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Classical and alternative nuclear factor-kappaB in epithelium: impacts in allergic airway disease and avenues for redox regulation

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CLASSICAL AND ALTERNATIVE NUCLEAR FACTOR-KAPPA B IN LUNG
EPITHELIUM: IMPACTS IN ALLERGIC AIRWAY DISEASE AND AVENUES FOR
REDOX REGULATION

A Dissertation Presented

by

Jane Tully

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The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
Specializing in Cellular, Molecular, and Biomedical Sciences

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Accepted by the Faculty of the Graduate College, The University of Vermont, in partial fulfillment of the requirements for the degree Doctor of Philosophy, specializing in Cellular, Molecular, and Biomedical Sciences.

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ABSTRACT

Nuclear Factor kappaB (NF- κ B) is a transcription factor whose activation is increased in settings of allergic asthma. At least two parallel NF- κ B pathways exist: the classical pathway, which plays a role in inflammation and cell survival, and the alternative pathway, which regulates lymphoid cell development and organogenesis. The classical NF- κ B pathway regulates inflammatory responses derived from lung epithelial cells; however, the role of the alternative pathway in lung epithelial cells remains unclear. We demonstrate that both classical and alternative NF- κ B are activated in lung epithelial cells in response to multiple pro-inflammatory agonists, and siRNA-mediated knockdown of alternative NF- κ B proteins largely attenuates pro-inflammatory cytokine production. Furthermore, simultaneous activation of both pathways leads to cooperative increases in pro-inflammatory responses, indicating a potential role for both classical and alternative NF- κ B in the regulation of epithelial-derived pro-inflammatory responses.

NF- κ B activation in the epithelium modulates allergic inflammation in mouse models of allergic airway disease, however, its role in the context of an allergen relevant to human asthma remains unknown. In order to address the impact of inhibition of NF- κ B in the epithelium *in vivo*, we utilized a House Dust Mite (HDM)-induced model of allergic airway disease. We demonstrate that HDM exposure activates classical and alternative NF- κ B in both murine lung epithelium and human bronchial epithelial cells. Furthermore, following exposure to HDM, airway hyperresponsiveness, neutrophilic inflammation, and remodeling are attenuated in transgenic CC10-NF- κ B_{SR} (airway epithelial specific inhibitor of classical and alternative NF- κ B) mice in comparison to wild type mice. Our data also demonstrate that specific knockdown of the alternative NF- κ B protein, RelB, in the lung partially protects against HDM-induced pro-inflammatory responses, indicating that both classical and alternative NF- κ B are important in HDM-induced responses.

NF- κ B proteins are modified by the redox-dependent post-translational modification, S-glutathionylation, under conditions of oxidative stress. S-glutathionylation of IKK β , an upstream kinase in the NF- κ B pathway, is known to decrease its catalytic activity; however, it is unknown how S-glutathionylation of IKK β occurs. GSTP is an enzyme that catalyzes protein S-glutathionylation under conditions of oxidative stress and has been associated with the development of allergic asthma. We aimed to determine whether GSTP regulates NF- κ B signaling, S-glutathionylation of IKK β , and pro-inflammatory cytokine production. We demonstrate that siRNA-mediated knockdown of GSTP modulates NF- κ B activation, NF- κ B transcriptional activity, and pro-inflammatory cytokine production in response to LPS, a component of a bacterial cell wall. Furthermore, we demonstrate that GSTP associates with IKK β in response to agonist stimulation and dampens IKK β -induced pro-inflammatory cytokine production, surprisingly, independent of its catalytic activity. We also show that GSTP associates with other proteins of the NF- κ B pathway, indicating a potential dual mechanism for repression of NF- κ B-induced signaling. These studies collectively demonstrate that classical and alternative NF- κ B contribute to epithelial-derived inflammatory responses, and GSTP may be a novel target by which NF- κ B can be regulated.

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Chapter 1

Comprehensive Literature Review

Asthma

Overview:

Asthma is a heterogeneous pulmonary disorder that affects approximately 300 million people worldwide. The pathogenesis of asthma is characterized by chronic inflammation, bronchoconstriction, smooth muscle hypertrophy, mucus metaplasia, and remodeling of the airways, all of which lead to breathlessness, tightening of the chest, and wheezing. Multiple endotypes of asthma exist, including atopic (allergic), non-atopic, and exercise-induced asthma. A wide variety of asthma triggers have been identified, such as allergens, exercise, cold air, cleaning solutions, pollutants, and others. While a genetic component is thought to contribute to the development of asthma, environmental impacts can increase the incidence of this disease. Children living in cities have a higher chance of developing asthma, which is hypothesized to be a result of increased air pollution and cockroach, mouse, and rat allergens in urban settings (Byrd & Joad, 2006). Conversely, children living on a farm have been shown to be protected from the development of atopic asthma (Braun-Fahrlander et al., 1999). In the latter case, the protective effects have been attributed to exposure to bacterial products, which lead to tolerance and decreased sensitization to allergens. As will be documented below, multiple therapeutics are effective in treating symptoms of asthma, yet still no effective cure for the disease exists. Therefore, substantial research has been conducted in order to understand the underlying mechanisms of asthma.

Characteristics and Treatments of Allergic Asthma:

Allergic asthma is frequently accompanied by chronic inflammatory processes. Inflammation is characterized by redness, heat, swelling, and pain, and is associated with pulmonary infiltration of leukocytes, or white blood cells. These cells include mast cells, lymphocytes, eosinophils, and neutrophils, all of which will be discussed in further detail later in this review (Lambrecht & Hammad, 2013; Lloyd & Hessel, 2010). Eosinophils have been identified as classic mediators of allergic asthma, while neutrophils have been associated with resistance to conventional therapies. Small signaling molecules (cytokines) are also associated with allergic inflammation and contribute to the recruitment of the aforementioned inflammatory cells. Another prominent feature of allergic airway disease is bronchoconstriction, which is characterized by mucus release, closure of small airways, smooth muscle constriction, and airway thickening (Dowell, Lavoie, Solway, & Krishnan, 2014). Bronchoconstriction is diagnosed via the exposure of a subject to the acetylcholine receptor agonist, methacholine, which leads to smooth muscle contraction. Following methacholine challenge, Forced Expiratory Volume in one Second (FEV1) measurements are taken. Although the pathogenesis of bronchoconstriction is not fully understood, multiple molecules have been identified as contributing factors. Histamine, an organic nitrogenous compound secreted by mast cells, was demonstrated to be important in potentiating bronchoconstriction in a murine model of allergic airway disease (De Bie et al., 1998). Additionally, leukotriene D4, a lipid mediator secreted mostly by leukocytes, has also been shown to initiate airway smooth muscle contractions (Dahlen, Hedqvist, Hammarstrom, & Samuelsson, 1980).

Mucus metaplasia, which is a change in mucosal membranes accompanied by the secretion of mucus, has also been shown to be a contributing factor to the pathogenesis of allergic asthma. Mucus is produced by goblet cells and functions to lubricate the epithelium and trap inhaled particles, pollutants, and other agents which damage the airway. During bacterial or viral infections, these responses are instrumental in clearing the infection; however, in allergic responses, mucus metaplasia can be detrimental as a result of airway obstructions, which leads to wheezing and coughing (James & Carroll, 1995). Furthermore, significant airway remodeling can occur in conjunction with smooth muscle hypertrophy and collagen deposition (Johnson et al., 2004; Roche, Beasley, Williams, & Holgate, 1989). Following repeated injury, both the lung epithelium and smooth muscle can increase in size, decreasing the caliber of the airways, which leads to a decrease in airflow.

No effective cure for asthma exists, however, several treatment options are available to alleviate symptoms associated with the disease. Glucocorticoids, which have been shown to decrease inflammation through a variety of mechanisms, including suppression of pro-inflammatory signaling cascades, are commonly utilized as a treatment modality for asthmatic patients to control the inflammatory process. Leukotriene receptor inhibitors are also used as therapeutics, although glucocorticoids have been shown to be more effective in alleviating asthma symptoms (Chauhan & Ducharme, 2012). Alternatives to steroids, such as cromolyn, a compound that stabilizes mast cells by inhibiting the release of histamine, and theophylline, a xanthine drug which inhibits leukotriene synthesis, have also been effective in alleviating symptoms (Kabra &

Lodha, 2003). Beta-adrenergic receptor agonists activate the enzyme, adenylate cyclase, which then leads to smooth muscle relaxation and serves to alleviate symptoms. In a population of severe asthmatics, a combination of glucocorticoids and long acting beta agonists was shown to be the only treatment that controlled symptoms (Currie, Lee, & Wilson, 2005). These compounds, while effective at alleviating symptoms, do not have an impact on the underlying biological mechanisms that drive allergic airway disease, and therefore do not represent effective “cures”.

Immunological Mechanisms in Allergic Asthma

The airway epithelium provides a physical barrier between inhaled air and lung tissue. It plays a major role in allergic airway immunological processes, such as allergic asthma, and therefore will be a main focus of this review. The epithelium consists of both proximal and distal airways, both of which have different structure and functions. The trachea, which is located past the vocal cord, consists of a ciliated pseudostratified columnar epithelium. Adjacent to the trachea, the airway bisects into primary and secondary bronchi, which remain ciliated and pseudostratified. As the airways narrow into bronchioles, the airway epithelium consists of both ciliated and cuboidal cell types. In its most distal regions, the airway epithelium is comprised of thin, squamous cells in the alveoli, which allow for efficient gas exchange (Crystal, Randell, Engelhardt, Vovnow, & Sunday, 2008). The different regions of the epithelium not only have different structure, but also different cell types and functions. The tracheal epithelium consists primarily of ciliated cells, which facilitate the removal of debris and particulates from the lung. Additionally, goblet cells, columnar cells that produce mucus, are also

prominent in the trachea and larger bronchi and also assist in the removal of inhaled debris. Basal cells are also present in the upper airway and it is thought that they repopulate epithelial cells following injury or aging events. Basal cell populations appear to be less prominent in distal regions of the lung, such as the small bronchioles. Bronchioles are primarily comprised of cuboidal ciliated cells as well as a population of nonciliated secretory endocrine cells, known as Club (previously Clara) cells. Club cells have been shown to secrete mucus and Club Cell Secretory Protein (CCSP), as well as having a role in surfactant production and metabolism of xenobiotics. In the alveoli, two main groups of cells exist; type I and type II cells. Type I cells are important in gas exchange and comprise up to 95% of the alveolar surface. Type II epithelial cells play a major role in surfactant production, and have been shown to have the capacity to repopulate injured lungs (Crystal et al., 2008; Franks et al., 2008). The epithelium plays a major role in defense against inhaled, potentially harmful, agents; however, it also plays a unique role in regulatory immune responses as will be described next.

The airway epithelium is crucial in the activation and potentiation of both innate and adaptive immune responses originating in the lung. Epithelial cells express Toll-Like Receptors, (TLRs) which are stimulated in response to Danger Associated Molecular Patterns (DAMPs) or Microbe Associated Molecular Patterns (MAMPs) and trigger innate host defense, leading to the expression of key mediators that regulate inflammatory immune responses. For example, TLR4, which recognizes the gram negative bacterial cell wall product, lipopolysaccharide (LPS), becomes activated upon stimulation and initiates activation of cell signaling cascades important for pro-

inflammatory and anti-bacterial responses (Becker, Mundandhara, Devlin, & Madden, 2005). TLR4 also recognizes the cleavage product, fibrin, as a result of the activity of allergen-derived proteinases. Fibrinogen, an antifungal protein, was found to be cleaved by fungal derived proteinases into fibrin, which facilitated sensitization to allergens (Millien et al., 2013). Following stimulation and activation, epithelial cells secrete mediators which are crucial to the recruitment of neutrophils, eosinophils, and lymphocytes into the lung (Perros, Lambrecht, & Hammad, 2011). Additionally, epithelial activation has been shown to be important in “alerting” cells of the immune system to inhaled agonists and is the first crucial step in activating adaptive immune responses.

It is commonly believed that the pathogenesis of allergic airway disease is regulated by the interplay between the lung epithelium and immune cells. The airway epithelium is crucial in activating both the innate and adaptive immunological responses which drive allergic airway disease. Upon activation of the epithelium with an adjuvant, epithelial cells release pro-inflammatory mediators which activate multiple cells of the immune system. Epithelial-derived signals are specifically crucial to the activation and maturation of dendritic cells which controls processing of antigens. Following stimulation by an allergen/agonist, such as House dust mite (HDM), epithelial cells secrete TSLP and CCL20, which then activate dendritic cells to engulf antigen (HDM) and migrate to the draining lymph nodes and present antigen to naïve T cells (B. Zhou et al., 2005). Another epithelial-derived cytokine demonstrated to be important in the activation of dendritic cells and have a role in allergic airway disease is GM-CSF.

Previous studies have implicated GM-CSF as an adjuvant in immunological responses (Disis et al., 1996). GM-CSF was also shown to activate bone marrow-derived macrophages to synthesize MCH class II molecules and increase antigen presentation and to be a critical component for dendritic cell proliferation (Sallusto & Lanzavecchia, 1994). Therefore, based upon its impact on immune cells and adjuvant activities, the role of GM-CSF was investigated in mouse models of allergic airway disease. Following GM-CSF overexpression in the airway prior to airway exposure with the antigen, OVA, a marked increase in the eosinophilic infiltration of the airways following OVA challenge was observed (Stampfli et al., 1998). Furthermore, overexpression of GM-CSF also led to increases in overall macrophage and dendritic cell activation in the lung, indicating that GM-CSF expression is important for antigen presentation (Stampfli et al., 1998). In a model of HDM-induced allergic airway disease, GM-CSF was also shown to be crucial for allergic inflammation (Cates et al., 2004). Therefore, it can be concluded from these studies that epithelial derived cytokines, particularly GM-CSF, are important in promoting sensitization to an antigen in the development of allergic airway disease. Other epithelial-derived cytokines that have been shown to be important in the activation of immune processes are IL-6, IL-1 α , IL-33, and members of the IL-17 family, some of which will be discussed in greater detail later in this review.

As previously indicated, dendritic cells engulf antigen and migrate to the draining lymph nodes. They then present antigen and release CC-chemokine ligand 17 (TARC/CCL17) and MDC/CCL22, which activate T Helper (TH)₂ cells via the CCR4 receptor (Imai et al., 1999) (Figure 1). This process primes the immune system to

recognize and respond to antigens to which a tissue is subsequently exposed. T lymphocytes have been shown to play a large role in the development of allergic disease in experimental models; however, different subtypes exhibit varying functions in the pathogenesis of the disease. Specific signaling events occur which polarize naïve CD4⁺ T cells to specific lineages, including TH1, TH2, and TH17, among others, each depending on specific transcription factors. GATA3 is a transcription factor which is critical in the development of TH2 cells and is activated more in asthmatic subjects versus normal subjects (Nakamura et al., 1999). Whereas TH2 cell activation has been implicated in driving the pathogenesis of asthma, TH1 differentiation has been demonstrated to be antagonistic towards TH2 differentiation. During TH1 development, the transcription factor, T-bet, is phosphorylated, and can inhibit GATA3-induced gene transcription. Not surprisingly, decreases in T-bet expression have been found in asthmatics in comparison to healthy controls (Finotto et al., 2002). Conversely, the primary function of TH1 cells is to secrete the cytokine, INF γ , which has been linked to more severe asthma and inflammation. A third lineage of T cell effector cells, the TH17 cell, has also been implicated in the pathogenesis of asthma. A main product of TH17 cells, IL-17A, has been found elevated in the sputum of asthmatic patients in comparison to healthy controls (Molet et al., 2001; Y. C. Sun, Zhou, & Yao, 2005). IL-17A presence has also been linked to more severe phenotypes of asthma in association with increases in neutrophilia and steroid resistance (Y. C. Sun et al., 2005). Additionally, an anti-inflammatory T cell subtype exists, the regulatory T cell (T Reg), which counteracts the pro-inflammatory nature of TH1, TH2, and TH17 cells via the secretion of the anti-

inflammatory cytokine, Interleukin 10 (IL-10). CD4⁺ T cells have been typically thought to contribute to and regulate adaptive immune responses. However, a distinct subpopulation exists, which are Innate Lymphoid Cells (ILCs). ILCs have been shown to become activated by epithelial-derived IL-25 and IL-33 in the settings of viral and bacterial infections and recent studies have implicated a role for ILCs in allergic asthma via the secretion of IL-5 and IL-13, cytokines important in potentiating the TH2 response, which will be discussed in further detail later in this review (Hung et al., 2013; Klein Wolterink et al., 2012). While it is known that multiple T cell lineages contribute to the pathogenesis of allergic airway disease, the extent and degree to which these cells orchestrate the respective phenotype of human asthma remains unknown.

In addition to T cell polarizing events described above, other mechanisms initiate and activate TH2 cell production. IL-33 is a cytokine that plays a prominent role in the pathogenesis of allergic airway disease and plays a crucial role in TH2 processes. IL-33 is a member of the IL-1 cytokine family, which signals through the Toll-like receptor-IL-1 receptor (TLR-IL-1R) superfamily. IL-33 was first identified as a potential ligand for the IL-1 receptor, ST2, and activator of the NF- κ B, ERK and JNK signaling pathways in mast cells (Schmitz et al., 2005). IL-33 is produced in a precursor form, which is then cleaved by caspase 1. The precursor form, Pro-IL-33, has also been demonstrated to associate with chromatin via a transcription factor-like mechanism (Carriere et al., 2007), illustrating both signaling and gene regulatory mechanisms for IL-33. Upon original identification of IL-33 as an IL-1 member and binding partner of ST2, numerous studies have been performed to delineate the mechanistic and biological effects of IL-33. The

IL-1 family has important functions in host defense, inflammation, and immune regulation. Therefore, the role of IL-33 was hypothesized to have roles in these biological processes as well. Early studies demonstrated that epithelial-derived IL-33 activated mast cells and also stimulated the production of IL-4, IL-5, and IL-13 from TH2 polarized cells *in vitro* (Schmitz et al., 2005). Additionally, IL-33 administration intraperitoneally in mice resulted in increased eosinophils, mononuclear cells, and plasma cell infiltration into the spleen, as well as increases in IgE and IgA in the serum (Schmitz et al., 2005). IL-33 was found highly expressed in the airway epithelium and macrophages of mice with OVA-induced inflammation (Kurowska-Stolarska et al., 2008) and it was elevated in the serum and lungs of asthmatic patients (Sakashita et al., 2008). Furthermore, IL-33 was demonstrated to stimulate eosinophil differentiation from CD117+ hematopoietic stem cells and had the ability to exacerbate eosinophil-mediated inflammation in a model of OVA-induced allergic airway disease (Stolarski, Kurowska-Stolarska, Kewin, Xu, & Liew, 2010). Therefore, mechanisms of transcriptional regulation, release, and activation of IL-33 in allergic airway disease remain a critical area of study.

In addition to the prominent role of CD4⁺ T lymphocytes in the pathogenesis of allergic asthma, B lymphocytes also significantly contribute to the disease. Following maturation in the bone marrow and subsequent activation with antigen, B lymphocytes interact with activated TH2 cells. TH2 cells then release IL-4, which initiates antibody class switching to IgE in the B cell (Vercelli, Jabara, Arai, & Geha, 1989). Subsequently, upon activation, B cells secrete IgE, which bind to mast cells and stimulate the release of

histamine, prostaglandin D₂, and leukotrienes; all of which lead to increases in smooth muscle constriction and potentiate airway hyperresponsiveness (Reuter, Stassen, & Taube, 2010). Therefore, the interaction and cooperation between T and B lymphocytes in allergic asthma is crucial to the progression of the disease.

Another function of TH₂ cells is the release of Interleukin-5 (IL-5), which is essential in the recruitment and maturation of eosinophils. Additionally, eotaxin, a cytokine secreted by the airway epithelium, is also important in the recruitment of eosinophils (Griffiths-Johnson, Collins, Rossi, Jose, & Williams, 1993). Eosinophils are an important component of the inflammatory response, as they degranulate and release cytokines, enzymes, and growth factors (Rosenberg, Dyer, & Foster, 2013), all of which contribute to asthma pathogenesis. Additionally, in response to cytokines, eosinophils undergo piecemeal degranulation, a method by which granule content is released into the extracellular space while the eosinophils remain intact and viable (Melo & Weller, 2010). Because of the substantial role for IL-5 in recruiting and activating eosinophils, considerable effort into targeting IL-5 has taken place. Mepolizumab and reslizumab, both of which are antagonists to IL-5R α , have been developed as potential therapies for asthma. Mepolizumab decreased eosinophilia in mild atopic asthmatics by 50% in conjunction with a decrease in TGF β 1, which is a pro-fibrotic cytokine (Flood-Page et al., 2003). Furthermore, Mepolizumab suppressed eosinophil maturation in the bone marrow and decreased the amount of eosinophil progenitors in the lungs of asthmatic patients in comparison to healthy controls (Menzies-Gow et al., 2003). Additionally, following administration of Mepolizumab to patients with persistent sputum eosinophilia,

patients were able to decrease their dependence on steroids (Nair et al., 2009). These studies confirmed that IL-5 is important in eosinophil activation and recruitment, and that blocking IL-5 can hold therapeutic potential for asthmatics with persistent eosinophilia.

While eosinophils are a prominent feature of allergic airway disease, reports also indicate significant increases in neutrophils and IL-8 (KC in the mouse) in the airways of asthmatics (Douwes, Gibson, Pekkanen, & Pearce, 2002). Activation of epithelial cells with lipopolysaccharide (LPS), a common adjuvant, leads to the release of IL-8, and subsequent recruitment/activation of neutrophils, whose early pulmonary infiltration is an important mechanism in the development of allergic airway disease (Monteseirin, 2009). Neutrophils are polymorphonuclear leukocytes that are important in the defense against bacterial and fungal infections. Their main functions include the release of enzymes, reactive oxygen species, and other cytotoxic agents; however, it is not specifically known how these functions contribute to the pathogenesis of allergic airway disease. Neutrophils classically were thought to contribute primarily to the innate immune response, however, their presence in the lungs of atopic asthmatics following allergen challenge and their ability to become activated by an IgE dependent mechanism (Vega et al., 2007) has led to substantial research investigating their role in allergic responses. It is hypothesized that a major function of neutrophils is to release matrix metalloproteinase-9 (MMP-9), which is elevated in both BAL and induced sputum in patients with asthma and these findings correlated positively with disease severity and resistance to glucocorticoids (Cundall et al., 2003). MMP-9 is believed to decrease pulmonary function via its role in promoting the movement of eosinophils to the lumen of the lung

(Wenzel, Balzar, Cundall, & Chu, 2003). Genetic ablation of MMP-9 in mouse models prevented allergic airway disease (Cataldo et al., 2002). Another mediator produced by neutrophils that was demonstrated to contribute to asthma pathogenesis is neutrophil elastase, which has been implicated in epithelial damage, vascularization, bronchial hyperresponsiveness, and other features of allergic disease. Furthermore, elastase has been shown to induce eosinophil cationic protein (ECP) release from eosinophils isolated from atopic asthmatics (H. Liu, Lazarus, Caughey, & Fahy, 1999). In addition to the production of pro-inflammatory, pro-injurious mediators, neutrophils have been associated with an increase in both TH17 and IL-17A responses in asthmatics (Cosmi, Liotta, Maggi, Romagnani, & Annunziato, 2011), which implicates multiple distinct pathways of allergic airway disease, in addition to aforementioned TH2-dependent mechanisms. Therefore, it appears critical to consider the phenotype of the underlying immunological process when treating allergic asthma.

