/+}$ mice at the same time after resting or swollen conidia challenge. ND=Not Detected (Units are ng/mg total protein)

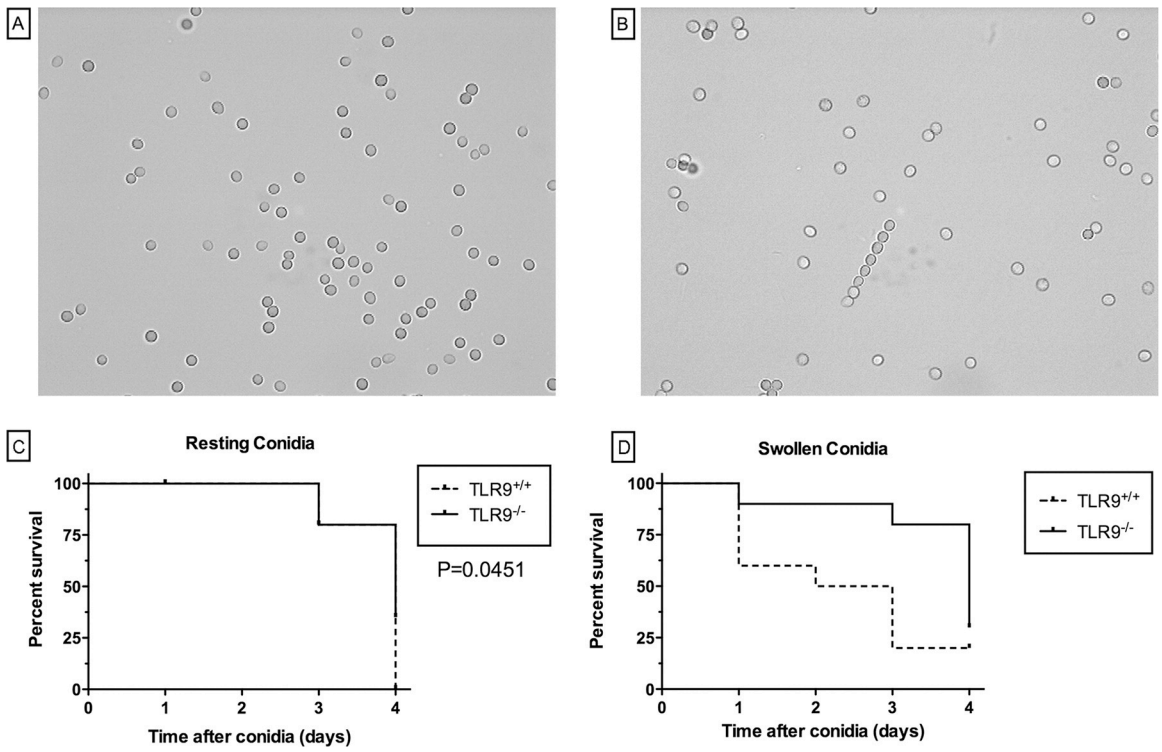

Figure 3.1. ***Neutrophil-depleted TLR9$^{-/-}$ mice are significantly less susceptible to resting conidia and succumb more slowly to swollen conidia compared with TLR9$^{+/+}$ mice.*** Unstained bright-field micrographs of resting **(A)** and swollen **(B)** *Aspergillus fumigatus* conidia. Kaplan-Meier survival curve analysis of α-GR-1 neutrophil depleted TLR9$^{+/+}$ **(C)** and TLR9$^{-/-}$ **(D)** mice after i.t. challenge with 5×10^6 conidia (N=10). To induce neutropenia, all mice were injected with 100 μg of α-GR-1 antibody 24 h prior to conidial challenge. The data are representative of two separate experiments. $P \leq 0.05$ for comparison between resting versus swollen conidia. Original magnification is 1000x for all micrographs.

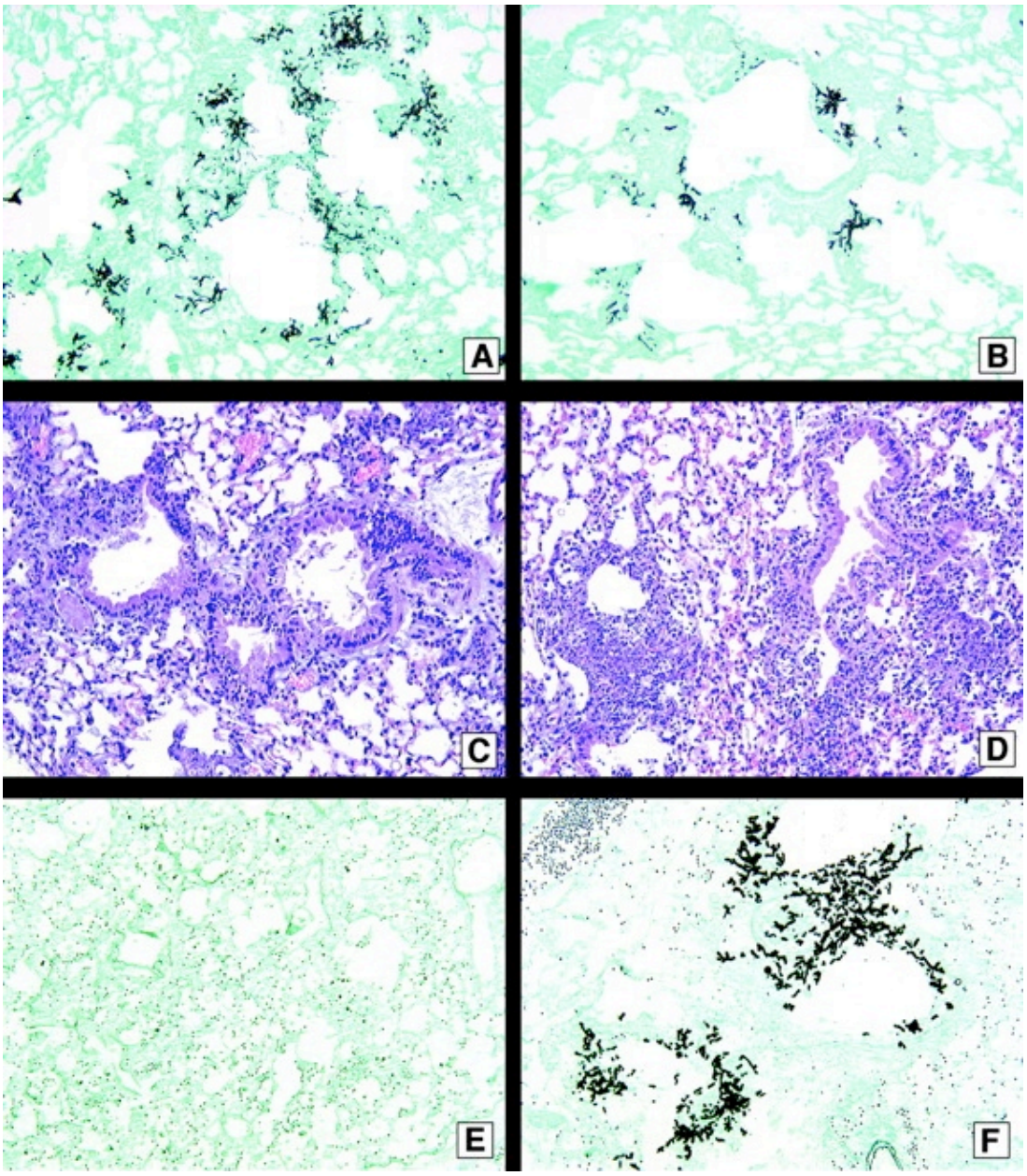

Figure 3.2. ***Neutrophil-depleted TLR9$^{-/-}$ mice exhibited less fungal growth and less tissue inflammation at day 2 after resting conidia challenge.*** TLR9$^{+/+}$ **(A)** and TLR9$^{-/-}$ **(B)** mouse whole-lung tissue sections from neutropenic mice processed via Gomori Methenamine Silver (GMS) stain two days post i.t. challenge with 5×10^6 resting conidia. *Aspergillus* conidia and hyphae stain black using the GMS staining procedure. Representative H&E stained TLR9$^{+/+}$ **(C)** and TLR9$^{-/-}$ **(D)** neutropenic mouse whole lung sections taken two days post i.t. challenge with 5×10^6 resting conidia. TLR9$^{+/+}$ **(E)** and TLR9$^{-/-}$ **(F)** mouse whole-lung tissue sections from neutropenic mice processed via Gomori Methenamine Silver (GMS) stain four days post i.t. challenge with 5×10^6 swollen conidia. The original magnification was ×200 for all photomicrographs.

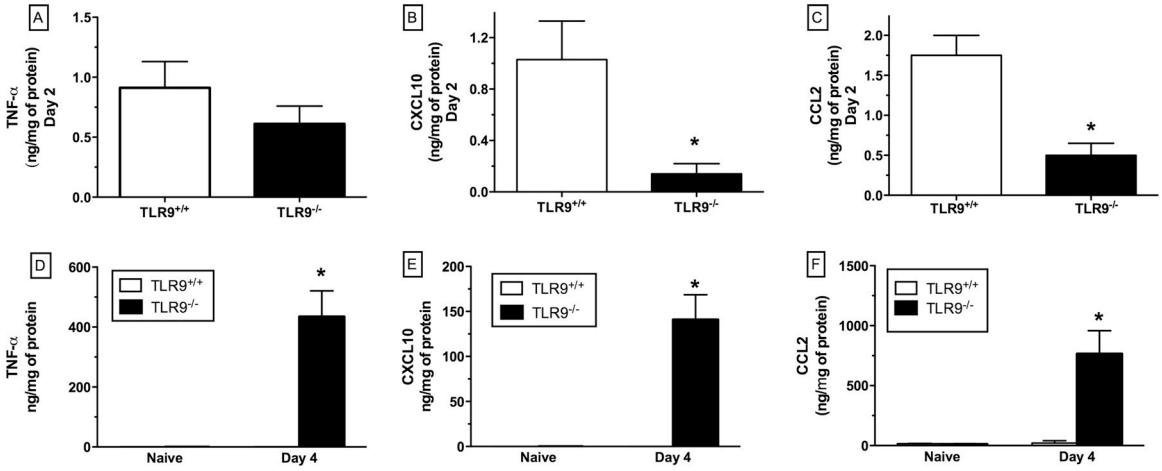

Figure 3.3. ***Dynamic changes in whole lung cytokines and chemokines were apparent in TLR9$^{+/+}$ and TLR9$^{-/-}$ mice during invasive aspergillosis induced by resting and swollen conidia.*** ELISA or Bio-Plex® of TNF-α **(A)**, CXCL10 **(B)**, and CCL2 **(C)**, comparing TLR9$^{+/+}$ to TLR9$^{-/-}$ whole lung protein levels from neutrophil depleted mice at Day 2 after challenge with 5×10^6 resting *Aspergillus* conidia. ELISA or Bio-Plex® of TNF-α **(D)**, CXCL10 **(E)**, and CCL2 **(F)**, comparing TLR9$^{+/+}$ to TLR9$^{-/-}$ whole lung protein levels from neutrophil depleted mice challenged with 5×10^6 swollen *Aspergillus* conidia. To induce neutropenia, mice were injected with α-GR-1 antibody 24 h prior to i.t. Data are expressed as mean ± SEM. *, $P \leq 0.05$.

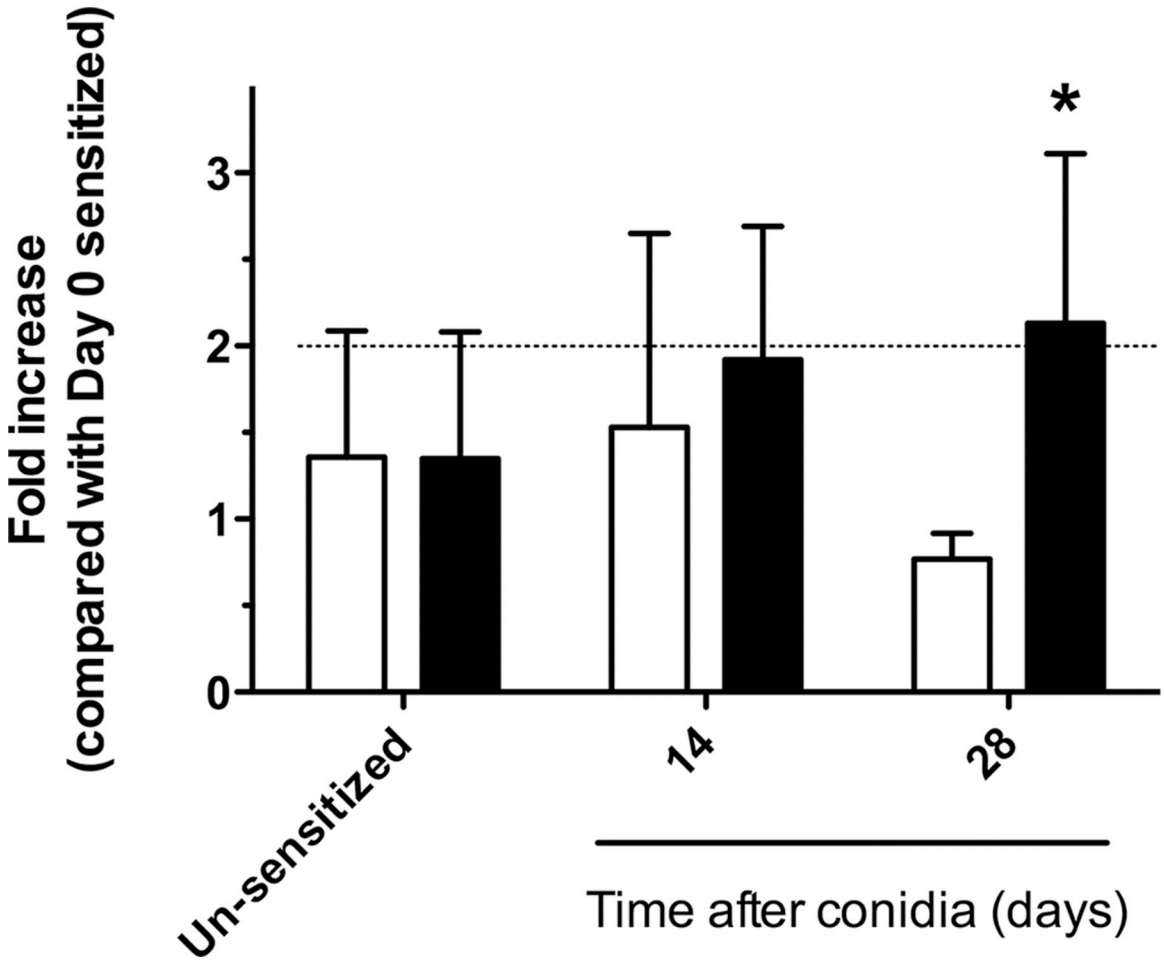

Figure 3.4. ***Sustained whole lung TLR9 transcript expression was observed in*** **A. fumigatus-*sensitized mice that received swollen conidia.*** TAQMAN analysis of whole lung TLR9 mRNA levels from *Aspergillus* sensitized mice receiving i.t. 5×10^6 resting or swollen conidia. Data are expressed as mean ± SEM; $n = 3$/group/time point. *, $P \leq 0.05$.

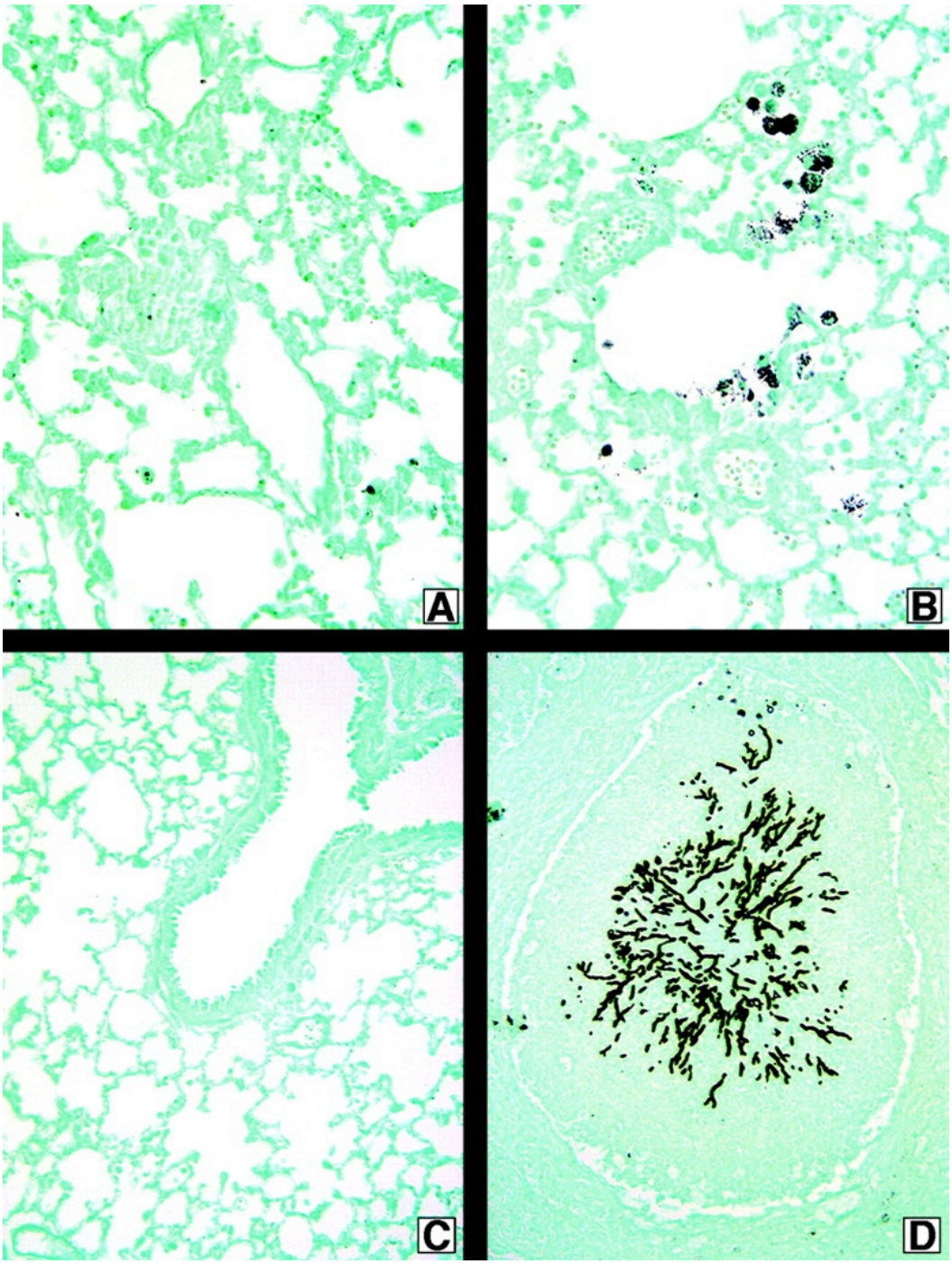

Figure 3.5. ***Swollen conidia grow in*** **A. fumigatus-*sensitized TLR9$^{-/-}$ mice.*** TLR9$^{+/+}$ **(A & C)** and TLR9$^{-/-}$ **(B & D)** mouse whole-lung tissue sections from sensitized mice challenged i.t. with 5×10^6 swollen *Aspergillus* conidia. Slides were processed via Gomori Methenamine Silver (GMS), which stains *Aspergillus* conidia and hyphae black. Lungs were harvested at day 14 **(A & B)** and at day 28 **(C & D)**. The original magnification was ×200 for all photomicrographs.

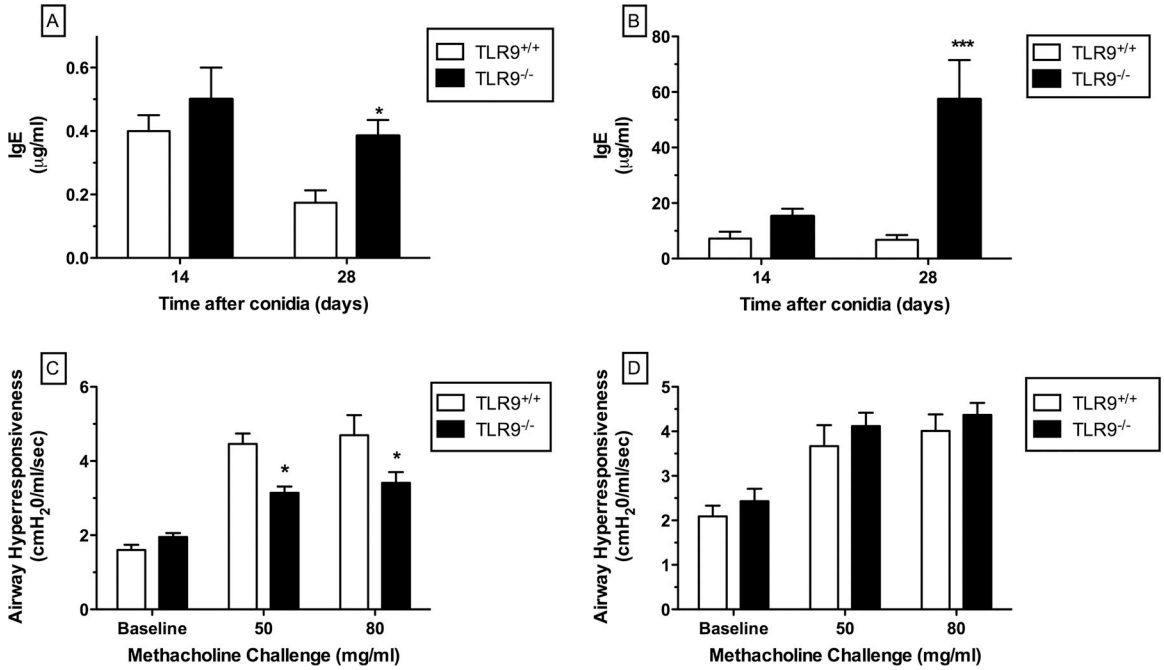

Figure 3.6. ***Conidia significantly augmented circulating total IgE levels in* A. fumigatus-*sensitized TLR9$^{-/-}$ mice regardless of germination status, but TLR9$^{-/-}$ mice showed significantly lower airway hyperresponsiveness following resting conidia challenge.*** ELISA for serum IgE from TLR9$^{+/+}$ and TLR9$^{-/-}$ sensitized mice challenged with 5×10^6 resting **(A)** or swollen **(B)** *Aspergillus* conidia. Airway hyperresponsiveness analysis from TLR9$^{+/+}$ and TLR9$^{-/-}$ sensitized mice infected with resting **(C)** or swollen **(D)** *Aspergillus* conidia. Peak increases in airway resistance or hyperresponsiveness (units = cm H_2O/mL/sec) were determined at each time point after the intravenous injection of methacholine. Data are expressed as mean ± SEM; n = 3/group/time point. *, $P \leq 0.05$; ***, $P \leq 0.001$.

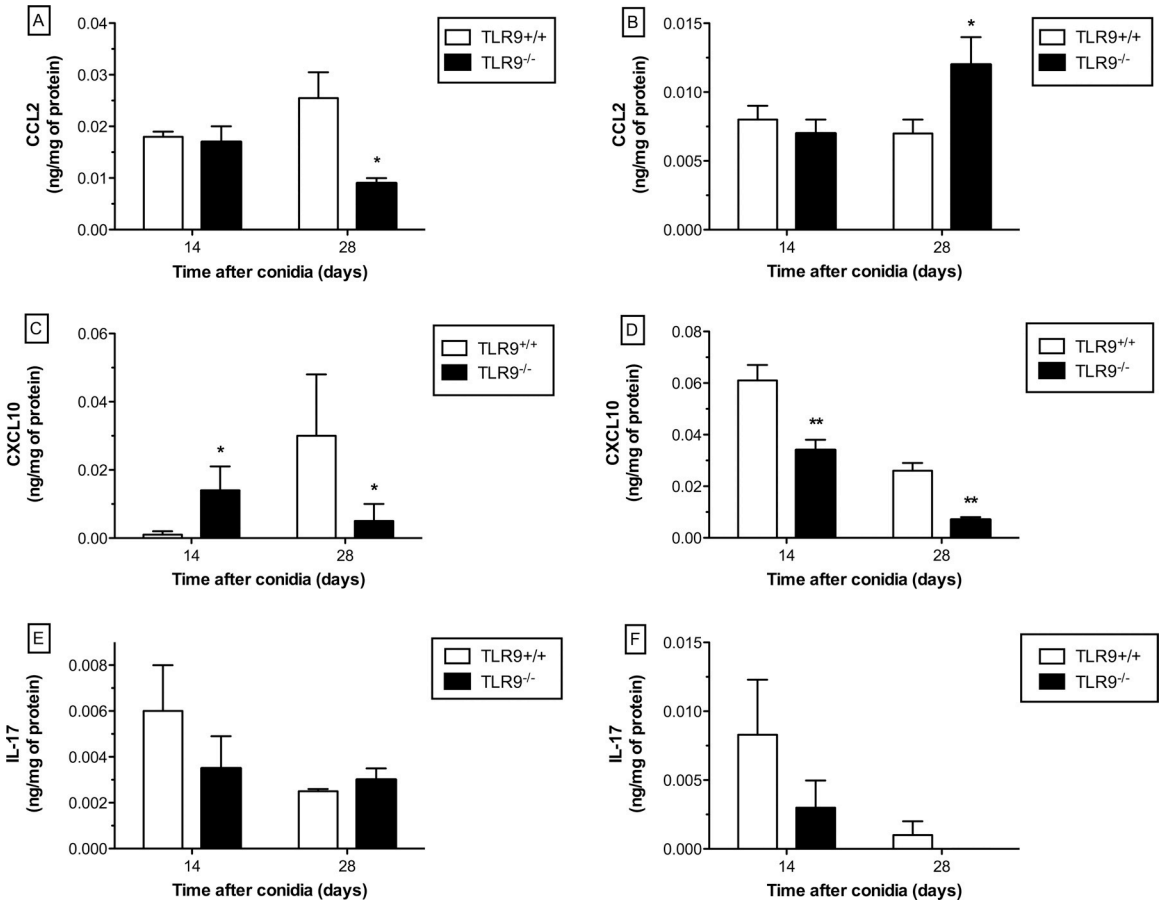

Figure 3.7. ***Divergent effects on whole lung cytokine and chemokine levels were observed in* A. fumigatus*-sensitized TLR9$^{-/-}$ mice following resting or swollen conidia challenge***. ELISA or Bio-Plex® of CCL2 **(A)**, CXCL10 **(B)**, and IL-17 **(C)**, comparing TLR9$^{+/+}$ to TLR9$^{-/-}$ whole lung protein levels from sensitized mice challenged i.t. with resting *Aspergillus* conidia. ELISA or Bio-Plex® of CCL2 **(D)**, CXCL10 **(E)**, and IL-17 **(F)**, comparing TLR9$^{+/+}$ to TLR9$^{-/-}$ whole lung protein levels from sensitized mice infected with swollen *Aspergillus* conidia. Data are expressed as mean ± SEM; n = 3/group/time point. *, $P \leq 0.05$; **, $P \leq 0.01$.