IL-17A has emerged recently as an important molecule in the pathogenesis of allergic airway disease. IL-17A levels in the airways are associated with increased neutrophilia in severe asthmatic patients (Linden, 2001). IL-17A is a cytokine primarily produced by $\alpha\beta\text{TCR}^+\text{TH17}$ cells; however, other cell types are known to produce IL-17A, such as $\gamma\delta\text{TCR}^+\text{TH17}$ cells. IL-17A was originally hypothesized to be involved in the pathogenesis of allergic airway disease, as $\alpha\beta\text{TCR}^+\text{TH17}$ cells are increased in the airways of asthmatics (Molet et al., 2001). Moreover, a recent study showed that IL-17A produced by $\alpha\beta\text{TCR}^+\text{TH17}$ contributed to AHR in mice exposed to both OVA and HDM (Kudo et al., 2012). However, recent studies have suggested other roles for IL-17A in

allergic airway disease. In a model of OVA/RSV-induced allergic airway disease (viral-induced allergic airway exacerbation), it was demonstrated that IL-17A knockout mice exhibited increased AHR and eosinophilic inflammation in comparison to WT littermate controls (Newcomb et al., 2012). Furthermore, it was shown that IL-13 can downregulate IL-17A production in an IL-10-dependent manner (Newcomb et al., 2012). Alternatively, mice that were instilled with recombinant IL-13 and IL-17A exhibited less AHR and inflammation compared to IL-13 alone, indicating that IL-17A inhibits the TH2-mediated allergic response (Kinyanjui, Shan, Nakada, Qureshi, & Fixman, 2013). These studies indicate that IL-17A may have a role in dampening classic TH2-mediated allergic airway disease. However, other mechanisms may exist by which IL-17A can modulate allergic airway disease. For example, IL-17A has been implicated in neutrophil recruitment, as it stimulates production of KC. IL-17A also has been shown to play a crucial role in pulmonary fibrosis (Y. Chen et al., 2013), suggesting a putative role for IL-17A in fibrotic airway remodeling observed in severe asthma.

Animal Models:

Animal models are instrumental in understanding the mechanisms that contribute to the pathogenesis of allergic asthma. Numerous species are utilized for asthma research; however, murine models will be the focus of this review. Substantial research has been performed in order to develop mouse models of allergic airway disease, which have likewise been instrumental in drug target discovery. A commonly used model is the aluminum hydroxide/ovalbumin (OVA) method of allergic airway disease. Aluminum salts were originally utilized as adjuvants in the preparation of vaccines based on their

ability to enhance antibody responses (Marrack, McKee, & Munks, 2009). Additionally, insoluble aluminum salts have been shown to activate innate immune cells, which then lead to T helper 2 (TH2) cell responses (Grun & Maurer, 1989). Recent studies have also shown a crucial role for uric acid following activation with aluminum salts, which has been hypothesized to activate the NLRP3 inflammasome and initiate innate immune responses (Kool et al., 2011). Following injection with aluminum hydroxide and chicken ovalbumin, mice are subsequently exposed to aerosolized ovalbumin via the airways. Common experimental results of the Alum/OVA method include eosinophilic and lymphocytic infiltration into the airways and the lung tissue, hyperresponsiveness to methacholine, and mucus metaplasia, which are all major hallmarks of allergic airway disease in humans (Kung et al., 1994). The OVA model has facilitated significant research on allergic airway disease; however, controversy regarding the physiological relevance of the method exists. The route of sensitization of the animal to ovalbumin is via intraperitoneal injection, whereas, an asthmatic patient is believed to be sensitized via the airway. Therefore, alternative models have been developed which have utilized more relevant adjuvants and sensitization methods.

As previously mentioned, airway epithelial cells are the first cells in contact with an inhaled allergen/adjuvant and are crucial in initiating activation of innate and adaptive immune responses. LPS is commonly found in allergens and is responsible for activating toll like receptor 4 (TLR4) on airway epithelial cells (Poltorak et al., 1998) and initiating downstream signaling of alveolar NF- κ B (Haddad & Land, 2001), p38, and c-Jun-N-terminal kinase (Jeyaseelan et al., 2005). Therefore, LPS has been utilized as an adjuvant

for current mouse models of allergic airway disease. Mice exposed to LPS and ovalbumin via the airways and challenged with aerosolized ovalbumin were shown to be hyperresponsive to methacholine, developed mucus metaplasia, and had increased eosinophils, lymphocytes, and neutrophils in the bronchoalveolar lavage fluid (BALF) (Wilson et al., 2009). In another model, mice exposed to flagellin, a TLR5 agonist, in conjunction with ovalbumin, had an even more robust response upon ovalbumin challenge in regards to these parameters (Wilson et al., 2012). Therefore, the strength and nature of responses to antigen challenge are strongly influenced by the methods which mice are sensitized.

In addition to models whereby specific adjuvant/antigen approaches are utilized, allergens themselves are also used as mouse models of allergic airway disease. As mentioned earlier, ovalbumin is an antigen utilized to induce allergic airway disease in mice, but it is not an allergen to which most asthmatics are allergic. In contrast, house dust mite (HDM) is a multifaceted allergen to which 50-85% of asthmatics are allergic (Gregory & Lloyd, 2011). HDM extract contains many components, including fecal matter, LPS, chitin (glucose derivative), and proteins, such as Derp1, all of which are hypothesized to be involved in the allergic response. Previous studies performed with HDM extract administration via the airway demonstrated strong hyperresponsiveness to methacholine, inflammation, mucus metaplasia, and fibrotic remodeling in the mouse lung (Johnson et al., 2004). Additionally, HDM induced allergic airway disease in mice via TLR4 triggering of airway structural cells, as well as production of pro-inflammatory cytokines TSLP, GM-CSF, IL-25, and IL-33 (Hammad et al., 2009). Utilization of the

HDM model has enabled the discovery of crucial early innate immune responses in the lung which lead to the development of allergic airway disease.

The Airway Epithelium in Asthma:

As stated earlier, the airway epithelium not only provides a protective physical barrier, but also is crucial in the control of innate and adaptive immune responses. Epithelial cells from allergic asthmatics, however, have been shown to exhibit characteristics that contribute to allergic asthma. As described below, the majority of the human population is exposed to “allergens” throughout their lives, however, specific features of the asthmatic epithelium provide an environment in which allergic asthma can develop and persist. Physical characteristics of the asthmatic lung epithelium include airway thickening, increased mucus secretion, and loss of barrier function (C. Xiao et al., 2011). The latter may facilitate the exposure of allergens to cells within the interstitial lung, including dendritic cells, which facilitates antigen presentation and adaptive immune responses. Another characteristic of asthmatics is the inability to clear viral infections efficiently, a feature that can be attributed to the asthmatic epithelium. Following rhinovirus infection, for example, the epithelium releases IFN γ to clear the virus, however, in the asthmatic epithelium, this response is defective (Hackett et al., 2011). The structure and characteristics of the airway epithelium are crucial to maintaining normal lung function and the deregulation of these processes in asthmatics may therefore contribute to disease pathogenesis.

Numerous cell signaling pathways have been shown to be deregulated in the asthmatic lung epithelium. As previously mentioned, mucus metaplasia is a common

feature of allergic asthma and contributes to disease pathogenesis. Polymorphisms of IL-13, a cytokine which stimulates epithelial cells to produce mucus, has been linked to atopy (Howard et al., 2001). Polymorphisms of its receptor, IL-13R, and downstream signaling component, STAT6, have also been linked to deregulation in asthmatic epithelium (Hoffjan, Nicolae, & Ober, 2003; Wills-Karp, 2000). Additionally, the developmental transcription factor, p63, has been linked to the development of allergic asthma. It has been reported that more asthmatic basal epithelial cells expressed p63 in comparison to healthy controls (Hackett et al., 2008) and these changes in expression have been linked to impaired wound repair and barrier function (Warner et al., 2013). Another prominent signaling pathway linked to the asthmatic epithelium is Nuclear Factor-kappaB (NF-κB), which has roles in inflammation, survival, and proliferation, as will be further described below. Due to the prominent features of inflammation in asthma and the role of NF-κB in inflammation, investigators have sought to examine the potential link between asthmatic epithelium and NF-κB. A more than two-fold increase in NF-κB transcription factor, RelA, content in the nucleus (indicative of NF-κB activation) of bronchial cells from asthmatics versus healthy controls was detected and a 44% increase of NF-κB DNA binding was observed (Hart, Krishnan, Adcock, Barnes, & Chung, 1998). Furthermore, Derp1, a component of the allergen, house dust mite, was shown to activate NF-κB more pronounced in asthmatic bronchial epithelial cells in comparison to normal bronchial epithelial cells (Stacey et al., 1997). Due to the strong evidence that NF-κB signaling is prominent in the asthmatic epithelium, its links to inflammation, and the prominent role for the epithelium in regulating allergic asthma, a

major focus of this review pertains to NF- κ B signaling in the epithelium and how it contributes to allergic asthma.

Nuclear Factor kappaB

Introduction:

Nuclear Factor kappaB (NF- κ B) is a transcription factor that contributes to and regulates multiple biological processes, including inflammation, proliferation, and cell survival. NF- κ B has been causally linked to the development of many diseases, such as cancer, COPD, and asthma and therefore considerable research has been performed to understand the mechanisms of its signaling and regulation. At least two main NF- κ B pathways have been described: the classical and the alternative pathway, also referred to as canonical and noncanonical NF- κ B (Figure 2). The pathway that leads to activation of NF- κ B consists of adaptor proteins which interact with receptors, and the Inhibitor of kappaB kinase (IKK) signalsome. Transcription factor/inhibitor complexes are important in the modulation of NF- κ B dependent transcription. Upon stimulation, adaptor complexes are recruited to the receptors and phosphorylate IKK. In turn, IKK phosphorylates the inhibitor of NF- κ B, which constitutively sequesters NF- κ B transcription factors in the cytoplasm. Upon phosphorylation, the inhibitor becomes ubiquitinated and subsequently degraded by the 26S proteasome, thus facilitating the nuclear translocation of transcription factors (mainly RelA and RelB) (Gilmore, 2006). This event, in addition to other post-translational modifications and associations with

cofactors, leads to NF- κ B-dependent transcription of genes involved in lymphoid organogenesis, inflammation, proliferation, and cell survival.

NF- κ B family members:

The NF- κ B family is comprised of three major groups of proteins: NF- κ B proteins, I κ B proteins, and IKK proteins. The NF- κ B proteins, consisting of RelA, RelB, cRel, p105/p50, and p100/p52, contain Rel homology domains (RHD) which facilitate DNA binding as well as protein-protein interactions with each other. The RHD is typically comprised of two beta barrels which facilitate the aforementioned associations. The I κ B group consists of I κ B α , I κ B β , I κ B ϵ , I κ B ζ , and BCL3; all contain ankyrin repeat domains, which are comprised of two alpha helices. The alpha helix domain also contributes to protein-protein interactions. In addition to the above mentioned I κ B proteins, p105/p50 and p100/p52 are also considered to be inhibitors of NF- κ B in addition to being members of the NF- κ B family, due to the presence of both ankyrin repeat domains and rel homology domains. IKK proteins, consisting of IKK α , IKK β , and IKK γ , comprise the IKK signalsome, whose formation is necessary for activation of NF- κ B. IKK α and IKK β contain catalytically active kinase domains which are important in phosphorylating the inhibitor of kappa B (I κ B) proteins. IKK γ (also known as NEMO), which does not contain a catalytically active domain, is crucial for the formation and aggregation of the IKK signalsome via associations with IKK α and IKK β (Ghosh & Hayden, 2008). The three families of proteins are all critical in the activation and signaling of NF- κ B, and their roles in these processes will be discussed in more detail in the next section.

Classical NF- κ B:

The IKK signalsome, which consists of IKK α , IKK β , and IKK γ , is a critical component for activation of the classical pathway. Upon stimulation with an agonist, such as Lipopolysaccharide (LPS), adaptor proteins, IRAK, TRAF6, and TRAF2 associate and initiate phosphorylation of TAK1 (Deng et al., 2000). IKK γ binds TRAF6 and assembles the IKK signalsome, whose complex is essential for classical signaling. Following phosphorylation of TAK1, IKK β is phosphorylated, which then in turn phosphorylates the Inhibitor of kappaB alpha (I κ B α) (Takaesu et al., 2003). I κ B α , which sequesters p65 (RelA) in the cytosol, is subsequently ubiquitinated and degraded by the 26S proteasome. This event facilitates the nuclear translocation of RelA, which can initiate transcription of target genes. Classical NF- κ B signaling has been causally linked to innate inflammation and proliferation, and its role in the epithelium has been crucial in regulating these processes (Pasparakis, 2009). Classical NF- κ B has been shown to contribute to diverse functions in many different cell types, organs, and diseases. Classical NF- κ B has been extensively studied, and its deregulation has been shown to play a role in cancers, inflammatory disorders, and diseases of the immune system. Over 100 known target genes of NF- κ B have been identified, many of which are involved in inflammation, immunity, proliferation, and cell survival. RelA^{-/-} mice are embryonic lethal at day 15-16 gestation and have severe deficiencies in liver function (Beg, Sha, Bronson, Ghosh, & Baltimore, 1995). Additionally, mice deficient in for IKK β in myeloid cells demonstrated enhanced susceptibility to endotoxin shock, suggesting a putative role for NF- κ B in defense against pathogens (Greten et al., 2007). Given the

importance of NF- κ B in regulating inflammation and its upregulation in the lung epithelium of asthmatics, the next section will discuss studies that have addressed the role of epithelial NF- κ B in mouse models of allergic airway disease.

NF- κ B in the lung epithelium:

Initial observations demonstrated that NF- κ B was elevated in the lungs of asthmatic patients and that this activity primarily occurred in the airway epithelium (Hart et al., 1998). To fully elucidate the complex role of airway epithelial NF- κ B, transgenic murine models whereby epithelial NF- κ B was either inhibited or activated in settings of inflammatory airways disease were utilized. Initial studies employed the use of a transgenic model whereby a mutant of the inhibitor of NF- κ B, I κ B α , was constitutively overexpressed in CC10 expressing cells (CC10-NF- κ B_{SR}), thus preventing the activation of NF- κ B. Using the alum/OVA model, the CC10-NF- κ B_{SR} transgenic mouse demonstrated reduced eosinophilia, mucus metaplasia, and inflammatory cytokines in comparison to wild type mice. However, no observed difference in AHR was detected, indicating that epithelial NF- κ B impacted many, but not all facets of Alum/OVA-induced allergic airway disease (Poynter et al., 2004).

To further unravel the complexities of epithelial NF- κ B activation in asthma models, a transgenic mouse was created in which IKK β was inducibly deleted from the airway epithelium using a floxp flanked allele of IKK β via the doxycycline/CC10-tet/op-cre system. Epithelial inhibition of IKK β , and thus NF- κ B, during the sensitisation phase of the ovalbumin model resulted in marked decreases in structural remodelling, mucus metaplasia, eosinophilia and AHR (Broide et al., 2005). Additional studies utilized a

mouse in which a phosphomimetic mutant of IKK β (CA-IKK β), which is constitutively active, was conditionally expressed under the CC10 promoter, facilitating increased activation of NF- κ B in the non-ciliated epithelium. Expression of the CA-IKK β transgene alone in the lung epithelium was sufficient to drive airway neutrophilia and airway hyperresponsiveness (Pantano et al., 2008). Following sensitization to ovalbumin, activation of NF- κ B in the epithelium during subsequent re-exposure resulted in enhanced AHR, neutrophilia, eosinophilia, and airway smooth muscle hyperplasia/hypertrophy (Pantano et al., 2008). Furthermore, NF- κ B activation within the epithelium during the initial exposure of ovalbumin was sufficient to cause sensitization to OVA in the absence of other adjuvants as well as a mixed TH2/TH17 immune response (Ather, Hodgkins, Janssen-Heininger, & Poynter, 2011). Together these findings, elicited through the comprehensive use of murine transgenic models, suggest an important role of NF- κ B activation in the airway epithelium in both sensitization and challenge to an antigen.

Alternative NF- κ B:

Alternative NF- κ B has been implicated in a variety of biological processes, many distinct from classical NF- κ B. Alternative NF- κ B activation results following stimulation with a variety of agonists, including of B cell activating factor (BAFF), CD-40 ligand, and Lymphotoxin- β . Upon stimulation, TRAF3, which constitutively ubiquitinates and degrades NF- κ B-inducing kinase (NIK), is ubiquitinated and degraded, thus allowing for accumulation of NIK. NIK accumulation is sufficient to phosphorylate a distinct pool of IKK α (separate from the IKK signalsome), which then initiates IKK α to

phosphorylate p100. P100 is a member of the inhibitor of kappaB family that has been shown to bind and sequester RelB in the cytosol in unstimulated cells. Upon phosphorylation of p100, it is ubiquitinated and partially processed into p52, which then facilitates RelB translocation into the nucleus. This event triggers transcription of alternative NF- κ B-dependent genes (S. C. Sun, 2011) (Figure 2).

In addition to the classical pathway, the alternative NF- κ B pathway has been shown to be important in a variety of different biological processes, including the development of peripheral lymphoid organs, and B and T cell maturation. NIK, IKK α , p100/p52, and RelB, all major components of the alternative pathway, have been shown to regulate both in normal biological processes and disease. In addition to its role in B and T cell development, alternative NF- κ B has been implicated in hyperglycemia, oxidative muscle metabolism, lymphoma, and diabetes-associated renal inflammation (G. Xiao, Rabson, Young, Qing, & Qu, 2006). Studies have also indicated a role for alternative NF- κ B in immune cells, such as dendritic cells and macrophages, whereby it appeared to play both pro and anti-inflammatory roles. RelB has been shown to play a role in tolerance to endotoxin in monocytes as well as providing protection against cigarette smoke-induced inflammation (McMillan et al., 2011; Yoza, Hu, Cousart, Forrest, & McCall, 2006). Additionally, it was demonstrated that RelB played an anti-inflammatory role in lung fibroblasts (Baglolle et al., 2008; Xia, Pauza, Feng, & Lo, 1997). IKK α , however, has been shown to enhance classical NF- κ B signaling via initiating phosphorylation of histones, thus enhancing classical NF- κ B-dependent gene transcription (Yamamoto, Verma, Prajapati, Kwak, & Gaynor, 2003). However, in direct

contrast to these findings, IKK α phosphorylates TAX1BP1, which then negatively regulates classical NF- κ B signaling in fibroblasts (Shembade, Pujari, Harhaj, Abbott, & Harhaj, 2011). The overall role of the alternative NF- κ B pathway differs among cell types and disease models, and its role in lung epithelial cells remains to be elucidated.

Despite the apparent differences in the roles of classical and alternative NF- κ B, numerous studies demonstrating cross talk between the two pathways have been described. It has been shown previously that a variety of stimuli, including LPS and TNF α , have the ability to activate both pathways simultaneously (Sasaki, Ghosh, & Longo, 2011; Souvannavong, Saidji, & Chaby, 2007). IKK α , the dominant kinase in the alternative pathway, is critical for prolonged classical NF- κ B-induced gene transcription via the phosphorylation of Histone H3 and enhanced transcription of NF- κ B dependent genes in mouse embryonic fibroblasts (Anest et al., 2003) through interactions with CREB binding protein (Yamamoto et al., 2003). In contrast, IKK α accelerates the removal of RelA and c-Rel from target gene promoters and dampens pro-inflammatory phenotypes in macrophages (Lawrence, Bebien, Liu, Nizet, & Karin, 2005) and IKK α also limits classical NF- κ B activation by phosphorylating TAX1BP1, a regulatory molecule which controls classical NF- κ B activation (Shembade et al., 2011). Additionally, it was shown in macrophages that following stimulation with LPS, RelA occupation with the IL-12p40 promoter was associated with enhanced transcription of IL-12p40 mRNA. Conversely, RelB was shown to associate with the same promoter after RelA; however, its occupancy was associated with decreased expression of the IL-12p40 mRNA (Saccani, Pantano, & Natoli, 2003). However, RelB recruitment to the GM-CSF

promoter was shown to enhance RelA-dependent gene transcription (Sasaki et al., 2011). RelA and RelB protein-protein interactions have been reported as well, which were associated with decreased gene transcription (Smale, 2012). The role of classical NF- κ B in regulating pro-inflammatory responses in lung epithelial cells has been demonstrated, however, given the controversial role of alternative NF- κ B in other cell types and crosstalk with classical NF- κ B, it is plausible that the alternative NF- κ B pathway also plays a role in orchestrating inflammatory responses from lung epithelial cells.

Regulation of NF- κ B

NF- κ B plays a crucial role in many cells and organs, and as such, mechanisms exist to tightly regulate its function. Phosphorylation, or the reversible conjugation of a phosphate group to a reactive hydroxyl group, has a major role in the activation of NF- κ B. Upon activation by a variety of agonists, multiple proteins, such as IKK β , and IKK α are phosphorylated at Serines 177/181 and 176/180, respectively, thus enhancing signaling (Carter, Geyer, Xie, Acevedo-Suarez, & Ballard, 2001; Y. Wang, Xiang, Kourouma, & Umar, 2006). Mutations of the aforementioned serine residues of IKK α and IKK β to glutamic acid residues was demonstrated to render the kinases constitutively active, and expression of IKK β -S177/181E in the lung was demonstrated to be sufficient to induce airway neutrophilia (Sadikot et al., 2003). In addition to phosphorylation of IKK β , a highly specific and conserved mechanism of regulation of NF- κ B is exerted via the phosphorylation of I κ B α . Following phosphorylation at serines 32 and 36, lysines 21 and 22 of I κ B α become ubiquitinated via K48-linked polyubiquitin chains (Rodriguez et al., 1996). The mechanisms regulating these reactions are highly specific and conserved

among species. Following phosphorylation at serines 32 and 36, a specific E3 ubiquitin ligase enzyme, identified as E3RS^{I κ B}, specifically recognizes the 6 amino acid peptide sequence of I κ B (Yaron et al., 1998). Therefore, the E3 ligase only associates with phosphorylated I κ B α , thereby catalyzing K48-linked ubiquitination at lysines 21 and 22. Mutation of I κ B α serines 32/36 to alanines renders I κ B α resistant to degradation and prevents activation of the NF- κ B pathway. This observation was used to create the CC10-NF- κ B_{SR} mouse model described earlier.

In addition to the specific regulation of I κ B α in the classical pathway, a similar mechanism exists in the activation of the alternative NF- κ B pathway. P100 sequesters RelB via masking the nuclear localization domain, thus rendering the alternative pathway inactive in an unstimulated cell. Following stimulation, NIK content accumulates and phosphorylates IKK α , which then in turn phosphorylates p100 at serines 866 and 870 (G. Xiao, Fong, & Sun, 2004). This event, as with phosphorylation of I κ B α , is required for the ubiquitination and degradation of p100. However, unlike I κ B α , p100 contains a processing inhibitor domain in its C-terminus, and therefore, rather than complete degradation of the protein, p100 is partially processed into p52. P52, the binding partner of RelB, then facilitates nuclear accumulation of RelB and DNA binding. In addition to activation, K48-linked ubiquitination also plays a role in limiting alternative NF- κ B activation. The E3 ubiquitin ligase, TRAF3, has been demonstrated to constitutively ubiquitinate, and, thus degrades, NIK. However, upon stimulation, TRAF3 itself is degraded, thus stabilizing NIK, and promoting the activation of the alternative pathway (Liao, Zhang, Harhaj, & Sun, 2004). Therefore, in addition to the phosphorylation events

that are required to activate kinases, phosphorylation is also required for ubiquitination and degradation of multiple inhibitors of NF- κ B.

Phosphorylation has been shown to activate NF- κ B associated kinases; however, mechanisms also exist in which phosphorylation can inhibit the kinase activity of numerous proteins. Autophosphorylation of both IKK α and IKK β in their C-terminal regions acts as a mechanism to shut down their kinase activity. Previous studies demonstrated that in IKK β mutants whereby 10 serines in the C-terminal tail were mutated to alanines, a fourfold prolonged signaling capacity of IKK β following stimulation with TNF- α was detected (Delhase, Hayakawa, Chen, & Karin, 1999). In contrast, replacement of these serines with glutamic acid, whose negative charge mimics phosphoserine, renders IKK β inactive, leading to greatly reduced TNF- α -induced IKK activity (Delhase et al., 1999). It is hypothesized that hyperphosphorylation of the C-terminus of IKK, which contains the helix-loop-helix domain, results in a conformational change that impairs its kinase activity. Another hypothesis is that this conformational change facilitates the recruitment of a phosphatase to remove the phosphate group from Serines 177 and 181. A phosphatase, PP2A, has been identified as a protein which has been shown to associate with IKK β , however, its impact on activity of IKK β has remained controversial (DiDonato, Hayakawa, Rothwarf, Zandi, & Karin, 1997). In addition to phosphorylation negatively regulating IKK β , phosphorylation also affects the alternative NF- κ B pathway through interactions between IKK α and NIK. Following NIK-induced activation and phosphorylation of IKK α , IKK α in turn phosphorylates NIK, thus destabilizing it (Razani et al., 2010). Therefore, the phosphorylation events that

govern the magnitude of both classical and alternative NF- κ B pathways are highly specific and context dependent.