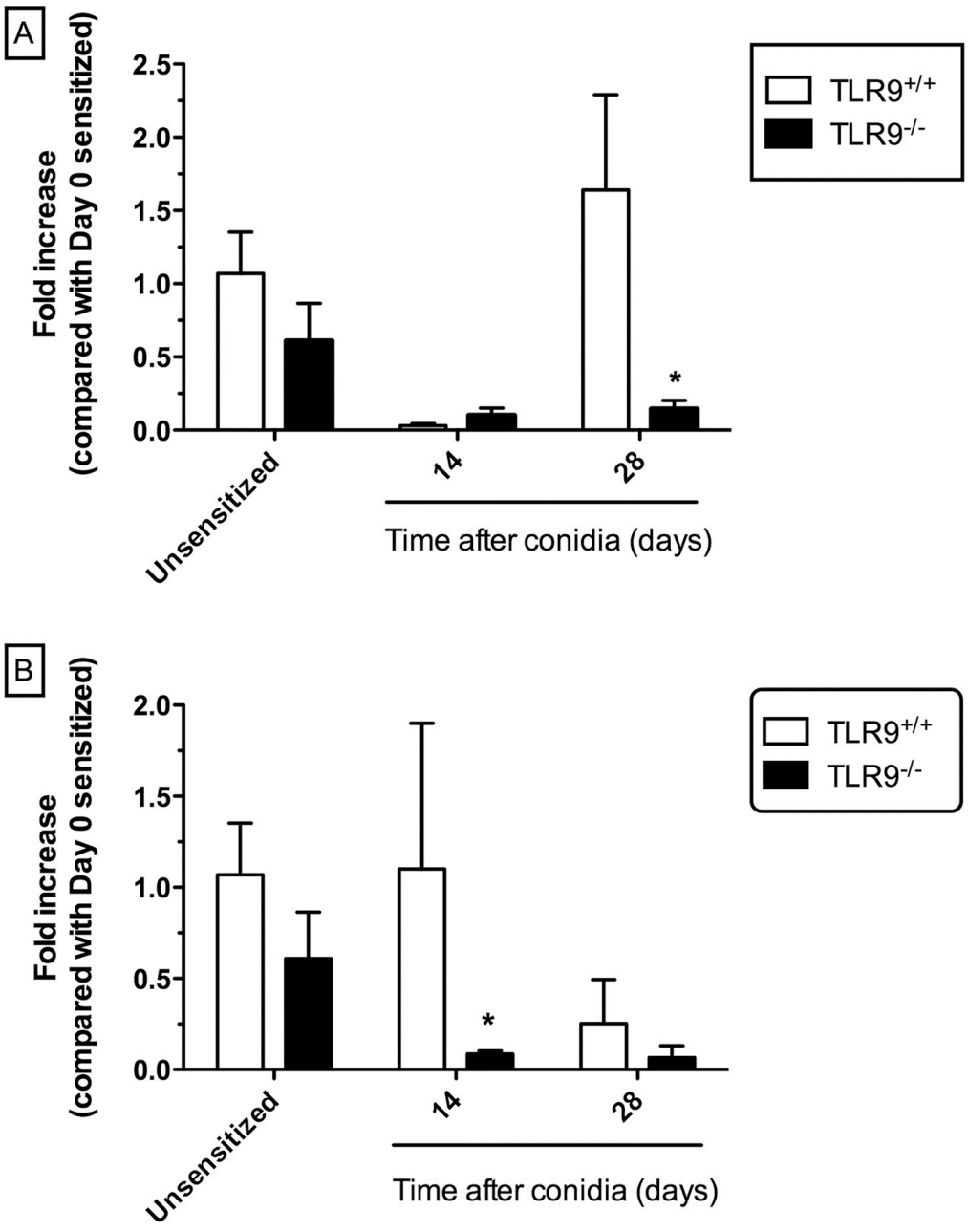

Figure 3.8. ***Non-sensitized and* A. fumigatus*-sensitized TLR9$^{-/-}$ mice exhibit lower whole lung dectin-1 expression relative to TLR9$^{+/+}$ mice.*** TAQMAN analysis of TLR9$^{+/+}$ and TLR9$^{-/-}$ mouse lung dectin-1 levels from *Aspergillus* sensitized mice infected i.t. with 5×10^6 resting **(A)** or swollen **(B)** *Aspergillus* conidia. Error bars indicate standard error means. Data are expressed as mean ± SEM; $n = 3$/group/time point. *, $P \leq 0.05$.

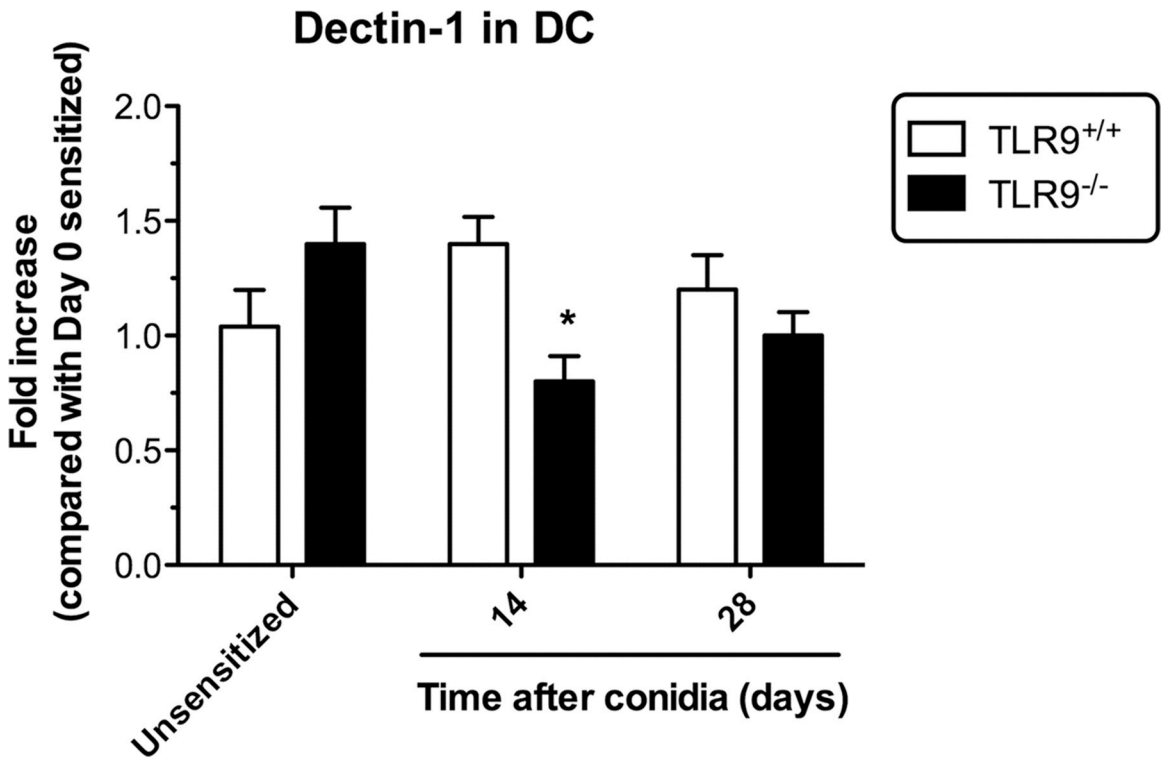

Figure 3.9. ***Bone marrow dendritic cells exhibit significantly lower dectin-1 expression at day 14 after swollen conidia challenge in TLR9$^{-/-}$ mice compared with TLR9$^{+/+}$ mice.*** TAQMAN analysis of dectin-1 expression by TLR9$^{+/+}$ and TLR9$^{-/-}$ mouse bone marrow dendritic cells from *Aspergillus* sensitized mice infected i.t. with 5×10^6 swollen *Aspergillus* conidia. Data are expressed as mean ± SEM; $n = 3$/group/time point. *, $P \leq 0.05$.

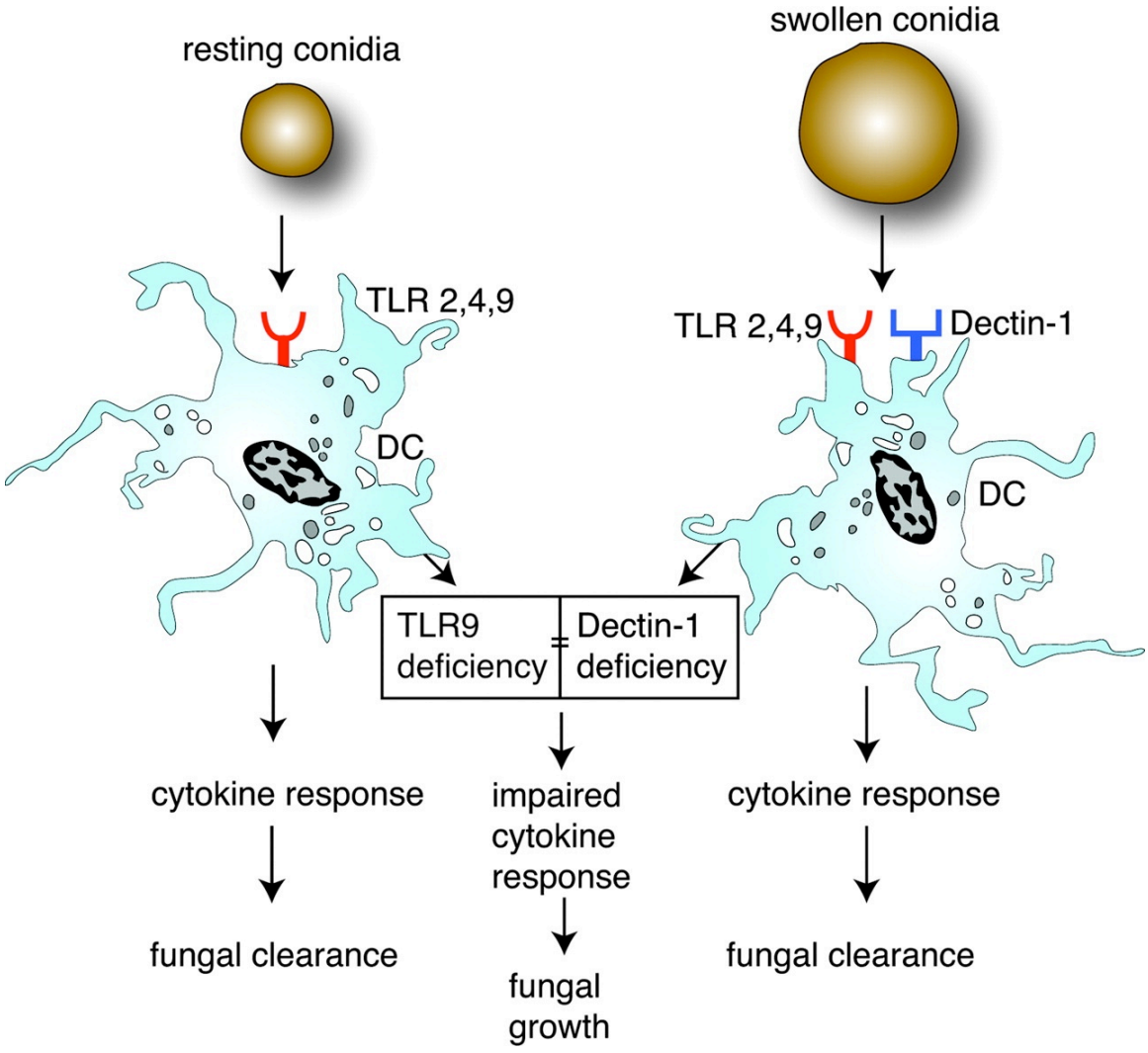

Figure. 3.10. ***Model for conidium recognition and innate immune responses in TLR9$^{+/+}$ and TLR9$^{-/-}$ mice.*** The recognition of resting conidia through TLR9 and swollen conidia through TLR9 and dectin-1 promotes cytokine responses leading to fungal clearance. The absence of TLR9, however, produces an impaired cytokine response and fungal growth in TLR9$^{-/-}$ mice challenged with resting conidia. Decreased levels of dectin-1 expression are observed in TLR9$^{-/-}$ mice. This decrease also produces a defective cytokine response in TLR9$^{-/-}$ mice challenged with swollen conidia and fungal growth.

CHAPTER 4

INTRANASAL CpG THERAPY ATTENUATED EXPERIMENTAL FUNGAL ASTHMA IN A TLR9-DEPENDENT AND INDEPENDENT MANNER.

Summary

CpG administration abolishes airway inflammation and remodeling in acute models of allergic airway disease. Herein, we investigated the therapeutic effect of CpG in a chronic fungal model of asthma. $TLR9^{+/+}$ and $TLR9^{-/-}$ mice were sensitized to soluble *Aspergillus fumigatus* antigens and challenged with live *A. fumigatus* conidia. Mice were treated with intraperitoneal (IP) CpG, intranasal (IN) CpG, or left untreated from days 14 to 28-post conidia challenge. All features of allergic airway disease were attenuated in $TLR9^{+/+}$ mice treated with IN CpG, including airway hyperresponsiveness (AHR), mucus production, and peribronchial fibrosis. $TLR9^{-/-}$ mice treated with IN CpG exhibited attenuated airway remodeling but not AHR. Whole-lung IL-12 levels were significantly elevated in both $TLR9^{+/+}$ and $TLR9^{-/-}$ mice receiving IN CpG but not in either group receiving IP CpG. Whole-lung IL-10 levels were significantly elevated in IN CpG-treated $TLR9^{+/+}$ mice but not in $TLR9^{-/-}$ mice receiving IN CpG. Increased whole-lung transcript and protein levels of the scavenger receptors SR-A and MARCO were observed in $TLR9^{-/-}$ mice compared with $TLR9^{+/+}$ mice, possibly accounting for the CpG responsiveness in the knockout group. Together, these data show that IN CpG has a therapeutic effect during established fungal asthma, which is TLR9-dependent and independent.

Introduction

Allergic asthma is characterized by Th2 inflammation, which drives physiologic and structural remodeling events in the lung (283, 284). Recent strategies to treat allergic airway disease have focused on limiting or reducing the Th2 inflammation. One immunotherapeutic strategy being developed for clinical therapy in asthma involves the use of hypomethylated oligodeoxynucleotides (ODN) containing CpG motifs, which mimic either bacterial or viral DNA (285-287). Prophylactic, systemic CpG administration in acute allergic airway models driven by ovalbumin (OVA), *Aspergillus* antigen, or house dust mite elicits major protective effects (288) (289) (290). Further, exogenously administered CpG have been shown to inhibit and/or reverse airway remodeling in an OVA model of allergic asthma even when administered after OVA challenge and establishment of airway disease (291) (292, 293). The mechanism through which CpG inhibits or reverses allergic airway disease is attributed, in part, to the inhibition of Th2 cytokines such as IL-4, IL-5, IL-9, and IL-13 and migration of Th2 cells into the lung environment (288, 291, 294-296). B cells and pDCs respond to CpG and generate Th1-type pro-inflammatory cytokines, interferons, and chemokines. Certain CpG motifs have also been demonstrated to activate NK cells and induce pDCs to secrete IFN-α (127, 297). Some controversy exists as to which Th1-type cytokines mediate the therapeutic effects of CpG in allergic airway disease. The beneficial effects of CpG in experimental allergic airway disease are partially inhibited after the immunoneutralization of IFN-α, IFN-β, and IL-12 (288). Subsequent studies identified that neither IL-12 nor IFN-γ were necessary for the therapeutic effect observed with CpG in allergic asthma models (298). More recently, the mechanisms behind the inhibitory

effects of CpG have been identified as involving the regulation of IL-5 through IL-10 synthesis (299). Thus, CpG administration prior to or during acute allergic airway disease markedly attenuates features of this disease via its immunomodulatory effects.

Although CpG is assumed to work via Toll like receptor 9 (TLR9), emerging evidence suggests that this ligand might exert immunomodulatory effects in a TLR9-independent manner (300-302). TLR9-independent CpG activation occurs through Src kinase signaling mechanisms, which produce tyrosine phosphorylation events leading to actin polymerization and chemokine generation (302). In neutrophils, TLR9-independent, but MyD88-dependent mechanisms have been identified as critical to the response to CpG *in vitro* (301). Macrophage receptor with collagenous structure (MARCO) and scavenger receptor-A (SR-A) have been recently identified as receptors for CpG and are expressed on lung macrophages and dendritic cells (303-305). Whether CpG exerts immunomodulatory effects in a TLR9-independent manner during experimental allergic airway disease has not been previously addressed.

In the present study, we investigated the therapeutic effect of CpG in a chronic fungal asthma model. While previous investigations of CpG in experimental asthma have investigated various modes of CpG delivery in wildtype ($TLR9^{+/+}$) mice with allergic airway disease, our present study examined the effects of both the systemic (IP) and local (IN) administration of CpG in $TLR9^{+/+}$ and $TLR9^{-/-}$ mice with established *A. fumigatus* conidia-induced fungal asthma. We recently reported that *A. fumigatus*-sensitized $TLR9^{-/-}$ mice exhibit a very severe form of fungal asthma characterized by fungal growth and profound tissue remodeling after these mice are challenge with swollen conidia (306). In the present study, IN CpG but not IP CpG administration to

TLR9$^{+/+}$ mice from day 14 to 28 after conidia challenge ameliorated AHR and airway remodeling at the day 28 time point. IN CpG but not IP CpG treatment over the same time ameliorated airway remodeling but not AHR in TLR9$^{-/-}$ mice with fungal asthma. While IN CpG induced whole lung levels of IL-12 in both TLR9$^{+/+}$ and TLR9$^{-/-}$ mice, only the TLR9$^{+/+}$ group exhibited an increase in whole lung levels of IL-10 when CpG was delivered in this manner. Responses to IN CpG in TLR9$^{-/-}$ mice were consistent with the markedly increased expression of MARCO and SR-A in the lungs of TLR9$^{-/-}$ mice with fungal asthma. Thus, TLR9-dependent and independent recognition of CpG in the lung are responsible for reversing airway remodeling in mice during chronic fungal asthma.

Results

Intranasal CpG attenuated airway hyperresponsiveness (AHR) in TLR9$^{+/+}$ but not TLR9$^{-/-}$ mice.

We have previously examined (AHR) responses in TLR9$^{+/+}$ and TLR9$^{-/-}$ mice (229). In this previous study we observed that the absence of TLR9 had no effect on methacholine-induced AHR since both wildtype and knockout mice showed similar airway responses to methacholine. Previous reports have shown that CpG inhibits AHR in experimental asthma (290). Our present results indicate that IN CpG from days 14 to 28 after conidia, but not IP CpG treatment over the same time period decreased AHR in TLR9$^{+/+}$ mice measured at day 28 after conidia challenge. We also observed that neither IN nor IP CpG affected allergic airway hyperreactivity in TLR9$^{-/-}$ mice **(Fig. 4.1A & B)**. Thus, local but not systemic CpG administration inhibited AHR in TLR9$^{+/+}$ mice and the inhibitory effects of IN CpG required TLR9 expression.

Exogenous CpG altered circulating Ig levels in TLR9$^{-/-}$ but not TLR9$^{+/+}$ mice.

We next assessed serum levels of IgE and IgG2a to determine whether the CpG therapies affected these immunoglobulins at day 28 after conidia. Elevation of serum IgE levels is a characteristic feature of atopy and allergic disease due to *Aspergillus* hypersensitivity (260, 261). *Aspergillus*-sensitized and -challenged TLR9$^{-/-}$ mice treated with IP CpG did not exhibit any significant increase in serum IgE levels compared with TLR9$^{+/+}$ mice. No differences in serum IgE levels between the untreated and IN CpG-treated TLR9$^{+/+}$ and TLR9$^{-/-}$ groups were observed **(Fig. 4.2A)**. IN CpG-treated TLR9$^{-/-}$ mice also did not demonstrate a significant change in serum IgG2a levels relative to

TLR9$^{+/+}$ mice **(Fig. 4.2B)**. No differences in serum IgG2a levels between the untreated and IP CpG-treated TLR9$^{+/+}$ and TLR9$^{-/-}$ groups were observed. It is important to note that neither IP nor IN CpG treatments in TLR9$^{+/+}$ mice produced any noticeable effect on serum Ig levels.

Intranasal CpG but not intraperitoneal CpG treatment of TLR9$^{+/+}$ and TLR9$^{-/-}$ asthmatic mice ameliorates peribronchial fibrosis and fungal growth.

We have previously reported that TLR9$^{-/-}$ mice were unable to control the growth of *A. fumigatus* leading to prominent fungal masses in the lungs of these mice at day 28 after conidia (306). Trichrome-stained lung sections from TLR9$^{+/+}$ and TLR9$^{-/-}$ mice at day 28 post intratracheal conidia challenge revealed that fibrosis was prominent around airways and fungal masses was prominent in control **(Fig. 4.3A & D; fibrotic tissue is stained light blue)** and IP CpG- **(Fig. 4.3C & F)** treated groups whereas peribronchial fibrosis was absent in both the TLR9$^{+/+}$ and TLR9$^{-/-}$ groups that received IN CpG **(Fig. 4.3B & E)**. Most remarkable, was the therapeutic effect of IN CpG on the fibrotic fungal masses in TLR9$^{-/-}$ mice; no fibrotic fungal masses were detected in IN CpG-treated TLR9$^{-/-}$ mice, and the histological appearance of whole lung sections in this group of mice was similar to that of IN CpG-treated TLR9$^{+/+}$ mice. Thus, these data indicate that IN CpG but not IP CpG reversed the peribronchial fibrotic remodeling in TLR9$^{+/+}$ and TLR9$^{-/-}$ mice, and eliminated fungus from TLR9$^{-/-}$ mice.

Intranasal CpG but not intraperitoneal CpG treatment reduced mucus production and fungal growth in both TLR9$^{+/+}$ and TLR9$^{-/-}$ mice.

Following our observations of route-specific effects of CpG administration on lung inflammation and remodeling, changes in airway mucus secretion through Periodic Acid Schiff (PAS) stain of lung histological sections was examined next. PAS-stained lung sections from $TLR9^{+/+}$ and $TLR9^{-/-}$ mice at day 28-post intratracheal conidia challenge demonstrated enhanced mucus production in untreated **(Fig. 4.4A & D)** and IP CpG **(Fig. 4.4C & F)** treated groups, but markedly decreased mucus was observed in lungs from IN CpG treated mice **(Fig. 4.4B & E)**. Further, GMS-stained lung sections from $TLR9^{+/+}$ and $TLR9^{-/-}$ mice at day 28-post intratracheal conidia challenge demonstrated fungal growth and macrophage engulfment of conidia in untreated **(Fig. 4.5A & D)** and IP CpG **(Fig. 4.5C & F)** treated groups, but markedly reduced fungal growth and macrophage phagocytosis of conidia was observed in lungs from IN CpG treated mice **(Fig. 4.5B & E)**. Thus, these data indicated that mucus metaplasia and fungal growth were markedly attenuated by IN CpG treatment in both $TLR9^{+/+}$ and $TLR9^{-/-}$ mice.

$TLR9^{-/-}$ mice exhibited significantly increased whole lung TLR3 transcript levels following IP CpG treatment.

We next examined whether IN or IP CpG altered whole lung TLR expression in $TLR9^{+/+}$ and $TLR9^{-/-}$ mice. Transcript levels of TLR4, 6, and 3 were analyzed in whole lung tissues at day 28 after conidia. CpG treatment did not alter levels of TLR 4 or TLR6 in the lungs of asthmatic mice **(Fig. 4.6A & B)**. IP CpG-treated $TLR9^{-/-}$ mice, however, demonstrated a significant increase in TLR3 levels relative to $TLR9^{+/+}$ mice **(Fig. 4.6C)**. Thus, these data suggest that IP CpG treatment in TLR9 deficient mice induced TLR3.

However, the CpG administration to TLR9$^{+/+}$ mice did not alter whole lung TLR4 or TLR6 levels in either wildtype or knockout mice.

TLR9$^{-/-}$ mice exhibit significantly increased whole lung IFN-α transcript levels following IP CpG treatment, but significantly elevated CXCL10 transcript levels following IN CpG treatment.

We next sought to characterize the mechanism through which IN CpG attenuated airway remodeling in TLR9$^{+/+}$ and TLR9$^{-/-}$ mice and analyzed the transcript levels of several cytokines and chemokines in whole lung tissues at day 28 after conidia. IP CpG-treated TLR9$^{-/-}$ mice demonstrated an approximately 10-fold increase in IFN-α levels relative to TLR9$^{+/+}$ mice **(Fig. 4.7A)**. It is significant to note that IFN-α was only induced with IP CpG treatment but this induction had no effect on any of the characteristics of allergic airway disease. The amount of IFN-α detected in TLR9$^{-/-}$ mice was markedly greater than that detected in untreated or IN CpG-treated mice. Further, CXCL10 transcript levels were significantly increased in IN CpG treated TLR9$^{-/-}$ mice and were 3-fold greater compared with the TLR9$^{+/+}$ group **(Fig. 4.7B)**. Thus, these data suggested that the levels of CpG administered to TLR9$^{+/+}$ mice did not alter whole lung Th1-type cytokine and chemokine levels. However, the amounts of CpG delivered either by the intranasal or intraperitoneal route were sufficient to alter levels of these cytokine and chemokines in TLR9$^{-/-}$ mice.

Pro-allergic Th2 cytokine and chemokine levels are suppressed following IN CpG treatment in TLR9$^{+/+}$ mice only.

Whole lung cytokine and chemokine protein levels were analyzed next in this model of fungal asthma in response to IN and IP CpG treatment at day 28 after conidia. No significant differences in IL-4, IL-5, IL-13, and IFN-γ were observed between the IN and IP CpG groups at day 28 after conidia **(Table 4.1).** Significant differences in the levels of the TGF-β, TSLP, and CCL17 (cytokines and chemokines that promote allergic airway disease, differentiation of Th2 cells, and chemotaxis of Th2 cells) were observed following IN CpG treatment. Specifically, a significant decrease in whole lung levels of TGF-β in $TLR9^{+/+}$ mice receiving IN CpG compared with untreated and IP CpG treated $TLR9^{-/-}$ mice **(Fig. 4.8A)**. Further, there was a decrease in whole lung levels of TSLP in $TLR9^{+/+}$ mice treated with IN CpG compared with untreated and IP CpG treated $TLR9^{-/-}$ mice **(Fig. 4.8B)**. Finally, a significant decrease in whole lung levels of CCL17 was observed in $TLR9^{+/+}$ mice that received IN CpG compared with untreated and IP CpG treated $TLR9^{-/-}$ mice **(Fig. 4.8C)**. It is important to note that IN CpG did not inhibit levels of these Th2-type cytokines and chemokines in $TLR9^{-/-}$ mice compared with untreated $TLR9^{-/-}$ mice. Further, IP CpG did not inhibit levels of these Th2-type cytokines and chemokines in either group of mice compared with the appropriate untreated group of mice. Thus, these data indicated that decreased allergic airway disease in IN CpG-treated $TLR9^{+/+}$ mice with fungal asthma might be a consequence of both decreased levels of TGF-β and lower levels of the Th2-associated cytokines TSLP and CCL17.

Asthmatic mice treated with intranasal CpG exhibited increased IL-12 levels regardless of TLR9 expression, but IL-10 induction following IN CpG was TLR9-dependent.

Changes in other cytokines, namely IL-12 and IL-10 were observed following CpG treatment in this model at day 28 after conidia. A significant elevation in IL-12 levels was observed in both $TLR9^{+/+}$ and $TLR9^{-/-}$ mice during IN CpG treatment compared with IP CpG treatment **(Fig. 4.9A)**. We also observed a significant elevation in whole lung IL-10 levels in IN CpG-treated $TLR9^{+/+}$ mice, but levels of this cytokine were not altered in $TLR9^{-/-}$ mice **(Fig. 4.9B)**. No immunoreactive IL-10/IL-12 was detected at this timepoint in untreated mice. These data indicated that IN CpG drove the expression of lung IL-12 in both $TLR9^{+/+}$ and $TLR9^{-/-}$ mice, but elicited lung IL-10 levels in only $TLR9^{+/+}$ mice.

SR-A and MARCO levels were elevated in $TLR9^{-/-}$ mice with chronic fungal asthma.

Given the altered histological and Th1-type cytokine/chemokine profile in CpG-treated $TLR9^{-/-}$ mice, we next sought to identify alternative receptors through which CpG was exerting its therapeutic effects and examined the transcript and protein levels of the scavenger receptors MARCO and SR-A at day 28 after conidia. Results indicated a significant elevation in SR-A transcript levels, but no significant elevation in transcript levels of MARCO in untreated $TLR9^{-/-}$ mice relative to $TLR9^{+/+}$ mice **(Fig. 4.10A & B)**. Further, immunohistochemical analysis of whole lung tissue sections revealed that both SR-A and MARCO were more abundantly expressed in the epithelium and mononuclear cells in untreated $TLR9^{-/-}$ mice compared with $TLR9^{+/+}$ mice at day 28 after conidia **(Fig. 4.11)**. To determine the effect of CpG treatment on whole lung levels of SR-A and MARCO an ELISA was employed. Lower levels of MARCO and SR-A were measured in whole lung samples from IN CpG-treated $TLR9^{+/+}$ mice compared with IP CpG-treated

TLR9$^{+/+}$ mice **(Fig. 4.12)**. SR-A levels were lower in IN CpG-treated TLR9$^{-/-}$ mice compared with IP CpG-treated TLR9$^{-/-}$ mice but MARCO levels were similar between the two treatment groups of TLR9$^{-/-}$ mice **(Fig. 4.12)**. Notably, significantly greater levels of MARCO were present in whole lungs samples from IN CpG-treated TLR9$^{-/-}$ mice compared with similarly treated TLR9$^{+/+}$ mice **(Fig. 4.12)**. Thus, greater transcript and protein levels of both SR-A and MARCO were present in TLR9$^{-/-}$ mice possibly accounting for the responsiveness of these mice to CpG.

Discussion

Herein, we characterized the asthmatic phenotype in *A. fumigatus*- sensitized and conidia-challenged TLR9$^{+/+}$ and TLR9$^{-/-}$ mice after treatment with IP or IN CpG from days 14 to 28 after conidia challenge. We observed that IN CpG but not IP CpG therapy significantly enhanced whole lung IL-12 and IL-10 levels, and significantly inhibited airway inflammation and hyperresponsiveness, lung remodeling, and Th2-associated cytokine levels in a model of allergic asthma in TLR9$^{+/+}$ mice. Surprisingly, we observed that IN CpG but not IP CpG therapy also significantly increased serum IgG2a levels and whole lung levels of CXCL10 and IL-12. Compared with untreated TLR9$^{-/-}$ mice, IN CpG treated TLR9$^{-/-}$ mice exhibited attenuated fungal growth and airway remodeling but not AHR. One explanation for the CpG responsiveness of *A. fumigatus*-sensitized and conidia challenged TLR9$^{-/-}$ mice might be derived from the observation that transcript and protein levels of the scavenger receptors MARCO and SR-A were dramatically elevated in these mice compared with similarly sensitized and challenged TLR9$^{+/+}$ mice. Thus, these data demonstrated that therapeutic responses to CpG during experimental fungal asthma were dependent upon the site of CpG administration, and CpG had TLR9-independent therapeutic effects in this model of fungal asthma, possibly due to CpG activation via MARCO and SR-A receptors.

Previous studies have demonstrated that the adjuvant or Th1-type cytokine response evoked by CpG appears to depend on the site of administration (307), with mucosal delivery being identified as the route promoting the optimal immunostimulatory effect (308, 309). Although the intranasal delivery of CpG promotes inflammatory changes in non-allergic mice (310), its delivery via this route in allergic mice has been

shown to markedly attenuate the pulmonary allergic inflammatory response in wildtype mice (119). The therapeutic effect of CpG-ODN treatment in the experimental fungal asthma model examined herein was dependent on the site of administration since the intranasal delivery of CpG provided a therapeutic effect in both wildtype and knockout mice whereas the intraperitoneal delivery of CpG did not. This difference in therapeutic outcome could be explained by the direct modulation of CpG-responsive cell types such as epithelial cells, macrophages, and dendritic cells, which are responsible for fungus-induced allergic inflammation. In fact, previous studies involving the priming of mice with *Aspergillus* antigens and CpG in a model of invasive aspergillosis have demonstrated that CpG promotes dendritic cell activation within the lung and the production of IL-12 and IFN-γ by these cells (311). Thus, the route of administration of CpG proved to be important for its therapeutic effect in experimental fungal asthma.

Immunostimulatory sequences containing CpG motifs induce interferons and IFN-inducible genes such as CXCL10 both in experimental models of allergic airway disease (119) and clinical asthma (312). CpG directly activates monocytes, macrophages, and dendritic cells to secrete IFN-α/β, IL-6, IL-12, and TNF-α leading to NK cell activation and secretion of IFN-γ (119, 309). Administration of neutralizing mAbs against type I cytokines such as IFN-α, have been found to attenuate the inhibitory effect of CpG-ODN on airway inflammation and Th2 cell migration into the lung (288). In the present study, IN CpG treatment in $TLR9^{+/+}$ mice was associated with significantly increased whole lung levels of IL-12 but this mode of CpG delivery did not alter whole lung levels of IFN-α and CXCL10. Given that IP CpG increased whole lung IFN-α but not IL-12 levels in $TLR9^{+/+}$ mice with fungal asthma, it is likely that the therapeutic

effects of CpG were mediated, in part, via IL-12 and not IFN-α or CXCL10 in $TLR9^{+/+}$ mice. Surprisingly, we saw more dynamic changes in Th1-type mediators in $TLR9^{-/-}$ mice with fungal asthma. IN CpG significantly enhanced serum levels of IgG2a, a Th1-associated Ig, and whole lung levels of CXCL10 and IL-12. IP CpG significantly enhanced whole lung levels of IFN-α. The latter finding in IP CpG-treated $TLR9^{-/-}$ mice might reflect the fact that TLR3 levels were significantly elevated in whole lung samples from this group of mice, but appears to confirm that this type 1 interferon does not mediate the therapeutic effects of CpG. Thus, the administration of CpG to TLR9-sufficient and -deficient mice with fungal asthma dynamically altered levels of Th1-type factors and mediators although only IN CpG enhanced the expression of whole lung IL-12.

Shifting the immune response away from Th2-type cytokine generation has been shown to effectively reduce asthmatic symptoms both experimentally and clinically (287). TGF-β and CCL17 have well described roles in Th2-mediated airway inflammation (259), and both are prominently expressed and appear to have clear roles in the experimental asthma model studied herein (228, 313). The cytokine TSLP has not been examined in this model previously but it is known to activate DCs and promotes the differentiation of Th2 T cells and secretion of Th2 cytokines and chemokines (314, 315). We observed that whole lung levels of TGF-β were reduced in CpG treated $TLR9^{+/+}$ mice and these findings are consistent with previous CpG studies in models of allergic airway disease (290, 292, 298). Inhibitory effects of IN CpG on whole lung TSLP and CCL17 were also apparent in $TLR9^{+/+}$ mice alone, consistent with reduced airway hyperresponsiveness and remodeling in these mice. Changes in IL-10 following IN CpG

were also TLR9-dependent. The inhibitory effects of IL-10 on allergic inflammation are well known and include regulation of eosinophilia (290), inhibition of the proliferation of various structural cell types (316), and regulation of airway inflammation through its inhibitory action on nitric oxide (NO), a potent mediator of airway hyperresponsiveness (317, 318). Recent studies have shown that the adoptive transfer of IL-10 over-expressing DCs into OVA-sensitized and challenged mice ameliorates allergic asthma symptoms (319). The contribution of macrophage-derived IL-10 driven by CpG activation of TLR9 has also shown to be an important therapeutic modality of CpG in allergic airway inflammation (320). Thus, IN CpG inhibited whole lung Th2 cytokines and chemokines and enhanced whole lung IL-10 levels in a TLR9-dependent manner, and these effects were consistent with the amelioration of all features of chronic fungal asthma in $TLR9^{+/+}$ mice.

The macrophage class A scavenger receptors SR-A and MARCO recognize CpG and promote IL-12 and NO synthesis (303, 305). SR-A levels were increased in an OVA-induced asthma model in wild-type mice, and both SR-A- and MARCO-deficient mice exhibited increased airway hyperresponsiveness and eosinophilic airway inflammation in this same model with a concomitant increase in the recruitment of dendritic cells in the lung (321). Recent studies have demonstrated that targeting scavenger receptors in a model of allergy produces a shift from a Th2 to a Th1 immune response (322). In the present study elevated SR-A and MARCO transcript and proteins levels were observed in whole lung from $TLR9^{-/-}$ mice compared with similar samples from $TLR9^{+/+}$ mice. We interpret these findings to indicate that scavenger receptors, such as SR-A and MARCO, play a compensatory role in the TLR9-deficient mouse and

recognize CpG. This interpretation is consistent with the enhanced Th1-type factors and mediators observed in TLR9$^{-/-}$ mice following CpG treatment. However, the full therapeutic effect of CpG required the presence of TLR9 indicating that SR-A and MARCO could not fully compensate for the absence of this toll like receptor. Future studies will address the identification and characterization of the TLR9-expressing cell, which is so critically important in the regulation of airway hyperresponsiveness.

While CpG therapies have proven very effective in experimental studies (323), clinical trials involving hypomethylated CpG oligodeoxynucleotides targeting TLR9 have failed to reproduce results observed in the laboratory. Specifically, clinical investigations into the efficacy of CpG in asthma have found that CpG increases in IFN-γ and IFN-inducible genes in asthmatics but has no impact on the characteristic airway hyperresponsiveness in these patients (312). Extrapolating the experimental findings in the present study to the clinical situation, it is plausible that the failure of CpG to work clinically is due in part to polymorphisms in TLR9 resulting in loss of function. Our previous study of the role of TLR9 during chronic fungal asthma showed that the absence of this receptor led to a severe form of allergic airway disease characterized by fungal growth (306) and thus it is possible that similar changes in severity related to the expression and function of TLR9 occur in clinical asthma. Investigations into the role of TLR9 polymorphisms in CpG-induced responses between individuals found major inter-individual differences of CpG-induced IFN-α production, however, these were not associated with common TLR9 variants (324). As mentioned above, CpG treatment in patients drove a Th1 response without improvement in airway hyperresponsiveness (312). Again, one might speculate that this scenario is similar to what we observed in

TLR9$^{-/-}$ mice given IN CpG, in which the presence of CpG recognizing scavenger receptors accounted for changes in Th1 cytokines without any attenuation of Th2 mechanisms driving AHR.

In summary, CpG appeared to have site-specific therapeutic effects on the maintenance of chronic fungal airway disease. Specifically, IN CpG but not IP CpG decreased airway hyperresponsiveness and airway remodeling in TLR9$^{+/+}$ mice consistent with its inhibitory effects on Th2-associated factors such as TGFβ, TSLP, and CCL17 and its stimulatory effects on IL-12 and IL-10. Surprisingly, IN CpG reduced airway remodeling in TLR9$^{-/-}$ mice, but airway hyperresponsiveness was not impacted in these mice. Together, these data highlight the importance of TLR9-dependent and –independent mechanisms in the modulation of all features of chronic fungal asthma.

Table 4.1. Whole lung cytokine and chemokine levels at day 28 after swollen conidia challenge in *A. fumigatus*-sensitized TLR9$^{+/+}$ and TLR9$^{-/-}$ mice.

Table 1						
	Untreated		IN CpG		IP CpG	
Cytokine/ Chemokine	TLR9$^{+/+}$	TLR9$^{-/-}$	TLR9$^{+/+}$	TLR9$^{-/-}$	TLR9$^{+/+}$	TLR9$^{-/-}$
IL-4	0.01±0.00	0.00±0.00*	0.02±0.00	0.00±0.00**	0.01±0.00	0.02±0.00
IL-5	ND	ND	ND	ND	ND	ND
IL-13	0.08±0.01	0.04±0.00*	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
IFN-γ	ND	ND	ND	ND	ND	ND

*P≤0.05, **P≤0.01, ***P≤0.001, compared with untreated TLR9$^{+/+}$ mice at the same time swollen conidia challenge. ND=Not Detected (Units are ng/mg total protein)

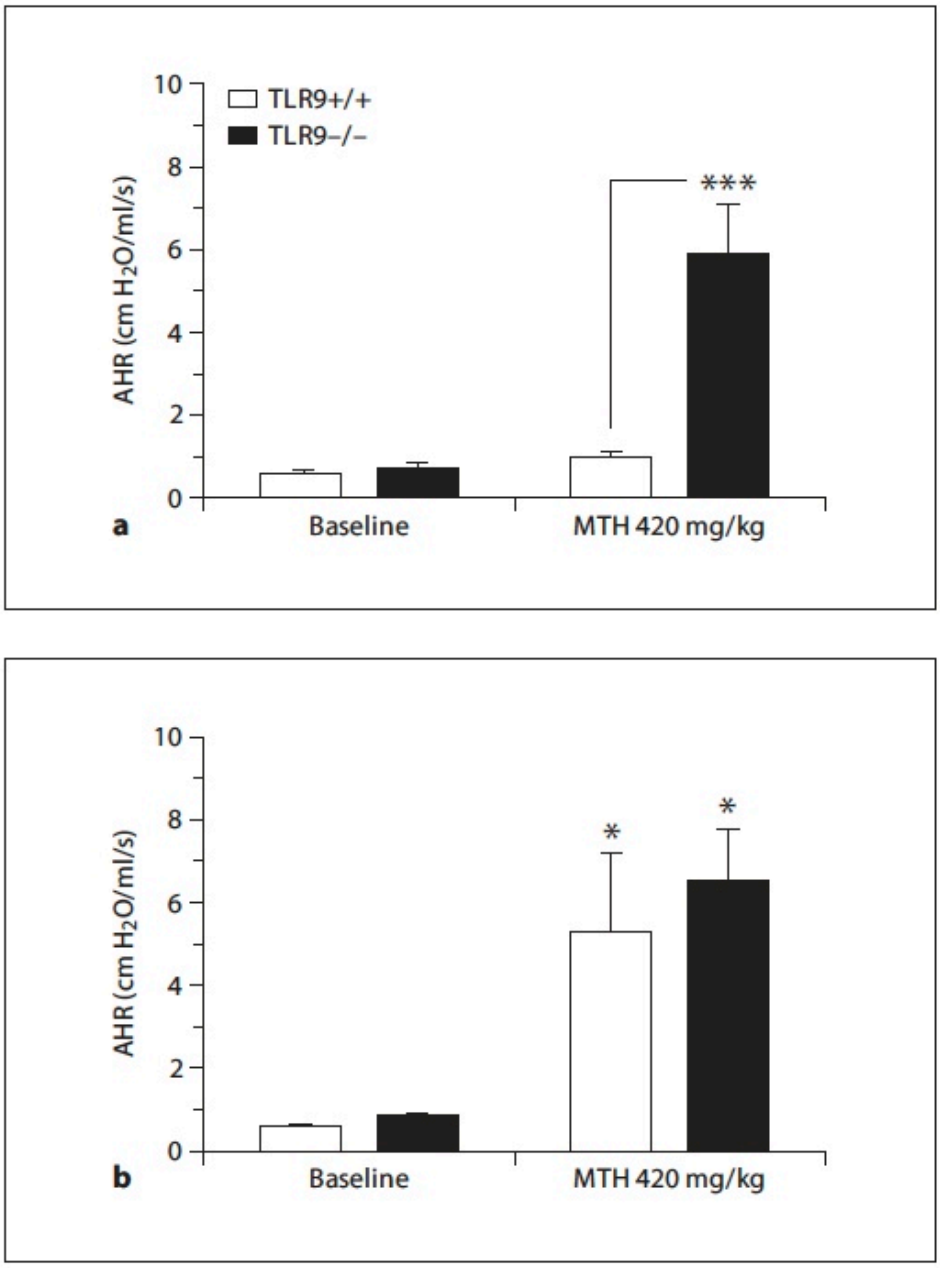

Figure 4.1. ***Intranasal CpG treatment attenuated airway hyperresponsiveness in TLR9$^{+/+}$ but not TLR9$^{-/-}$ mice.*** Airway resistance analysis in TLR9$^{+/+}$ and TLR9$^{-/-}$ sensitized and conidia challenged mice treated with intranasal CpG **(A)** or intraperitoneal CpG **(B)** on alternate days from day 14 to day 28 after conidia challenge. Peak increases in airway resistance or hyperresponsiveness (units = cm H2O/mL/sec) were determined at each time point after the intravenous injection of methacholine. Airway resistance in untreated TLR9$^{+/+}$ mice was 1.89 ± 0.11 cm H2O/mL/sec and 16.96 ± 3.86 cm H2O/mL/sec post IV administration of methacholine. Data are expressed as mean ± SEM; n = 5/group/time point. *, $P \leq 0.05$; **, $P \leq 0.01$; ***$P \leq 0.001$ compared with the appropriate baseline measurement or the indicated treatment group following methacholine challenge.

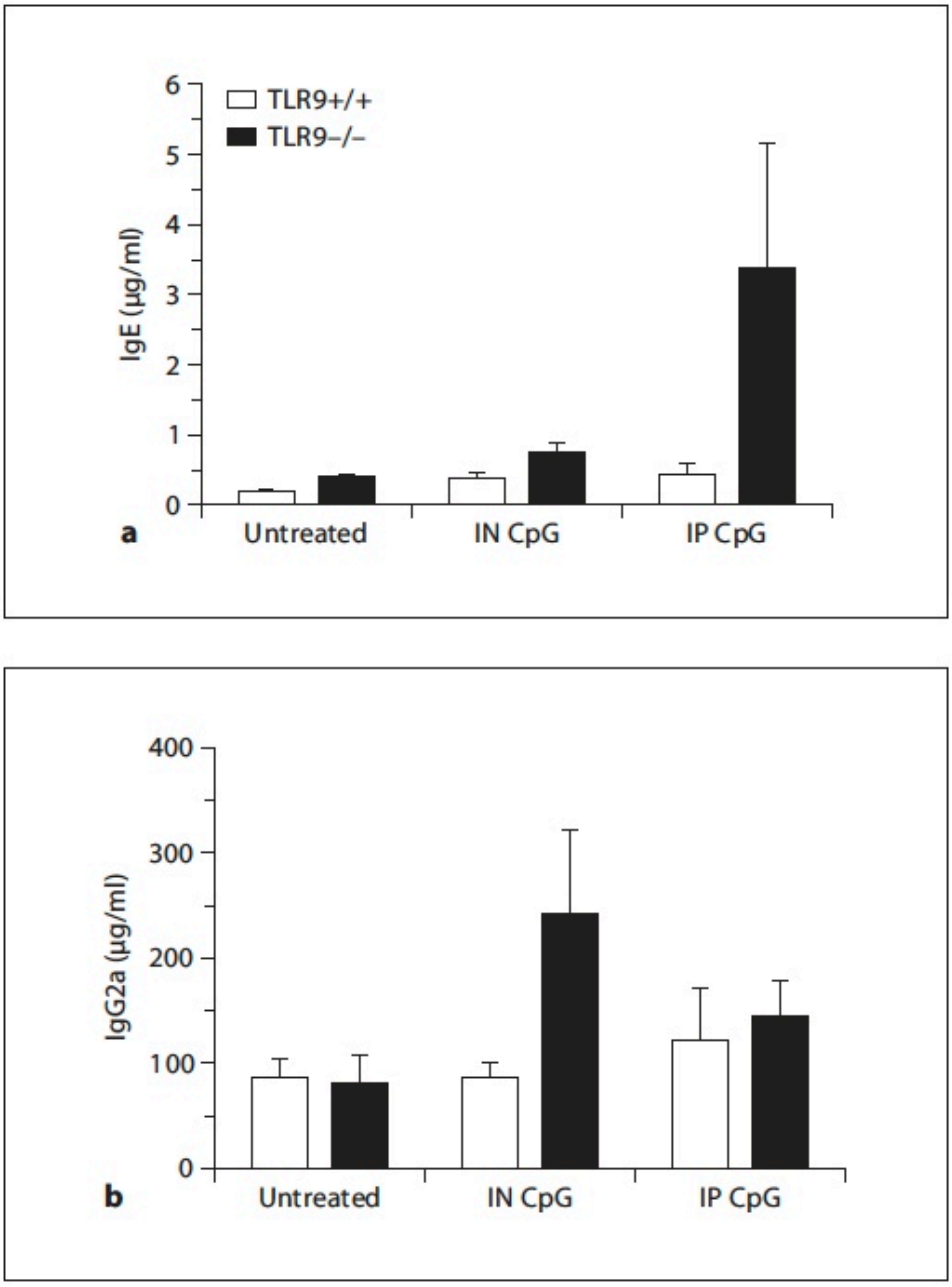

Figure 4.2. ***CpG treatments altered systemic Ig levels in TLR9$^{-/-}$ mice alone.*** ELISA analysis of serum IgE **(A)** and IgG2a **(B)** from *Aspergillus*-sensitized and conidia challenged TLR9$^{+/+}$ and TLR9$^{-/-}$ mice either untreated, treated with intranasal CpG, or intraperitoneal CpG. Serum was removed from all groups of mice at day 28 after conidia. Data are expressed as mean ± SEM; n = 10/group/time point.

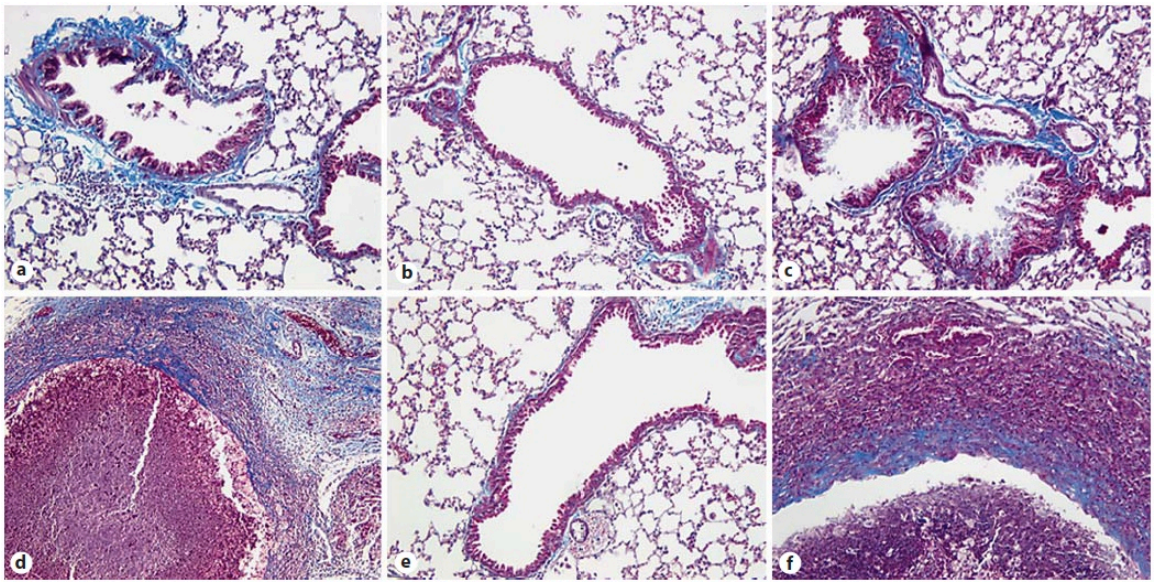

Figure 4.3. ***Peribronchial fibrosis and peri-fungal fibrotic capsules were markedly decreased or absent following intranasal but not intraperitoneal CpG treatment in TLR9$^{+/+}$ and TLR9$^{-/-}$ mice.*** Whole-lung tissue sections from *Aspergillus*-sensitized and challenged TLR9$^{+/+}$ and TLR9$^{-/-}$ mice were stained with Masson trichrome stain. Panels shown are representative of whole lung sections from TLR9$^{+/+}$ mice that were given no treatment **(A)**, intranasal CpG **(B)**, or intraperitoneal CpG **(C)**. Panels shown are representative of whole lung sections from TLR9$^{-/-}$ mice given no treatment **(D)**, intranasal CpG **(E)**, or intraperitoneal CpG **(F)**. Note that no fungal masses were detected in whole lung sections from intranasal CpG-treated TLR9$^{-/-}$ mice. Collagen deposition is stained blue in these photomicrographs. The original magnification was ×200 for all photomicrographs.

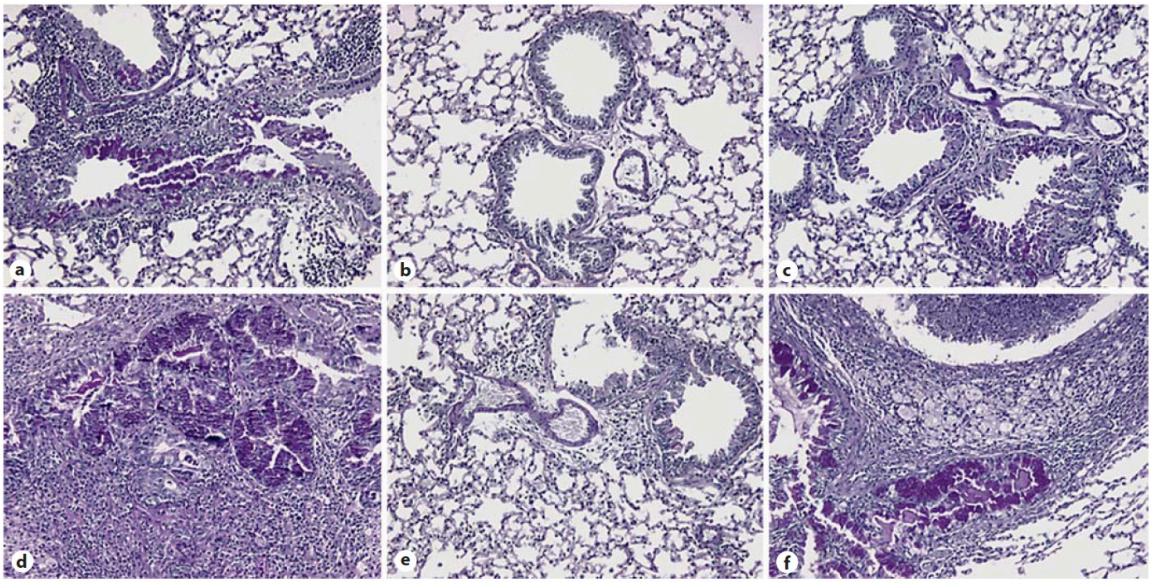

Figure 4.4. ***Mucus was markedly reduced following intranasal but not intraperitoneal CpG treatment in TLR9$^{+/+}$ and TLR9$^{-/-}$ mice.*** Mouse whole-lung tissue sections from *Aspergillus*-sensitized and challenged TLR9$^{+/+}$ and TLR9$^{-/-}$ mice stained with Periodic Acid Schiff (PAS). Panels shown are representative of whole lung sections from TLR9$^{+/+}$ mice that were given no treatment **(A)**, intranasal CpG **(B)**, or intraperitoneal CpG **(C)**. Panels shown are representative of whole lung sections from TLR9$^{-/-}$ mice given no treatment **(D)**, intranasal CpG **(E)**, or intraperitoneal CpG **(F)**. Mucus stains purple using the PAS staining procedure. The original magnification was ×200 for all photomicrographs.