Phosphorylation of transcription factor, RelA, is a key event in mediating its transcriptional activity. Following phosphorylation of RelA at serine 276 after LPS stimulation, RelA interacts with coactivator, CBP/300, and transcriptional activity of RelA becomes enhanced (Zhong, SuYang, Erdjument-Bromage, Tempst, & Ghosh, 1997). Additionally, stimulation with TNF- α has been shown to increase phosphorylation of RelA at serine 529 (D. Wang & Baldwin, 1998). However, the best described phosphorylation of RelA occurs at serine 536, which has been shown to be initiated by IKK itself in response to a variety of stimuli (Sakurai et al., 2003; F. Yang, Tang, Guan, & Wang, 2003). In contrast, RelB phosphorylation has also been documented in settings in which it facilitates ubiquitination and degradation of RelB of itself. In both immortalized and primary T cells, it has been shown that RelB is phosphorylated by GSK3 β , an enzyme which regulates other facets of NF- κ B signaling. It was demonstrated that GSK3 β phosphorylated RelB on threonine 84 and serine 552 and that these events were important for degradation of RelB (Neumann et al., 2011).

K48-linked ubiquitination has been shown to be an important process by which proteins are marked for degradation by the 26S proteasome, however, other types of ubiquitination exist that are important in biological processes. For example, K63 and linear ubiquitination have been shown to be important mechanisms by which NF- κ B is activated. IKK γ , or NEMO, contains ubiquitin binding domains which facilitate binding of NEMO, IKK α , and IKK β to upstream adaptor proteins whose interactions are crucial

for NF- κ B activation. K63-linked ubiquitination has been shown to be critical for the congregation of the IKK complex as well as the activation of the IKK β kinase (Z. J. Chen, 2012). Multiple receptors and pathways activate NF- κ B via ubiquitination, however, for the purpose of clarity; this review will emphasize IL-1R/TLR signaling. Following stimulation, the IL-1R/TLR receptor rapidly recruits adaptor proteins IRAK1/4 and MyD88, which in turn recruits the E3 ligase, TRAF6. Following its recruitment, TRAF6, in conjunction with E2 enzymes Ubc13 and Uev1a, catalyze K63-linked ubiquitination of IRAK1, NEMO, and TRAF6 itself (Z. J. Chen, 2012). A complex containing the proteins TAB2/3 and TAK1 is then recruited and via ubiquitin binding domains on TAB2/3, associates with the adaptor complex. Additionally, NEMO, which also contains a ubiquitin binding domain, associates with K63-linked ubiquitin chains on proteins, such as TRAF6 and IRAK1, which facilitates the congregation of the TAB and IKK complexes. Following these recruitment events, IKK β can then be phosphorylated by TAK1 (member of the TAB complex) and thus potentiate NF- κ B signaling. Additionally, deubiquitinases exist, such as CYLD1 and A20, which then can act to remove the K63-linked ubiquitin chains, thereby shutting down NF- κ B signaling (Z. J. Chen, 2012). As previously mentioned, this mechanism is central to the activation of NF- κ B and serves as a dynamic regulatory mechanism.

Linear ubiquitination is another type of regulatory process that is essential for NF- κ B activation. The linear ubiquitin chain, in which the C-terminal glycine of ubiquitin is conjugated to the α -amino group of the amino-terminal methionine of another ubiquitin, is a complex created by the ubiquitin ligase complex, known as LUBAC.

LUBAC, which is comprised of two proteins, HOIL-1L and HOIP, has been shown to be important in NF- κ B signaling via direct binding to NEMO following stimulation with agonists, such as TNF α (Tokunaga et al., 2009). LUBAC-catalyzed linear ubiquitination of NEMO occurs at lysine 285 and 309 has been hypothesized to impact NF- κ B signaling via a few different mechanisms (Niu, Shi, Iwai, & Wu, 2011). It is possible that ubiquitinated NEMO can bind other NEMO molecules and facilitates trans-autophosphorylation of IKK α and IKK β . Alternatively, it is also possible that linear ubiquitinated NEMO may allow for the recruitment of an unidentified kinase that activates the IKKs. Whether these putative scenarios occur remains to be determined, however, it has become clear that linear ubiquitination of NEMO is crucial for NF- κ B activity. The posttranslational modifications phosphorylation and ubiquitination are critical in maintaining proper activity and regulation of NF- κ B. However, other redox dependent posttranslational modifications that affect cysteines, such as protein S-glutathionylation and S-nitrosylation, are critical regulatory mechanisms that strongly impact NF- κ B signaling. The role of oxidants in lung biology, the role of S-glutathionylation in regulating NF- κ B signaling, and key enzymes in these processes will be discussed in the next section of this review.

Redox Biology

Oxidants and lung biology:

Asthma and other chronic inflammatory diseases have been associated with elevated levels of oxidants, such as nitric oxide and hydrogen peroxide, which have been

detected in the exhaled breath condensates from patients with asthma in comparison to healthy controls (Antczak et al., 1997; Emelyanov et al., 2001; Horvath et al., 1998; Kharitonov & Barnes, 1996). Oxidants are molecules that accept electrons from other molecules or compounds, and in doing so, become reduced themselves. Hydrogen peroxide and superoxide are common examples of oxidants. Oxidants can be produced endogenously, for example, by the electron transport chain in mitochondria, the major cellular source of ATP generation (Rigoulet, Yoboue, & Devin, 2011). In addition, enzymes, such as NADPH oxidase and nitric oxide synthase, can generate oxidants (Dikalov, 2011). “Oxidative stress” is a generic term that refers to an imbalance of oxidants/antioxidants that can potentially be damaging to a cell or surrounding tissue. Lungs are susceptible to both exogenous-derived oxidants, such as inhaled particles, cigarette smoke, pollen, nitrogen dioxide, and oxidant gases, and endogenous-derived oxidants, which are in part produced from inflammatory cells recruited to the lungs (Ciencewicky, Trivedi, & Kleeberger, 2008). Two common sources of ROS in the setting of pulmonary inflammation are eosinophils and neutrophils, whose pulmonary infiltration have been demonstrated to be prominent in allergic airway disease, infections, and lung injury, and have the potential to play diverse functions in multiple settings of pulmonary diseases. Under normal physiological conditions, neutrophils are protective against invading pathogens via a mechanism known as a respiratory burst, or the rapid release of ROS, such as superoxide radicals and hydrogen peroxide (Grommes & Soehnlein, 2011). Neutrophils generate ROS, whose release is critical in the clearance of infection, from the NADPH oxidase complex. One main defense mechanism against

bacterial infection consists of the release of HOCl, which is an oxidant that reacts with sulfhydryls and promotes disulfide bonds. However, while neutrophil-derived ROS are crucial in the defense against pathogens, they can also be damaging to the lung. Additionally, eosinophils contribute to the increase in ROS observed in the lungs of asthmatics. Eosinophils generate the enzyme, eosinophil peroxidase (EPO), which exists primarily in the granules of the eosinophil and is not released until degranulation of the eosinophil occurs. Following eosinophil activation, EPO is released and amplifies the oxidizing potential of hydrogen peroxide by producing highly reactive oxidants, including HOBr (Andreadis, Hazen, Comhair, & Erzurum, 2003), in addition to several other pro-oxidant enzymes. In addition to neutrophils and eosinophils, other inflammatory cells have been shown to contribute to ROS production in asthma, such as mast cells; however, they will not be discussed in further detail in this review.

Oxidants are also produced by structural cells of the lung, such as endothelial and epithelial cells (Barnes, 1995). Environmental agents relevant to human asthma, such as pollen, cigarette smoke, and nitrogen dioxide, whose exposure has been associated with asthmatic exacerbations, also contribute to oxidative stress in the lung. Antioxidants, which prevent or reverse the oxidation of other molecules, have been shown to protect against oxidant-induced damage. For example, mice with targeted deletion of the antioxidant enzyme, glutathione peroxidase 2, display enhanced susceptibility to allergic airway disease (Dittrich et al., 2010). It has also been shown that antioxidant enzymes, such as catalase, are inactivated and levels of glutathione, an antioxidant tripeptide consisting of glutamic acid, alanine, and cysteine, are diminished in

lungs of asthmatic patients (Ghosh et al., 2006). It is known that the presence of oxidants correlates with asthma and disease severity (Saleh, Ernst, Lim, Barnes, & Giaid, 1998); however, their functional contribution to the disease is unknown. An emerging field, coined “redox biology”, investigates the functional contribution of oxidants to the regulation of multiple biological processes. Subtle changes in the redox environment have been shown to impact biological processes, such as protein function, which consequently can affect signaling pathways. The mechanisms that regulate these processes will be discussed in further detail in this review.

Cysteine Modifications:

The amino acid, cysteine, contains a thiol group, and can be susceptible to oxidant-induced post-translational modifications. Nitric oxide and hydrogen peroxide have both been shown to catalyze post-translational modifications on selective cysteines (Spadaro et al., 2010). Due to a negatively charged environment, select cysteines have a low pKa, typically exist as thiolates, the deprotonated S^- form, and are readily susceptible to oxidative modifications. Nitric oxide, capable of conjugating to a reactive cysteine in a process known as S-nitrosylation through the formation of S-nitrosothiol, can impact the function and properties of target proteins as well as giving rise to other redox-based post-translational modifications, such as Protein S-glutathionylation (PSSG). Hydrogen peroxide also has been shown to react with target cysteines and facilitate the formation of sulfenic acid (SOH), which is an unstable intermediate, and subsequent PSSG. PSSG, the conjugation of a glutathione moiety to a reactive cysteine of a protein, is another common oxidant-induced post-translational modification associated with lung disease

(Janssen-Heininger et al., 2009). PSSG can modify the function of a target protein by changing its size, charge, and conformation and protects against further oxidation. The sulfenic acid intermediate can also be further oxidized to sulfinic (SO₂H) and sulfonic (SO₃H) acids (Klomsiri, Karplus, & Poole, 2011). The latter modifications are considered to be irreversible in most circumstances, affecting the integrity of the protein and leading to its degradation; however, PSSG has the ability to protect target proteins from these irreversible oxidations and PSSG can be reduced to the original sulfhydryl group, restoring the protein.

Redox regulation of NF-κB:

Substantial research has been conducted in order to elucidate the contributions of oxidants in regulating NF-κB signaling, and to date, multiple studies have implicated a role for oxidants in modulating NF-κB signaling. Following exposure to TNF-α, a pro-inflammatory cytokine, IKKβ activity increased (Reynaert et al., 2004); however, upon addition of S-nitrosothiols, IKKβ activity decreased, in association with increased S-nitrosylation. Following mutation of IKKβ Cysteine 179 to Alanine, S-nitrosylation was no longer detected and inhibition of kinase activity no longer occurred following addition of S-nitrosothiols (Reynaert et al., 2004). P50, a binding partner of RelA, is also S-nitrosylated at cysteine 62, which has been associated with inhibition of NF-κB function (Marshall & Stamler, 2001). Nitric oxide is also known to facilitate S-nitrosylation of RelA, which leads to a decrease in its DNA binding and NF-κB dependent gene transcription (Kelleher, Matsumoto, Stamler, & Marshall, 2007). Indeed, a conserved cysteine within the rel homology domain of Rel proteins exists, suggesting that S-

nitrosylation of that motif also can affect other Rel proteins (Kelleher et al., 2007). Depletion of arginase, a competitor of nitric oxide synthase, enhances nitric oxide production and S-nitrosylation in conjunction with decreased in NF- κ B DNA binding (Ckless, van der Vliet, & Janssen-Heininger, 2007). These data indicate that enhanced oxidant production has the ability to repress NF- κ B signaling via the nitric oxide-dependent S-nitrosylation of IKK β at cysteine 179 as well as S-nitrosylation of RelA and p50.

Hydrogen peroxide has been extensively studied for its role in modulating NF- κ B. Hydrogen peroxide inhibits TNF- α induced IKK activity and DNA binding, and this impact was attributed directly to oxidation of the IKK complex (Korn, Wouters, Vos, & Janssen-Heininger, 2001). Notably, inactivation of the IKK signalsome by hydrogen peroxide was due to S-glutathionylation of IKK β at Cysteine 179, the same targeted for S-nitrosylation (Reynaert et al., 2006). IKK α , the dominant kinase in the alternative NF- κ B pathway, is glutathionylated in response to cigarette smoke stimulation, which leads to increased association with chromatin (Chung, Sundar, Yao, Ho, & Rahman, 2010). Glutathionylation of RelA and p50 also occurs in response to hypoxia and oxidative stress, respectively, which correlates with inactivation of NF- κ B and decreased DNA binding (Pineda-Molina et al., 2001; Qanungo, Starke, Pai, Mieryal, & Nieminen, 2007). I κ B α , the inhibitor of NF- κ B, can also be S-glutathionylated, an event which has also been associated with decreased NF- κ B activation (Kil, Kim, & Park, 2008; Seidel et al., 2011). In contrast to aforementioned findings, which demonstrated direct oxidation of NF- κ B inhibit signaling, a wealth of studies have shown that oxidants can positively

regulate NF- κ B activity in a context dependent manner, contributing to the concept that NF- κ B is a prototypic redox-sensitive transcription factor. As an example, one study demonstrated that hydrogen peroxide inhibited TNF- α induced nuclear translocation of NF- κ B and subsequent gene transcription, while hydrogen peroxide exposure alone increased IKK activity (Jaspers, Zhang, Fraser, Samet, & Reed, 2001). In another study, hydrogen peroxide was administered either prior to or post exposure of lung epithelial cells with TNF- α . Pre-exposure with hydrogen peroxide inhibited TNF- α induced activation of NF- κ B; however, administration of hydrogen peroxide following TNF- α stimulation prolonged NF- κ B activation and IKK phosphorylation, in conjunction with inhibition of PP2A, hypothesized to be a phosphatase for IKK (Loukili et al., 2010). Furthermore, hydrogen peroxide can inhibit the ability of Cezanne, a deubiquitinating cysteine protease, to stabilize I κ B α content, thereby facilitating its degradation and NF- κ B activation (Enesa et al., 2008). Collectively, these studies demonstrated that oxidants strongly impact NF- κ B signaling via multiple mechanisms, however, in a context specific manner.

Glutaredoxin and NF- κ B activation:

Oxidant-induced post-translational modifications have been shown to strongly influence NF- κ B signaling. Key enzymes have evolved to control the extent of cysteine oxidation. Thioredoxins, sulfiredoxins, and glutaredoxins are enzymes commonly classified as oxidoreductases, which catalyze deglutathionylation reactions and reduce disulfide bridges in target proteins. Under physiological conditions, glutaredoxins have been shown to be primarily responsible for deglutathionylation of target proteins. Four

isoforms of glutaredoxin have been described in mammals: Grx 1, 2, 3, and 5, and Grx1 has been most extensively described and characterized. Grx1 catalyzes the reversible reduction of protein-glutathionyl mixed disulfides back to the protein's free sulfhydryl state. During this reaction, Grx1 is itself glutathionylated at Cysteine 22 and is subsequently reduced by glutathione disulfide reductase (Y. Yang et al., 1998). Grx1 is primarily expressed in the trachea, bronchiolar epithelial cells, alveolar macrophages, and the alveolar epithelium (Aesif et al., 2009; M. Peltoniemi et al., 2004). Alterations in Grx1 content have been associated with inflammatory diseases of the lung, such as COPD and asthma (Kuipers et al., 2013; M. J. Peltoniemi et al., 2006). Additionally, Grx1 content and mRNA are increased in mouse models of allergic airway disease (Reynaert, Wouters, & Janssen-Heininger, 2007). The association of Grx1 with allergic airway disease and its ability to deglutathionylate proteins warrants further research to delineate the role of Grx1 in regulating allergic inflammation and the NF- κ B pathway.

As previously mentioned, NF- κ B is a redox sensitive pathway that is modulated by oxidant-induced post-translational modifications. Specifically, S-nitrosylation and S-glutathionylation of IKK β at Cysteine 179 have been shown to decrease its catalytic activity and downstream NF- κ B activation. To date, very little is known about the role of enzymes in catalyzing glutathionylation or deglutathionylation of target NF- κ B proteins, specifically IKK β . Grx1 deglutathionylates IKK β and restores catalytic activity of IKK β , as well as downstream NF- κ B activity (Figure 3). Furthermore, in Grx1^{-/-} primary mouse tracheal epithelial cells exposed to TNF- α , reduced RelA nuclear translocation and NF- κ B dependent gene transcription were observed (Reynaert et al., 2006). Additionally,

following Grx1 overexpression in alveolar epithelial cells, increases in NF- κ B activation and NF- κ B-dependent pro-inflammatory cytokine production was observed (Aesif, Kuipers, et al., 2011). The role of Grx1 in models of inflammatory diseases of the airway has also been explored. Following acute exposure to LPS, Grx1^{-/-} mice displayed reduced inflammation and alveolar macrophage NF- κ B activation in comparison to WT mice (Aesif, Anathy, et al., 2011). In response to sensitization and challenge with OVA, to model allergic airway disease, Grx^{-/-} mice displayed reduced eosinophilic inflammation and mucus metaplasia in association with enhanced resolution of airway hyperresponsiveness to methacholine and increased overall protein S-glutathionylation levels in the lung in comparison to WT mice (Hoffman et al., 2012). Therefore, Grx1 potentially plays a pro-inflammatory role in the settings of allergic airway disease via the regulation of NF- κ B.

Glutathione S-transferases:

Glutathione S-transferases (GST's) are a family of enzymes known for their ability to catalyze the conjugation reduced GSH to xenobiotic substrates for the purpose of detoxification. Numerous GST's exist, including GST alpha (GSTA), omega (GSTO), theta (GSTT), mu (GSTM), and pi (GSTP). GSTs have been implicated in a variety of diseases, including cancer, Parkinson's, and diabetes; however their functional contributions to these diseases remain unknown. Interestingly, GST expression and polymorphisms correlate to incidence and severity of allergic asthma. GSTA and GSTP expression is increased in the sputum of asthmatics compared to healthy controls (Sohn et

al., 2013), and GSTM deletion polymorphisms are associated with increases in inflammatory lung diseases (W. Wu, Peden, & Diaz-Sanchez, 2012).

The GST member, Glutathione S-transferase pi (GSTP) was recently demonstrated to catalyze S-glutathionylation reactions (Townsend et al., 2009), and at least two domains have been characterized that describe the major functions of GSTP: the catalytic domain in the N-terminus and the ligandin domain in the C-terminus. GSTP exists in many forms, such as monomers, catalytically active dimers, and can form heterodimers with other proteins. GSTP contains a tyrosine at amino acid 7, which enhances the nucleophilicity of a bound GSH molecule by abstraction of a proton and enables a thioether linkage on electrophilic substrates (Dirr, Reinemer, & Huber, 1994; Townsend et al., 2009). Within the C-terminus, a domain exists which facilitates binding of GSTP to hydrophobic substrates. GSTP can associate with numerous protein targets, such as c-JUN N-terminal kinase (JNK) and TRAF2, leading to their inhibition (Y. J. Kim et al., 2006; Y. Wu et al., 2006). The catalytic domain of GSTP, however, is not required for associations with target proteins.

GSTP is a known biomarker of many types of cancers, including lung and esophageal and polymorphic variants of GSTP have been linked to the susceptibility of disease (Vasieva, 2011). Additionally, the GSTP gene maps to the chromosome 11q3, a locus linked to the development of asthma. Therefore, studies have been performed in order to elucidate the relationship between GSTP and allergic asthma. Multiple polymorphic variants of GSTP have been identified, and the substitution of Valine 105 with Isoleucine 105 was shown to confer a 6-fold higher association with bronchial

hyperresponsiveness and atopy (Spiteri, Bianco, Strange, & Fryer, 2000). In contrast, asthmatics with GSTP Val¹⁰⁵/Val¹⁰⁵ compared with asthmatics with Val¹⁰⁵/Ile¹⁰⁵ or Ile¹⁰⁵/Ile¹⁰⁵ had greater acute inflammatory cytokine production and IgE levels after allergen challenge, however no differences existed between the groups in hyperresponsiveness to methacholine (Hoskins, Wu, Reiss, & Dworski, 2013). GSTP^{-/-} mice subjected to the Alum/OVA model of allergic airway disease elucidated an anti-inflammatory role for GSTP (J. Zhou et al., 2008), and downregulation of GSTP in models of allergic airway disease was shown to be associated with increased levels of oxidative stress (Schroer et al., 2011). Furthermore, GSTP mRNA levels were increased in the lungs of mice exposed to Ova and induced sputum from asthmatics (Sohn et al., 2013). However, while studies have been shown to correlate GSTP content and haplotype with asthma, the functional contribution remains to be elucidated.

Despite the known role for classical NF- κ B signaling in lung epithelial cells and the emerging function for alternative NF- κ B in inflammatory processes, virtually no studies have been performed in order to elucidate alternative NF- κ B signaling in lung epithelial cells. Furthermore, despite the observed increases in the activation of classical NF- κ B in the lung epithelial cells of asthmatic patients in response to HDM, the role of NF- κ B in the epithelium in response to HDM in *in vivo* models of allergic airway disease remains to be determined. Finally, given the critical role for NF- κ B signaling in diverse biological processes, it has been reported that the oxidant-induced post-translational modification, PSSG, inhibits the kinase activity of IKK β . Despite the significant impact of PSSG on IKK β activity, mechanisms that catalyze PSSG of IKK β are still unknown.

Statement of Hypothesis and Scope of Thesis

As mentioned in the literature review, activation of NF- κ B in the lung epithelium is crucial in regulating pro-inflammatory responses in models of allergic airway disease and stimulation of cells with pro-inflammatory mediators *in vitro*. The term “NF- κ B” is typically interchangeable with the “classical NF- κ B pathway”; however, emerging studies have revealed an important role for the alternative NF- κ B pathway. Studies have also demonstrated crosstalk between the classical and alternative NF- κ B pathways, indicating that they are not as distinct as previously thought. Virtually no studies exist which have investigated the role of alternative NF- κ B in the lung epithelium in the context of inflammatory stimuli either *in vitro* or *in vivo*. Furthermore, it is known that the redox status of a cell can have a strong influence on NF- κ B, via tightly regulated oxidative modifications of target proteins. The mechanisms of these signaling events and functional implications remain to be fully elucidated. Therefore, the hypothesis of this thesis is that: classical and alternative NF- κ B pathways collectively contribute to the inflammatory phenotype of lung epithelial cells in both *in vitro* and *in vivo* models and that these pathways can be regulated by GSTP-catalyzed S-glutathionylation of NF- κ B proteins.

The goal of chapter 2 in this thesis was to evaluate the potential role of both classical and alternative NF- κ B in lung epithelial cells following pro-inflammatory stimuli *in vitro*. We investigated activation of both classical and alternative NF- κ B pathways, as well as their functional contribution via siRNA-mediated knockdown of

specific target proteins. Finally, we explored the impact of simultaneous activation of both classical and alternative NF- κ B on the production of lung epithelial-derived pro-inflammatory cytokines.

The goal of chapter 3 in this thesis was to evaluate the role of epithelial NF- κ B *in vivo* utilizing a transgenic model whereby both classical and alternative NF- κ B signaling is inhibited. We utilized a house dust mite- induced mouse model of allergic airway disease in order to test the role of epithelial NF- κ B inhibition in both acute and repeated house dust mite induced inflammation, airway hyperresponsiveness, mucus metaplasia, and airway remodeling. We also evaluated activation of classical and alternative NF- κ B in response to house dust mite utilizing human bronchial and nasal epithelial cells in order to strengthen the notion of the importance of these pathways to human disease.

The goal in chapter 4 of this thesis was to explore the role of GSTP in regulating both classical and alternative NF- κ B activation via S-gluthionylation of components of the NF- κ B pathway. The prior demonstration that GSTP has the ability to glutathionylate and is associated with allergic asthma, in addition to the fact that NF- κ B, can be inhibited by S-glutathionylation, indicates a potential relationship between NF- κ B and GSTP; to date, these putative links have yet to be explored. Therefore, a focus of this dissertation is to elucidate potential mechanisms by which GSTP may regulate NF- κ B in the epithelium in settings of pulmonary inflammation. We utilized siRNA targeted to GSTP as well as plasmids expressing either wild type or catalytically inactive GSTP and assessed activation and S-glutathionylation of both NF- κ B pathways.

Finally, the goal of chapter 5 in this thesis was to discuss the potential role of the importance of not only classical NF- κ B in the lung epithelium, but also alternative, in the context of regulation of inflammatory responses. Furthermore, we discuss the role of redox perturbations, specifically through the modification of GSTP in influencing aforementioned pathways and how these concepts contribute to the study of allergic asthma. Finally, we propose future directions of this research and potential studies that could unravel further the role of redox regulation of NF- κ B in the lung epithelium.

Figure Legends

Figure 1: Schematic depicting role of lung epithelium in allergic airway disease.

Upon stimulation by agonists, such as allergens or pollutants, NF- κ B becomes activated in the airway epithelium. The epithelium then releases cytokines important in dendritic cell maturation and subsequent T cell polarization, leading to the allergic inflammatory response.

Figure 2: Schematic depicting classical and alternative NF- κ B pathways.

Following stimulation by a variety of stimuli, IKK β is phosphorylated, which then in turn phosphorylates the Inhibitor of kappaB alpha (I κ B α). I κ B α , which sequesters p65 (RelA) in the cytosol, is subsequently ubiquitinated and degraded by the 26S proteasome. This event facilitates the nuclear translocation of RelA, which can initiate transcription of target genes. In the alternative NF- κ B pathway, NIK accumulation phosphorylates a distinct pool of IKK α (separate from the IKK signalsome), which then initiates IKK α to phosphorylate p100. Upon phosphorylation of p100, it is ubiquitinated and partially processed into p52, which then facilitates RelB translocation into the nucleus. This event triggers transcription of alternative NF- κ B-dependent genes.

Figure 3: Schematic depicting structure of NF- κ B proteins. Three main groups of NF- κ B proteins have been described: the NF- κ B proteins, inhibitor proteins, and kinase proteins. NF- κ B proteins consist of RelA, RelB, c-Rel, p105/p50 and p100/p52, all of which contain a rel homology domain. Furthermore, p105/p50 and p100/p52 contain ankyrin repeat domains, which mark proteins for partial processing and degradation. The inhibitor proteins, consisting of I κ B α , I κ B β , I κ B ϵ , and BCL-3, all contain ankyrin repeat

domains. The kinase proteins, IKK α and IKK β each contain a kinase domain, helix loop helix, and nemo binding domains, which assist in the congregation of the IKK signalsome. IKK γ , the scaffolding protein, contains domains crucial in protein binding, but lacks the kinase domains of the other IKK's.

Figure 4: Schematic depicting redox regulation of NF- κ B pathway. Following stimulation with a variety of stimuli, IKK β is phosphorylated and NF- κ B is activated. Following this phosphorylation event, IKK β is then S-glutathionylated by an as yet unknown mechanism, and NF- κ B signaling is inhibited. Subsequently, Grx1 catalyzes the deglutathionylation of IKK β and restores its kinase activity, and thus NF- κ B.

Figures

Figure 1:

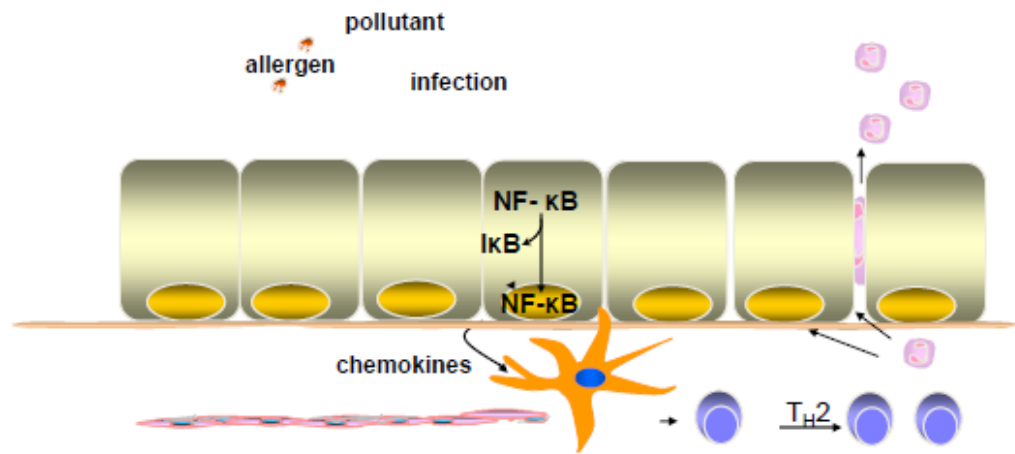


Figure 2:

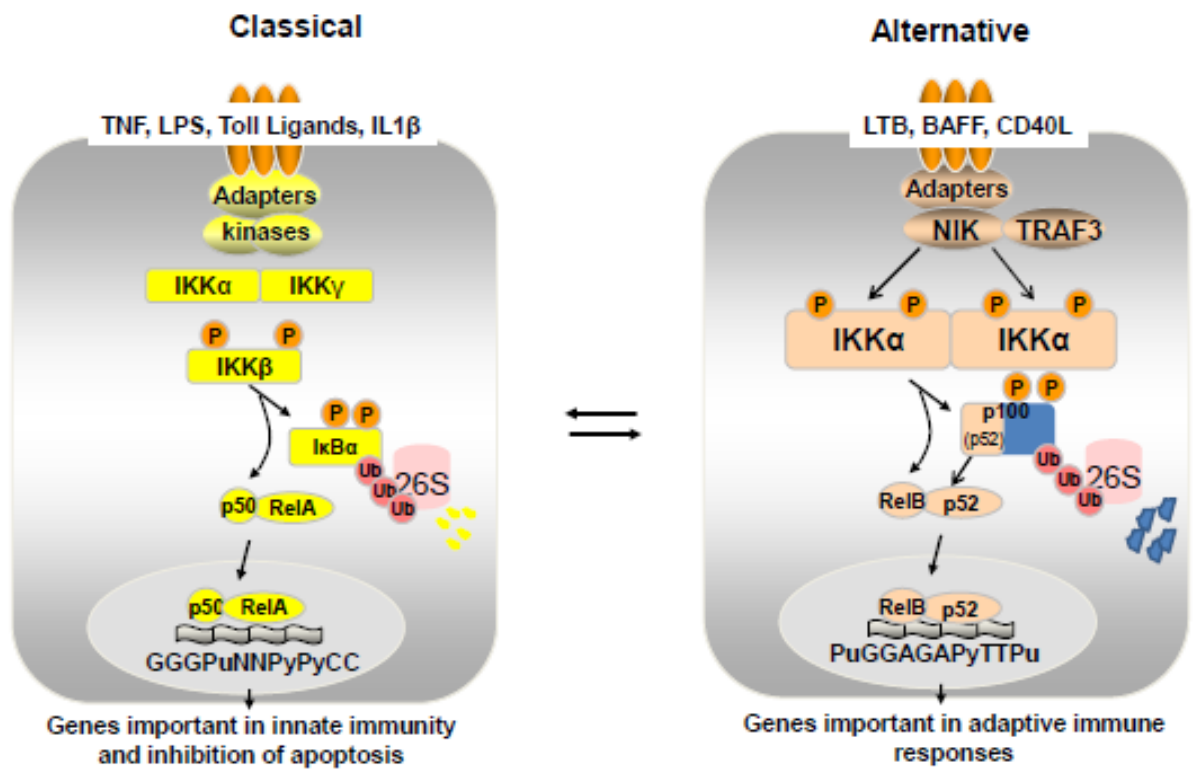
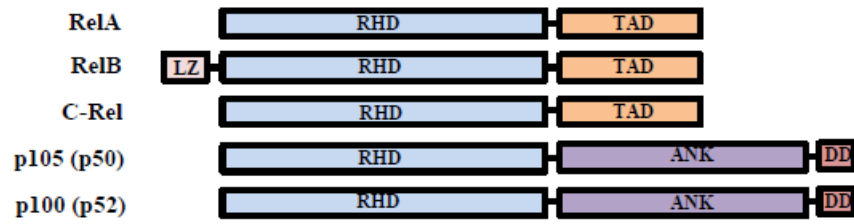
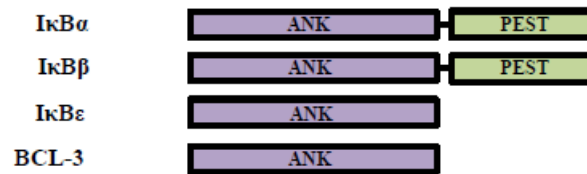


Figure 3:

A.



B.



C.

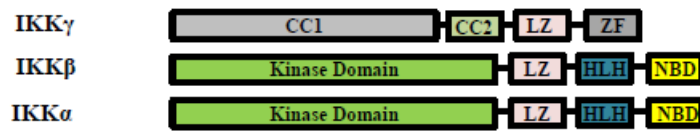
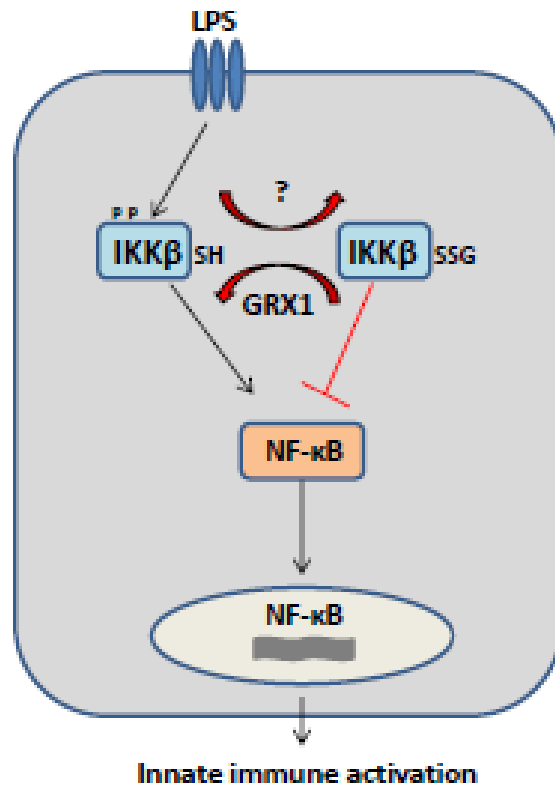


Figure 4:



List of Abbreviations:

TSLP: Thymic Stromal Lymphopoietin

CCL20: Chemokine (C-C motif) ligand 20

GM-CSF: Granulocyte Macrophage-Colony Stimulating Factor

MHC: Major Histocompatibility Complex

OVA: Ovalbumin

IL-6: Interleukin-6

IL-1 β : Interleukin-1 β

IL-33: Interleukin-33

IL-17: Interleukin-17

TH1: T helper Type 1

TH2: T helper Type 2

TH17: T helper Type 17

GATA3: Trans-acting T-cell-specific transcription factor

T-bet: T box transcription factor

INF γ : Interferon- γ

T Reg: Regulatory T cell

IL-25: Interleukin-25

IL-5: Interleukin-5

IL-13: Interleukin-13

NF- κ B: nuclear factor kappa-light-chain-enhancer of activated B cells

ERK: Extracellular signal-regulated kinase

JNK: c-Jun N-terminal kinase

IgM: Immunoglobulin M

IgD: Immunoglobulin D

IgE: Immunoglobulin E

TGF β 1: Transforming Growth Factor β 1

IL-8: Interleukin-8

KC: Keratinocyte Chemokine

RSV: Respiratory Syncytial Virus

COPD: Chronic Obstructive Pulmonary Disease

I κ B α : nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha

I κ B β : nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta

I κ B ϵ : nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon

I κ B ζ : nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta

BCL3: B-cell lymphoma 3-encoded protein

IKK α : Inhibitor of nuclear factor kappa-B kinase subunit alpha

IKK β : Inhibitor of nuclear factor kappa-B kinase subunit beta

IKK γ : Inhibitor of nuclear factor kappa-B kinase subunit gamma

IRAK: Interleukin-1 receptor-associated kinase

TRAF6: TNF receptor associated factor 6

TRAF2: TNF receptor associated factor 2

TAK1: TGF β 1-activated kinase

AHR: Airway hyperresponsiveness

TRAF3: TNF receptor associated factor 3

TAX1BP1: human T-cell leukemia virus type I binding protein 1

CBP/300: Binding protein 300/CREB binding protein

GSK3 β : Glycogen synthase kinase 3 beta

Ubc13: Ubiquitin-conjugating enzyme E2 N

Uev1a: Ubiquitin-conjugating Enzyme Variant 1a

CYLD1: Cylindromatosis 1

LUBAC: linear ubiquitin chain assembly complex

HOIL-1L: Heme-oxidized IRP2 ubiquitin ligase 1

HOIP: HOIL-1L interacting protein

TNF α : Tumor Necrosis Factor alpha

NADPH: nicotinamide adenine dinucleotide phosphate

PP2A: Protein Phosphatase 2A

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Chapter 2

Cooperation between classical and alternative NF- κ B pathways regulates pro-inflammatory responses in epithelial cells

Abstract

The transcription factor, Nuclear Factor kappa B, (NF- κ B) has been causally linked to inflammatory lung diseases. Recent studies have unraveled the complexity of NF- κ B activation by identifying two parallel activation pathways; the classical NF- κ B pathway, which is controlled by IKK β and RelA/p50, and the alternative pathway, which is controlled by IKK α and RelB/p52. The alternative pathway regulates adaptive immune responses and lymphoid development, yet its role in the regulation of innate immune responses remains largely unknown. In this study we determined the relevance of the alternative NF- κ B pathway in pro-inflammatory responses in lung epithelial cells. Exposure of C10 mouse alveolar lung epithelial cells to diverse stimuli, or primary mouse tracheal epithelial cells to lipopolysaccharide (LPS) resulted in activation of both NF- κ B pathways, based upon nuclear translocation of RelA, p50, RelB, and p52. Increases in nuclear content of RelA occurred rapidly, but transiently, while in contrast, increases in nuclear RelB content were protracted. siRNA-mediated knockdown of IKK α , RelA, or RelB resulted in decreases in multiple LPS-induced pro-inflammatory cytokines. Surprisingly, siRNA ablation of IKK α or RelB led to marked increases in production of IL-6 in response to LPS. Simultaneous expression of CA-IKK α and CA-IKK β caused synergistic increases in pro-inflammatory mediators. Lastly, disruption of the IKK signalsome inhibited activation of both NF- κ B pathways. These results demonstrate that

coordinate activation of both NF- κ B pathways regulates the magnitude and nature of pro-inflammatory responses in lung epithelial cells.

Introduction

Nuclear Factor-kappa B (NF- κ B) is a transcription factor that plays a cardinal role in multiple cellular processes, including survival, proliferation, and inflammation. In unstimulated cells, NF- κ B dimers RelA and p50 are sequestered in the cytosol by the inhibitor of kappa B (I κ B α). The I κ B kinase complex (IKK) consists of two catalytic subunits, IKK β and IKK α , and the regulatory protein, IKK γ (also known as NF- κ B essential modulator). Upon ligation by a variety of stimuli, such as tumor necrosis factor- α , or toll like receptor agonists, IKK β is phosphorylated and in turn phosphorylates I κ B α , leading to its subsequent ubiquitination and degradation by the 26S proteasome (Hayden & Ghosh, 2004; Scheidereit, 2006). The processing of I κ B α promotes nuclear translocation of RelA/p50, leading to transcriptional activation of NF- κ B-dependent genes. NF- κ B activates transcription of many pro-inflammatory cytokine and chemokine genes which initiate and propagate innate immune responses (Hayden & Ghosh, 2004; Scheidereit, 2006).

The airway epithelium, classically regarded as the first line of defense against inhaled agents, toxic factors, and physical trauma, is now recognized as a key component of the innate immune system, and plays an active role in the orchestration of acute inflammatory and adaptive immune responses (Fahy & Locksley, 2011; Swamy, Jamora, Havran, & Hayday, 2010). Upon stimulation, epithelial cells secrete pro-inflammatory mediators such as interleukin-6 (IL-6), keratinocyte-derived chemokine (KC), regulated on activation normal T cells expressed and secreted (RANTES), granulocyte monocyte-

colony stimulating factor (GM-CSF), CCL20, and many others, all of which have been implicated in the pathogenesis of inflammatory disorders via interactions with dendritic cells, macrophages, T cells, and B cells (Swamy et al., 2010). NF- κ B activation in lung epithelial cells has been shown to be crucial in regulating these pro-inflammatory responses (Ather et al., 2011; Broide et al., 2005; Pantano et al., 2008; Poynter et al., 2004; Poynter, Irvin, & Janssen-Heininger, 2003).

In addition to the classical NF- κ B pathway, a parallel, alternative NF- κ B activation pathway has been identified. The alternative NF- κ B pathway is activated by distinct ligands, such as CD40 ligand and B cell activating factor (BAFF) via NF- κ B inducing kinase (NIK)-induced phosphorylation of IKK α . IKK α in turn phosphorylates the I κ B domain of p100, leading to its partial processing and release of RelB and p52 into the nucleus, and transcriptional activation of unique subsets of NF- κ B-dependent genes important in the adaptive immune response (Oeckinghaus, Hayden, & Ghosh, 2011; Senftleben et al., 2001). Classically, the alternative pathway has been described to be important for B and T lymphocyte development and formation of peripheral lymphoid organs (G. Xiao et al., 2006), however, its role in orchestrating pro-inflammatory responses in lung epithelial cells remains largely unknown.

Given the importance of the classical NF- κ B pathway in driving expression of pro-inflammatory mediators in epithelial cells, and recent reports demonstrating cross talk between both NF- κ B activation pathways (Anest et al., 2003; Lawrence et al., 2005; Shembade et al., 2011; Yamamoto et al., 2003), we sought to determine whether the alternative NF- κ B pathway is activated in lung epithelial cells upon stimulation with a

variety of agonists. Furthermore, we evaluated the functional importance of classical and alternative NF- κ B pathway activation in LPS-induced pro-inflammatory responses, and whether crosstalk between activation of both NF- κ B activation pathways exists in lung epithelial cells.

Materials and Methods

Cell Culture: Primary mouse tracheal epithelial cells (MTEC) were isolated and cultured according to previously described methods (Alcorn et al., 2008; R. Wu & Smith, 1982). MTEC were infected with constitutively active adeno IKK α at 1×10^5 virions per transwell and harvested at the indicated times. A spontaneously transformed type II mouse lung alveolar epithelial cell line (Malkinson, Dwyer-Nield, Rice, & Dinsdale, 1997) (C10) was cultured, as described previously (Alcorn et al., 2008). 16 h before treatment, cells were starved in medium containing 0.5% FBS. Cells were exposed to 1 μ g/mL Lipopolysaccharide (LPS, List Biological Laboratories, Inc), 50ng/mL Interleukin 17A (IL-17A, R&D Systems), 1 μ g/mL mouse Tumor Necrosis Factor- α (TNF- α , Sigma), 100ng/ml CD-40 ligand (CD-40L, eBioscience), 10 μ g/mL polyinosinic acid (poly IC, Calbiochem) and 1 μ g/mL lipoteichoic acid (LTA, Sigma) for indicated times. Cells were exposed to 10 μ M NEMO binding domain (NBD) peptides (Calbiochem) or scrambled peptide controls 2 h prior to indicated treatments.

Mice: C57Bl/6 mice were anesthetized and given 5 μ g LPS oropharyngeally (List Biological Laboratories, Inc., Campbell, CA) and 24 h later, euthanized by a lethal pentobarbital injection. The right lobe was flash frozen in liquid nitrogen and pulverized

and the left lobe was inflated with 4% paraformaldehyde. 5 μ m sections were used to assess localization of RelB in situ via immunofluorescence, according to previously described methods (Poynter et al., 2004). RelB was detected in nuclear extracts prepared from whole lung according to previously published procedures (Chung et al., 2010). All studies were approved by the institutional Animal Care and Use Committee at the University of Vermont.

Western Blot Analysis: Cells were washed with cold PBS before harvest. Protein concentration was determined by the Bio-Rad DC Protein Assay kit (Bio-Rad, Hercules, CA), and 20 μ g protein was used for Western Blotting. RelA, RelB, p50, and β -Actin antibodies were from Santa Cruz biotechnology, Santa Cruz, CA. Antibodies p100/p52, I κ B α , IKK β , phosphoserine RelA 536, tubulin, and Histone H3 were from Cell Signaling Technology, Danvers, MA. The IKK α antibody was purchased from Upstate. Cytosolic and nuclear extracts were prepared as previously described (van der Velden, Schols, Willems, Kelders, & Langen, 2008).

RelA, RelB, and IKK α siRNA: C10 cells were incubated with Dharmacon SMARTpool control non-targeting small interfering (si)RNA or Dharmacon SMARTpool siRNA against RelA, RelB, and IKK α (all 100nM) (Dharmacon, Lafayette, CO) and subsequently harvested and analyzed as indicated.

Transfections and Plasmids: Plasmid transfections were performed with Nanofectin (PAA, Pasching, Austria). CA-IKK β containing serine 177/181 to glutamic acid mutations and CA-IKK α containing serine 176/180 to glutamic acid mutations were cloned into pcDNA3.1 and pRc β -actin plasmids, respectively.

Enzyme-Linked Immunosorbent Assays (ELISA): C10 cells were treated, and medium was assessed for MIP-2, KC, RANTES, CCL-20, IL-6, and GM-CSF cytokines by R & D Systems, Minneapolis, MN according to manufacturer's instructions.

Gene Expression: Total RNA was isolated from C10 cells using the RNeasy kit (Qiagen, Valencia, CA) and reverse transcribed for taqman gene analysis using SYBR green (Bio-Rad, Hercules, CA).

Primers: Primers for quantitative reverse transcriptase polymerase chain reaction are provided in the online supplement.

Statistical Analysis: Data were evaluated by GraphPad Prism 5 Software using one-way analysis of variance (ANOVA) with Bonferroni to adjust for multiple comparisons. Results with $p < 0.05$ or smaller were considered statistically significant.

Results

Activation of classical and alternative NF- κ B signaling in response to diverse agonists in lung epithelial cells: Previous work in our laboratory has demonstrated the importance of classical NF- κ B signaling in lung epithelium in acute inflammatory and allergic disease (Ather et al., 2011; Poynter et al., 2004; Poynter et al., 2003). To date, no studies exist to determine whether activation of alternative NF- κ B signaling occurs in lung epithelial cells. We therefore analyzed activation of both classical and alternative NF- κ B signaling after stimulation with a variety of agonists. C10 cells were exposed to Toll-like receptor (TLR) 4 agonist, lipopolysaccharide (LPS), for varying times prior to evaluation of the nuclear content of RelA, RelB, p50, and p52. Results in Fig. 1 demonstrate rapid increases in nuclear content of RelA, and p50, components of the

classical NF- κ B pathway, which decreased by 2 h. Increases in nuclear content of RelB/p52, components of the alternative NF- κ B pathway also were observed in response to LPS. Increases in RelB occurred gradually, and were still apparent 8 h following LPS exposure. In order to determine whether increases in RelB/p52 are unique to LPS, we exposed C10 lung epithelial cells to the TLR2 agonist, lipoteichoic acid (LTA), the TLR3 agonist, Polyinosinic acid (PolyIC), Tumor Necrosis Factor- α (TNF- α), and interleukin-17A (IL-17A). Results in Fig. 1 demonstrate similar patterns of NF- κ B activation in response to all agonists. Densitometric evaluation of RelA or RelB nuclear content, normalized to histone H3, demonstrated that in response to all stimuli, early increases in nuclear content of RelA were apparent, while increases in nuclear RelB tended to be protracted (Fig. 1A and 1B). Comparative evaluation of nuclear p50 and p52 demonstrated more variable increases with apparent biphasic fluctuations in response to all agonists (Fig. 1A and 1C). Upon stimulating cells with CD-40 ligand, a molecule known to activate the alternative pathway in myeloid cells (Ramakrishnan, Wang, & Wallach, 2004), increases in nuclear content of RelA/p50 and RelB/p52 occurred (Fig. 1A-C), demonstrating that an agonist implicated in activation of the alternative NF- κ B pathway, in fact, induces both NF- κ B pathways in epithelial cells.