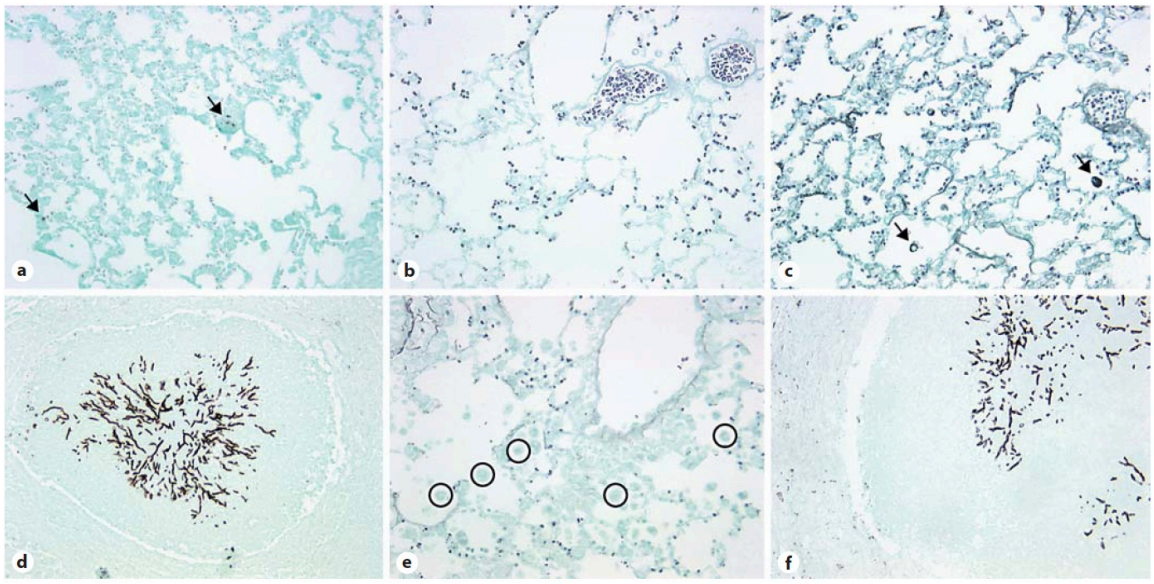

Figure 4.5. ***Fungal growth is markedly reduced following intranasal but not intraperitoneal CpG treatment in TLR9$^{+/+}$ and TLR9$^{-/-}$ mice.*** Mouse whole-lung tissue sections from *Aspergillus*-sensitized and challenged TLR9$^{+/+}$ and TLR9$^{-/-}$ mice stained with Gomori Methenamine Silver (GMS) stain. *Aspergillus* conidia and hyphae stain black using the GMS staining procedure. Panels shown are representative of whole lung sections from TLR9$^{+/+}$ mice that were given no treatment **(A)**, intranasal CpG **(B)**, or intraperitoneal CpG **(C)**. Panels shown are representative of whole lung sections from TLR9$^{-/-}$ mice given no treatment **(D)**, intranasal CpG **(E)**, or intraperitoneal CpG **(F)**. Black arrows highlight conidia engulfed by macrophages. Open circles denote macrophages that have not engulfed conidia. No macrophages containing engulfed conidia were visible in TLR9$^{+/+}$ mice given IN CpG treatment. The original magnification was ×200 for photomicrographs in panels **(A, C, D, & F)** and ×400 for photomicrographs in panels **(B & E)**.

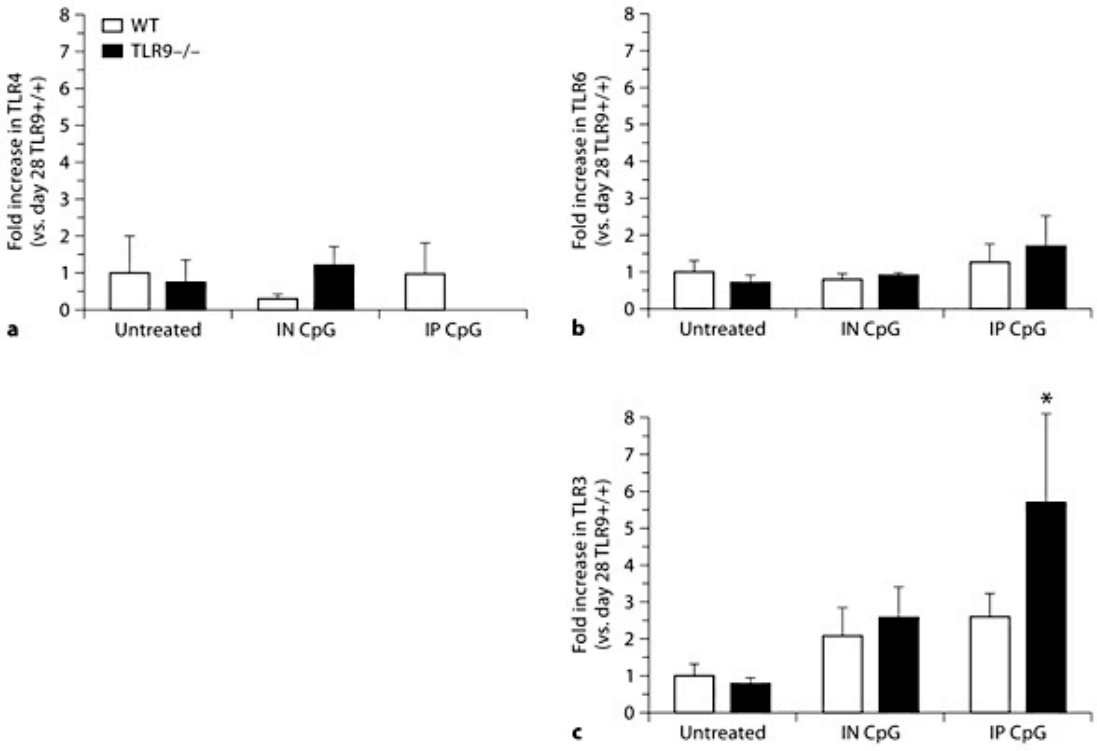

Figure 4.6. ***Elevated TLR3 levels were observed in intraperitoneal CpG treated TLR9$^{-/-}$ mice.*** TAQMAN analysis of TLR9$^{+/+}$ and TLR9$^{-/-}$ mouse lung TLR4 **(A)**, TLR6 **(B)**, and TLR3 **(C)** levels from *Aspergillus*-sensitized and conidia challenged TLR9$^{+/+}$ and TLR9$^{-/-}$ mice that were either untreated, treated with intranasal CpG, or treated with intraperitoneal CpG. Data are expressed as mean ± SEM; n = 5/group/time point. *, $P \leq 0.05$ compared with the appropriate untreated control group.

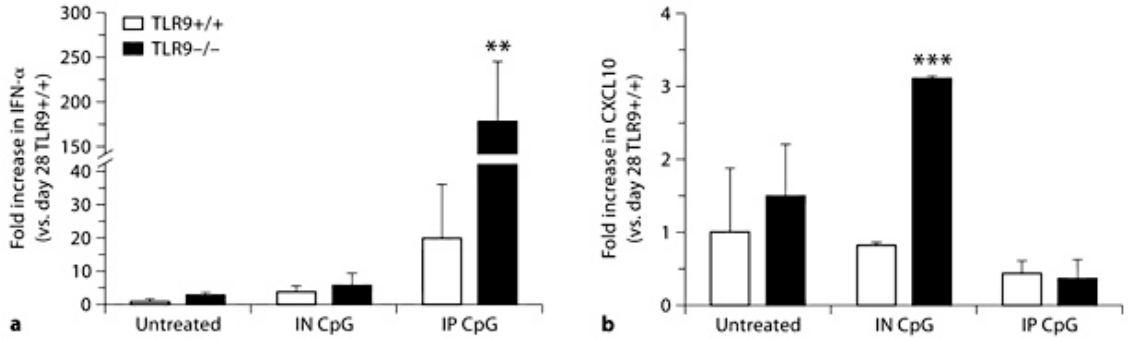

Figure 4.7. ***Elevated IFN-α levels were observed in intraperitoneal CpG treated TLR9$^{-/-}$ mice, and CXCL10 levels were increased with intranasal CpG treatment in TLR9$^{-/-}$ mice.*** TAQMAN analysis of TLR9$^{+/+}$ and TLR9$^{-/-}$ mouse lung IFN-α **(A)** and CXCL10 **(B)** levels from *Aspergillus*-sensitized and conidia challenged TLR9$^{+/+}$ and TLR9$^{-/-}$ mice that were either untreated, treated with intranasal CpG, or treated with intraperitoneal CpG. Data are expressed as mean ± SEM; $n = 5$/group/time point. **, $P \leq 0.01$; *** $P \leq 0.001$ compared with the appropriate untreated control group.

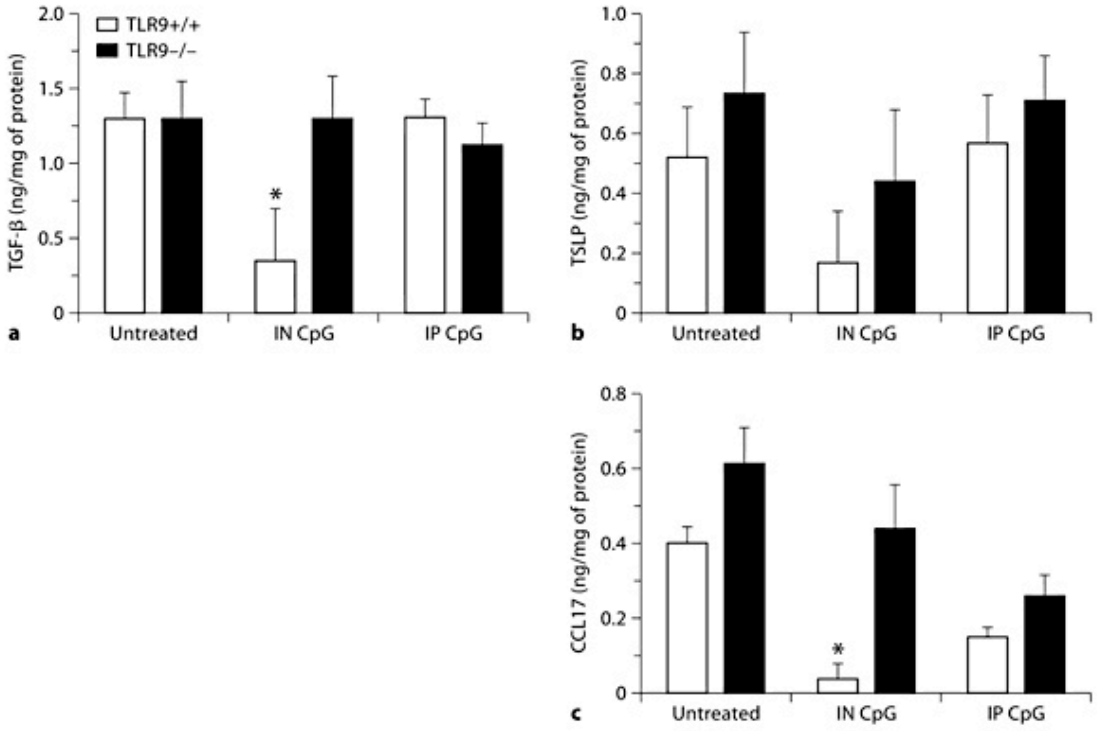

Figure 4.8. ***Intranasal CpG inhibited Th2-associated cytokine levels in a TLR9-dependent manner in chronic fungal asthma.*** ELISA or Bio-Plex® of TGF-β **(A)**, TSLP **(B)**, and CCL17 **(C)** in whole lung samples from *Aspergillus*-sensitized and conidia challenged mice $TLR9^{+/+}$ and $TLR9^{-/-}$ mice. Both groups of mice were left untreated or treated with intranasal CpG, or intraperitoneal CpG on alternate days from day 14 to day 28 after conidia challenge. Data are expressed as mean ± SEM; n = 5/group/time point. *, $P \leq 0.05$ compared with appropriate untreated control group.

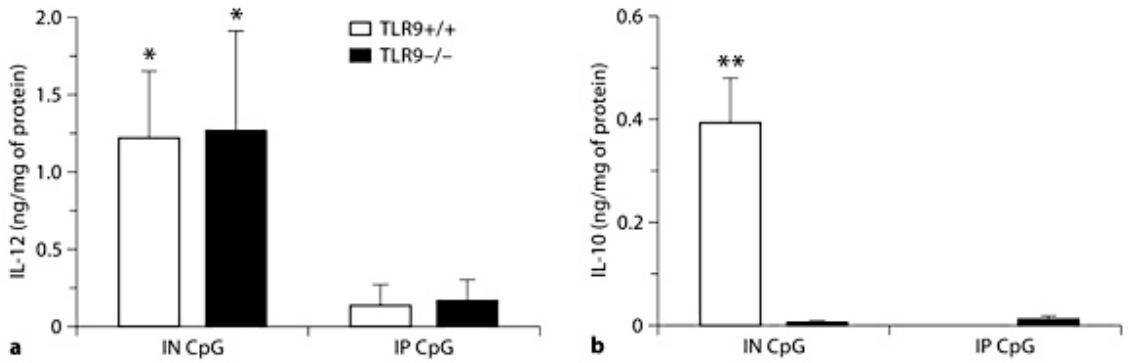

Figure 4.9. ***TLR9-independent induction of IL-12 and TLR9-dependent induction of IL-10 following intranasal CpG treatment in chronic fungal asthma***. ELISA or Bio-Plex® of IL-12 **(A)**, and IL-10 **(B)** in whole lung samples from *Aspergillus*-sensitized and conidia challenged mice $TLR9^{+/+}$ and $TLR9^{-/-}$ mice. Both groups of mice were left untreated or treated with intranasal CpG, or intraperitoneal CpG on alternate days from day 14 to day 28 after conidia challenge. Note that the levels of IL-12 and IL-10 in untreated $TLR9^{+/+}$ and $TLR9^{-/-}$ mice were below the limits of detection for these assays. Data are expressed as mean ± SEM; $n = 10$/group/time point. *, $P \leq 0.05$; **, $P \leq 0.01$ compared with appropriate intraperitoneal CpG group.

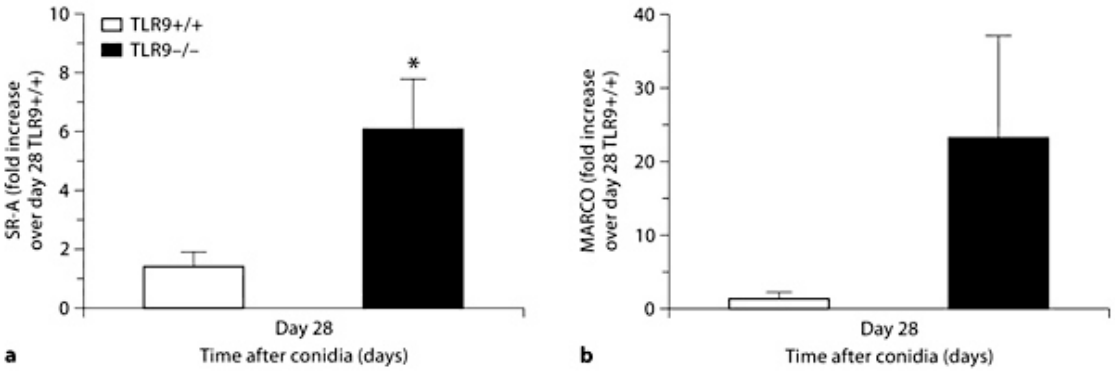

Figure 4.10. ***Scavenger receptor transcript levels were elevated in TLR9$^{-/-}$ mice with chronic fungal asthma.*** TAQMAN analysis of TLR9$^{+/+}$ and TLR9$^{-/-}$ mouse lung SR-A **(A)** and MARCO **(B)** levels in *Aspergillus*-sensitized and conidia challenged mice TLR9$^{+/+}$ and TLR9$^{-/-}$ mice. Both groups of mice were left untreated and transcript levels for these scavenger receptors were determined from whole lung samples taken at day 28 after conidia challenge. Error bars indicate standard error means. Data are expressed as mean ± SEM; n = 5/group/time point. *, $P \leq 0.05$ compared with TLR9$^{+/+}$ group.

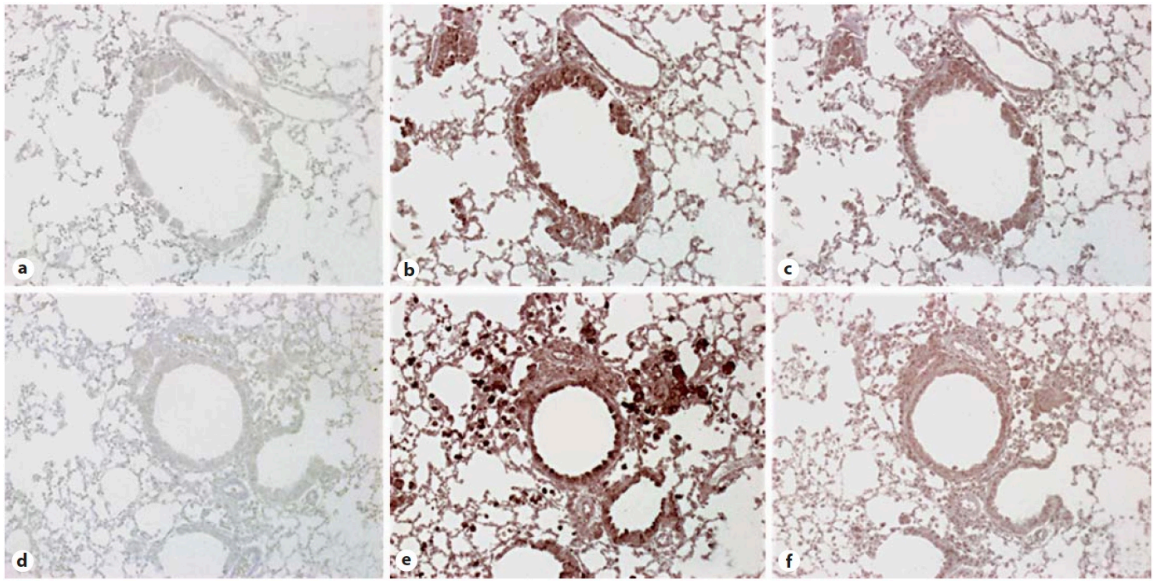

Figure 4.11. ***Immunohistochemical analysis of SR-A and MARCO expression in whole lung sections from TLR9$^{+/+}$ and TLR9$^{-/-}$ mice at day 28 after conidia challenge.*** Whole-lung tissue sections from *Aspergillus*-sensitized and challenged TLR9$^{+/+}$ and TLR9$^{-/-}$ mice were stained using routine immunohistochemical techniques. Panels shown are representative of whole lung sections from TLR9$^{+/+}$ mice stained with IgG control **(A)**, anti-SR-A antibody **(B)**, or anti-MARCO antibody **(C)**. Panels shown are representative of whole lung sections from TLR9$^{-/-}$ mice stained with IgG **(D)**, anti-SR-A antibody **(E)**, or anti-MARCO antibody **(F)**. Receptor expression stains brown with this immunohistochemical procedure. The original magnification was ×200 for all photomicrographs.

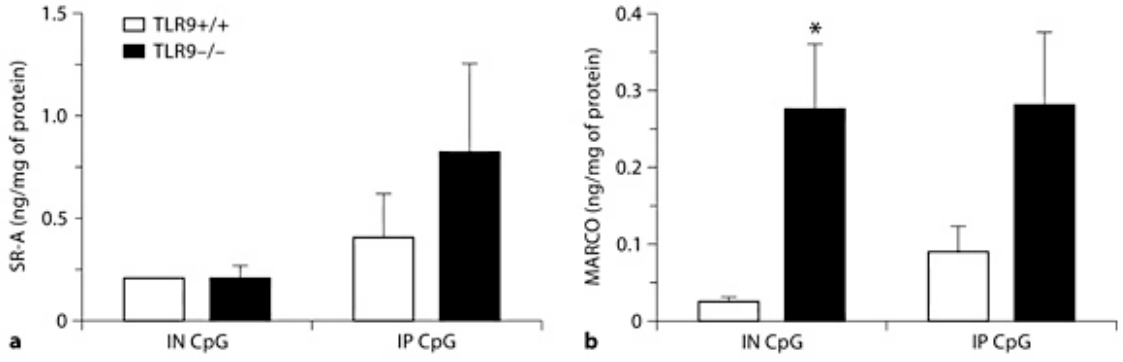

Figure 4.12. ***Immunoreactive levels of MARCO and SR-A remain elevated in TLR9$^{-/-}$ mice compared with TLR9$^{+/+}$ mice after CpG treatment.*** ELISA analysis of SR-A **(A)** and MARCO **(B)** in whole lung samples from *Aspergillus*-sensitized and conidia challenged mice TLR9$^{+/+}$ and TLR9$^{-/-}$ mice. Both groups treated with intranasal CpG or intraperitoneal CpG on alternate days from day 14 to day 28 after conidia challenge. Data are expressed as mean ± SEM; $n = 5$/group/time point. *, $P \leq 0.05$ compared with intranasal CpG TLR9$^{+/+}$ group.

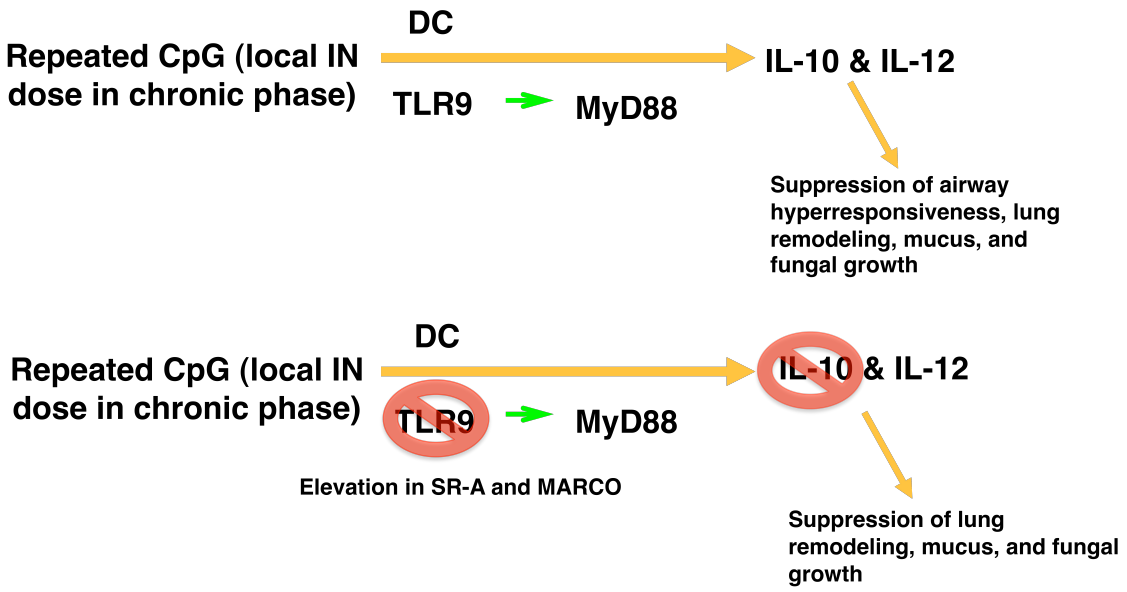

Figure 4.13. ***Model for CpG-ODN TLR9-dependent and -independent effects in fungal asthma.*** TLR9$^{+/+}$ mice are able to recognize IN CpG-ODN and produce IL-10 and IL-12 cytokine responses leading to suppression of airway hyperresponsiveness (AHR), lung remodeling, mucus, and fungal growth. In TLR9$^{-/-}$ mice, repeated IN CpG dosing is unable to activate TLR9 signaling, however elevation in the scavenger receptors SR-A and MARCO allow for CpG-ODN recognition and IL-12 production, leading to suppression of lung remodeling, mucus, and fungal growth without impacting AHR.

CHAPTER 5

ANTI-ST2L TREATMENT AUGMENTS TLR9-MEDIATED DENDRITIC CELL ACTIVATION AND INHIBITS THE MAINTENANCE OF CHRONIC FUNGAL ASTHMA.

Summary

CpG treatment abolishes airway inflammation and remodeling in murine models of allergic airway disease, but not in human trials. Intranasal, but not systemic CpG therapy has been demonstrated to be effective in mice. We seek to define the role of the IL-1R-like receptor ST2L in regulating TLR9 signaling in a fungal model of asthma. Herein, we investigated the therapeutic efficacy of blocking ST2L activity and combining this with CpG therapy in a chronic fungal model of asthma. C57BL/6 mice were sensitized to soluble *Aspergillus fumigatus* antigens and challenged with live *A. fumigatus* conidia. Mice were treated with IgG, α-ST2L, CpG, IgG+CpG, or α-ST2L+CpG from days 14 to 28-post conidia challenge with α-ST2L administered at a 10μg dose and CpG at a 5μg dose. α-ST2L therapy was also tested alone at a higher 100μg dose. We identified that ST2L and TLR9 levels in the lung increase with time post conidia challenge in the model. All features of allergic airway disease were attenuated in mice treated with 10μg α-ST2L+CpG and with 100μg α-ST2L, including airway hyperresponsiveness, mucus production, and peribronchial fibrosis. Whole-lung MIG levels were significantly elevated in both 10μg α-ST2L+CpG and 100μg α-ST2L treated mice but not in control groups. Bone marrow derived dendritic cells from

asthmatic mice exhibited elevated IL-12 levels in mice treated with 10μg α-ST2L+CpG, and STAT3 phosphorylation was decreased in BMDCs from these mice. Together, these data show that α-ST2L therapy has a synergistic effect with systemic CpG therapy, such that combination therapy attenuates all features of fungal asthma.

Introduction

Th2 inflammation is a characteristic feature of allergic asthma, and is an immune response that drives physiologic and structural remodeling events in the lung (283, 284). Recent strategies to treat allergic airway disease have focused on limiting or reducing the Th2 inflammation. One such strategy has been by targeting the first recognition phase of the immune response and the cells and receptors that mediate this process. Dendritic cells are an antigen-presenting cell (APC) type that recognize allergens through pathogen recognition receptors. Activation of these receptors promotes further innate and adaptive immune responses through the activation of cells such as T cells or recruitment of cells to the asthmatic lung through cytokine signaling (42, 325). The toll-like receptors (TLRs) are one receptor type found on dendritic cells that recognize pathogen signals. Toll-like receptor 9 (TLR9) is a toll-like receptor that has been targeted therapeutically in experimental models of asthma and in human patients. Clinical trials involving targeting TLR9 with its agonist, CpG-ODN, however, have been unsuccessful even though experimental models have shown promising results (312). TLR9 activation has been found to shift immune responses away from Th2 immunity to Th1-type responses, even though it has been unable to affect the characteristic airway hyperresponsiveness found in asthma (127). Further investigation is thus warranted to clarify how TLR9 signaling is regulated so that its utility in animal models can be translated into an effective human therapy for asthma.

Dendritic cells activated with the cytokine IL-33 produce atypical Th2 responses characterized by increased expression of MHC II and CD86 and production of IL-6, but not IL-12 (49). ST2L, the receptor for IL-33, negatively regulates IL-1RI and TLR4 but

not TLR3 signaling by sequestering the adaptors MyD88 and Mal (326). It has also been shown that TRAF6 is required for ST2L dependent signaling as TRAF6 deficient MEFs are unable to demonstrate IL-33 induced activation of NF-κB (327). IL-33 administration has been shown to cause significant changes in mucosal tissues, including lung, esophageal, and intestinal surfaces, and has been shown to induce splenomegaly (190). It has been shown that inhibition of IL-33, either with α-ST2 antibody or ST2 immunoglobulin, blocks secretion of IL-4, IL-5, and IL-13 and suppresses eosinophilic inflammation in recipients of ovalbumin-specific Th2 cells (328-330). ST2L expression is not dependent on either IL-5 or IL-10 (330). Previous investigations have shown that treatment of human and murine dendritic cells with the tolerance inducing drug rapamycin promotes IL-1β-induced ST2L expression and unresponsiveness to proinflammatory, maturation-inducing stimuli (331). These previous findings are significant, since inhibition of TLRs promotes a T_H2 response and has also helped to identify ST2L as a key regulator of endotoxin tolerance (332). It is therefore logical to infer that targeting ST2L activity in dendritic cells can improve TLR signaling and can lessen allergic asthma through downregulating the T_H2 immune response.

The *ST2* gene was originally identified as a gene induced by serum or oncogene expression in mouse fibroblasts (333, 334). In the mouse, differential mRNA splicing within the *ST2* gene generates two mRNAs of 2.7 and 5 kb. These fragments correspond to a shorter secreted form (soluble ST2 or sST2) and a longer, membrane-anchored form (ST2L) of the protein. Therefore, sST2 is identical to the extracellular region of ST2L except for the addition of nine amino acids present at the C terminus of the molecule (335, 336). Soluble *ST2* is expressed in embryonic tissues, mammary tumors and

fibroblasts (333-336), while ST2L expression was originally detected in T-cell lines, macrophage, erythroid and bone-marrow stem cell lines and primary mastocytes (335, 336). Transcription of ST2 is controlled by two distinct promoters: an upstream promoter directs transcription in hematopoietic cells such as mast cells, while a promoter 10.5 kb downstream directs expression in fibroblasts (336). Regulation of *ST2* expression between mice and humans appears to be conserved, since two promoters also control *ST2* expression in human cells (337). The widespread expression of *ST2* gene in different cell types indicates that it might serve important functions across a broad range of biological systems. In addition to the proposed involvement of *ST2* in cell growth regulation and embryogenesis, the presence of *ST2* in hematopoietic cell lines and its homology with the members of Toll/IL-1 receptor family indicates its potential functional role in immune responses. A number of recent reports on ST2-mediated modulation of various immune functions support this perspective (338).