Lipopolysaccharide activates classical and alternative NF- κ B signaling in primary mouse lung epithelial cells and in the mouse lung: Given that LPS resulted in increases in both RelA/p50 and RelB/p52, and that TLR4 signaling is important in acute and allergic inflammation (Hammad et al., 2009; Wilson et al., 2009), we sought to further characterize the relevance of coordinate activation of both NF- κ B pathways in

lung epithelial cells in response to LPS. We first determined the timing and duration of increases in RelA/p50 and RelB/p52 content in response to LPS with further detail. Results in Fig. 2A demonstrate that increases in nuclear RelA/p50 content occurred robustly by 30 min and strongly decreased toward control levels by 2 h post LPS administration. Increases in nuclear RelB also occurred by 30 min, albeit somewhat less robustly as RelA, but increases in nuclear RelB were sustained at least for 48 h post LPS exposure. Increases in p52 nuclear content were observed rapidly in response to LPS, and decreased 16 h post exposure. In order to determine whether similar patterns of activation of classical and alternative NF- κ B pathways exist in primary epithelial cells, mouse tracheal epithelial cells (MTEC) were exposed to LPS. Results in Fig. 2B demonstrate increases in nuclear RelA and p50 content 15 min or 30 min, respectively, following stimulation of MTEC with LPS, with peak increases apparent by 1 h, and decreases by 4 h. Nuclear content of RelB and p52 increased following 1 h of exposure to LPS, and remained elevated by 4 h. Phosphorylation of RelA at Ser536, reflective of activation of IKK, was apparent by 15 min, and decreased by 2h, while degradation of I κ B α occurred between 30 min and 1 h post stimulation of cells with LPS. While in control cells p100 was undetectable, increases in overall p100 content occurred by 1 h and were sustained for at least 4 h following LPS. Overall, these findings demonstrate that in response to a TLR-4 agonist, activation of both classical and alternative NF- κ B pathways occur in lung epithelial cells, based upon increases in nuclear content. Increases in RelA/p50 were transient, while notably, increases in RelB and p52 occurred in a protracted manner in response to LPS.

It has been shown previously that LPS activates classical NF- κ B signaling in lung tissue (Poynter et al., 2003). In order to confirm whether increases in nuclear RelB occurred in lung tissue in response to LPS, C57Bl/6 mice were given 5 μ g LPS oropharyngeally and harvested 24 h later. Compared to PBS controls following exposure to LPS, increases in nuclear RelB were apparent (Fig. 2C). Assessment of RelB in lung tissue via confocal laser scanning microscopy, demonstrated that in control mice RelB (red) was predominantly localized in the cytoplasm of bronchiolar epithelial cells. 24 h after LPS administration, RelB immunoreactivity (red) was markedly enhanced in the bronchiolar epithelium, and strong co-localization with the DNA stain, Sytox Green was observed, reflected by the yellow color, indicative of nuclear localization of RelB in the bronchiolar epithelium. In mice exposed to LPS, select cells in the parenchyma, that require further identification, also started to express detectable RelB.

Impact of classical and alternative NF- κ B signaling on cytokine production in response to LPS: In order to determine the relative contribution of RelA and RelB for the regulation of LPS-induced pro-inflammatory responses, we utilized siRNA mediated knockdown of RelA or RelB, and assessed the content of NF- κ B dependent pro-inflammatory mediators in supernatants. RelA siRNA decreased RelA content in C10 lung epithelial cells, albeit not completely (Fig. 3A). As expected, LPS-induced increases in CCL-20, RANTES, KC, and IL-6 in the medium were significantly attenuated in cells transfected with RelA siRNA, compared to scrambled control (Fig. 3B). SiRNA-mediated knock-down of RelB (Fig. 4A) also resulted in marked decreases in LPS-induced production of the pro-inflammatory mediators, CCL-20, RANTES, and KC

compared to scrambled siRNA controls (Fig. 4B). Surprisingly, knockdown of RelB led to a significant increase in LPS-induced IL-6 content in comparison to scrambled siRNA controls at all time points. In order to determine whether IKK α , which activates alternative NF- κ B signaling, had a similar impact on LPS-induced pro-inflammatory mediators as RelB, we next ablated IKK α with siRNA (Fig. 5A). Results in Fig. 5B demonstrate decreases in LPS-induced CCL-20 and RANTES content following ablation of IKK α , and no effect on KC. Conversely, siRNA mediated knock-down of IKK α led to increases in basal and LPS- stimulated production of IL-6, similar to the effects of RelB siRNA. In aggregate, these findings demonstrate that both the classical and alternative NF- κ B pathways contribute to LPS-induced production of diverse pro-inflammatory cytokines that include CCL-20 and RANTES, but that IKK α and RelB repress the production of IL-6.

Impact of constitutively active IKK α and IKK β on expression of pro-inflammatory mediators in lung epithelial cells: In order to evaluate the role of activation of IKK α and IKK β directly, C10 lung epithelial cells were transfected with 1 μ g constitutively active (CA) variants of IKK α or IKK β . Results in Fig. 6A demonstrate that expression of IKK α or IKK β individually led to increases in nuclear content of RelA, p50, RelB, and p52. Expression of CA-IKK α or CA-IKK β individually led to increases in mRNA expression and production of CCL-20, MIP-2, RANTES, and GM-CSF in C10 lung epithelial cells (Fig. 6B). In contrast, no clear increases in content of IL-6 and KC were observed under these conditions (data not shown). Co-transfection of C10 cells with 0.5 μ g of IKK α and IKK β together led to increases in nuclear RelA and RelB, which were not observed in

response to transfection of individual constructs at these lower concentrations (Fig. 6C). Dual expression of 0.25 or 0.5 μg CA-IKK α and CA-IKK β each resulted in striking increases in production of CCL-20, MIP-2, GM-CSF, and RANTES which were not observed in cells transfected with individual constructs (Fig. 6D) at these lower concentrations, in contrast to earlier experiments with 1 μg of plasmid (Fig. 6B). These findings demonstrate clear cooperation between both kinases in eliciting pro-inflammatory responses in lung epithelial cells. In order to determine the role of RelA and RelB in CA-IKK α or CA-IKK β -dependent pro-inflammatory responses, we knocked down either RelA or RelB with siRNA prior to transfection of epithelial cells with CA-IKK α or CA-IKK β . Results in Fig. 6E demonstrate that the ability of CA-IKK α or CA-IKK β to induce expression of pro-inflammatory mediators depended partially on both RelA and RelB, dependent upon the individual pro-inflammatory mediator. RelA and RelB each contributed to CA-IKK α or CA-IKK β -mediated increases in CCL-20, RANTES, and GM-CSF. In contrast RelA, but not RelB contributed to CA-IKK α or CA-IKK β -induced expression of MIP-2, as the content of this cytokine was unchanged after RelB siRNA (Fig 6E). These findings demonstrate that both RelA and RelB are functionally important in driving expression of CA-IKK α or CA-IKK β -induced pro-inflammatory genes, and that the exact contribution of these NF- κ B subunits depends on the target gene.

In order to confirm that CA-IKK α elicits pro-inflammatory responses in primary lung epithelial cells, we infected MTEC with adenovirus expressing CA-IKK α for 48 and 72 hours in comparison to control virus (Fig. 6F). Transduction of CA-IKK α led to

increases in production of diverse pro-inflammatory mediators, including KC, MIP-2, CCL-20, and GM-CSF, compared to control virus (Fig. 6G). All together these findings demonstrate that both IKK α and IKK β are capable to elicit pro-inflammatory responses in lung epithelial cells, and that cooperation between both kinases exists.

Requirement of IKK γ for activation of classical and alternative NF- κ B pathways in lung epithelial cells: We next sought to further explore the mechanism that underlies activation of classical and alternative NF- κ B pathways in epithelial cells exposed to LPS. The mechanism of activation of IKK α in response to diverse ligands is not fully known. Although IKK α has been shown to be part of the classical NF- κ B signalsome, consisting of IKK α , IKK β and IKK γ (DiDonato et al., 1997), during activation of the alternative pathway IKK α is activated via NF- κ B inducing kinase (NIK) in an IKK γ -independent manner (Oeckinghaus et al., 2011). In order to determine whether increases in nuclear content of RelB/p52 in response to LPS occur in an IKK γ (NEMO)-dependent manner, we incubated epithelial cells with NEMO binding domain peptide which disrupts the classical NF- κ B signalsome (May et al., 2000), and determined the impact for LPS-induced nuclear accumulation of RelA/p50, and RelB/p52. Results in Fig. 7A demonstrate that disruption of the IKK signalsome with NBD peptide not only attenuated LPS-induced nuclear accumulation of RelA/p50, but also blocked increases in nuclear RelB/p52, demonstrating that activation of the alternative pathway by LPS in lung epithelial relies, at least in part, on the classical IKK signalsome containing IKK γ (Fig. 7B).

Discussion

The transcription factor, NF- κ B, has been shown to play a crucial role in the orchestration of pro-inflammatory responses in the lung. Previous work performed in our laboratory demonstrated nuclear presence of RelA in acute models of LPS-induced inflammation (Poynter et al., 2003) and in the ovalbumin-model of allergic airways disease (Poynter, Irvin, & Janssen-Heininger, 2002). Furthermore, transgenic mice expressing a dominant negative version of I κ B α in bronchial epithelial cells to inhibit the classical NF- κ B pathway, exhibited marked protection from inflammation associated with those agents (Poynter et al., 2004; Poynter et al., 2003). Conversely, in multiple independently generated transgenic lines, inducible activation of the IKK β transgene in bronchiolar epithelial cells resulted in marked neutrophilic inflammation (Cheng et al., 2007; Pantano et al., 2008), enhanced sensitization to and inflammatory responses to ovalbumin (Ather et al., 2011; Pantano et al., 2008; Sheller et al., 2009), and carcinogenesis (Zaynagetdinov et al., 2011), demonstrating the functional significance of activation of the classical NF- κ B pathway in lung epithelial cells. Despite this knowledge, little information is available about the functional requirement for activation of the alternative NF- κ B pathway in regulating transcription of pro-inflammatory genes in lung epithelial cells.

Results from the present study demonstrate that classical and alternative NF- κ B activation pathways are coordinately activated in lung epithelial cells in response to diverse stimuli, as evidenced by increases in nuclear content of RelA, p50, RelB, and p52. Notably, increases in RelA tended to occur rapidly, while RelB increased more

gradually, but in a protracted manner. Although we did not detect p100 protein in lung epithelial cells under basal conditions, in response to LPS, p100 content increased in primary tracheal epithelial cells, with corresponding increases in nuclear p52. Since p100 is an NF- κ B dependent gene (Liptay, Schmid, Nabel, & Nabel, 1994), it is plausible that activation of classical NF- κ B leads to induction of p100, which is subsequently processed by IKK α , leading to alternative NF- κ B signaling. Validation of this possible scenario will require additional studies. The functional significance of the differential timing of activation of classical and alternative NF- κ B signaling in lung epithelial cells remains unknown and requires additional assessment of presence of NF- κ B components bound to regulatory regions of target genes, and correlations with transcriptional activation.

In C10 epithelial cells stimulated with LPS, RelA, RelB, and IKK α all were demonstrated to play a role in controlling the strength and nature of pro-inflammatory cytokine production. Additionally, we demonstrate here that cooperation exists between both pathways, indicated by the synergistic increases in cytokine production upon co-expression of IKK α and IKK β , illuminating an important role for both NF- κ B signaling pathways in dictating pro-inflammatory responses in lung epithelial cells. Recent work has shown activation of NIK and IKK α in A549 lung epithelial cells in response to respiratory syncytial virus, and demonstrated a causal role for this pathway in the activation of RANTES (Choudhary, Boldogh, Garofalo, Jamaluddin, & Brasier, 2005).

Our findings demonstrating that LPS can activate both classical and alternative NF- κ B pathways in both primary lung epithelial cells and in lung tissue are supported by previous work demonstrating activation of both classical and alternative NF- κ B pathways

by LPS in B lymphocytes (Souvannavong et al., 2007). Similarly, TNF α , an agonist of the classical NF- κ B pathway, has been shown to activate the alternative NF- κ B pathway in mouse embryonic fibroblasts (Adli, Merkhofer, Cogswell, & Baldwin, 2010; J. Y. Kim et al., 2011). These findings demonstrate that multiple ligands previously considered as unique activators of either classical or alternative NF- κ B indeed can activate both pathways. Results from the present study show simultaneous activation of both NF- κ B pathways in lung epithelial cells in response to diverse stimuli, and demonstrate the functional relevance of both pathways in regulating pro-inflammatory signals activated by LPS.

IKK α has been demonstrated to play diverse roles, both in regulation of activation of NF- κ B in the cytosol, as well as remodeling of chromatin, and previous studies have demonstrated both positive and negative roles of IKK α in the classical NF- κ B pathway. For example, the requirement of IKK α for activation of NF- κ B responsive genes, such as IL-8 and I κ B α , was demonstrated in response to TNF- α (Yamamoto et al., 2003) or cigarette smoke (S. R. Yang et al., 2008), in association with increased phosphorylation of histone H3. Conversely, IKK α also has been shown to shut down the classical NF- κ B pathway, via phosphorylation of Tax1bp1 (Shembade et al., 2011), or Protein Inhibitor of Activated STAT1 (PIAS) (B. Liu et al., 2007). Loss of IKK α has been shown to prolong the activity of IKK β and to promote inflammation (Lawrence et al., 2005). Additionally, accelerated removal of RelA/p50 NF- κ B subunits from pro-inflammatory gene promoters by IKK α has been demonstrated to limit activation of NF- κ B (Yamamoto et al., 2003). Furthermore, IKK α can inhibit the alternative pathway NF- κ B via the phosphorylation

and subsequent degradation of NIK (Razani et al., 2010). Lastly, IKK α also can mediate transcriptional activation of genes independently of NF- κ B (Oeckinghaus et al., 2011). In aggregate, these reports demonstrate the multifaceted roles of IKK α in regulating the transcriptional output and duration of NF- κ B activation in diverse cell types exposed to a variety of agonists. Our present results, demonstrating that siRNA-mediated ablation of IKK α paradoxically leads to increases in IL-6 content, but decreases in RANTES and CCL-20, indicate that the functional contribution of IKK α not only depends on stimulus and cell type, but also on the actual target genes. Additional studies will be required to formally assess the impact of IKK α on the transcriptional activation potential of individual NF- κ B-regulated genes in lung epithelial cells.

Our present results demonstrate that ablation of RelB with siRNA affected production of pro-inflammatory mediators in response to LPS in a similar manner as siRNA-mediated ablation of IKK α , suggesting that IKK α may exert its effects in lung epithelial cells via the regulation of RelB. Numerous studies have demonstrated anti-inflammatory roles for RelB. For example, RelB knockout animals develop spontaneous inflammation in multiple organs, including the lung (Weih et al., 1995), which were further exacerbated by concomitant loss of p50 (Weih et al., 1997). Aryl hydrocarbon receptor-deficient fibroblasts exposed to cigarette smoke showed increased expression of cyclooxygenase-2 and prostaglandins, in association with a loss of RelB (Baglolle et al., 2008). Indeed, an anti-inflammatory role for RelB in cigarette smoke-induced inflammation was recently demonstrated, evidenced by decreased neutrophilic infiltration, and diminished content of pro-inflammatory mediators following adenovirus-

mediated transduction of RelB (McMillan et al., 2011). In response to LPS, RelB^{-/-} fibroblasts showed prolonged increases in pro-inflammatory cytokines compared to WT controls, findings that suggest a role for RelB in the resolution of inflammatory responses (Xia et al., 1997). Lastly, RelB also has been shown to promote tolerance to LPS, and diminish pro-inflammatory responses in macrophages (El Gazzar, Liu, Yoza, & McCall, 2010; Yoza et al., 2006). These previous findings contrast with the results of the present study, demonstrating that siRNA mediated knockdown of RelB diminishes LPS-induced production of KC, CCL-20, and RANTES. However, RelB knockdown resulted in a significant increase in IL-6 production in response to LPS, illustrating a dynamic and highly specific role for RelB in controlling gene expression in epithelial cells exposed to LPS.

While previous reports have demonstrated cross-regulation between classical and alternative NF- κ B pathway activation (Oeckinghaus et al., 2011), it is unknown whether inducible activation of both pathways can cooperatively enhance production of NF- κ B-dependent cytokines. Results in the present study demonstrate that co-expression of the constitutively active kinases, IKK α and IKK β , in the absence of any other ligands, lead to a cooperative increase in expression of pro-inflammatory cytokines, which correspond to increases in nuclear RelA and RelB. Additionally, we showed in the present study that nuclear translocation of RelA, RelB, p50, and p52 all are, in part, regulated by the IKK γ -containing signalsome complex, indicating at least one common regulatory mechanism whereby IKK α and IKK β facilitate NF- κ B-dependent gene activation.

Interactions between RelA and RelB have been described previously, and have been shown to regulate transcription. In response to priming of cells with TNF- α , and lymphotoxin beta, both RelA and RelB were recruited to the GM-CSF promoter, in association with increased transcription (Sasaki et al., 2011). However, RelB has also been shown to replace RelA at the IL-12p35 promoter, shutting down transcription (Saccani et al., 2003). In response to stimulation of dectin-1, Raf-1-induced phosphorylation of RelA was shown to lead to inactive RelA-RelB dimers that inhibit transcription (Gringhuis et al., 2009), and similar inhibitory effects of RelA on RelB-mediated transcription following phosphorylation of RelA were reported in response to TNF α (Jacque, Tchenio, Piton, Romeo, & Baud, 2005). Future studies are needed to determine whether enhanced association between RelA and RelB contribute to gene specific stimulatory (CCL-20, RANTES, KC) or inhibitory effects (IL-6) observed in the present study.

Convincing studies exist to demonstrate the contribution of classical NF- κ B to lung inflammation. However, the role of the alternative NF- κ B activation pathway in the regulation of pulmonary inflammation is only emerging. Adenovirus expressing phosphomimetic, and hence constitutively IKK α (CA-IKK α) introduced into airways, was sufficient to induce neutrophilic infiltration, and increased mRNA expression of a variety of pro-inflammatory genes, similar to adenovirus expressing CA-IKK β (Sadikot et al., 2003). In support of those findings, the present study demonstrates similar pro-inflammatory effects of CA-IKK α in primary tracheal epithelial cells. In support of a contribution of both IKK α and IKK β to pulmonary inflammation, a recent study showed

increased activity of IKK β and IKK α in both healthy smokers and COPD patients (Gagliardo et al., 2011). SiRNA-mediated knockdown of IKK α in peripheral blood mononuclear cells isolated from these patients resulted in decreases in IL-8 production, illustrating a pro-inflammatory role for IKK α . Based upon those findings, results from the present study, and recent reports that have illuminated the cardinal role of epithelial cells in orchestration of inflammation (Swamy et al., 2010), additional studies that further unravel the mechanisms whereby IKK α and RelB facilitate and/ or repress inflammatory signaling in lung epithelial cells are well warranted.

Figure Legends

Figure 1: Activation of classical and alternative NF- κ B signaling in response to diverse agonists in lung epithelial cells. (A) Western blot analysis of nuclear extracts from mouse type II alveolar lung epithelial (C10) cells stimulated with LPS (1 μ g/ml), lipoteichoic acid (LTA 1 μ g/ml), Polyinosinic acid (PolyIC, 10 μ g/mL), TNF- α (1 μ g/ml), CD-40L (100ng/ml), or IL-17A (50ng/ml) for the indicated times. 20 μ g nuclear protein was separated by SDS-PAGE and analyzed for RelA, RelB, p50, p52 or Histone H3 (H3, nuclear loading control). Densitometric evaluation of nuclear RelA and RelB (B), p50 and p52 (C). Data shown are normalized to Histone H3. Values on the left y-axis correspond to RelA/H3 (B) or p50/H3 (C) and values on the right y-axis correspond to RelB/H3 (B) or p52/H3 (C), and reflect arbitrary units.

Figure 2: Lipopolysaccharide activates the classical and alternative NF- κ B pathways in C10 lung epithelial cells, primary mouse tracheal epithelial cells, and lung

tissue. (A) Western blot analysis of nuclear extracts from C10 cells treated with LPS for the indicated times. Blots were evaluated for content of RelA, RelB, p50, p52, and β -Actin is shown as a loading control, due to changes in Histone H3 content over time in culture. Right panel: Confirmation of cellular fractionation into nuclear and cytosolic fractions, by analysis of tubulin (cytosol) and Histone H3 (nuclear). β -Actin: loading control. (B) Western blot analysis of nuclear and cytosolic extracts prepared from primary mouse tracheal epithelial cells (MTEC) treated with 10 μ g/ml LPS for the indicated times. Nuclear extracts were evaluated for content of RelA, RelB, p50, p52, and Histone H3, as a loading control. Cytosolic extracts were assayed for content of p100, phosphorylated RelA (serine 536 P), I κ B α , and β -Actin as a loading control. (C) Western blot of nuclear extracts (20 μ g protein per lane) prepared from lung homogenates of C57Bl/6 mice (n=3/group) oropharyngeally instilled with 5 μ g LPS or sterile PBS as a control for 24 h. Blots were analyzed for nuclear content of RelB and Histone H3 (nuclear loading control). Note that samples were run on the same gel, and blotted onto the same membrane, and that lanes were reassembled for consistency. Data are representative of at least 2 separate experiments. (D) Immunofluorescence analysis of nuclear RelB, 24 h after oropharyngeal instillation of LPS. De-paraffinized lung sections were stained for RelB (**red**), and nuclei were counterstained with Sytox Green. Images were captured by laser scanning confocal microscopy, using identical instrument settings. Images are representative results of three independent experiments. **IgG control:** As a negative control, primary antibody was omitted. RelB, **red**; DNA, **green**; Nuclear RelB, yellow

Figure 3: LPS-induced production of pro-inflammatory mediators is dependent on RelA. (A) Verification of RelA knockdown in C10 cells. Whole cell lysates were prepared from cells transfected with either scrambled siRNA or RelA siRNA. 20µg protein was separated by SDS-PAGE, and Western blotting, for evaluation of content of RelA protein by Western blotting. β-Actin served as a loading control. (B) Enzyme-Linked Immunosorbent assay (ELISA) performed on cell supernatants from C10 cells transfected with either scrambled (sc) siRNA or RelA siRNA. Cells were treated with LPS for the indicated times, and evaluated for content of IL-6, CCL-20, RANTES, and KC. * p< 0.05 (ANOVA), compared to untreated controls: † p<0.05 (ANOVA) compared to scrambled siRNA groups at the same timepoint. Results are representative of at least 3 independent experiments.

Figure 4: Impact of RelB of LPS-induced production of pro-inflammatory mediators. (A) Verification of RelB knockdown in C10 cells. Whole cell lysates were prepared from cells transfected with either scrambled siRNA or RelB siRNA. 20µg protein was separated by SDS-PAGE, and Western blotting, for evaluation of content of RelB protein by Western blotting. β-Actin served as a loading control. (B) Enzyme-Linked Immunosorbent assay (ELISA) performed on cell supernatant from C10 cells transfected with either scrambled (sc) siRNA or RelB siRNA, treated with LPS for the indicated times, and evaluated for content of IL-6, CCL-20, RANTES, and KC. * p< 0.05 (ANOVA), compared to untreated controls: † p<0.05 (ANOVA) compared to scrambled siRNA groups at the same timepoint. Results are representative of at least 3 independent experiments.