The use of hypomethylated oligodeoxynucleotides containing CpG motifs, which mimic either bacterial or viral DNA has been identified as one possible therapy for asthma (285-287). Prophylactic, systemic CpG administration in acute allergic airway models induced by ovalbumin (OVA), *Aspergillus* antigen, or house dust mite elicits major protective effects (288-290). In OVA model of allergic asthma, exogenously administered CpG has been shown to inhibit and/or reverse airway remodeling, even when administered after OVA challenge and establishment of airway disease (291) (292, 293). The mechanism through which CpG inhibits or reverses allergic airway disease is attributed, in part, to the inhibition of Th2 cytokines such as IL-4, IL-5, IL-9, and IL-13 and migration of Th2 cells into the lung environment (288, 291, 294-296). B cells and

pDCs respond to CpG and generate Th1-type pro-inflammatory cytokines, interferons, and chemokines. Certain CpG motifs have also been demonstrated to activate NK cells and induce pDCs to secrete IFN-α (127, 297). Secretion of IFN-α in response to CpG-ODN administration has also been implicated in the induction of catatonic levels of TNF-α in rodents, but not humans (339). For this reason dosing of administered CpG must be carefully controlled to prevent side effects or tissue damage. While some controversy exists as to which Th1-type cytokines mediate the therapeutic effects of CpG in allergic airway disease, the beneficial effects of CpG in experimental allergic airway disease are partially inhibited after immunoneutralization of IFN-α, IFN-β, and IL-12 (288). One mechanism behind the inhibitory effects of CpG has been identified as involving the regulation of IL-5 through IL-10 synthesis (299). Recent studies have identified that dendritic cell maturation is driven by autocrine secretion of IL-10 leading to activation of STAT3, a transcription factor critical for immune tolerance (233).

STAT3 has been identified as a negative regulator of dendritic cell function (340). While STAT3 plays critical roles in the differentiation and maturation of DCs, it hinders activation of dendritic cells and thus plays a regulatory role in the pathogenesis of asthma (233). STAT3 has been identified in bone marrow derived dendritic cells as a factor that regulates the recruitment of NF-κB to the IL-12p40 promoter. This response appears to be mediated by IL-10 and its ability to induce activation of STAT3, as shown by increased NF-κB recruitment and induction of IL-12p40 gene expression observed in BMDCs from IL-$10^{-/-}$ mice (226).

In the present study, we investigated the therapeutic effect of CpG in a chronic fungal asthma model with combined α-ST2L therapy. Our previous investigations of

CpG in experimental asthma have investigated CpG delivery through both the systemic (IP) and local (IN) administration of CpG in $TLR9^{+/+}$ and $TLR9^{-/-}$ mice with established *A. fumigatus* conidia-induced fungal asthma and found therapeutic AHR attenuation only in $TLR9^{+/+}$ mice with IN CpG administration. We seek here to investigate the role of ST2L in regulating TLR9 signaling and the impact of this action on dendritic cells in a model of fungal asthma to understand why systemic CpG-ODN therapy has been ineffective both in our model and in clinical trials (230, 312). We recently reported that *A. fumigatus*-sensitized $TLR9^{-/-}$ mice exhibit a very severe form of fungal asthma characterized by fungal growth and profound tissue remodeling after these mice are challenge with swollen conidia (306). In the present study, CpG therapy in conjunction with α-ST2L therapy enhanced the therapeutic effects of CpG observed previously in our fungal asthma model and we present evidence that regulation of STAT3 phosphorylation may play a role in this process.

Results

ST2L induction during the course of chronic fungal asthma.

We have previously examined the role of the TLR9 agonist CpG-ODN in fungal asthma (230). In this study we observed that intranasal CpG-ODN therapy attenuated airway hyperresponsiveness and remodeling, but systemic CPG therapy affected only airway remodeling. Previous reports have indicated that TLR signaling can be regulated through the action of the negative regulator ST2L through sequestration of MyD88 (332, 341). Further, mice deficient in the *ST2* gene have been identified to produce impaired type 2 responses (342). Our present results through immunohistochemical staining and ELISA of whole-lung homogenates for ST2L indicate that ST2L levels increase in the lung with time from D0 to D28 post intratracheal administration of conidia **(Fig. 5.1 A-H, J)**. We have also observed that serum ST2 levels are also elevated in a sustained manner from D0 to D28 post IT challenge in fungal asthma **(Fig. 5.1 I)**. Thus, ST2L is significantly elevated in fungal asthma during the establishment of the chronic phase of the model and may play a role in regulating the response to CpG-ODN therapy.

IL-33 levels are elevated in the lungs of asthmatic mice.

The ligand for ST2L, IL-33, is critical to the action of this cell surface receptor. Recent reports have provided evidence that IL-33 expression is increased in epithelial cells in bronchial asthma (343). Previous reports have indicated that IL-33 is elevated in the lungs of asthmatic patients and that elevation in lung IL-33 levels produces alternative activation of lung macrophages (342). Asthmatic mice demonstrate increased IL-33 levels in the epithelial cells surrounding the airways at day 7 post intratracheal

conidia challenge that decreases slowly with time **(Fig. 5.2 A-H)**. We have also observed this to be the case with ELISA analysis of whole-lung homogenates from asthmatic mice **(Fig. 5.2 I)**. Thus, IL-33 levels are elevated in the lung in our fungal model of asthma in a manner that reflects clinical observations of this factor in the lungs of asthmatic patients.

TLR9 levels increase with time in the lung in fungal asthma and ST2 levels increase with systemic CpG therapy, but not intranasal CpG.

Our previous studies identified that TLR9 is unique in fungal asthma in that it is the only TLR we observed whose transcripts were elevated in fungal asthma (229). We have been able to bolster these findings with the observation that increased TLR9 levels are visible with immunohistochemical staining for TLR9 in lungs from asthmatic mice **(Fig. 5.3 A-H)**. Our results indicate that TLR9 levels increase in the lung with time from D0 to D14 post intratracheal administration of conidia, but decrease slightly by D28 from the D14 peak. Further, in our previous studies of CpG-ODN administration in a site-specific manner, we observed that in a fungal asthma model in Balb/c mice that ST2 levels demonstrated an elevation trend in IP CpG treated mice, but not IN CpG treated mice **(Fig. 5.3 I)**. Thus, TLR9 is elevated in the lungs of mice in a model of fungal asthma by the time the chronic phase of the model is established, and CpG-ST2L combination therapy may be best initiated at this peak of TLR9 levels in the lung. Further, these data provide our earliest rationale for targeting ST2L as it the transcript levels of this protein appear elevated in the lungs of IP CpG treated mice, but not IN CpG

treated mice, a finding that correlates with the observation of a decrease in AHR only in the IN CpG treated group.

Dendritic cells from asthmatic mice demonstrate altered levels of ST2L and IL-33.

Previous reports have indicated a role for IL-33 activated dendritic cells in the development of an atypical Th2 response. IL-33 stimulated dendritic cells demonstrate increased MHC II expression and incubation of these cells with CD4+ T cells demonstrates robust production of IL-13 and IL-5, but not IL-4 or IFN-γ (344). Recent reports have also indicated that dendritic cells generated in the presence of IL-33 are maturation resistant and GM-CSF production is triggered by IL-33 (345). Our results indicate that asthmatic BMDC ST2 transcript levels significantly increase with time with TLR 2,3,4, and 9 stimulation and peak at D28 post IT conidia challenge **(Fig. 5.4A)**. Further, ST2 protein levels increase with time and peak at D28 in asthmatic BMDCs stimulated with TLR3 & 4 stimulation **(Fig. 5.4B)**. Our results also indicate that dendritic cells from asthmatic mice demonstrate robust increases in IL-33 transcript levels in response to TLR2 and TLR4 stimulation at the D14 and D28 timepoints post IT challenge. TLR9 and TLR3 agonists elicit maximal IL-33 transcript levels at the D7 in asthmatic BMDCs **(Fig. 5.4C)**. We also observed that naïve BMDCs respond to IL-33 *in vitro* and that IL-33 tends to decrease STAT3 and pSTAT3 Tyr705 levels and decrease SOCS3 levels at higher concentrations **(Fig. 5.4D)**. Thus, BMDCs levels of ST2 transcripts and IL-33 increase with time post conidia challenge in fungal asthma. This increase in BMDC ST2 and IL-33 may produce responses different from non-asthmatic naïve BMDCs as evidenced by the decrease in factors such as STAT3 that impact DC

activation in naïve DCs treated with IL-33. Decreases in the inhibitory SOCS3 protein in naïve BMDCs treated with IL-33 also indicate that BMDCs treated with higher concentrations of IL-33 may be more receptive to producing pro-inflammatory cytokines.

Bone marrow derived dendritic cells from asthmatic mice demonstrate decreasing TLR9 transcripts and IL-12 levels post conidia challenge in fungal asthma.

We next analyzed the TLR9 transcript levels from BMDCs from asthmatic mice at the D0-D28 timepoints post conidia challenge in order to assess any changes in the receptor for CpG-ODN as the axis of regulation for its effects as an asthma therapeutic in our model. We observed that BMDC TLR9 transcript levels peaked at D7 post conidia challenge when stimulated *in vitro* with CpG-ODN and decreased significantly thereafter **(Fig. 5.5 A)**. A similar pattern was observed with TLR3 stimulation. We also analyzed IL-12p70 levels from BMDCs from asthmatic mice that were cultured with TLR agonists and found that the levels of IL-12p70 also decreased at the later timepoints post-conidia challenge and were significantly decreased relative to BMDCs from naïve C57BL/6 mice **(Fig. 5.5 B)**. Thus, TLR9 levels decrease in asthmatic mice with increasing time post conidia challenge in response to CpG-ODN stimulation and this occurs in tandem with a decrease in IL-12p70 levels.

α-ST2L and CpG-ODN combined treatment enhances CpG-induced responses in fungal asthma and alters whole-lung and BAL cytokine levels.

We next sought to investigate the therapeutic utility of combination α-ST2L and CpG therapy. We utilized a low dose of 10μg of α-ST2L alone or in combination with a

5μg dose of CpG. We did not observe a significant change in airway hyperresponsiveness with IgG, 10μg α-ST2L, or CpG+IgG therapy. We did, however, observe a significant decrease in the airway response with combination CpG & 10μg α-ST2L therapy that suggests that a synergistic action may occur with both together **(Fig. 5.6A)**.

Whole lung cytokine and chemokine protein levels were analyzed next in this model of fungal asthma in response to 10μg α-ST2L & CpG combination therapy. ELISA and Bio-Plex® analysis of whole-lung homogenates demonstrated significant increases in MIG with combination therapy, but no such increase with control IgG, CpG, IgG+CpG, or 10μg α-ST2L **(Fig. 5.6B)**. We also noted a trend toward an increase in IL-12p40/70 levels in the BAL of combination treated mice **(Fig. 5.6C)**. Thus, these data indicated that decreased allergic airway disease and airway hyperresponsiveness in α-ST2L+CpG combination treated mice with fungal asthma might be a consequence of increased Th1-associated factors such as increased MIG levels and perhaps increased IL-12.

α-ST2L and CpG-ODN combined therapy alters dendritic cell cytokines.

BMDCs isolated from asthmatic mice treated with a 10μg dose of α-ST2L alone or in combination with CpG were next analyzed in this model of fungal asthma for cytokine and chemokine protein levels. ELISA and Bio-Plex® analysis of BMDC culture media demonstrated a significant increase in MIG and IL-12p70 levels in BMDCs from combination treated mice that was not observed in IgG, IgG & CpG, or IgG & CpG + *in vitro* CpG. One important observation was that combination stimulation of BMDCs,

either *in vivo* prior to analysis or α-ST2L *in vivo* followed by CpG *in vitro* stimulation, produced significant increases in MIG levels. Further, IL-12p70 levels were enhanced with CpG *in vitro* stimulation **(Fig. 5.6 D & E)**. We also observed significant increases in the Th1-associated factors IP-10 (CXCL10), MIP1α, and TNFα levels in α-ST2L + CpG *in vivo* treated mice relative to CpG alone, IgG, or 10μg α-ST2L alone **(Fig. 5.6 F-H)**. Thus, α-ST2L + CpG therapy *in vivo* enhances constitutive BMDC production of Th1-associated cytokines such as MIG, IL-12, IP-10, MIP1α, and TNFα. Also, CpG *in vitro* restimulation further enhances IL-12 production by BMDCs from α-ST2L + CpG *in vivo* treated mice.

α-ST2L and CpG-ODN combination therapy reduces inflammation, mucus metaplasia, and lung remodeling, while increasing fungal clearance in fungal asthma better than monotherapy.

We next further investigated the impact of α-ST2L & CpG combination therapy on changes in airway inflammation, lung remodeling, mucus secretion, and fungal growth **(Fig. 5.7)**. Lung sections from 10μg of IgG **(Fig 5.7 panels A,F,K,P),** 10μg α-ST2L **(Fig. 5.7 panels B,G,L,Q)**, 5μg of CpG **(Fig. 5.7 panels C,H,M,R)**, IgG & CpG **(Fig. 5.7 panels D,I,N,S)**, or α-ST2L & CpG **(Fig. 5.7 panels E,J,O,T)** treated mice on day 28 after IT conidium challenge were stained with Hematoxylin & Eosin **(Fig. 5.7 A-E)**, Trichrome **(Fig. 5.7 F-J)**, Period Acid Schiff **(Fig. 5.7 K-O)**, and Gomori Methenamine Silver (GMS) **(Fig. 5.7 P-T)** stains. Mice treated with α-ST2L & CpG therapy demonstrated attenuated airway inflammation, lung remodeling, mucus secretion, and fungal growth relative to all other groups. Thus, these data indicated that α-ST2L + CpG

combination therapy decreases the lung pathology of asthmatic mice, better than low doses of α-ST2L or CpG alone.

α-ST2L & CpG-ODN treatment increases fungal clearance in fungal asthma.

We next qPCR quantitated the fungal burden in lung samples from 10μg of IgG, 10μg α-ST2L, 5μg of CpG, IgG & CpG, or α-ST2L & CpG treated mice on day 28 after IT conidium challenge to assess quantitatively our observations of increased fungal clearance from our GMS stained lung sections. We observed significantly increased fungal clearance in 10μg α-ST2L, 5μg of CpG, IgG & CpG, or α-ST2L & CpG treated mice with the greatest clearance observed in the α-ST2L & CpG treated groups **(Fig. 5.8)**. Thus, α-ST2L or CpG therapy increased clearance of fungal conidia from the lungs of *Aspergillus* sensitized and challenged mice with the greatest clearance observed in the lungs of α-ST2L & CpG treated mice.

Splenic dendritic cell responses are altered in α-ST2L & CpG-ODN treated.

We also investigated the activation of splenic DCs isolated from combination treated mice through flow cytometric staining and analysis after MACS sorting for CD11c+ cells in order to determine if already differentiated DCs in circulation exhibited the changes observed in the BMDCs. We observed a significant change in the percentage of MHCII+ cells between the α-ST2L & CpG *in vivo* treated groups and all other groups **(Fig. 5.9 A)**. Most cells that were CD11c+ were also found to be CD11b+, indicating these cells were myeloid dendritic cells as they expressed markers corresponding to this cell type **(Fig. 5.9 B)**. Thus, these data indicated that decreased allergic airway disease in

α-ST2L+CpG combination treated mice with fungal asthma might be a consequence of increased numbers of MHCII+, CD11c+, CD11b+ cells.

α-ST2L and CpG-ODN therapy alters STAT3 activation in BMDCs from asthmatic mice.

The transcription factor STAT3 has been identified to negatively regulate the recruitment of NF-κB to the IL-12p40 promoter such that transcription can occur in BMDCs (226, 340). Further, it has been identified that airway epithelial STAT3 is required for allergic inflammation in a murine model of asthma (346). This negative regulatory role of STAT3 is one reason why we next investigated the role of STAT3 in the regulation of the IL-12 induction we observed in α-ST2L & CpG treated mice. We performed western blot analysis of bone marrow derived dendritic cells from asthmatic mice **(Fig. 5.10 A & B)** treated with 10μg IgG, 10μg α-ST2L, CpG, 10μg of IgG+CpG, or 10μg of α-ST2L+CpG on alternate days from day 14 to day 28 after conidia challenge. BMDCs isolated from asthmatic mice were either cultured in media **(Fig. 5.10 A)**, or restimulated for 24h with CpG in the culture media **(Fig. 5.10 B)**. Upon quantitation of the bands for STAT3, pSTAT3 Tyr 705, and SOCS3 relative to GAPDH control bands we observed that STAT3 and pSTAT3 levels were decreased in BMDCs from α-ST2L & CpG treated mice and this was further observed in the CpG restimulated BMDCs. In order to assess the mechanistic significance of these findings, we stimulated BMDCs from naïve mice with controls, CpG, STAT3 siRNA, CpG-STAT3 fusions, 10μg α-ST2L, or combinations thereof **(Fig. 5.10 C)**. We observed that CpG-STAT3 fusion constructs that have been demonstrated to be internalized into dendritic cells (234) were

able to downregulate SOCS3 and STAT3 levels and that these cells produced more IL-12p70 **(Fig. 5.10 D)**. We also observed that BMDCs treated with 100ng/mL IL-33, CpG, & α-ST2L were able to demonstrate a trend toward more IL-12 than BMDCs treated with IL-33 & CpG, and IL-33 & α-ST2L (which produced no detectable IL-12p70). It is of note to mention that SOCS3 levels were low to undetectable in all asthmatic BMDC lysates. This appears to indicate one mode in which asthmatic BMDCs may be able to produce more effective intracellular signaling, as the suppressive factor SOCS3 is not present in these cells. SOCS3 has several different targets it suppresses, among them TRAF6, an intracellular mediator of TLR9 signaling (347, 348). Thus, it appears that in asthmatic BMDCs that decreased STAT3 levels correspond to an increase in IL-12 production and that SOCS3 also plays a role in reducing IL-12 levels from BMDCs. It is interesting to note the CpG-ODN has been observed to elevate SOCS3 levels in APCs (348). Thus, absence of this factor in asthmatic BMDCs is a unique regulatory change in these cells due to asthmatic sensitization.

ST2L targeting alters airway responses, serum IgE, and eosinophilia in fungal asthma.

Following our observations of the impact of 10μg α-ST2L & CpG therapy on airway responses, inflammation, remodeling, mucus secretion, and fungal growth, we were interested in examining if high dose therapy with 100μg α-ST2L therapy would be able to produce a therapeutic effect in the absence of exogenous TLR9 agonist administration. H&E-stained lung sections from 100μg α-ST2L treated mice on day 28 after IT conidium challenge demonstrated reduced lung inflammation **(Fig. 5.11 A & E)** and decreased lung remodeling **(Fig. 5.11 B & F)**. Further, 100μg α-ST2L treated

asthmatic mice demonstrated decreased mucus metaplasia **(Fig. 5.11 C & G)**, and fungal growth **(Fig. 5.11 D & H)**. Thus, these data indicated that 100μg α-ST2L therapy decreases the lung pathology of asthmatic mice. Previous reports have shown that CpG inhibits AHR in experimental asthma (290). Our results also indicated that 100μg α-ST2L therapy without the addition of CpG-ODN on alternate days from days 14 to 28 after conidia challenge, decreased AHR in asthmatic mice measured at day 28 after conidia challenge compared to IgG administration **(Fig. 5.11 I)**. This therapy also decreased serum IgE levels and BAL eosinophil counts **(Fig. 5.11 J & K)**. Further, 100μg α-ST2L therapy demonstrated a trend toward decreased mucus associated genes Muc 5 and Gob 5 **(Fig. 5.11 L & M)**. We also observed that this therapy significantly decrease fungal counts in the lungs of mice as measured through qPCR for 18s ribosomal DNA of lung sections from asthmatic mice **(Fig. 5.11 N)**. Thus, high dose blockade of ST2L exhibits therapeutic effects on several of the characteristic features of asthma. It is important to note that the 100μg IgG control used in these studies was also able to produce some reduction in inflammation in the lung as observed from the histological images in comparison to the histological images of lung samples from 10μg IgG treated mice presented earlier **(Fig. 5.7)**. This IgG control did not, however, impact the airway responses in a specific manner as we observed a significant decrease only with 100μg α-ST2L therapy.

Whole-lung cytokines are altered by 100μg α-ST2L therapy in fungal asthma.

We next sought to characterize the specific cytokines affected by 100μg α-ST2L therapy. We first analyzed the transcript levels of several cytokines and chemokines in

whole lung tissues at day 28 after conidia in the treated and IgG control mice. IL-12 transcript levels were approximately 6-fold elevated relative to IgG control treated mice **(Fig. 5.12A)**. MIG transcript levels in treated mice demonstrated an approximately 10-fold increase in 100μg α-ST2L treated mice relative to IgG treated mice **(Fig. 5.12B)** and MIG protein levels increased by about 4-fold in α-ST2L treated mice **(Fig. 5.12F)**. IL-4 transcript levels were undetectable in α-ST2L treated mice relative to IgG control treated mice **(Fig. 5.12C)** and IL-4 protein levels were reduced about 8-fold in treated mice **(Fig. 5.12G)**. Also, IL-33 protein levels were reduced by 100μg α-ST2L therapy to a quarter of their level in the lungs of IgG treated mice **(Fig. 5.12E)**, but transcript levels were unchanged **(Fig. 5.12D)**. Thus, these data suggested that the levels of Th2 associated cytokines like IL-4 are reduced in response to 100μg α-ST2L therapy, with Th1 associated cytokines such as MIG elevated with therapy. The pro-inflammatory and asthma-associated "alarmin" protein IL-33 is reduced in level in α-ST2L treated mice, further providing evidence that α-ST2L therapy reduces pro-asthma cytokines.

α-ST2L therapy impacts BMDC cytokine levels significantly only in conjunction with CpG therapy.

BMDCs isolated from asthmatic mice treated with a 100μg dose of α-ST2L were next analyzed in this model of fungal asthma for cytokine and chemokine protein levels. ELISA and Bio-Plex® analysis of BMDC culture media demonstrated a significant increase in IL-12p70 levels and MIG **(Fig. 5.13 A & B)** in BMDCs from α-ST2L *in vivo* treated mice that were restimulated *in vitro* with CpG. No difference, however, was observed in BMDCs with 100ug α-ST2L therapy, even upon *in vitro* restimulation. Thus,

these data indicate that decreased allergic airway disease in α-ST2L treated mice with fungal asthma might be a consequence of increased IL-12 and MIG responses from BMDCs, but that these responses require the inclusion of CpG therapy *in vivo* in order to occur.

Discussion

The manner in which TLR9 signaling is regulated in the context of asthma is an important area of inquiry that demands exploration if CpG-ODN therapy is to become an effective therapy against asthmatic disease in human patients. The identification of IL-33, the ligand for ST2L and its association with inflammation in asthma has led to a significant amount of research into the impact this factor and signaling pathway have on the pathogenesis of asthma (190, 349). Herein, we characterized the asthmatic phenotype in *A. fumigatus*- sensitized and conidia-challenged C57BL/6 mice after treatment with systemic CpG and blockade of ST2L from days 14 to 28 after conidia challenge. We observed that CpG therapy was therapeutically effective in reducing AHR in $TLR9^{+/+}$ but not $TLR9^{-/-}$ mice in previous studies and we observed that systemic CpG therapy was ineffective at reducing AHR, while intranasal CpG therapy was effective in this regard (230). These observations coupled with the observation that ST2L transcript levels were elevated in whole-lung samples from asthmatic mice treated with intraperitoneal CpG, but not intranasal CpG led us to investigate this avenue of regulation further (230). Both intraperitoneal and intranasal CpG significantly inhibited airway remodeling and inflammation in $TLR9^{+/+}$ mice, so our studies presented here were designed to identify the factors involved in the inability for TLR9 agonists administered systemically to attenuate the airway response (AHR).

In this study we observed that a low dose of CpG therapy in conjunction with a 10μg dose of α-ST2L produced a synergistic therapeutic effect. Compared with IgG & CpG treated mice, 10μg dose α-ST2L & CpG treated mice exhibited attenuated fungal growth, airway remodeling, and AHR. These findings were replicated with a high 100μg

dose of α-ST2L therapy. 100μg α-ST2L therapy was observed to produce significantly elevated MIG levels in the lungs, however it appears that TLR9 activation is necessary for significant elevation of IL-12 and MIG production from BMDCs as observed in BMDCs taken from these mice and treated *in vitro* with CpG. The IgG control used in these experiments appears to have a small activating effect that was observed and at the higher 100μg dose of α-ST2L, some of the activating effects are likely due to the presence of antibody. We were able to observe some attenuation of inflammation even in the 100μg IgG treated mice in the histological data, so it appears that the optimum dose of α-ST2L for its specific effects on TLR9 signaling is a lower 10μg dose. At a lower 10μg dose of antibody the differences between the α-ST2L & CpG and IgG & CpG treated groups become evident and the therapeutic effects of the α-ST2L & CpG become visible at this dose along with the impact of this therapy on BMDCs.

The elevation of IL-33 we observed in the airway epithelium in our fungal model of asthma is consistent with what has been observed in lung biopsy samples from human patients (343). While we also investigated IL-33 levels as produced from BMDCs from asthmatic mice, no significant amounts of IL-33 were observed from BMDCs at any timepoint post IT conidia challenge (unpublished results). These findings indicate that the IL-33 observed upon immunohistochemical staining is primarily from the epithelial tissue and any impact this cytokine has on the DCs within the lung environment is likely due to fungal sensitization of the bronchial epithelial tissue. Several recent publications have provided evidence that IL-33 affects the differentiation of DCs by triggering the production of GM-CSF by other bone marrow cells (350). DCs generated through this mechanism are maturation resistant and thus are less likely to respond to external stimuli

such as CpG. This is further established by evidence that GM-CSF generated BMDCs in the presence of rapamycin overexpress ST2L (351). It is therefore possible that increased IL-33 levels in the asthmatic lung leads to the production of CpG resistant BMDCs by the action of IL-33 driving the differentiation of BMDCs in a GM-CSF enriched bone marrow environment such that the resultant BMDCs express high levels of ST2L on their surface. The mechanisms behind such a process in the bone marrow have yet to be investigated.

The induction of IL-12 in allergic asthma has potent immunoactivating and therapeutic effects on the pathology of allergic airway disease (352). We have made observations that the bone marrow derived dendritic cell can produce significant amounts of IL-12 in response to TLR stimulation in allergic asthma. IL-12 secretion, however, can be regulated through the ST2 pathway, as we have observed decreased IL-12 synthesis from asthmatic BMDCs in mice with elevated ST2 levels at later timepoints in the fungal asthma model. This decrease in IL-12 in a fungal model of asthma has also been observed in a fungal model of asthma involving the fungus *Alternaria* and in BMDCs that have been exposed to *Alternaria* conidia (353). The *in vivo* ramifications of targeting this IL-12 regulatory pathway have as yet been uninvestigated, and thus have proven to be an important avenue of investigation.

We observed that pSTAT3, a negative regulator of DC function through its action negatively regulating NF-κB recruitment to the IL-12p40 promoter (226), is decreased in the lysates of BMDCs from asthmatic mice that were treated with α-ST2L & CpG therapy. STAT3 levels were also decreased in α-ST2L & CpG treated mice. These observations are the reverse of what has been observed with just CpG activation of DCs

in which STAT3 levels are induced by CpG and appear to constrain the action of CpG (354). This downregulation of STAT3 provides a potential mechanism of action for α-ST2L blockade in asthma. We also examined *in vitro* the mechanism behind this STAT3 decrease in α-ST2L & CpG treated mice by treating naïve BMDCs with STAT3 siRNA fused to CpG with a C3 carbon linker. We were able to observe that IL-12p40 levels increased in the culture media of these cells in response to both CpG therapy and STAT3 knockdown in a similar manner to what we observed with α-ST2L & CpG combined therapy. We also observed a trend toward an increased in IL-12 levels in naïve BMDCs treated with IL-33, CpG, and α-ST2L relative to BMDCs treated with only IL-33 and α-ST2L or just IL-33 and CpG. How changes in systemic cytokines mediating inflammation due to α-ST2L & CpG therapy affect the levels of STAT3 phosphorylation and ST2L levels on BMDCs is one important area for future investigation that has been opened up by these findings.