Figure 5: Impact of IKK α for LPS-induced production of pro-inflammatory mediators. (A) Verification of IKK α knockdown in C10 cells. Whole cell lysates were prepared from cells transfected with either scrambled siRNA or IKK α siRNA. 20 μ g protein was separated by SDS-PAGE, and Western blotting, for evaluation of content of IKK α protein by Western blotting. β -Actin served as a loading control. (B) Enzyme-Linked Immunosorbent assay (ELISA) performed on cell supernatant from C10 cells transfected with either scrambled (sc) siRNA or IKK α siRNA, treated with LPS for the indicated times, and evaluated for content of IL-6, CCL-20, RANTES, and KC. * $p < 0.05$ (ANOVA), compared to untreated controls; † $p < 0.05$ (ANOVA) compared to scrambled siRNA groups at the same timepoint. Results are representative of at least 3 independent experiments.

Figure 6: Expression of constitutively active (CA) IKK α and IKK β in lung epithelial cells increases production of pro-inflammatory mediators in a cooperative manner. (A) Confirmation of expression of CA-IKK α and CA-IKK β in C10 lung epithelial cells. C10 cells were transfected with either 1 μ g pcDNA3, CA-IKK α , or CA-IKK β . 24 h later, cytosolic extracts were prepared and 20 μ g protein was evaluated via Western Blot analysis for content of IKK α and IKK β . β -Actin: loading control. Lower panels: Assessment of nuclear content of RelA, RelB, p50, and p52 following expression of CA-IKK α , or CA-IKK β . Histone H3: loading control. (B) Assessment of mRNA expression (left) and overall content (right) of CCL-20, MIP-2, RANTES and GM-CSF in C10 cells 24 h after transfection with pcDNA3, CA-IKK α , or CA-IKK β , as described in A. mRNA expression was determined via real time PCR analysis, while chemokine

content was evaluated by Enzyme-Linked Immunosorbent assay (ELISA) performed on cell supernatants. * $p < 0.05$ (ANOVA), compared to pcDNA3-transfected controls. (C) Western blot of C10 cells transfected with indicated amounts of CA-IKK α and/or CA-IKK β , or pcDNA3 as a control. All transfections were equalized to 1 μ g DNA/dish with pcDNA3. 24 h later cytosolic extracts were prepared for analysis of IKK α and IKK β to confirm enhanced expression. β -Actin: loading control. Lower Panels: Evaluation of content of RelA, RelB, p50 and p52 in nuclear extracts. Histone H3: Loading control. (D) ELISA analysis to assess content of CCL-20, MIP-2, RANTES, and GM-CSF in cell supernatants from transfection conditions indicated in (C). * $p < 0.05$ (ANOVA), compared to pcDNA3 transfected controls. (E) Assessment of overall content of CCL-20, MIP-2, RANTES, and GM-CSF in C10 cells transfected with CA-IKK α or CA-IKK β following ablation of RelA or RelB with siRNA. Chemokine content was assessed by ELISA. * $p < 0.05$ (ANOVA), compared to respective pcDNA3-transfected controls, † $p < 0.05$ (ANOVA), compared to CA-IKK α or CA-IKK β , and scrambled siRNA-transfected conditions. (F) Primary mouse tracheal epithelial cells were infected with either adeno-null (control) vector or adenovirus expressing CA-IKK α . After 48 or 72 h whole cell lysates were prepared to confirm increased expression of Hemeagglutinin-tagged constitutively active IKK α . (G) Assessment of content of KC, GM-CSF, MIP-2, and CCL-20 in supernatants from cells transduced with adenovirus expressing CA-IKK α or control virus as described in (F). All data are representative of at least 3 independent experiments. * $p < 0.05$ (ANOVA), compared to control vector.

Figure 7: LPS-mediated activation of classical and alternative NF- κ B pathways is mediated via IKK γ . (A) C10 cells were exposed to NEMO binding domain peptide (NBD) or mutant peptide as a control. 2 h later, cells were exposed to LPS for 30 min or 4 h. Nuclear extracts were prepared for evaluation of I κ B α , nuclear RelA, RelB, p52 and p50. Histone H3: loading control. (B) Schematic summarizing the coordinate requirement of classical and alternative NF- κ B pathways, controlled by IKK β /RelA, and IKK α /RelB, respectively in regulating pro-inflammatory signaling in lung epithelial cells.

Figure 1: Classical and alternative NF- κ B activation

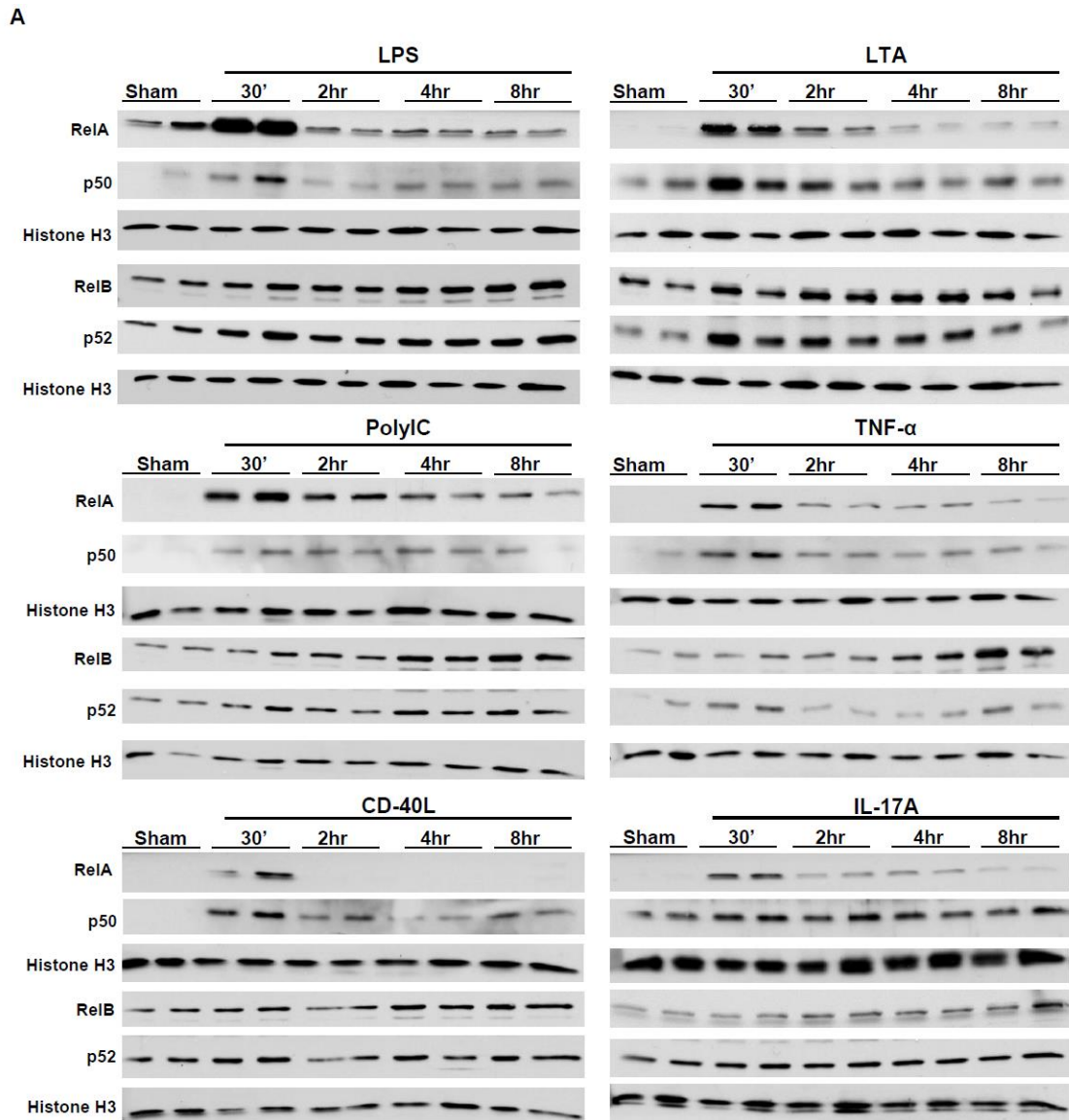


Figure 1 cont: Classical and alternative NF- κ B activation

B

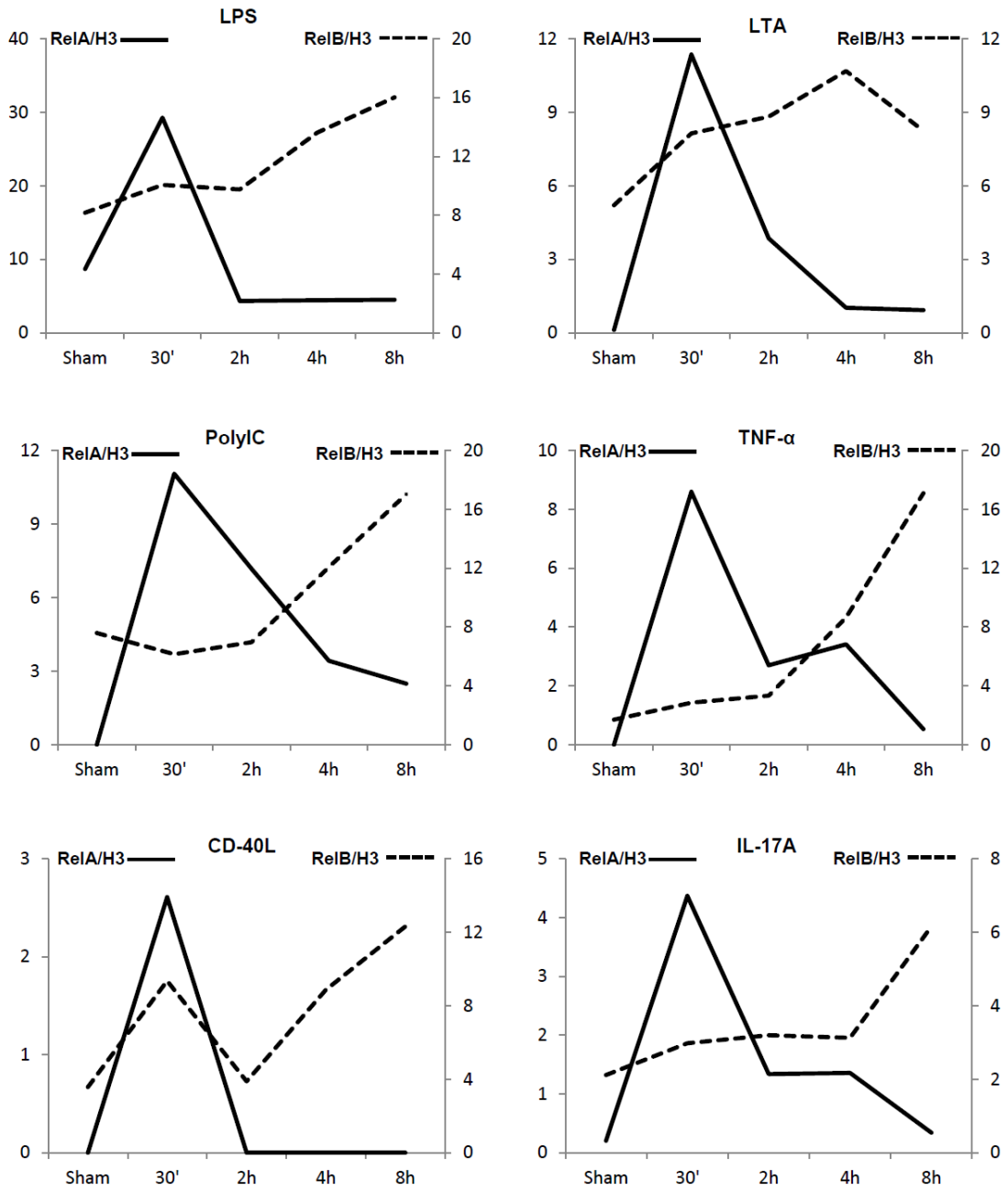


Figure 1 cont: Classical and alternative NF- κ B activation

C

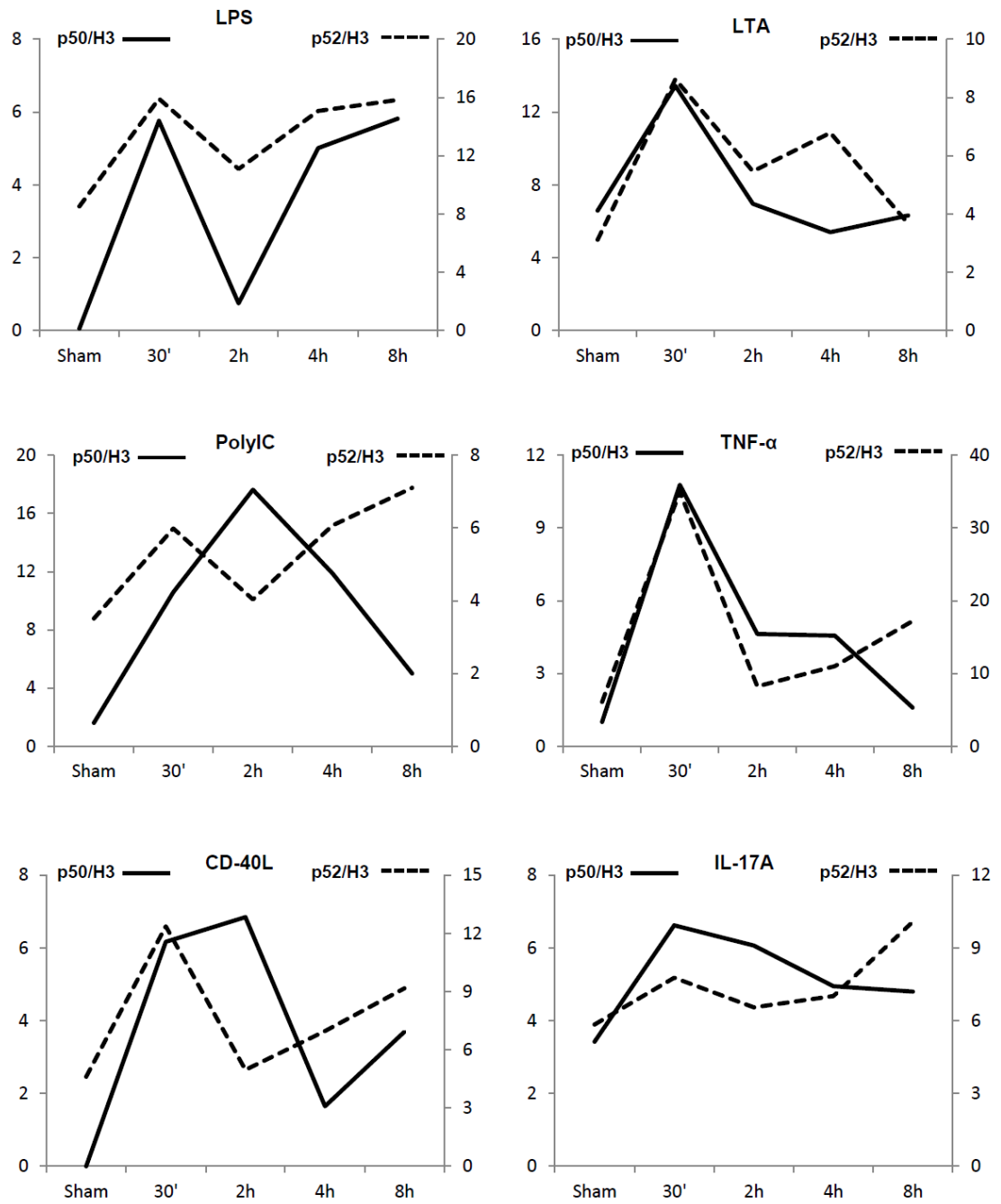
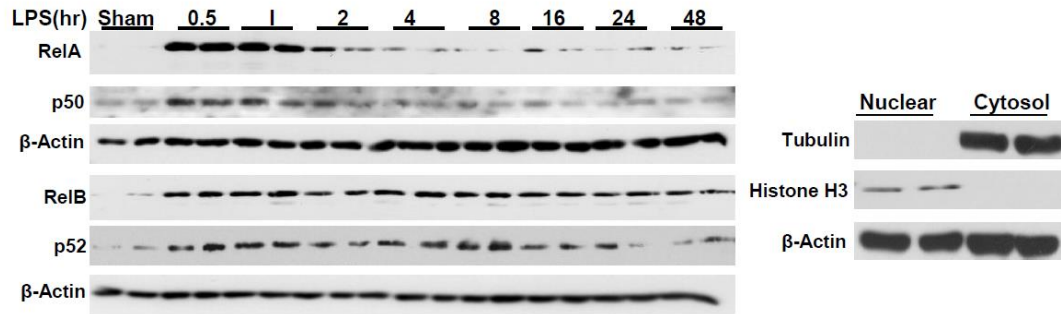
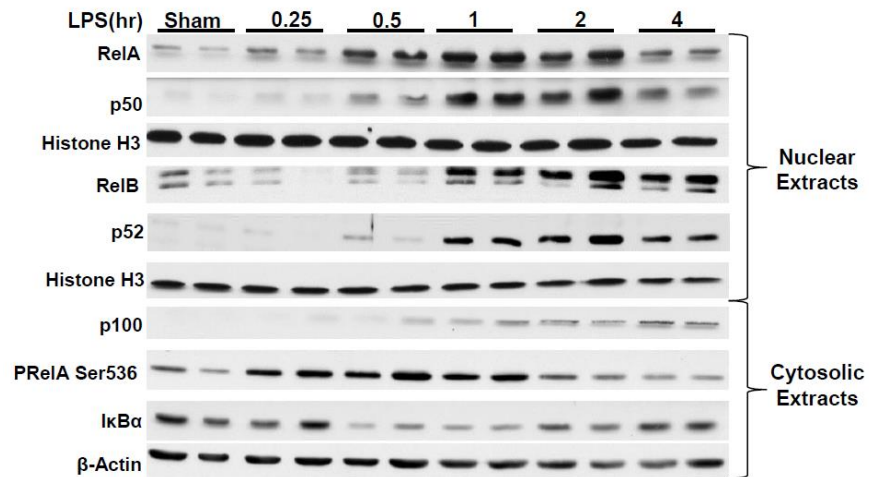


Figure 2: LPS-induced activation of Classical and alternative NF- κ B

A



B



C

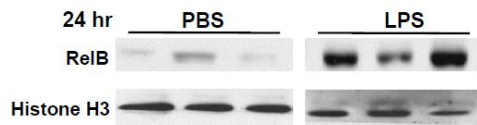


Figure 2 cont: LPS-induced activation of Classical and alternative NF- κ B

D

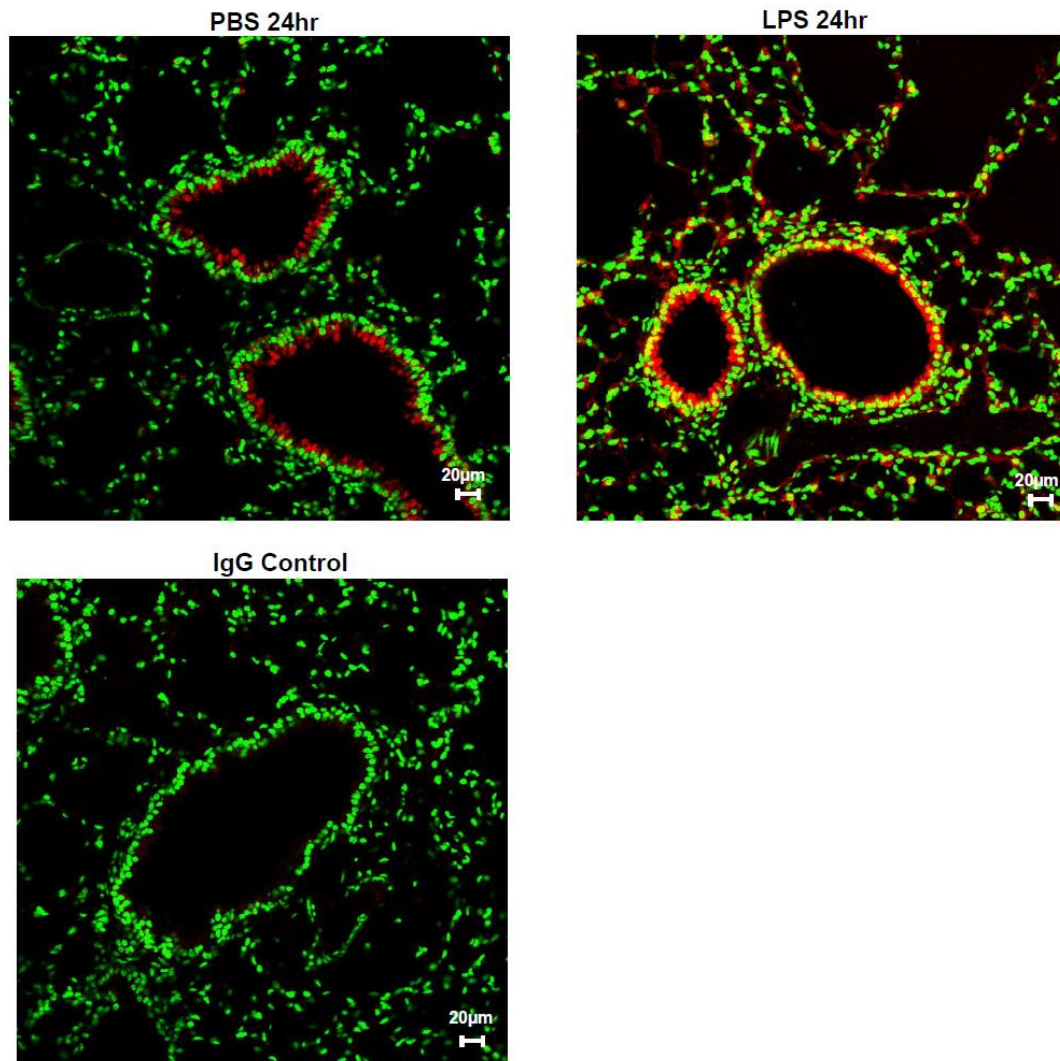
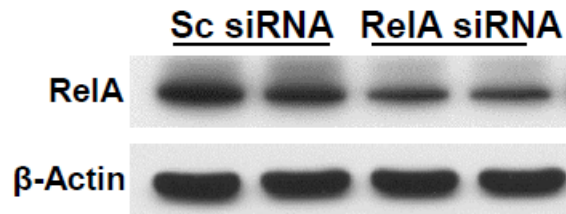


Figure 3: RelA-induced pro-inflammatory cytokine production

A

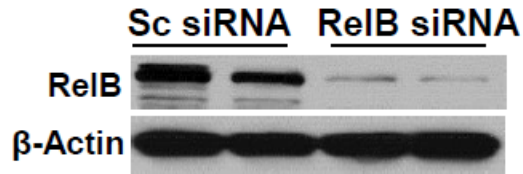


B

| <u>LPS</u> | <u>Sc siRNA</u> | <u>RelA siRNA</u> |
|-----------------------|--------------------|--------------------|
| <u>IL-6 (pg/ml)</u> | | |
| - | 10.9 \pm 1.1 | 8.6 \pm 0.9 |
| 6 | 31.8 \pm 1.4* | 19.6 \pm 1.5*† |
| 24 | 39.4 \pm 2.5* | 33.7 \pm 1.2* |
| <u>CCL-20 (pg/ml)</u> | | |
| - | 11.0 \pm 1.7 | 8.1 \pm 0.3 |
| 6 | 323.9 \pm 34.7* | 90.9 \pm 7.7*† |
| 24 | 452.0 \pm 21.1* | 142.7 \pm 6.5*† |
| <u>RANTES (pg/ml)</u> | | |
| - | 89.1 \pm 6.9 | 37.3 \pm 3.0 |
| 6 | 492.4 \pm 2.1* | 91.2 \pm 5.3† |
| 24 | 1290.0 \pm 78.0* | 232.0 \pm 19.0*† |
| <u>KC (ng/ml)</u> | | |
| - | 4.7 \pm 0.6 | 2.5 \pm 1.0 |
| 6 | 26.4 \pm 1.3* | 7.6 \pm 1.3 |
| 24 | 82.9 \pm 7.3* | 34.0 \pm 4.8*† |