We also examined the number of $MHCII^{+}$ $CD11c^{+}$ $CD11b^{+}$ cells present in freshly isolated splenic cells from asthmatic mice that received α-ST2L & CpG therapy. We observed a small, but significant increase in the percentage of MHCII+ cells that were both $CD11c^{+}$ and $CD11b^{+}$ in mice that were treated with α-ST2L & CpG. Turnquist et. al. have mentioned that BM cells differentiated in the presence of GM-CSF through exogenous administration leads to the production of $CD11c^{Hi}CD11b^{Hi}$ cells that are MHC I/II^{Hi} and TLR responsive. They have also indicated that IL-33 induced GM-CSF from basophils or precursors leads to the production of $CD11c^{Hi}CD11b^{Hi}$ cells that are MHC II^{Lo} and are TLR unresponsive. They have indicated that an as yet unknown factor is responsible for this shift in TLR responsiveness in the presence of IL-33 (345, 350). Our

results indicate that this factor could be ST2L induced in the lungs and BMDCs of asthmatic mice that produce increased levels of IL-33 and ST2L mediated effects on STAT3 phosphorylation. Induction of increased pSTAT3 in asthmatic mice could be one reason why this suppression of TLR signaling at the level of NF-κB could occur in addition to the sequestration of MyD88 that ST2L can perform. Our findings provide one possible explanation for this effect on BMDCs and future efforts to understand the intracellular signaling occurring in the bone marrow will help to elucidate this phenomenon further.

There are a significant number of studies investigating the differentiation of immune cells from bone marrow precursors. Few studies, however, have investigated the mechanisms directing bone marrow local innate immune responses in connection with TLR signaling. Future studies investigating the observations made in these studies can be directed toward understanding the interactions between cell types in the bone marrow precursor cells in asthmatic mice in response to α-ST2L & CpG therapy. Lamb et. al. recently identified that bone marrow cells from naïve mice that were $DEC205^{+}Gr\text{-}1^{low}$ exhibited increased IL-12p70 and TNF-α level relative to $DEC205^{+}Gr\text{-}1^{high}$ cells (355). One possibility is that α-ST2L & CpG is enriching the bone marrow cell population of $DEC205^{+}Gr\text{-}1^{low}$ cells leading to the enhancement of IL-12p70 and TNF-α from BMDCs we observed in our model.

In summary, ST2L targeting enhances BMDC activation in asthmatic mice leading to enhanced IL-12 production and ultimately a reduction in AHR, airway inflammation, remodeling, mucus metaplasia, and fungal growth. This effect appears to be mediated by an increase in not just IL-12, but also other Th1 associated factors such as

MIG, IP-10, MIP1α, and TNFα produced from BMDCs. Myeloid dendritic cells derived from the bone marrow of asthmatic mice undergo changes in response to the development of allergic sensitization in the model that leads to enhanced expression of ST2L along with enhanced TLR9 and IL-33 expression in the lung. The enhanced IL-33 levels we observed in the lung are consistent with clinical observations of enhanced IL-33 levels in the airway epithelium of asthmatic patients and recent observations of enhanced IL-33 with TLR9 stimulation provide further evidence that blockade of the IL-33 receptor, ST2L, is one way to enhance TLR9 signaling (356, 357). Our observations fit with this hypothesis and provide a rationale for clinically targeting ST2L in conjunction with CpG therapy. We have also observed that pSTAT3, which has a negative regulatory activity on DC activation, is decreased in the lysates of BMDCs from asthmatic mice that were treated with α-ST2L & CpG therapy. This downregulation of STAT3 provides one possible mechanism of action for α-ST2L blockade in asthma.

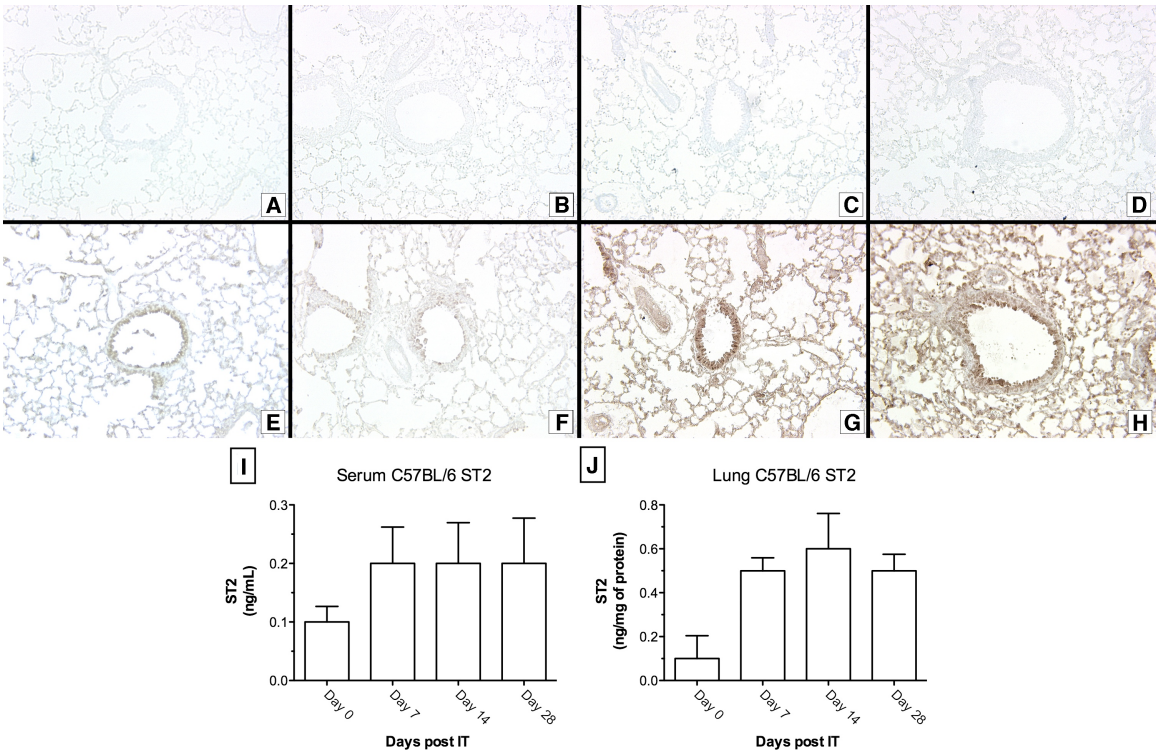

Figure 5.1. ***ST2L levels increase in lung and serum in asthmatic C57BL/6 mice.*** Whole-lung tissue sections from *Aspergillus*-sensitized and challenged C57BL/6 mice at D0 **(A, E)**, D7 **(B, F)**, D14 **(C, G)**, and D28 **(D, H)** post IT conidia challenge were stained using routine immunohistochemical techniques. Panels shown are representative of whole lung sections from mice stained with IgG control **(A-D)** or anti-ST2L antibody **(E-H)**. Receptor expression stains brown with this immunohistochemical procedure. The original magnification was ×200 for all photomicrographs. ELISA analysis of serum **(I)** and whole-lung ST2L from *Aspergillus*-sensitized and conidia challenged mice from D0-D28 post IT challenge. Data are expressed as mean ± SEM; n = 5/group/time point.

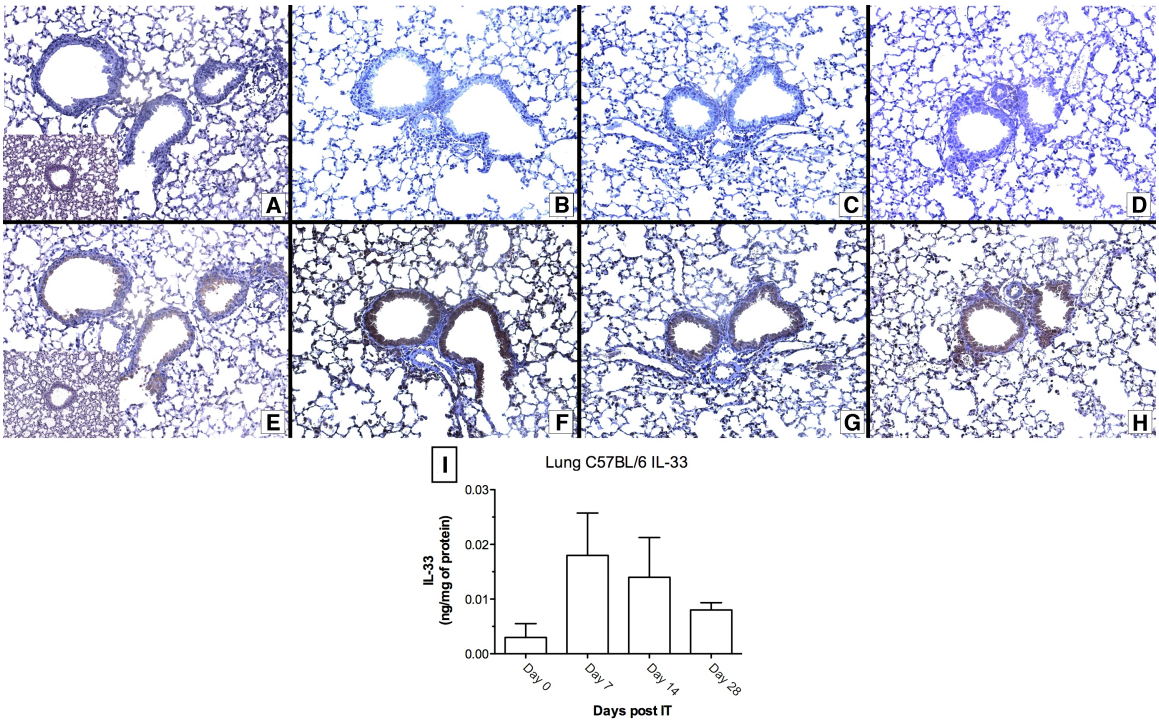

Figure 5.2. ***IL-33 levels in whole-lung in asthmatic mice.*** Whole-lung tissue sections from *Aspergillus*-sensitized and challenged C57BL/6 mice at D0 **(A, E)**, D7 **(B, F)**, D14 **(C, G)**, and D28 **(D, H)** post IT conidia challenge were stained using routine immunohistochemical techniques. Panels shown are representative of whole lung sections from mice stained with IgG control **(A-D)** or anti-IL-33 antibody **(E-H)**. Receptor expression stains brown with this immunohistochemical procedure. The original magnification was ×200 for all photomicrographs. ELISA analysis of whole-lung **(I)** IL-33 levels from *Aspergillus*-sensitized and conidia challenged C57BL/6 mice from D0-D28 post IT conidia challenge. Data are expressed as mean ± SEM; n = 5/group/time point.

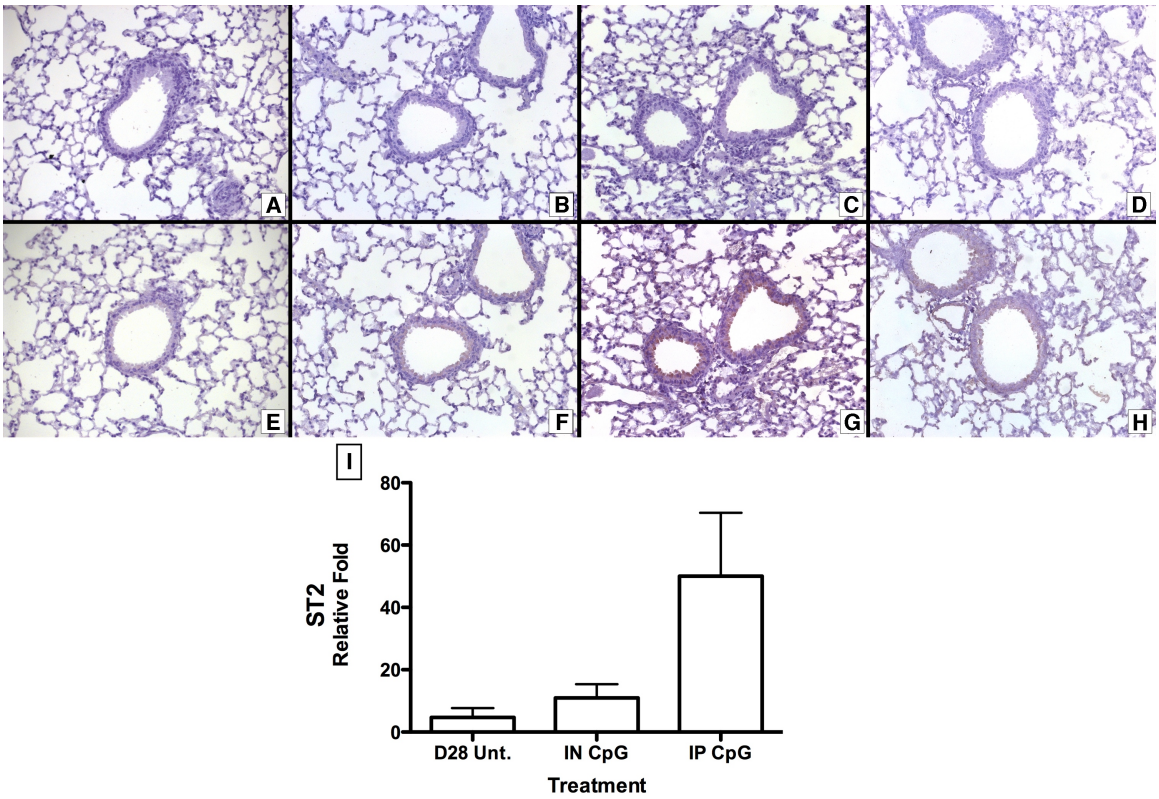

Figure 5.3. ***TLR9 levels increase with time in the lung in fungal asthma and ST2 levels increase with systemic CpG therapy, but not intranasal CpG.*** Whole-lung tissue sections from *Aspergillus*-sensitized and challenged C57BL/6 mice at D0 **(A, E)**, D7 **(B, F)**, D14 **(C, G)**, and D28 **(D, H)** post IT conidia challenge were stained using routine immunohistochemical techniques. Panels shown are representative of whole lung sections from mice stained with IgG control **(A-D)** or anti-TLR9 antibody **(E-H)**. TAQMAN analysis of BALB/c mouse lung ST2 expression in untreated, intranasal CpG (IN) treated, or systemic intraperitoneal CpG (IP) treated mice **(I)**. Receptor expression stains brown with this immunohistochemical procedure. The original magnification was ×200 for all photomicrographs.

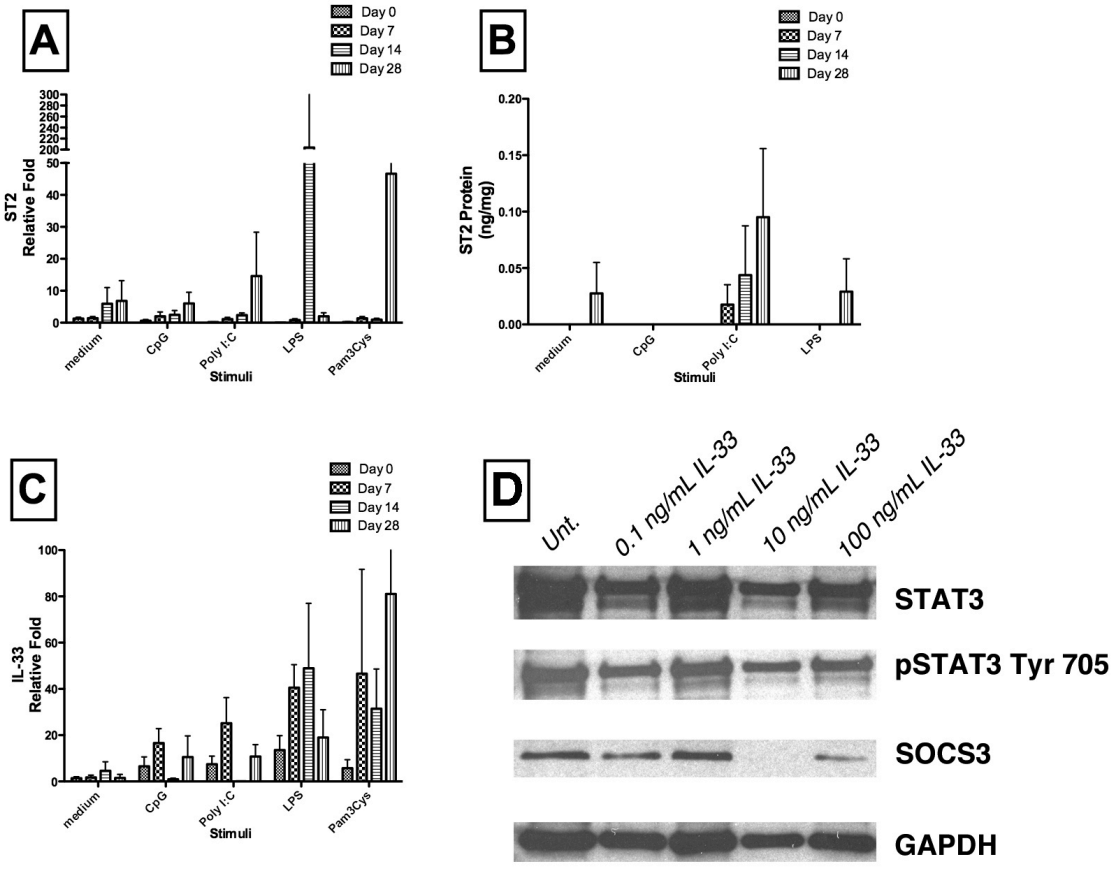

Figure 5.4. ***Allergic dendritic cells demonstrate altered levels of ST2L and IL-33.*** TAQMAN analysis of C57BL/6 mouse BMDC ST2 **(A)** and IL-33 **(C)** levels in *Aspergillus*-sensitized and conidia challenged C57BL/6 mice from D0-D28 post-conidia challenge. ELISA analysis of BMDC IL-33 **(B)** levels from asthmatic mice. Error bars indicate standard error means. Data are expressed as mean ± SEM; n = 5/group/time point. *, $P \leq 0.05$ compared with TLR9$^{+/+}$ group. Western blot analysis of STAT3, pSTAT3 Tyr705, and SOCS3 with GAPDH control of naïve BMDCs treated with IL-33 *in vitro* **(D)**. Data are expressed as mean ± SEM; n = 5/group/time point.

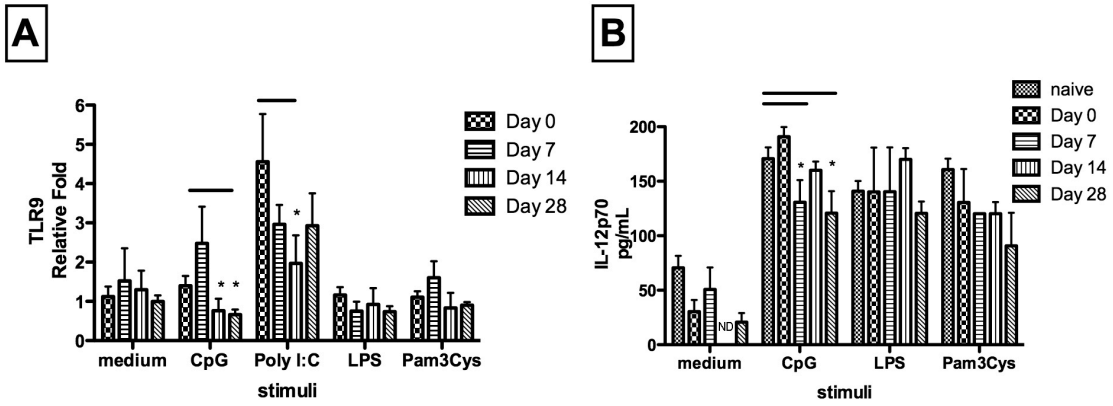

Figure 5.5. ***BMDC TLR9 transcripts and IL-12p70 levels decrease with time post conidia challenge in fungal asthma.*** TAQMAN analysis of asthmatic mouse BMDC TLR9 **(A)**, and ELISA for IL-12p70 **(B)** levels from *Aspergillus*-sensitized and conidia challenged C57BL/6 mice that were either from day 0 to day 28 post conidia challenge. TLR9 TAQMAN levels are normalized to naïve control. Data are expressed as mean ± SEM; n = 5/group/time point. *, $P \leq 0.05$.

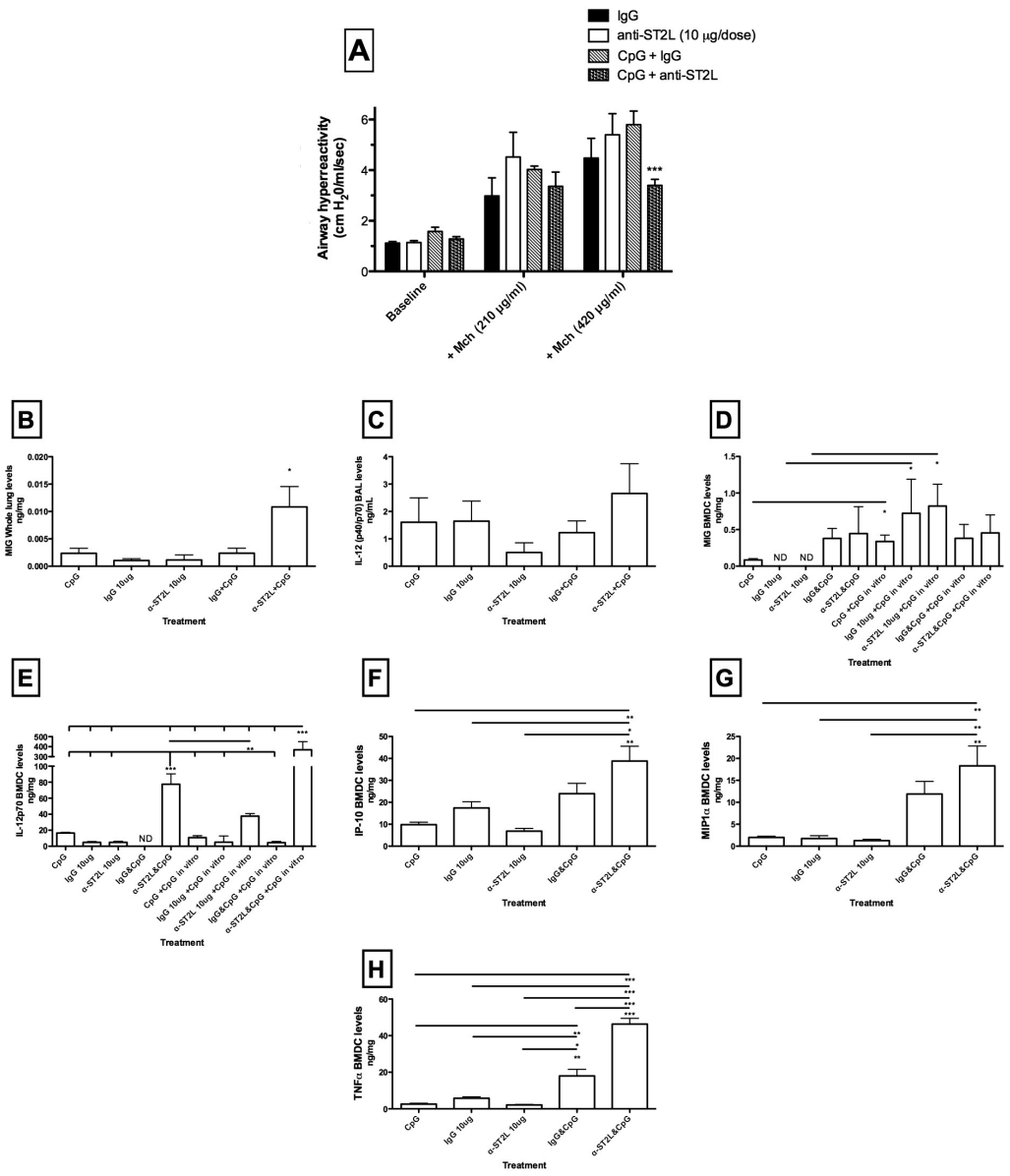

Figure 5.6. ***α-ST2L and CpG-ODN combined treatment enhances CpG-induced responses in fungal asthma and alters whole-lung and BAL cytokines.*** Airway resistance analysis **(A)**, and ELISA analysis of MIG in whole-lung **(B)**, IL-12(p40/p70) in BAL fluid **(C)**, MIG from BMDCs **(D)**, IL-12p70 from BMDCs **(E)**, IP-10 from BMDCs **(F)**, MIP1α from BMDCs **(G)**, and TNFα in BMDCs **(H)** in sensitized and conidia challenged C57BL/6 mice treated with CpG, 10μg IgG, 10μg α-ST2L, 10μg of IgG+CpG or 10μg of α-ST2L+CpG on alternate days from day 14 to day 28 after conidia challenge. BMDCs were also restimulated with CpG *in vitro* **(D, E)**. Peak increases in airway resistance or hyperresponsiveness (units = cm H2O/mL/sec) were determined at each time point after the intravenous injection of methacholine. Data are expressed as mean ± SEM; n = 5/group/time point. *, $P \leq 0.05$; **, $P \leq 0.01$; ***$P \leq 0.001$ compared with the appropriate baseline measurement or the indicated treatment group following methacholine challenge. Cytokine levels from BMDCs are measured from culture media.

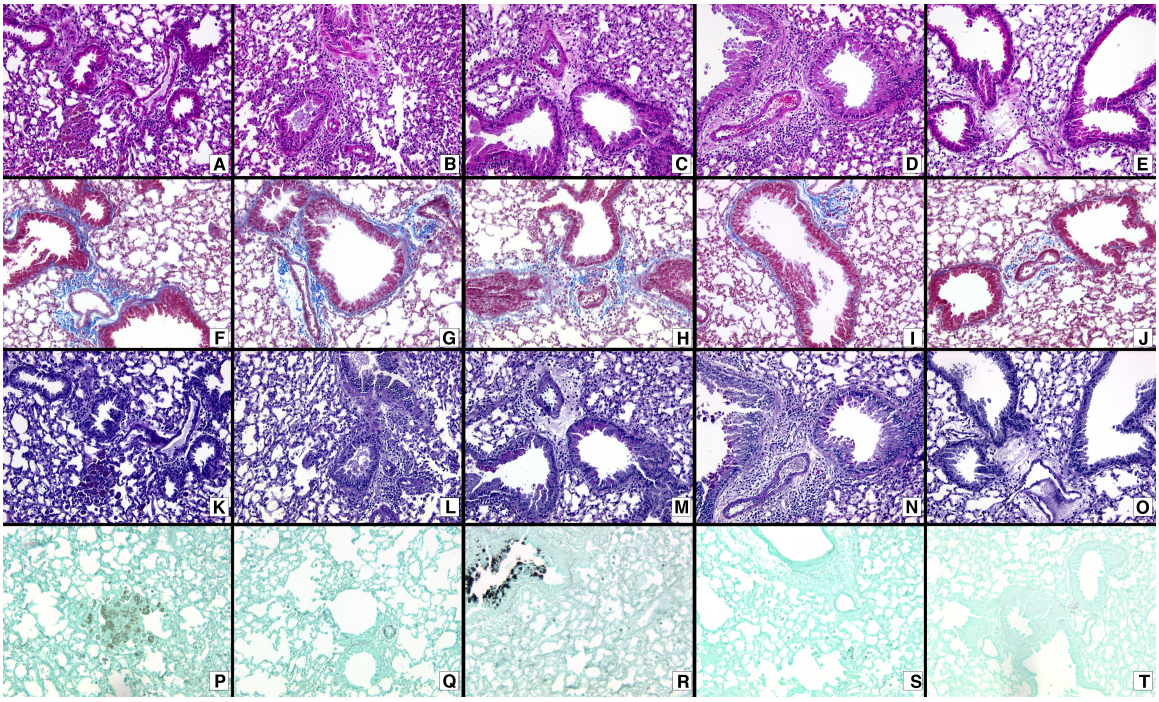

Figure 5.7. ***α-ST2L and CpG-ODN combination therapy reduces inflammation, mucus metaplasia, and lung remodeling, while increasing fungal clearance in fungal asthma better than monotherapy.*** Mouse whole-lung tissue sections from *Aspergillus*-sensitized and challenged C57BL/6 mice treated with 10μg of IgG **(panels A, F, K, P)**, α-ST2L **(panels B, G, L, Q)**, 5μg of CpG **(panels C, H, M, R)**, IgG & CpG **(panels D, I, N, S)**, or α-ST2L & CpG **(panels E, J, O, T)** on alternate days from day 14 to day 28 after conidia challenge. Tissues were stained with Hematoxylin & Eosin **(A-E)**, Trichrome **(F-J)**, Period Acid Schiff **(K-O)**, and Gomori Methenamine Silver (GMS) **(P-T)** stains. The original magnification was ×200 for all photomicrographs.

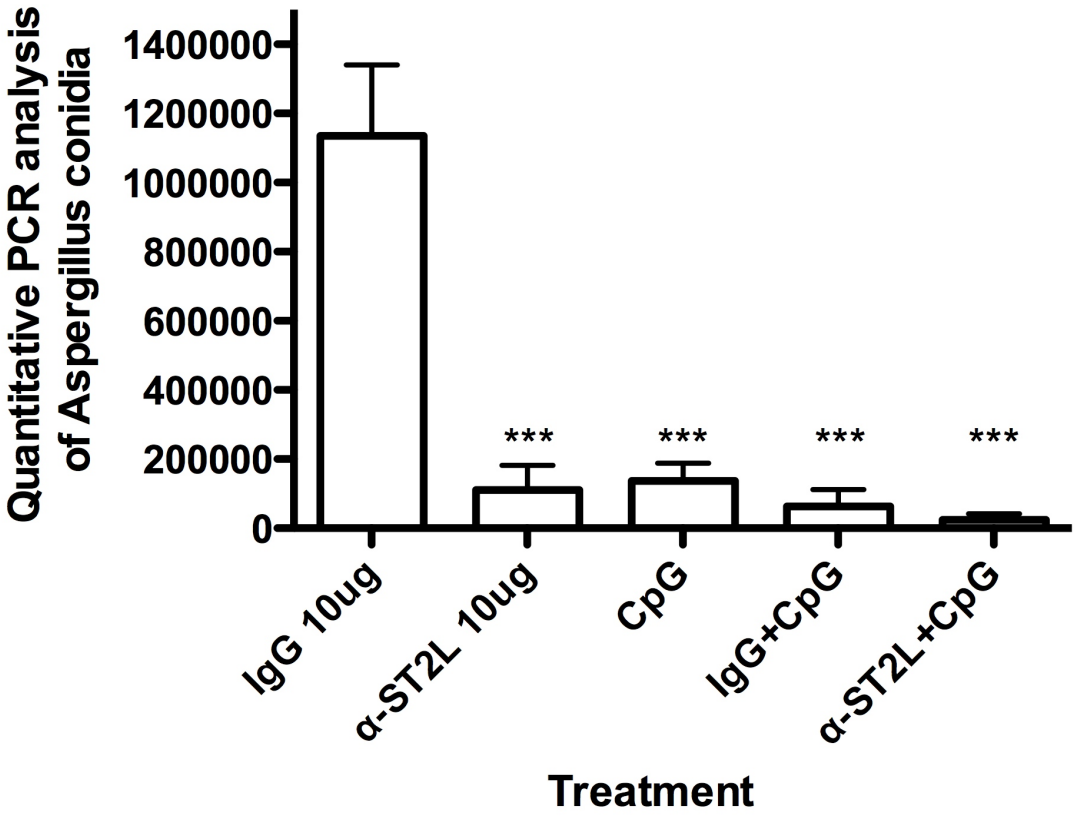

Figure 5.8. Aspergillus *conidia are significantly reduced in number in the lungs of asthmatic mice treated with α-ST2L, CpG, IgG&CpG, and α-ST2L&CpG.* TAQMAN quantitation using primers for 18s ribosomal DNA of *Aspergillus fumigatus* conidia in the lungs of asthmatic mice treated with 10μg IgG, 10μg α-ST2L, CpG, 10μg of IgG+CpG, or 10μg of α-ST2L+CpG on alternate days from day 14 to day 28 after conidia challenge. Data are expressed as mean ± SEM; n = 5/group/time point. ***, $P \leq 0.001$.

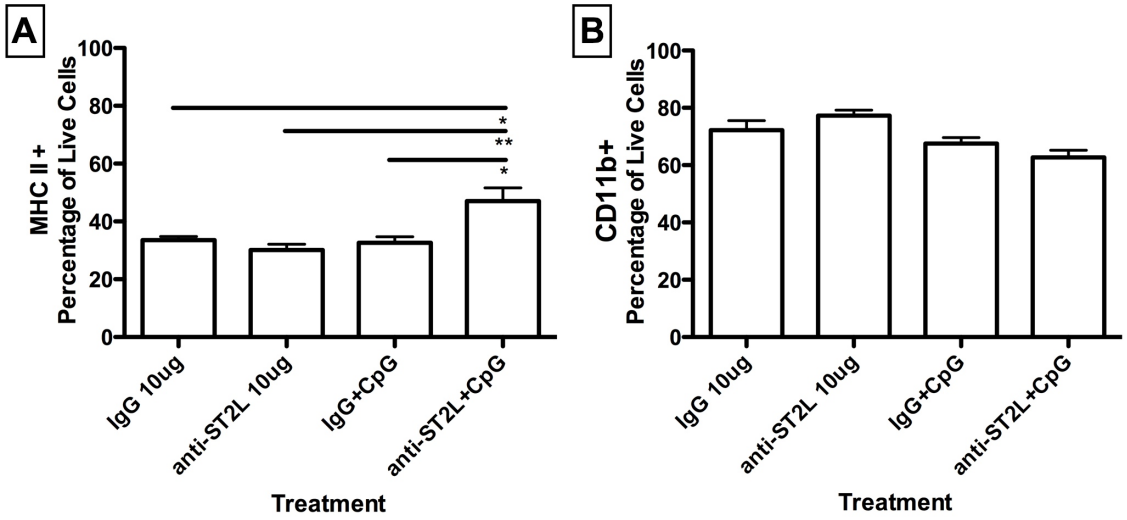

Figure 5.9. ***MHCII+ splenic DCs increase with α-ST2L & CpG combination therapy in fungal asthma.*** Flow cytometric analysis of percentages of MHC II+ **(A)** and CD11b+ **(B)** cells in freshly isolated splenic cells from asthmatic mice MACS sorted for CD11c+ cells and treated *in vivo* with 10μg IgG, 10μg α-ST2L, CpG, 10μg of IgG+CpG, or 10μg of α-ST2L+CpG on alternate days from day 14 to day 28 after conidia challenge and MACS sorted for CD11c+ cells. Data are expressed as mean ± SEM; $n = 5$/group/time point. *, $P \leq 0.05$; **, $P \leq 0.01$.

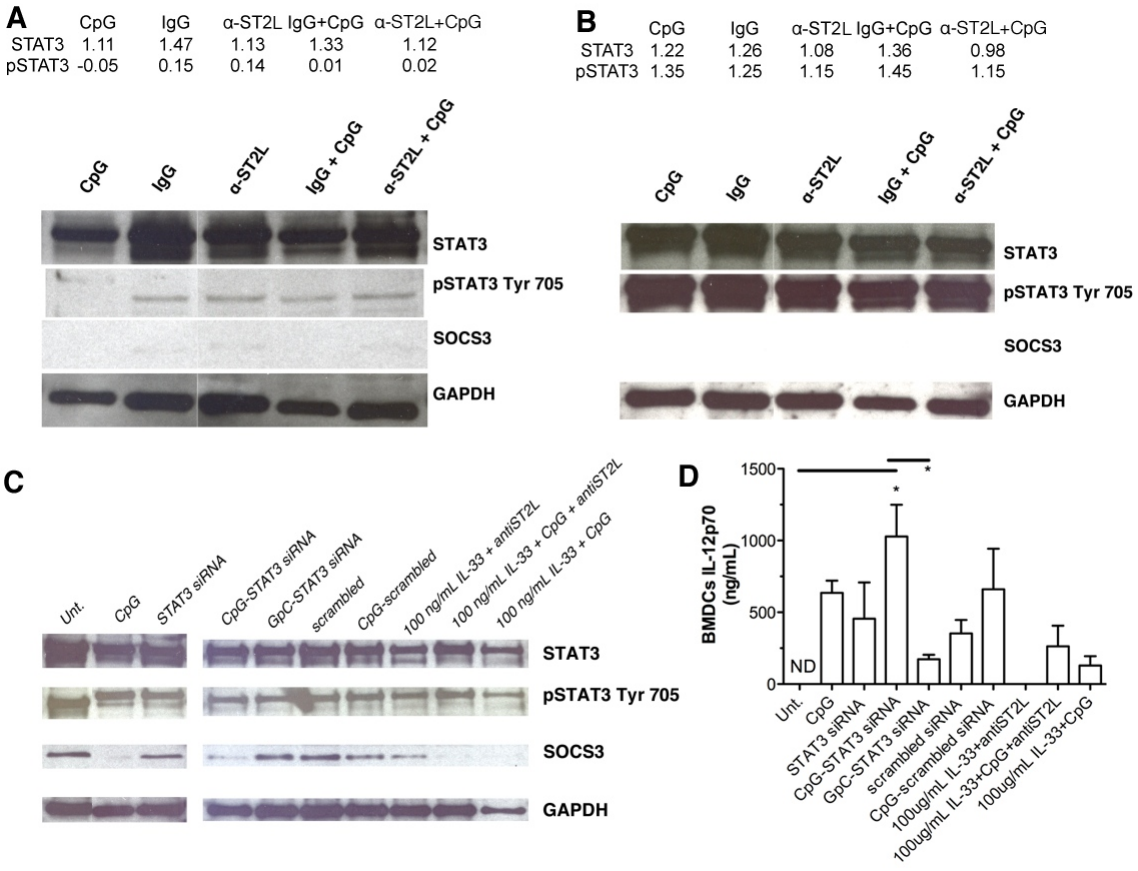

Figure 5.10. ***α-ST2L and CpG-ODN therapy alters STAT3 activation in BMDCs from asthmatic mice.*** Western Blot analysis of bone marrow derived dendritic cells from asthmatic mice **(A & B)** treated with 10μg IgG, 10μg α-ST2L, CpG, 10μg of IgG+CpG, or 10μg of α-ST2L+CpG on alternate days from day 14 to day 28 after conidia challenge. BMDCs were either cultured in media **(A)**, or restimulated for 24h with CpG in the culture media **(B)**. Bands were quantitated relative to GAPDH control bands. BMDCs from naïve mice were treated *in vitro* with controls, CpG, STAT3 siRNA, CpG-STAT3 fusions, 10μg α-ST2L, or combinations thereof and lysates were subjected to western blot analysis **(C)** or culture media of cells to ELISA analysis for IL-12p70 **(D)**. Data are expressed as mean ± SEM; n = 5/group/time point. *, $P \leq 0.05$.

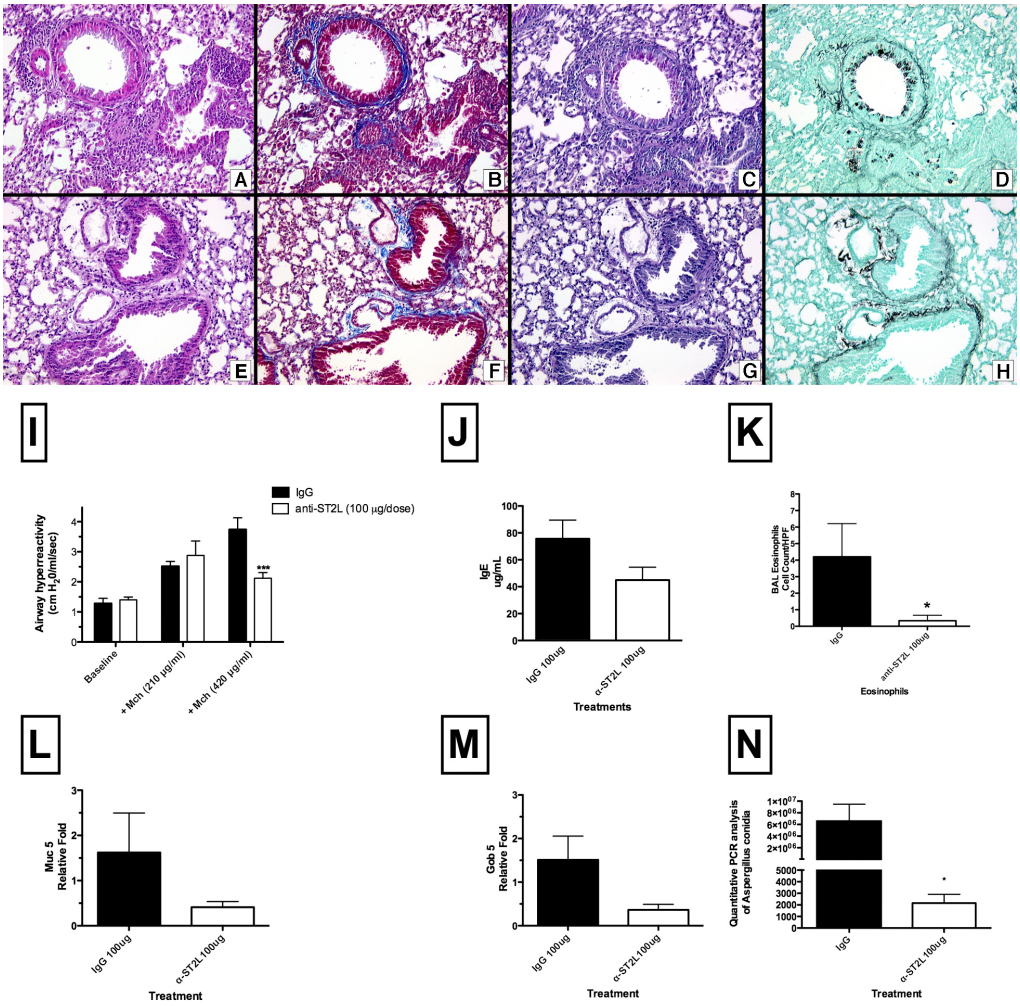

Figure 5.11. ***100µg α-ST2L therapy reduces inflammation, lung remodeling, and mucus metaplasia, alters airway responses, serum IgE, and eosinophilia in fungal asthma, while enhancing fungal clearance in fungal asthma.*** Mouse whole-lung tissue sections from *Aspergillus*-sensitized and challenged C57BL/6 mice treated with 100µg of IgG **(panels A-D)** or α-ST2L **(panels E-H)** on alternate days from day 14 to day 28 after conidia challenge. Tissues were stained with Hematoxylin & Eosin **(A & E)**, Trichrome **(B & F)**, Period Acid Schiff **(C & G)**, and Gomori Methenamine Silver (GMS) **(D & H)** stains. Airway resistance analysis **(I)**, ELISA analysis of serum IgE **(J)**, and BAL eosinophil counts **(K)** of these groups. TAQMAN analysis of mouse lung Muc 5 **(L)**, Gob 5 **(M)**, and quantitative PCR for *Aspergillus* conidia in lung sections **(N)**. Peak increases in airway resistance or hyperresponsiveness (units = cm H2O/mL/sec) were determined at each time point after the intravenous injection of methacholine. Data are expressed as mean ± SEM; $n = 5$/group/time point. *, $P \leq 0.05$; AHR is compared with the appropriate baseline measurement or the indicated treatment group following methacholine challenge. The original magnification was ×200 for all photomicrographs.

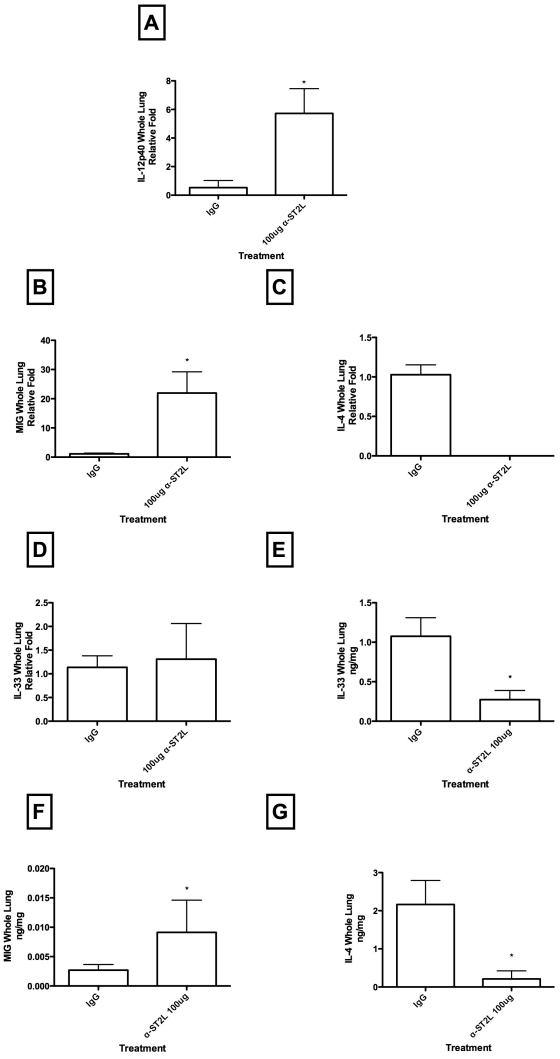

Figure 5.12. ***100μg α-ST2L therapy alters whole-lung cytokine levels.*** TAQMAN analysis of IL-12p40 **(A)**, MIG **(B)**, IL-4 **(C)**, and IL-33 **(D)** in whole-lung. ELISA analysis of IL-33 **(E)**, MIG **(F)**, and IL-4 **(G)** in whole-lung. Samples are from sensitized and conidia challenged C57BL/6 mice treated with 100μg IgG or 100μg α-ST2L on alternate days from day 14 to day 28 after conidia challenge. Data are expressed as mean ± SEM; n = 5/group/time point. *, $P \leq 0.05$.

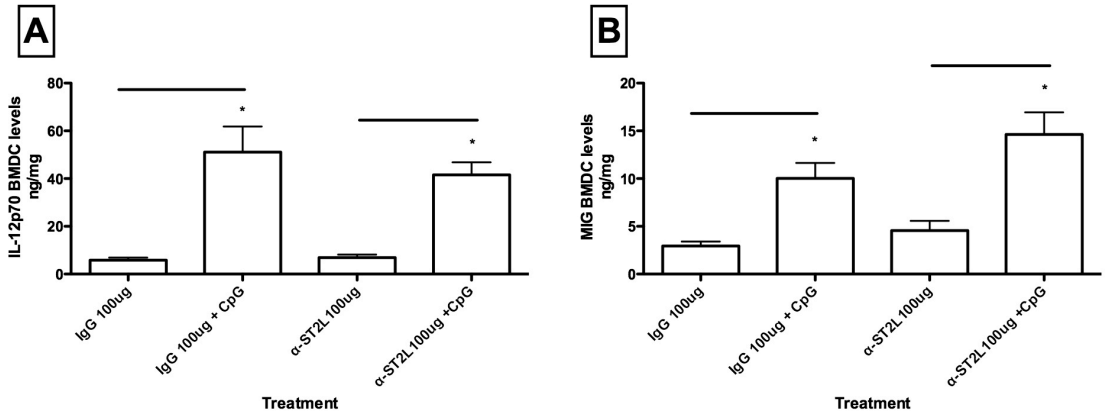

Figure 5.13. ***α-ST2L therapy impacts BMDC cytokine levels significantly only in conjunction with CpG therapy.*** ELISA analysis of asthmatic mouse BMDC IL-12p70 **(A)**, and MIG **(B)** levels from *Aspergillus*-sensitized and conidia challenged C57BL/6 mice that were treated with 100μg IgG or 100μg α-ST2L on alternate days from day 14 to day 28 after conidia challenge and either cultured in media alone or for 24h with CpG *in vitro*. Data are expressed as mean ± SEM; n = 5/group/time point. *, $P \leq 0.05$.

CHAPTER 6

CONCLUSION

Asthma is a chronic inflammatory disease of increasing prevalence in the western world that afflicts an increasing number of individuals each year. While therapies exist to control asthma, the associated side effects of long-term use of the currently available therapies and the resistance of severe forms of the disease to effective management of symptoms demand the development of a new generation of therapeutics (358, 359). The innate immune system plays the initiating role in the development of inflammation in asthma. Development of therapeutics that target the cell types and receptors responsible for initiating allergic inflammation in asthma are therefore the current focus of research in this field. There is a significant amount of evidence that targeting innate immune receptors such as TLR9 is an effective method of altering immune responses in animal models of allergic airway disease (360, 361). Human trials of CpG-ODN therapy, however, have been unable to impact the characteristic airway hyperresponsiveness that is associated with asthma (312). Recent research efforts have identified components of the immune response that trigger inflammation in asthma. Cytokines such as IL-33 and its corresponding receptor, ST2L, have been recognized to play a significant role in mediating the initiation of the innate immune response against allergen in a manner that helps to commence the adaptive immune response and the pathology associated with

allergic airway disease (362). The aim of this body of work has been to identify the role of TLR9 signaling within the lung in a model of asthma and to understand the interplay between the factors that regulate its signaling. We have focused on the role of TLR9 and its regulation through the IL-33-ST2L axis in the dendritic cells that initiate immune responses to allergen in the hope of understanding how to harness TLR9 signaling towards effective treatment of asthma.

TLR9 mediates antifungal responses in fungal asthma

In our studies we initially investigated the role of TLR9 in antifungal responses in a model of invasive aspergillosis involving neutrophil depletion and a model of fungal asthma, both in $TLR9^{+/+}$ and $TLR9^{-/-}$ mice. These studies were focused on investigating the role of TLR9 in fungal conidia recognition in an already defined model of fungal infection and asthma (228). During the course of these studies we were able to identify that a difference in fungal conidia recognition existed between conidia that were administered through intratracheal injection just after isolation (resting conidia), and conidia that were allowed to incubate for several hours and thus begin the process of germination (swollen conidia). According to the literature, this additional incubation time allowed for fungal conidia to express increased β-glucan moieties on their surface, altering the recognition of the conidia to include the receptor dectin-1 in the pulmonary environment (99, 271). We observed that the absence of TLR9 had a minor but significant protective effect in invasive aspergillosis and allergic asthma following conidia challenge. We also observed that $TLR9^{-/-}$ mice were susceptible to *A. fumigatus* growth when given swollen conidia. The observed effect was most dramatic when $TLR9^{-}$

$^{/-}$ mice were sensitized to this fungus and challenged with swollen conidia in our model of fungal asthma. These initial data demonstrated that the absence of TLR9 markedly impaired the immune responses required to contain the growth of fungal conidia in allergic mice.

We made several early observations with regard to the differences between the innate immune response to resting versus swollen *Aspergillus* conidia that allowed us to refine our model to use only swollen *Aspergillus* conidia in our later studies to better model fungal asthma in mice. Significantly we observed that serum IgE levels were as a whole higher in *Aspergillus* sensitized mice that were challenged with swollen conidia and that more robust CCL2, CXCL10, and IL-17 cytokine responses were present.

Appropriate recognition of allergens such as those derived from *Aspergillus fumigatus* lead to a balanced immune response and clearance of fungal conidia. Inappropriate responses in asthma, however, lead to excessive inflammation and lung remodeling that can lead to permanent changes in lung function. TLR9 has been identified to recognize fungal DNA (111). MyD88-dependent signaling on dendritic cells has also been identified as crucial for priming antifungal Th1 responses (113). Allergic asthma is a Th2 cytokine dominated disease (258). During ABPA and fungal asthma, persistence of conidia drives airway hyperresponsiveness and airway remodeling. We therefore focused on understanding the factors that can help drive the immune response away from the pro-asthmatic Th2 immune response while allowing for clearance of fungus within the lungs? Our studies have suggested that TLR9 has a major role in controlling fungal growth in the allergic lung and in our initial studies with the asthma model we identified that TLR9 levels increase in the lungs of asthmatic mice with

increasing time post conidia challenge (229). These findings helped set the stage for our efforts to target TLR9 activation in our model of fungal asthma.

Dendritic cells mediate the immune response against *Aspergillus* and help initiate the adaptive immune response involved in fungal asthma (104). Dendritic cell recruitment occurs initially through recognition of *Aspergillus* conidia by alveolar macrophages in the lung and secretion of chemokine and cytokine signals (281). Dendritic cells also recognize and engulf *Aspergillus* conidia and aid in development of Th and neutrophil responses to the fungus (104, 107). In our first studies we were able to identify that TLR9 and dectin-1 play roles in the recognition of fungal conidia and that in TLR9 deficient mice dectin-1 is significantly decreased at day 14 in the asthma model in both the lung and on BMDCs. We were thus able to conclude that TLR9 activation can also have a significant impact on signaling through other cell surface receptors, such as dectin-1, that are critical for fungal recognition.

TLR9 is critical for pulmonary responses against fungal allergens in allergic asthma and is important for regulating inflammation that aids in the containment and clearance of *Aspergillus*. TLR9 also is important for fungal recognition through other receptors such as dectin-1 that recognize swollen fungal conidia β-glucans. TLR9 is also important for Th1-associated cytokines such as CXCL10 (IP-10) as shown by our first studies. Thus, from these initial studies identifying the role that TLR9 plays in the fungal model of asthma, we were able to determine that therapeutic strategies targeting TLR9 would be a logical next step to take in the model.

CpG-ODN therapy of asthma is TLR9 dependent and site specific

CpG-ODN therapy has been tested in clinical trials and has been observed to produce a Th1-type milieu of cytokines, but has been unable to decrease AHR in asthma patients (312). Upon examination of the literature, we first surmised that the inability of CpG to work clinically was due to polymorphisms in TLR9 resulting in loss of function (324). Our initial studies of the role of TLR9 during chronic fungal asthma showed that the absence of this receptor led to a severe form of allergic airway disease characterized by fungal growth (306) and thus we first thought it possible that similar changes in severity were related to the expression and function of TLR9 in clinical asthma. Recent investigations into the role of TLR9 polymorphisms in CpG-induced responses between individuals found major inter-individual differences of CpG-induced IFN-α production, however, these were not associated with common TLR9 variants (324). This discrepancy in the understanding of TLR9 function led us to investigate CpG-ODN therapy ourselves in a fungal asthma model. Previous studies have demonstrated that the adjuvant or Th1-type cytokine response evoked by CpG appears to depend on the site of administration (307), with mucosal delivery being identified as the route promoting the optimal immunostimulatory effect (308, 309). Although the intranasal delivery of CpG promotes inflammatory changes in non-allergic mice (310), its delivery via this route in allergic mice has been shown to markedly attenuate the pulmonary allergic inflammatory response in wildtype mice (119). We therefore set about exploring CpG-ODN therapy in our model through site-specific administration (either intranasally or systemically through the intraperitoneal route) in both $TLR9^{+/+}$ and $TLR9^{-/-}$ mice.

The findings of our second set of studies allowed us to observe that TLR9$^{-/-}$ mice given IN CpG allowed for the production of Th1 cytokines without any attenuation of Th2 mechanisms driving AHR and that this effect may be due to the effects of scavenger receptors recognizing CpG. The therapeutic effect of CpG-ODN treatment in the experimental fungal asthma model examined in our studies was dependent on the site of administration since the intranasal delivery of CpG provided a therapeutic effect in both wildtype and knockout mice whereas the intraperitoneal delivery of CpG did not. This difference in therapeutic outcome would serve as a primer for our third set of studies. CpG-responsive cell types such as epithelial cells, macrophages, and dendritic cells interact through signaling messengers, one of which is IL-33, a pro-inflammatory "alarmin" protein that is produced in instances of cellular necrosis. IL-33 has recently been found to impact the activation and maturation of dendritic cells (345, 350). Previous studies involving the priming of mice with *Aspergillus* antigens and CpG in a model of invasive aspergillosis demonstrated that CpG promotes dendritic cell activation within the lung and the production of IL-12 and IFN-γ by these cells (311). These observations led us to further refine our focus on the dendritic cell specifically as the innate immune recognition mechanisms of this cell type help to initiate the adaptive immune response that involves Th2 cells and IgE production B cells that are important in the immune response to allergens.