Figure 4: RelB-induced pro-inflammatory cytokine production

A

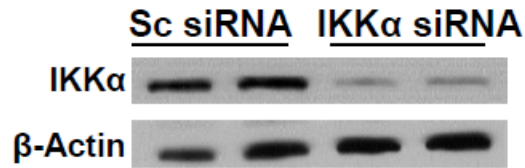


B

| <u>LPS</u> | <u>Sc siRNA</u> | <u>RelB siRNA</u> |
|-----------------------|------------------|---------------------|
| <u>IL-6 (pg/ml)</u> | | |
| - | 10.9 \pm 1.1 | 38.3 \pm 9.4 |
| 6 | 31.8 \pm 1.4* | 103.9 \pm 23.6*† |
| 24 | 39.4 \pm 2.5* | 304.8 \pm 37.8*† |
| <u>CCL-20 (pg/ml)</u> | | |
| - | 7.9 \pm .74 | 0.4 \pm .04 |
| 6 | 606.0 \pm 8.5* | 183.0 \pm 7.1*† |
| 24 | 635.0 \pm 9.9* | 205.0 \pm 4.6*† |
| <u>RANTES (pg/ml)</u> | | |
| - | 89.1 \pm 6.9 | 36.5 \pm 8.0 |
| 6 | 492.4 \pm 2.1* | 349.0 \pm 2.0* |
| 24 | 1290.0 \pm 78* | 1099.0 \pm 94.0*† |
| <u>KC (ng/ml)</u> | | |
| - | 4.7 \pm 0.6 | 4.8 \pm 1.5 |
| 6 | 26.4 \pm 1.3* | 22.5 \pm 5.4 |
| 24 | 82.9 \pm 7.3* | 53.6 \pm 1.8*† |

Figure 5: IKK α -induced pro-inflammatory cytokine production

A



B

| LPS | Sc siRNA | IKK α siRNA |
|-----------------------|------------------|--------------------|
| IL-6 (pg/ml) | | |
| - | 0.4 \pm 0.2 | 3.9 \pm 0.9 |
| 6 | 2.6 \pm 0.8* | 24 \pm 3.5*† |
| 24 | 7.8 \pm 1.7* | 33 \pm 2.4*† |
| CCL-20 (pg/ml) | | |
| - | 7.2 \pm 2 | 3.9 \pm 3 |
| 6 | 285 \pm 7.6* | 110 \pm 17*† |
| 24 | 407 \pm 53* | 156 \pm 2.0*† |
| RANTES (pg/ml) | | |
| - | 434 \pm 50 | 421 \pm 282 |
| 6 | 1155 \pm 51.1* | 200 \pm 18† |
| 24 | 2139 \pm 91.5* | 479 \pm 4.2† |
| KC (ng/ml) | | |
| - | 5.8 \pm .05 | 4.3 \pm .01 |
| 6 | 21 \pm .90* | 21 \pm 1.2* |
| 24 | 32 \pm 1.0* | 32 \pm .08* |

Figure 6: Classical and alternative NF- κ B cooperatively enhance pro-inflammatory cytokines

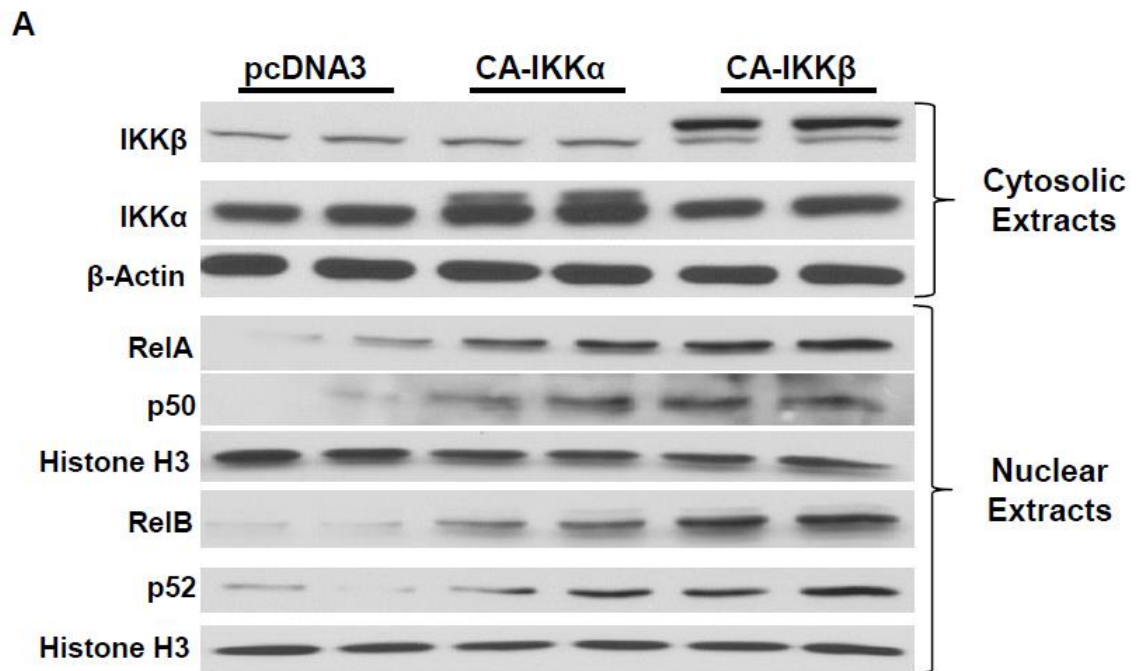


Figure 6: Classical and alternative NF- κ B cooperatively enhance pro-inflammatory cytokines

B

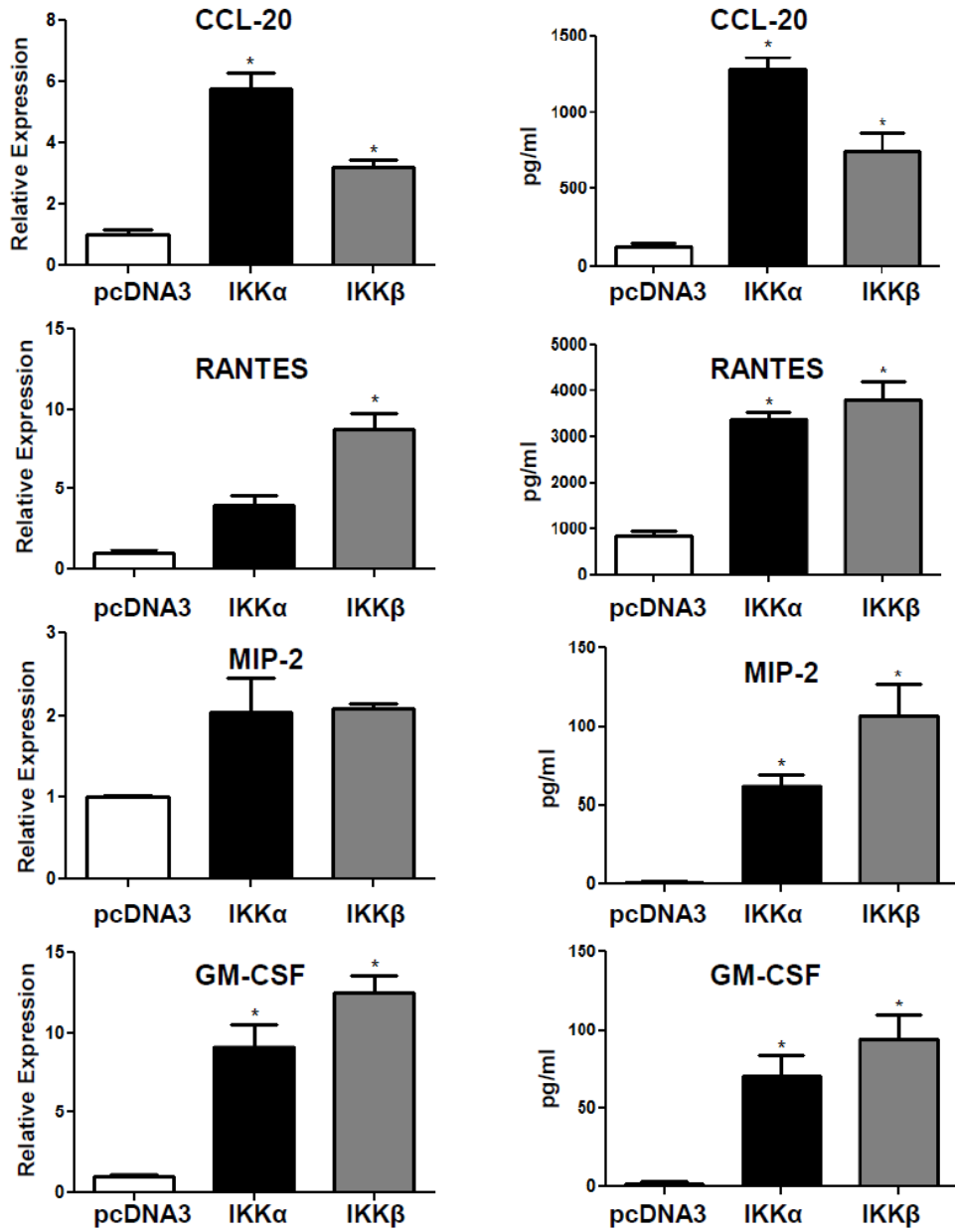
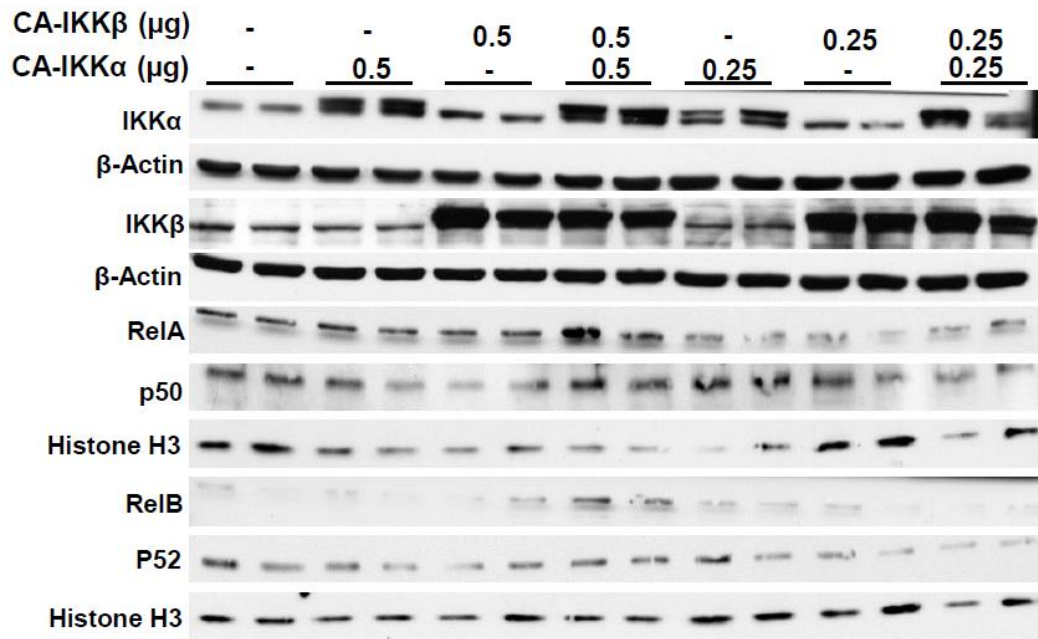


Figure 6 cont: Classical and alternative NF- κ B cooperatively enhance pro-inflammatory cytokines

C



D

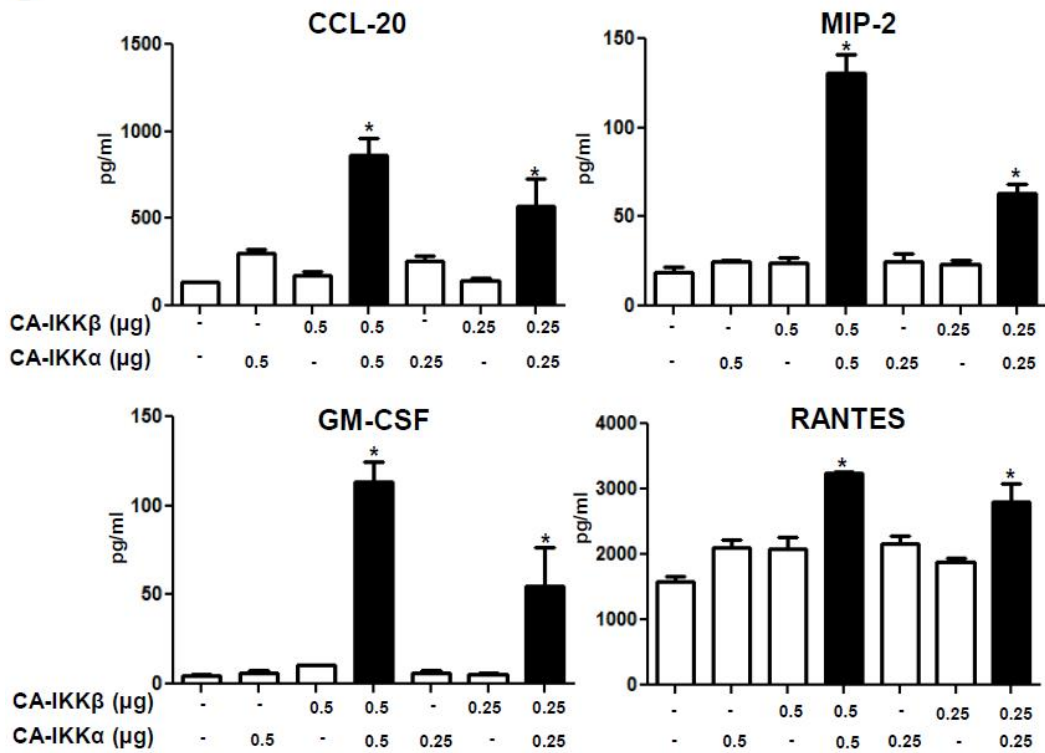


Figure 6 cont: Classical and alternative NF- κ B cooperatively enhance pro-inflammatory cytokines

E

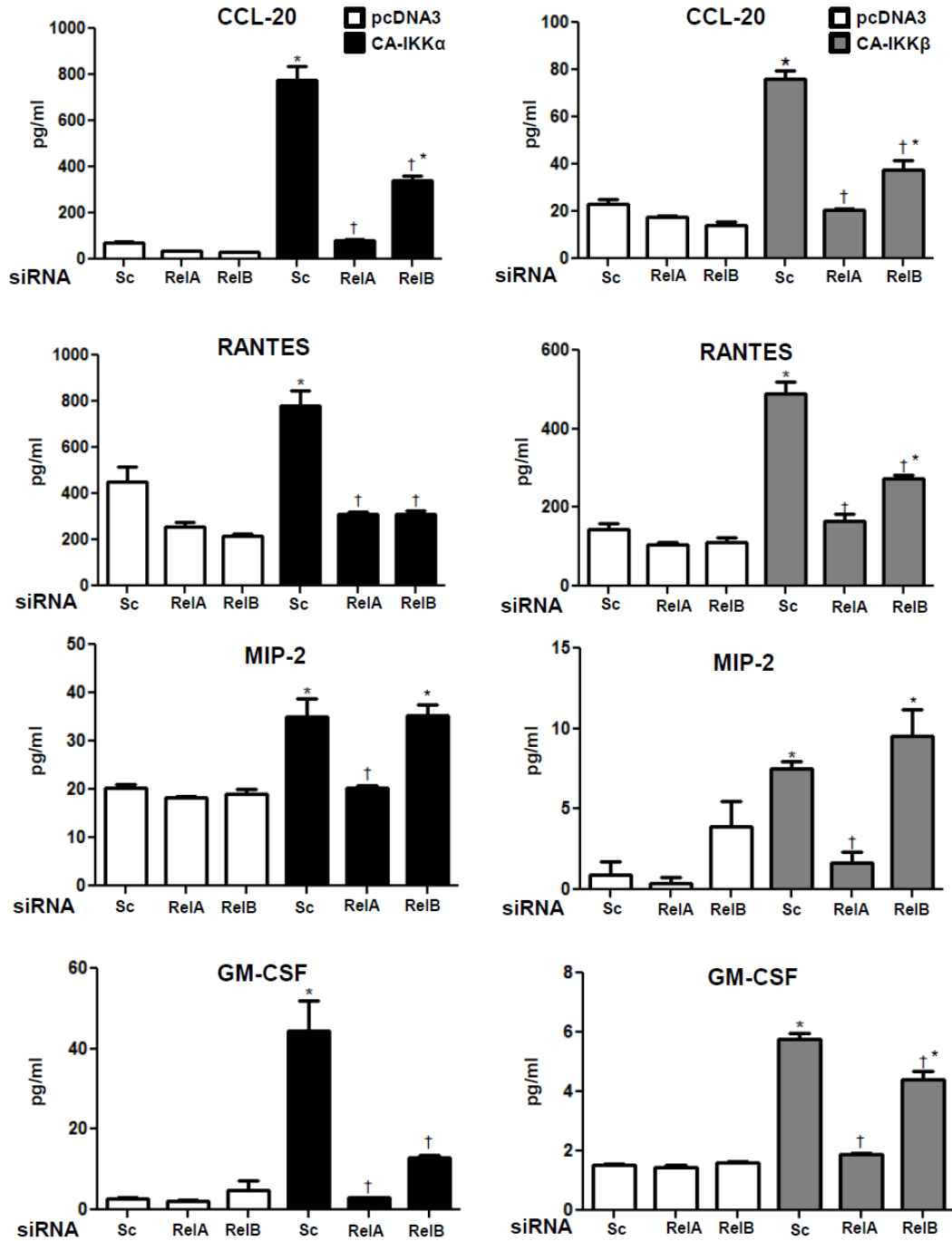


Figure 6 cont: Classical and alternative NF- κ B cooperatively enhance pro-inflammatory cytokines

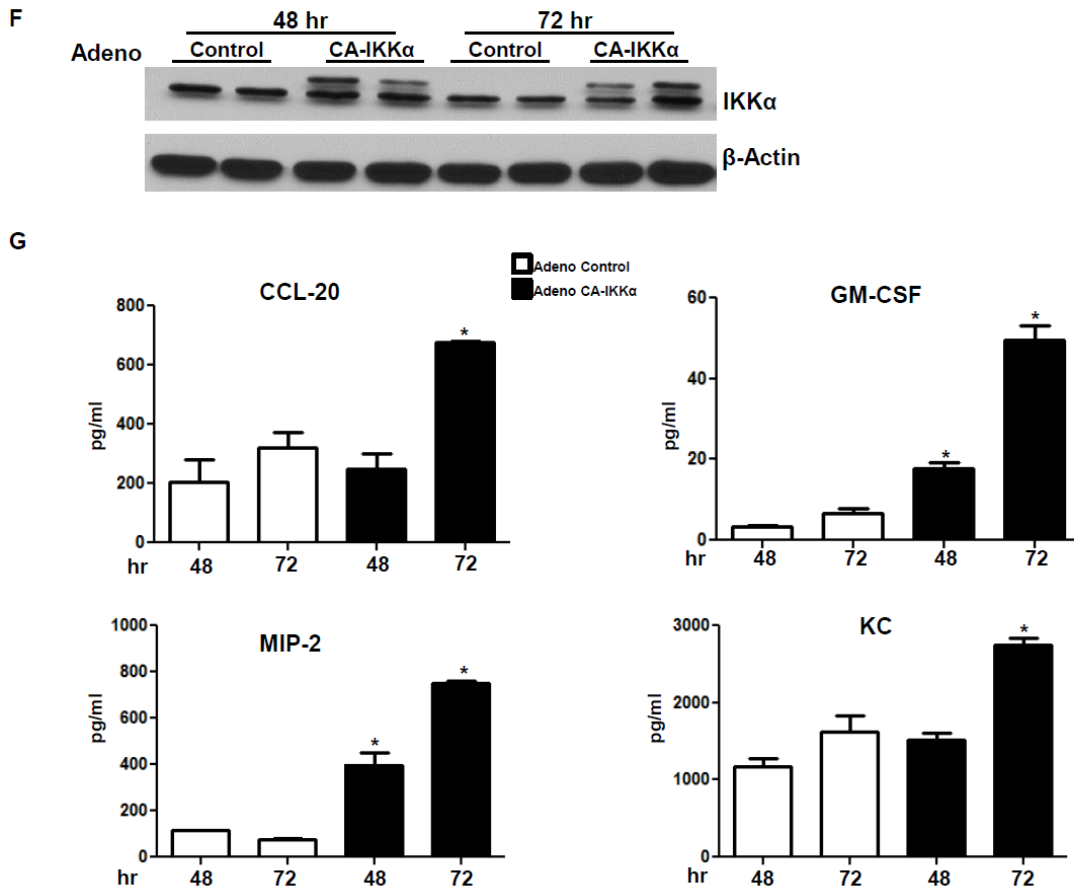
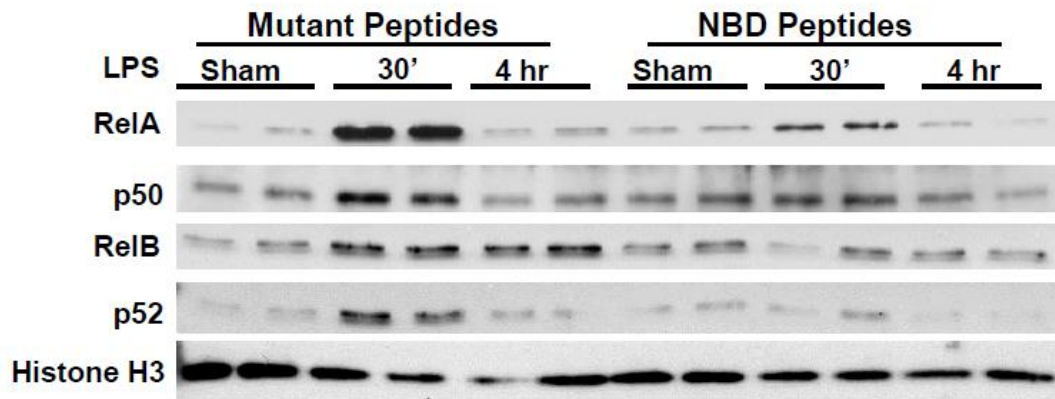
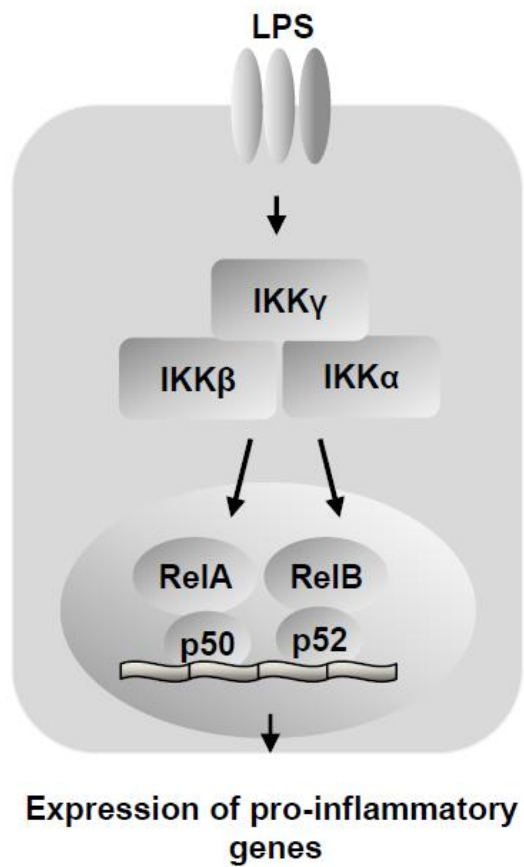


Figure 7: IKK signalsome regulation of classical and alternative NF- κ B

A



B



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Chapter 3

Epithelial Nuclear Factor- kappa B orchestrates house dust mite-induced airway inflammation, hyperresponsiveness, and fibrotic remodeling

Abstract

NF- κ B activation within the epithelium has been implicated in the pathogenesis of asthma, yet the exact role of epithelial NF- κ B in allergen-induced inflammation and airway remodeling remains unclear. In the present study, we utilized an intranasal House Dust Mite (HDM) extract exposure regimen time course in BALB/c mice to evaluate inflammation, NF- κ B activation, airway hyperresponsiveness (AHR), and airway remodeling. We utilized CC10-I κ B α_{SR} transgenic mice to evaluate the functional importance of epithelial NF- κ B in response to HDM. After a single exposure of HDM, mRNA expression of pro-inflammatory mediators was significantly elevated in lung tissue of WT mice, in association with increases in nuclear RelA and RelB, components of the classical and alternative NF- κ B pathway, respectively, in the bronchiolar epithelium. In contrast, CC10-I κ B α_{SR} mice displayed marked decreases in nuclear RelA and RelB and mRNA expression of pro-inflammatory mediators compared to WT mice. After 15 challenges with HDM, WT mice exhibited increases in inflammation, airway hyperresponsiveness, mucus metaplasia and peri-bronchiolar fibrosis. CC10-I κ B α_{SR} transgenic mice displayed marked decreases in neutrophilic infiltration, tissue damping, and elastance parameters, in association with less peri-bronchiolar fibrosis and decreases in nuclear RelB in lung tissue. However, central airway resistance and mucus metaplasia remained elevated in CC10-I κ B α_{SR} transgenic mice, in association with continued

presence of lymphocytes, and partial decreases in eosinophils and IL-13. The current study demonstrates that following airway exposure with an asthma-relevant allergen, activation of classical and alternative NF- κ B pathways occur within the airway epithelium and may coordinately contribute to allergic inflammation, AHR and fibrotic airway remodeling.