We found that IN CpG significantly enhanced serum levels of IgG2a, a Th1-associated Ig, and whole lung levels of CXCL10 and IL-12. Thus, the administration of CpG to mice with fungal asthma dynamically altered levels of Th1-type factors and mediators although only IN CpG enhanced the expression of whole lung IL-12. This

increase in IL-12 correlated with our observations of resolution of asthma pathology in the lungs of asthmatic mice. Thus, our investigation of CpG administered in a site-specific manner helped to direct our next studies towards the investigation of the regulation of IL-12 production by dendritic cells within the context of asthma.

Shifting the immune response away from Th2-type cytokine generation has been shown to effectively reduce asthmatic symptoms both experimentally and clinically (287). In our fungal model of asthma we were able to observe the airway inflammation-associated Th2 type cytokines TGF-β and CCL17 present in control vehicle treated asthmatic mice in our second set of studies (228, 259, 313). We observed that whole lung levels of TGF-β were reduced with IN CpG treated $TLR9^{+/+}$ mice in manner consistent with previous CpG studies in models of allergic airway disease (290, 292, 298). TSLP, another cytokine known to activate DCs and promote the differentiation of Th2 T cells the secretion of Th2 cytokines and chemokines, was also observed (314, 315). We were able to observe that IN CpG played an inhibitory role on whole lung TSLP and CCL17 in $TLR9^{+/+}$ mice. These findings correlated with our observation of reduced airway hyperresponsiveness and remodeling in IN CpG treated $TLR9^{+/+}$ mice.

In our investigation of therapeutic TLR9 activation, CpG appeared to have site-specific therapeutic effects on the maintenance of chronic fungal airway disease. In particular, IN CpG but not IP CpG decreased airway hyperresponsiveness and airway remodeling in $TLR9^{+/+}$ mice in a manner consistent with its inhibitory effects on Th2-associated factors such as TGFβ, TSLP, and CCL17 and its stimulatory effects on IL-12 and IL-10. Surprisingly, IN CpG reduced airway remodeling in $TLR9^{-/-}$ mice, but airway hyperresponsiveness was not impacted in these mice, again confirming the findings of

our first studies that TLR9 activation was necessary for clearance of fungus and resolution of inflammation in the asthma model. Our second set of studies thus highlighted the potential of TLR9 directed therapies to effect modulation of all features of chronic fungal asthma, but a revealed a need to understand how TLR9 signaling is regulated such that CpG-ODN is unable to produce the same results with systemic therapy as it does with intranasal therapy.

ST2L hinders TLR9 mediated asthma therapy

The intracellular mechanisms through which TLR9 signaling is regulated in the context of asthma is an area that must be well understood if the discrepancies between CpG-ODN therapy in animal models versus human trials are to be addressed. In our second set of studies involving CpG-ODN we were able to observe that AHR attenuation in response to CpG therapy only occurred with IN CpG administration. We were also able to observe that ST2 transcript levels demonstrated an elevated trend in these animals. Further examination of the literature yielded that ST2 was a Th2 associated factor expressed on Th2 cells that are prevalent in allergic asthma (167). ST2L is also described as a factor that can sequester MyD88, an important component of the intracellular signaling pathway of TLR9 (326). We therefore reasoned that targeting this pathway would be an appropriate next step to take in identifying the blockade in TLR9 signaling that prevented systemic CpG-ODN therapy from reducing AHR. IL-33 was recently identified as the ligand for ST2L and is associated with inflammation in asthma (190, 349). Upon examination of this factor in our model, we were able to replicate the findings of IL-33 immunohistochemical staining in human lung biopsy samples in our

model, observing elevation in IL-33 in the airway epithelium of asthmatic mice at increasing time post conidia challenge. We were also able to observe increases in ST2L levels in the airways of asthmatic mice. These findings led us to investigate blockade of ST2L in the asthma model.

In our third set of studies, we observed that a low dose of CpG therapy in conjunction with a 10μg dose of α-ST2L produced a synergistic therapeutic effect. In comparison with IgG & CpG treated mice, 10μg dose α-ST2L & CpG treated mice exhibited attenuated fungal growth, airway remodeling, and AHR. These findings were replicated with a high 100μg dose of α-ST2L therapy. In histological sections from mice administered 100μg α-ST2L therapy we observed significantly elevated MIG levels in the lungs, however, we did not observe IL-12 and MIG production from BMDCs taken from these mice until they were treated *in vitro* with CpG. Further, at the 100μg dose the IgG control used was observed to attenuate inflammation to some extent, so we believe that the optimum dose of α-ST2L is a lower 10μg dose and that TLR9 activation is necessary for the full therapeutic effect.

We closely examined the role of BMDCs in the therapeutic effects observed with α-ST2L & CpG therapy. Upon close examination of IL-33 levels from BMDCs from asthmatic mice, no significant amounts of IL-33 were observed from BMDCs at any timepoint post IT conidia challenge, leading us to conclude that the IL-33 playing the major role in the exacerbation of inflammation in the asthmatic lung was epithelial in origin. These findings helped to forward the perspective that the epithelial tissue that first encounters allergen activates DCs in the vicinity through alarmin proteins such as IL-33, leading to exacerbation of inflammation through DC mediated mechanisms. In

lysates from BMDCs from α-ST2L & CpG treated mice we were able to observe a decrease in STAT3 and pSTAT3 levels. The presence of significant amounts of pSTAT3 inhibits the ability of NF-κB, an important transcription factor activated by TLR9 signaling, to be recruited to the IL-12p40 promoter (226). This was significant in light of the fact that we were able to observe significant amounts of IL-12 production only from BMDCs produced from asthmatic mice treated with both α-ST2L & CpG.

The results of our third set of studies revealed to us that ST2L targeting enhances BMDC activation in asthmatic mice leading to enhanced IL-12 production and ultimately a reduction in AHR, airway inflammation, remodeling, mucus metaplasia, and fungal growth with systemic CpG-ODN therapy. This effect appears to be mediated by an increase in not just IL-12, but also other Th1 associated factors such as MIG, IP-10, MIP1α, and TNFα produced from BMDCs. The myeloid dendritic cells we derived from the bone marrow of asthmatic mice were observed to undergo changes in our model of fungal asthma leading to enhanced expression of ST2L with increased time post conidia challenge. This in concert with enhanced TLR9 and IL-33 expression in the lung and the observed decrease in TLR9 expression and IL-12 levels in asthmatic BMDCs at the late timepoints post conidia challenge indicated that therapy to increase IL-12 levels from the BMDC through the TLR9 pathway may be one way to reverse the characteristic features of fungal asthma. Our observations fit with this hypothesis and provide a rationale for clinically targeting ST2L in conjunction with CpG therapy. We have also observed that pSTAT3, which has a negative regulatory activity on DC activation, is decreased in the lysates of BMDCs from asthmatic mice that were treated with α-ST2L & CpG therapy. This downregulation of STAT3 provides a possible mechanism of action for α-ST2L

blockade in asthma. Thus, from the first stage of our studies to this third and most recent set of investigations, we have been able to characterize the role of TLR9 in our model of fungal asthma and one factor, ST2L, which regulates its function. Our efforts targeting ST2L have provided evidence that stimulating TLR9 in concert with ST2L blockade may be one way to produce effective reversal of the inflammation, remodeling, and AHR in asthma and that this signaling pathway on BMDCs can play a role in mediating the ability for asthmatic BMDCs to produce cytokines that can draw the immune response away from the Th2 immune response found in asthma. Future studies to investigate the signaling occurring in the bone marrow between precursor cell types to produce TLR responsive BMDCs with α-ST2L & CpG therapy is a logical next step for this work. The findings presented here have helped to define a role for TLR9 signaling on BMDCs in the context of asthma and have presented one way in which TLR9 signaling can potentially be harnessed to treat allergic airway disease.

Future directions and implications

These studies collectively have provided evidence that there is a role for TLR9 in the therapy of fungal asthma and that the current limitations observed in CpG-ODN therapy in clinical trials has potentially been due to the action of negative regulators of TLR function such as ST2L. We utilized a well-characterized animal model of chronic fungal asthma to investigate the role of TLR9 in knockout mice, the effects of activating TLR9 with CpG-ODN therapy in fungal asthma through different routes of administration **(Fig. 6.1)**, and the impact that blocking ST2L in conjunction with IP CpG-ODN therapy would have on inflammation, remodeling, fungal clearance, and AHR in

the model **(Fig. 6.2 & 6.3)**. We were able to remove the hindrance observed with only systemic (IP) CpG-ODN therapy with regard to AHR attenuation by targeting ST2L specifically. We were also able to identify a cell type, the bone-marrow derived dendritic cell that was impacted by systemic α-ST2L & CpG therapy. Future efforts will be necessary in order to understand exactly how the BMDC is altered in the asthmatic mouse and how cell populations in the bone marrow are altered in response to therapy.

There are a significant number of studies investigating the differentiation of immune cells from bone marrow precursors. Few studies, however, have investigated the mechanisms directing bone marrow local innate immune responses in connection with TLR signaling. Future studies investigating the observations made in these studies can be directed toward understanding the interactions between cell types in the bone marrow precursor cells in asthmatic mice in response to α-ST2L & CpG therapy. Bone marrow cells from naïve mice that were $DEC205^{+}Gr\text{-}1^{low}$ exhibited increased IL-12p70 and TNF-α level relative to $DEC205^{+}Gr\text{-}1^{high}$ cells (355). One possibility is that α-ST2L & CpG is enriching the bone marrow cell population of $DEC205^{+}Gr\text{-}1^{low}$ cells leading to the enhancement of IL-12p70 and TNF-α from BMDCs we observed in our model. IL-12 can also contribute to allergic inflammation in asthma if produced in excessive quantities (363). Further investigation of the regulation of this cytokine can help to reveal how IL-12 synthesis by DCs can be regulated such that it can be maintained in the therapeutic range. Research into the regulation of the signaling between the bone marrow precursors may be able to help address this question.

A secondary motive for further investigation of IL-12 is that the IL-12p40 subunit identified to be regulated by the action of STAT3 on NF-κB is also a component of the

cytokine IL-23 (364, 365). IL-23 has been identified to enhance Th2 polarization in a model of allergic asthma (366). IL-12p40, on the other hand has been identified to down-regulate bronchial hyperresponsiveness in a mouse model of bronchial asthma with prolonged antigen exposure (367). Signaling through P2 receptors on human dendritic cells has been identified to inhibit IL-12 expression and enhance IL-23 expression through a pathway involving extracellular ATP, inhibiting the ability of DCs to induce Th1 responses (368, 369). The role of TLR9 and ST2L in mediating the balance between these two cytokines has not yet been investigated and is therefore another future direction that should be examined in allergic asthma.

TLR9 polymorphisms within patient populations may make targeting TLR9 more relevant for some individuals and less so for others (370). We recognize that targeting TLR9 in patients may require understanding how variation within the receptor affects its function. Polymorphisms in TLR9, for example, were associated with airway hyperresponsiveness in a recent study (370) and with pulmonary aspergillosis infection (256). Other studies, however, have not been able to find a correlation between common TLR9 variants and CpG-induced cytokine changes (324). Further work must be done to clarify if TLR9 polymorphisms do play a role in the pathogenesis of asthma. Similarly, investigation of ST2L polymorphisms and the role of the recently discovered other isoforms of this protein would also be an important future area of inquiry. Targeting ST2L impacts the signaling of all TLRs that signal through MyD88. Thus, these findings may serve as a platform upon which to continue investigation of targeting the intracellular factors that mediate TLR signaling in asthma. Future studies to understand

the impact of TLR9 and ST2L variants may help to further understand how variation in these receptors could predispose some individuals to develop asthma.

These studies have investigated the role of a mouse variant of CpG-ODN similar to CpG-B DNA, however, the impact that other types of CpG-ODN have in asthma in a site-specific manner needs to be addressed. CpG-ODN has 3 types: A, B, and C. The cell-type specificity of the different types of CpG DNA hint that further levels of complexity underlie the role of this type of oligonucleotide as a therapeutic option. Recent studies have identified that CpG-B DNA inhibits type-1 interferon production by dendritic cells, but does not affect TNF-α or IL-12p40 secretion. CpG-A DNA, however, is able to induce DCs to produce IFN-α and IFN-β (371). The role of these differing sequences of CpG-ODN may play an even more dramatic role if taken within the context of variation in the structure and sequence of TLR9 and its gene within the population. Further efforts are necessary to fully understand the contribution that the different CpG-ODN sequences provide in the context of asthma.

The use of non-CpG-ODN DNA as a control for CpG-ODN therapy is one angle we did also consider and test (unpublished data). The non-CpG DNA for the CpG-ODN used in these studies, however, did exhibit an activating effect in the asthma model and thus could not be considered a non-specific DNA control. With non-CpG we were able to observe some degree of attenuation of airway responses and inflammation in the lung, thus the control DNA was not immune neutral. In future studies a more refined non-CpG control could be used to provide more accurate comparison between therapeutic and control groups.

The existence of steroid-resistant asthma is one reason why alternative therapies for asthma are needed. Within this context, the interaction of other antigen-presenting cell types in the infiltrating cell mileu within the asthmatic lung needs further understanding to fully comprehend the mechanisms behind the pathogenesis of asthma resistant to the currently available therapies. A recent study identified that interaction between the IFN-γ and TLR4/MyD88 pathways plays a role in the development of steroid resistant asthma (358). In these studies, only mice that received OVA-specific Th1 cells exhibited steroid resistance, whereas mice that received Th2 cells were not. Therefore, this ability for the presence of Th1 skewed cells without a decrease in AHR provides further justification for studies investigating the underlying pathways behind why CpG-ODN has not worked in human clinical trials even though a Th1 skew was observed with treatment (312). The similarity between these clinical observations and the observations of steroid resistance in a murine model of asthma suggests a possible common mechanism behind the inability for CpG-ODN to treat human asthma and the pathogenesis of steroid resistant asthma.

TLR9 agonist can contribute to tissue remodeling at appropriate concentrations (372). For this reason, the safe dose of CpG-ODN must be as low as is therapeutically effective. Our studies investigating the role of α-ST2L allowed us to use a lower dose of CpG-ODN than in previous studies in combination with α-ST2L antibody and still observe a therapeutic effect. Repeated large doses of CpG-ODN can cause a wasting-like syndrome and the activation of monocytes and macrophages has also been observed with CpG-ODN therapy (373). These cytotoxic effects of higher doses of CpG necessitate further investigation into the cell types that CpG-ODN therapy impacts and additional

efforts at understanding the mechanisms behind CpG-ODN mediated effects on immune responses are needed to clarify both the beneficial and detrimental effects of therapy.

The recent finding that IL-33 is present in both a pro-IL-33 form that is functional and can enhance inflammation and a form cleaved by caspase-1 further complicates our understanding of the IL-33-ST2L axis (374). The synthesis of IL-33 continues to be enigmatic and the exact roles of these two forms of the protein continue to be poorly characterized (375). Macrophages and even cultured mast cells have also recently been identified to be able to produce IL-33 (376). Further work is necessary to fully understand how this protein enhances and initiates inflammatory processes.

Future studies investigating the role of the ST2L-IL-33 axis in the regulation of dendritic cell activation through TLR9 will be directed at understanding the specific cell types involved in the lung and the mechanisms underlying the activation of these cells through the TLR9 pathway and regulated through ST2L. While the number of DCs available through digestion of lung tissue are small, studies could be directed at flow cytometric analysis of DC in the asthmatic lung to understand the percentage of ST2L+ DCs in the asthmatic lung with increasing time post conidia challenge. Analysis of TLR9 levels in these same cells could throw further light on the changing significance of each of these factors with increasing time post conidia challenge.

Data presented in these studies has included siRNA knockdown of STAT3 in naïve BMDCs and the increased IL-12 responses of these cells when this knockdown is performed with STAT3 siRNA fused to CpG-ODN. Current studies underway will analyze the levels of STAT3 in asthmatic BMDCs treated with siRNA in a similar manner to understand the alterations in STAT3 in asthmatic BMDCs and how these cells

respond to STAT3 knockdown. The studies presented in this dissertation investigated the changes in Tyr705 phosphorylation that occur in asthmatic mice that are treated with both CpG-ODN and α-ST2L or control treatments. STAT3 also has a second phosphorylation site at Ser727 that enhances the ability of this transcription factor to bind to promoter sequences in the nucleus. Tyr705 plays the dominant role in STAT3 function as it is necessary for STAT3 dimerization and entry into the nucleus. Future studies could also focus on the alternative Ser727 phosphorylation site to investigate changes at this regulatory site in response to therapy.

Additional studies could also involve investigation of the role of the epithelial cells and IL-33 originating from epithelium in the pathogenesis of asthma. Cell culture studies involving epithelial cell lines cultured with *A. fumigatus* conidia could provide further evidence as to how proteases from fungal conidia can promote tissue damage and thus the production of IL-33 that effects dendritic cell TLR9 signaling through ST2L. While we were able to draw a connection between IL-33 levels from epithelium tissue in the asthmatic lung through immunohistochemical staining, isolation of some of these cells and quantitative analysis of their transcriptional changes in IL-33 would help to extend the findings presented here.

The studies presented here involve the use of inbred strains of mice such as the C57Bl/6 mouse that demonstrates a Th1 skew in its immune responses. The Balb/c mice we used in our first studies demonstrate Th2 skew to their immune responses and are also an inbred strain. Future studies could involve the use of outbred strains of mice to investigate the role of TLR9 signaling and ST2L regulation in the absence of such strain-specific effects.

In closing, TLR9 directed therapeutic strategies proved effective in resolving the characteristic features of asthma in a fungal model of allergic airway disease when applied in conjunction with ST2L blockade. While the etiology of asthma is multifactorial, we believe that the findings presented in this body of work have provided new mechanistic details in one model of allergic airway disease that shed new light on the therapeutic possibilities for treatment of this disease. It is our hope that our findings will spur further investigation of how innate immune receptors instruct dendritic cells to initiate and regulate the inflammatory processes found in allergic asthma and that this research will one day lead to an effective treatment for this disease.

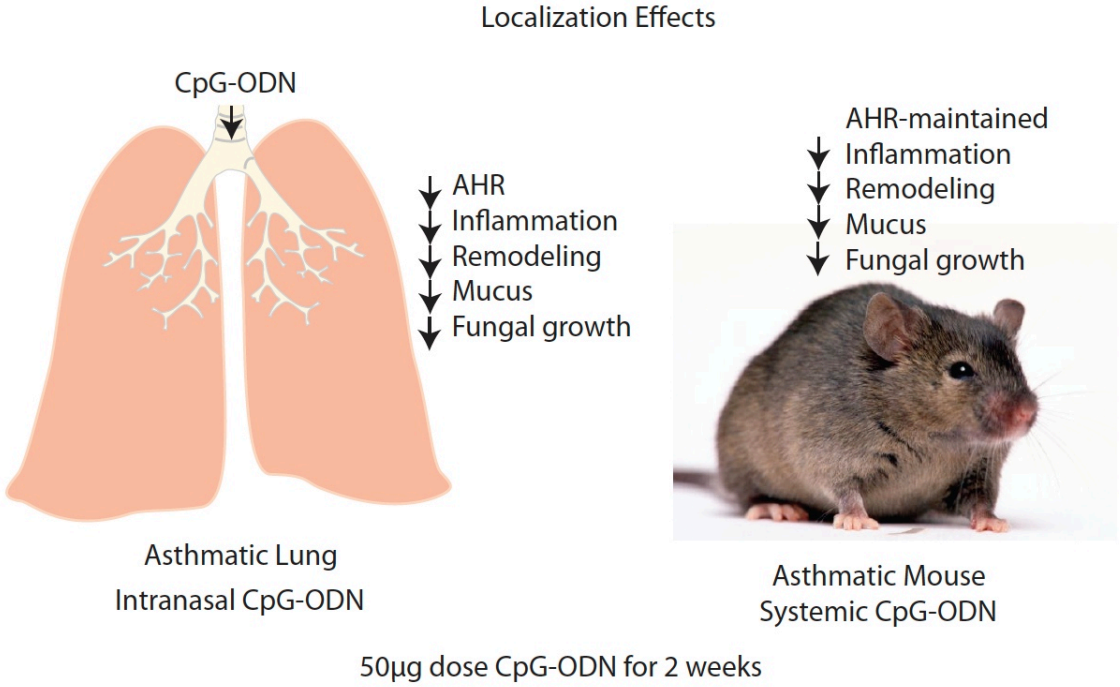

Figure 6.1. ***Model for site-specific effects of intranasal (IN) and intraperitoneal (IP) administration of CpG-ODN in the asthmatic mouse.*** Model for CpG-ODN site-specific effects in the asthmatic mouse. We observed attenuation of airway hyperresponsiveness (AHR), inflammation, remodeling, mucus, and fungal growth with IN CpG-ODN therapy and some degree of attenuation of all of these factors, except for AHR in the Systemic (IP) CpG-ODN treated mice. Note that a high 50ug dose was used in these studies.

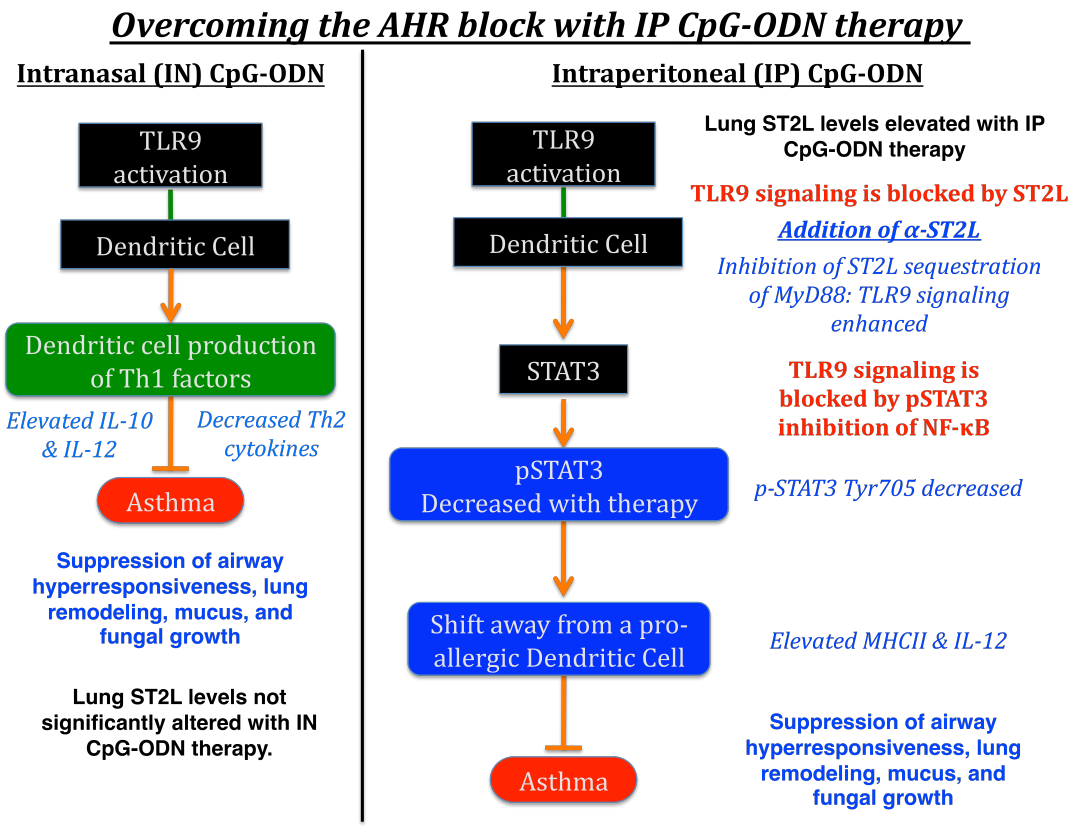

Figure 6.2. ***Overcoming the AHR block with intraperitoneal (IP) CpG-ODN therapy.*** IN CpG-ODN promotes the production of Th1 factors by dendritic cells and decreases in Th2 cytokines leading to suppression of airway hyperresponsiveness, lung remodeling, mucus, and fungal growth. ST2L levels are not significantly changed in the lungs of IN CpG-ODN treated mice. IP CpG-ODN treated mice, however, exhibit elevated transcript levels of ST2L in their lungs. Treatment with IP CpG-ODN in conjunction with α-ST2L antibody removes ST2L inhibition of TLR9 signaling through MyD88 sequestration. Combination therapy also decreases pSTAT3 levels in BMDCs, further removing a block on TLR9 signaling through pSTAT3 inhibition of NF-κB binding to the IL-12 promoter. IP CpG-ODN with α-ST2L therapy leads to suppression of airway hyperresponsiveness, lung remodeling, mucus, and fungal growth.

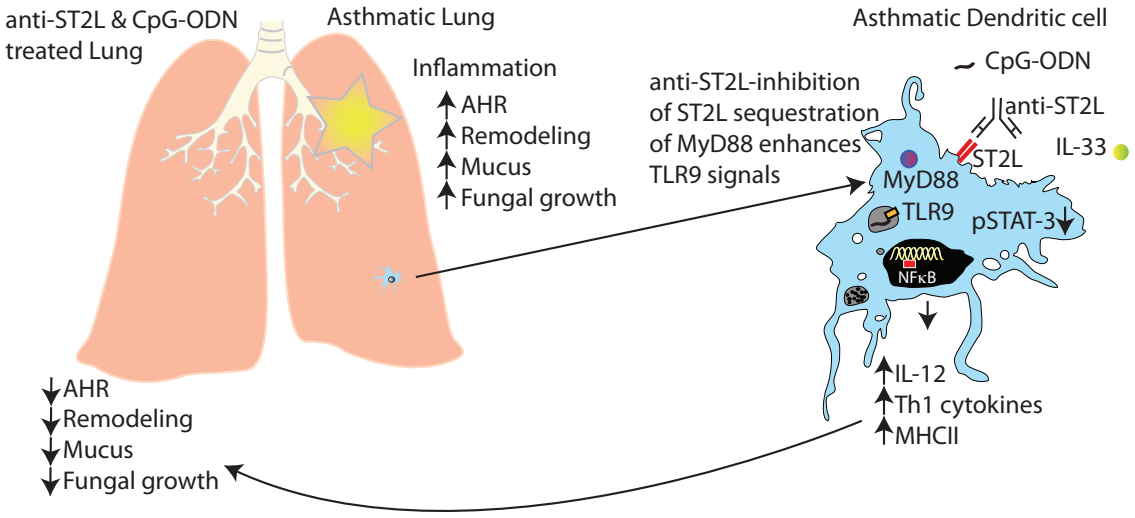

Figure 6.3. ***Model for α-ST2L and CpG-ODN combined treatment in the asthmatic mouse.*** Model for α-ST2L and CpG-ODN mediated therapeutic effects in the asthmatic mouse lung. In the asthmatic lung, we observed AHR, remodeling, mucus, and fungal growth. The dendritic cells originating from the bone marrow present in the lungs demonstrated elevated levels of ST2L, thus inhibition of this factor with the α-ST2L antibody blocks the activation of this receptor by IL-33, also present within the asthmatic lung. α-ST2L therapy frees MyD88 to allow for signaling through TLR9 using CpG-ODN. Dual therapy with CpG-ODN and α-ST2L also decreases pSTAT3 levels in BMDCs, removing the block on NF-κB at the nuclear level preventing transcription of IL-12. Together, CpG-ODN therapy with α-ST2L antibody treatment increases IL-12, Th1 cytokines, and increases MHCII on the BMDCs leading to decreased AHR, remodeling, mucus, and fungal growth.

APPENDIX

Chapter 3

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Chapter 4

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Chapter 5

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General

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