Introduction

The NF- κ B pathway is a critical regulator of both innate and adaptive immune responses in a wide variety of cell types. Upon stimulation, the I kappa B kinase (IKK) complex, consisting of IKK β , IKK α , and IKK γ , is activated, leading to IKK β -mediated phosphorylation of I κ B α . Phosphorylation of I κ B α in turn leads to its subsequent ubiquitination and degradation by the 26S proteasome, thus allowing for transcription factor, RelA, to translocate to the nucleus. This event results in RelA-dependent transcription of genes important in cell survival, proliferation, and inflammation (Hayden & Ghosh, 2004; Scheidereit, 2006). A wide variety of agonists can activate the classical NF- κ B pathway in lung epithelial cells and the resultant release of pro-inflammatory mediators crucial in the recruitment and activation of dendritic cells, lymphocytes, neutrophils, and many other cells in the lung (Swamy et al., 2010). Additionally, an alternative NF- κ B pathway exists, which requires activation of NF- κ B inducing kinase (NIK) and subsequent phosphorylation of IKK α . IKK α in turn phosphorylates p100, leading to its partial processing to p52. This allows subsequent nuclear translocation of RelB/p52 and transcriptional activation of a subset of NF- κ B dependent genes (Oeckinghaus et al., 2011; Senftleben et al., 2001). It was originally thought that the alternative NF- κ B pathway played a predominant role in lymphocyte activation and lymphoid organ development (G. Xiao et al., 2006). However, recent work from our laboratory demonstrated that both classical and alternative NF- κ B pathways are activated in lung epithelial cells in response to diverse pro-inflammatory stimuli and that both pathways coordinately regulate pro-inflammatory responses (Tully et al., 2012).

Activation of the classical NF- κ B pathway within the airway epithelium has been demonstrated to play a critical role in acute inflammation and allergic airways disease. CC10-I κ B α_{SR} transgenic mice, which are refractory to I κ B α degradation and NF- κ B activation in the lung epithelium, were demonstrated to be strongly protected from airway inflammation induced by lipopolysaccharide (Poynter et al., 2003). Following intraperitoneal sensitization and challenge with ovalbumin (Ova), CC10-I κ B α_{SR} transgenic mice showed a marked diminution of airway inflammation compared to WT littermate controls, although Ova-induced airways hyperresponsiveness (AHR) was unaffected in CC10-I κ B α_{SR} transgenic mice compared to controls (Poynter et al., 2004). A similar protection against Ova-induced allergic inflammation and peri-bronchiolar fibrosis has been observed in mice following epithelial-specific ablation of IKK β (Broide et al., 2005).

It remains unclear to date whether activation of NF- κ B within epithelial cells plays a role in the orchestration of inflammatory responses *in vivo* to an asthma-relevant allergen following sensitization via the airways. It also remains unknown whether both NF- κ B pathways are activated following exposure to an antigen. House dust mite (HDM) is a multifaceted allergen to which up to 85% of asthmatics are allergic (Gregory & Lloyd, 2011). HDM has been shown to signal through the classical NF- κ B pathway in human bronchial epithelial cells *in vitro* (Osterlund et al., 2009). Therefore, the goal of the present study was to determine the activation of classical and alternative NF- κ B in epithelial cells *in vivo* in response to HDM, and to address its effect on HDM-triggered airway inflammation, remodeling, mucus, and AHR. Our results demonstrate the

functional importance of epithelial NF- κ B in HDM-induced acute inflammatory responses, AHR, and airway remodeling. We also demonstrate activation of both classical and alternative NF- κ B pathways in response to HDM. These findings illustrate the complexity of activation of the NF- κ B pathways in settings of allergic airways disease and suggest a broader role for epithelial NF- κ B in lung disease pathogenesis.

Materials and Methods

Animal Studies: CC10-I κ B α_{SR} mice were generated as previously described (Poynter et al., 2003) and backcrossed onto the BALB/cJ (N=10) (The Jackson Laboratories, Bar Harbor, ME) background. Transgene-negative littermates were used as a control. All experiments were approved by the University of Vermont Institutional Animal Care and Use Committee.

Cell Culture: A human bronchial epithelial cell line (HBE) was kindly provided by Dr. Albert van der Vliet and cultured as described previously (Olson et al., 2009; R. Wu, Zhao, & Chang, 1997) and primary human nasal epithelial cells were cultured as described previously (Jaspers et al., 2005). Human cell lines were exposed to either PBS or 25 μ g HDM (Greer, Lenoir, NC). All protocols that utilize primary human nasal epithelial cells were approved by the Institutional Review Board.

HDM exposure: BALB/cJ mice (Jackson Laboratories, Bar Harbor, ME) were subjected to daily intranasal instillation with 50 μ g HDM (35 endotoxin units/mg) extract resuspended in PBS, or PBS alone as a vehicle control. Briefly, mice were instilled with either 1 dose of HDM and euthanized: 2 h, 6 h, or 24 h later or instilled with 3 doses of

HDM on 3 consecutive days and euthanized 24 h thereafter. In addition, mice were exposed to HDM 5 days/week for 1, 2 or 3 weeks, (5, 10 or 15 instillations, respectively), and harvested 72 h after the final exposure (Fig. 1A).

Assessment of AHR: Mice subjected to 5, 10, or 15 administrations of HDM were anesthetized with an intraperitoneal injection of pentobarbital sodium (90mg/kg), tracheotomised, and mechanically ventilated at 200 breaths/minute and assessed in response to increasing doses of methacholine (0, 3.125mg, 12.5mg, and 25mg). Respiratory mechanics were assessed with a forced oscillation technique on a computer controlled small animal ventilator (Flexi-vent™, SCIREQ, QC, Canada), as previously described (Pantano et al., 2008; Riesenfeld et al., 2012), and the parameters Newtonian resistance (R_n), tissue damping (G), and elastance (H) were calculated.

Serum IgG1 and IgE: Following euthanization, blood was collected by heart puncture and immediately spun through a microtainer and serum was separated. Analysis of serum IgG1 and IgE was performed via enzyme-linked immunosorbent assay (ELISA) methods, using 1 µg/ml HDM to coat the 96 well plates.

Bronchoalveolar lavage: Following euthanization, bronchoalveolar lavage (BAL) was collected using 1ml of PBS. Cell counts were determined (Advia 120 automated hematology analyser), and differential cells were analysed by the Hema3 kit (Fisher Scientific, Kalamazoo, MI) by counting a minimum of 300 cells per mouse as previously described (Pantano et al., 2008).

mRNA/protein analysis: Right lung lobes were flash frozen and pulverized for protein and mRNA. Total mRNA was isolated using the RNeasy kit (Qiagen, Valencia,

CA). 1 µg of mRNA was used to generate cDNA, followed by quantitative polymerase chain reaction (qPCR) using SYBR green (BioRad, Hercules, CA) via in order to assess expression of: Muc5ac, KC, MIP-2, GM-CSF, IL-6, CCL20, and IL-33. Primers were:

KC (Fwd:5'-GCTGGATTCACCTCAAGAA-3', Rv:5'-TGGGGACACCTTTTAGCATC-3'), IL-6 (Fwd:5'-CTGATGCTGGTGACAACCAC-3', Rv:5'-CAGAATTGCCATTGCACAAC-3'), CCL20 (Fwd:5'-AAGACAGATGGCCGATGAAG-3', Rv:5'-AGCCCTTTTCACCCAGTTCT-3'), Muc5ac (Fwd:5'-CAGTGAATTCTGGAGGCCAACAAGGTAGAG-3', Rv:5'-AGCTAAGCTTAGATCTGGTTGGGACAGCAGC-3'), MIP-2 (Fwd:5'-AGTGAAGTGCCTGTCAATG-3', Rv:5'-TTCAGGGTCAAGGCAAACCTT-3'), GM-CSF (Fwd:5'-GGCCTTGGAAGCATGTAGAGG-3', Rv:5'-GGAGAAGTTCGTTAGAGACGACTT-3'), and IL-33 (Fwd:5'-GCTGCGTCTGTTGACACATT-3', Rv:5'-GACTTGCAGGACAGGGAGAC-3'). The expression of all genes was normalized to the housekeeping gene, cyclophilin, (Fwd:5'-TTCCTCCTTTCACAGAATTATTCCA-3', Rv:5'-CCGCCAGTGCCATTATGG-3') and calculated via the ddCt method. Antibodies for Western blot analysis were: RelB and NIK (Santa Cruz Biotchnologies, Santa Cruz, CA), Histone H3, p52, and phospho RelA536 (Cell Signaling Technology, Danvers, MA) and β-Actin (Sigma-Aldrich, St. Louis, MO). Enzyme-linked Immunosorbent assays (ELISA) were performed according to manufacturer's instructions (R & D Systems, Minneapolis, MN) on either BAL or homogenized lung extracts. Protein was equalized before analysis.

Histopathology/immunofluorescence/ α SMA immunohistochemistry: Following euthanization, the left lobe was inflated with 4% paraformaldehyde and mounted in paraffin embedded 5 μ m sections. Hematoxylin and Eosin (H&E) and Periodic Acid Schiff (PAS) imaging were all performed with 3 small bronchioles (x20 magnification) per animal. Mucus metaplasia was assessed with a blinded scoring system by two independent investigators with the following scale: 0, no reactivity; 1, minimal staining; 2, moderate staining; and 3, prominent staining. Scores were averaged according to treatment group. Immunofluorescence was performed as previously described to detect nuclear RelA and RelB *in situ* (Poynter et al., 2002). RelA and RelB antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Staining for α -SMA was performed on lung sections following incubation of slides for 20 minutes in 0.01M sodium citrate, pH 6.0 at 95°C. Slides were then blocked with 2% normal goat serum for 30 minutes, incubated with monoclonal mouse antibody against α -SMA (1:5000 dilution; Sigma Aldrich, St. Louis, MO) overnight at 4°C, and then incubated in biotinylated anti-mouse IgG for 30 minutes at room temperature. Subsequently, the slides were incubated in avidin-biotin- complex-alkaline phosphatase (Vectastain ABC-AP, Vector Laboratories, Burlingame, CA) for another 30 minutes at room temperature. After rinsing the sections in PBS, the substrate, Vector Red (Vector Laboratories), was added for 20 minutes, which reacts with the bound alkaline phosphatase, thus producing an intense red color. The slides were then counterstained in hematoxylin and imaged (x20 magnification) for analysis.

Assessment of collagen content: Collagen was assessed from the upper right lobe of the lung after an overnight digestion with 10mg pepsin in 0.5M acetic acid. Quantification was performed by the Sircol Assay according to manufacturer's instructions (Accurate Chemical and Scientific corp, Westbury, NY). Masson's Trichrome reactivity was evaluated in 3 bronchioles (x20 magnification) per animal, and analyzed by two blinded investigators with the following scale: 0, no reactivity; 1, minimal staining; 2, moderate staining; and 3, prominent staining.

Statistical Analysis: All data were evaluated using Graphpad Prism 6 Software (Graphpad, Inc, San Diego, CA). A one-way ANOVA was used with Bonferoni corrections to adjust for multiple comparisons and all p-values <0.05 were considered statistically significant. Histopathological/ α SMA IHC scores were analyzed using the Kruskal-Wallis test and Dunn's multiple comparison post hoc tests.

Results

Inflammatory response in BALB/c mice exposed to HDM: Because of the well-known role of NF- κ B in the orchestration of inflammation, we first sought to assess the extent and kinetics of the HDM-induced inflammatory response through a time-course analysis illustrated schematically in Fig. 1A. Neutrophils in bronchoalveolar lavage fluid (BAL) were significantly increased 6 h following a single intranasal challenge with HDM, and remained elevated until 24 h post 3 challenges, as compared to PBS controls (Fig. 1B). It is important to note that neutrophils were elevated 24 h following exposure to the vehicle PBS, potentially indicative of a non-specific response to the intranasal

instillation. Neutrophils were also detectable in BAL 72 h post 5, 10, or 15 challenges, with statistically significant increases occurring in response to HDM 72 h post 15 challenges. Although macrophages in BAL tended to increase in response to HDM, these trends were not statistically significant. No eosinophils or lymphocytes were detected in BAL up to 24 h post 3 challenges. In contrast, robust increases in eosinophils and lymphocytes occurred in BAL 72 h post 10 and 15 challenges with HDM compared to PBS controls (Fig. 1B). Although some fluctuations in the total number of macrophages in BAL were observed throughout these time points, these were not statistically significant (Fig. 1B).

Increases in nuclear localization of RelA and RelB in airway epithelium in response to HDM: We next evaluated a potential role for epithelial NF- κ B in response to HDM. We investigated kinetics of activation of both classical and alternative pathways in the bronchiolar epithelium via the assessment of nuclear presence of RelA and RelB. Nuclear presence of RelA and RelB (indicated by yellow staining) within the bronchiolar epithelium was increased 2 and 6 h following a single challenge with HDM, compared to PBS controls in the nuclei (Fig. 2). Nuclear RelA decreased to control reactivity by 24 h, but increased again 24 h post- 3 challenges with HDM, indicative of a bi-modal activation pattern (Fig. 2). In contrast, increases in nuclear RelB were apparent throughout the time course evaluated here (Fig. 2). No clear evidence for nuclear localization of RelA or RelB within the airway epithelium was apparent 72 h after 5, 10 or 15 challenges with HDM compared to PBS (data not shown).

Pro-inflammatory mediator expression following acute HDM exposures: In order to investigate the early inflammatory response after HDM exposure, we evaluated mRNA expression of Interleukin-33 (IL-33), Keratinocyte Derived Chemokine (KC), Granulocyte Macrophage Colony Stimulating-Factor (GM-CSF), Macrophage Inflammatory Protein-2 (MIP-2), Chemokine (C-C motif) ligand 20 (CCL20), Interleukin-6 (IL-6), Interleukin-25 (IL-25) and Thymic Stromal Lymphopoietin (TSLP), pro-inflammatory mediators shown to be produced by epithelial cells. Expression levels of all genes analyzed were significantly increased following 2 h exposure HDM, with the exception of IL-25 and TSLP, whose expression did not change (data not shown). mRNA expression of CCL20, GM-CSF, MIP-2, KC, and IL-6 remained elevated 6 h after a single administration of HDM, and tended to decrease towards control levels thereafter (Fig S1).

Role of epithelial NF- κ B in pro-inflammatory cytokine expression induced by HDM: We next sought to address the role of epithelial NF- κ B in HDM-induced pro-inflammatory responses utilizing CC10-I κ B α_{SR} transgenic mice. While nuclear RelA and RelB content increased in the bronchiolar epithelium in WT mice in response to HDM, these increases were not observed in CC10-I κ B α_{SR} mice exposed to HDM (Fig 3A). HDM-mediated increases in mRNA expression of CCL20, GM-CSF, MIP-2, KC, IL-33, and IL-6 in WT mice were strongly attenuated in CC10-I κ B α_{SR} mice (Fig. 3B), demonstrating the importance of NF- κ B activation in the bronchiolar epithelium in the orchestration of the acute pro-inflammatory responses to HDM.

Impact of RelB knockdown on HDM-induced pro-inflammatory cytokine expression: We demonstrate that classical and alternative NF- κ B are both inhibited via expression of CC10-I κ B α_{SR} transgene; therefore, we aimed to specifically evaluate the role of the alternative pathway in HDM-induced inflammation. BALB/c mice were given indicated siRNA oropharyngeally 2 and 4 days prior to 2 h HDM exposure. Immunofluorescence analysis demonstrated knockdown of RelB in the bronchiolar epithelium (Fig. 4A). Evaluation of pro-inflammatory cytokine expression showed a significant increase in CCL20, GM-CSF, MIP-2, KC, IL-33, and IL-6 in scrambled siRNA HDM groups compared to PBS controls (Fig. 4B-G). Interestingly, in RelB siRNA HDM groups, pro-inflammatory gene expression did not increase over PBS controls, indicating a strong role for alternative NF- κ B in regulating HDM-induced inflammatory responses.

Effects of repeated HDM exposure on AHR, mucus metaplasia, and remodeling: We next subjected mice to 5, 10, and 15 challenges of HDM. Serum content of HDM-specific IgG1 and IgE showed no apparent increases 72 h following 5 days challenge. Marked increases in HDM-specific IgG1 and IgE were apparent following 10 and 15 challenges with HDM, compared to PBS controls, indicative of activation of adaptive immune responses (Fig. S2A). AHR, a feature of allergic airways disease, was assessed via forced oscillation mechanics using ascending doses of methacholine. Newtonian resistance (Rn) increased significantly over PBS controls after 10 and 15 challenges with HDM, while no changes were apparent in mice subjected to 5 challenges with HDM (Fig S2B). Tissue damping (G), which is indicative of tissue resistance and small airway

dysfunction, was increased following 5 challenges HDM, and remained increased throughout 10 and 15 challenges. Elastance (H) was also increased following 5, 10, and 15 challenges of HDM. Increases in elastance were most prominent after 10 challenges of HDM and tended to decrease after 15 challenges (Fig. S2B). Histo-pathological evaluation revealed a robust inflammatory response to HDM, with prominent peri-bronchiolar and perivascular cellular infiltrates being apparent following 10 and 15 challenges with HDM (Fig. S2C). Additionally, mucus metaplasia was apparent following 5, 10, and 15 challenges HDM, based upon staining with Periodic Acid Schiff reagent and Muc5ac expression (Fig. S2C and S2D). Another hallmark of severe allergic airways disease is peri-bronchiolar fibrotic remodeling. Following 15 challenges with HDM, overall collagen content increased in the lung tissue (Fig S2E), consistent with increases in peri-bronchiolar collagen deposition evaluated via Masson's Trichrome staining (Fig S2C). These results collectively demonstrate that the HDM exposure regimen used herein induces a number of the hallmark features of allergic airway disease.

Role of epithelial NF- κ B in HDM-induced inflammation: We next determined the role of epithelial NF- κ B on the inflammatory response induced following 15 challenges of HDM. HDM-mediated increases in total cell counts in BAL observed in WT mice were significantly decreased in CC10-I κ B α_{SR} mice (Fig. 5A). BAL eosinophils were significantly decreased in CC10-I κ B α_{SR} transgenic mice exposed to HDM, while BAL lymphocytes remained elevated in HDM exposed CC10-I κ B α_{SR} transgenic mice compared to WT littermates (Fig. 5B). Total macrophage number in BAL remained unchanged in CC10-I κ B α_{SR} mice (Fig. 5B). In contrast HDM-mediated increases in BAL

neutrophils in WT mice were completely attenuated in CC10-I κ B α_{SR} mice. Consistent with these observations, increases in BAL content of the neutrophil chemoattractant, KC, in WT mice exposed to HDM were absent in CC10-I κ B α_{SR} mice (Fig. 5C). HDM-specific IgG1 and IgE levels in serum were decreased slightly in CC10-I κ B α_{SR} HDM-exposed mice, although not significantly, compared to littermate controls, but remained elevated over PBS controls (Fig. 5D). Levels of IFN- γ , IL-13, and IL-17A in homogenized lung tissue were significantly decreased in CC10-NF- κ B $_{SR}$ mice exposed to HDM, compared to WT animals (Fig. 5E), collectively suggesting an attenuation of Th-mediated responses to HDM following epithelial-specific inhibition of NF- κ B. We next sought to assess inhibition of nuclear RelA and RelB in lung tissue in CC10-NF- κ B $_{SR}$ mice exposed to HDM. After 15 challenges with HDM, no consistent increases in nuclear RelA or RelB were detected in the bronchiolar epithelium (data not shown), in contrast to earlier time points at which increases in nuclear RelA and RelB were readily observed (Fig 3). Assessment of homogenized lung tissue also showed variable and inconsistent patterns of nuclear RelA (data not shown). However, clear increases in nuclear RelB content were observed in WT mice, which were absent in the CC10-I κ B α_{SR} transgenics (Fig. 5F).

Role of epithelial NF- κ B in HDM-induced AHR and remodeling: WT and CC10-I κ B α_{SR} mice subjected to 15 challenges with HDM demonstrated equivalent increases in Newtonian resistance (Rn). In contrast, HDM-mediated increases in tissue damping (G) and elastance (H) were significantly decreased in CC10-I κ B α_{SR} mice compared to littermate controls (Fig. 6A). Consistent with attenuated inflammatory cell profiles in

BAL, peri-bronchiolar infiltrates were attenuated CC10-I κ B α_{SR} mice compared to controls (Fig. 6B). In contrast, mucus metaplasia was not observed to be decreased in HDM-exposed CC10-I κ B α_{SR} mice, compared to WT animals (Fig. 6B+D). These findings are consistent with a lack of attenuation of HDM-induced Muc5AC mRNA (Fig. 6C) and modest decreases in IL-13 content (Fig. 5E). Biochemical analysis of collagen demonstrated that HDM-mediated increases in WT mice were significantly decreased in CC10-I κ B α_{SR} transgenic mice (Fig.6E), consistent with less peri-bronchiolar collagen detected via histopathology (Fig. 6B+F). Furthermore, α -Smooth Muscle Actin (α -SMA), a known marker of airway thickening and remodeling, was assessed via immunohistochemistry. Peri-bronchiolar α -SMA staining was significantly increased following HDM administration in WT mice, as compared to PBS controls. In contrast, no increases in peri-bronchiolar α -SMA reactivity were detected in CC10-I κ B α_{SR} mice exposed to HDM, in comparison to PBS controls (Fig. 6G+H).

HDM-induced activation of the classical and alternative NF- κ B pathways in human nasal and bronchial epithelial cells: Since we demonstrated increases in nuclear RelB in the bronchial epithelium in response to HDM administration, we sought to determine whether the alternative NF- κ B pathway could be activated directly by HDM in human epithelial cells. Results in Fig. 7A demonstrate increased levels of NIK and p52 in response to HDM in Primary Human Nasal Epithelial Cells (PHNEC) obtained from two independent donors, and similar increases were observed (Fig. 7B) in human bronchial epithelial cells (HBEC), albeit less robust, demonstrating activation of the alternative NF- κ B pathway. In addition, phosphorylation of RelA at serine 536, a separate parameter of

NF- κ B activation, was also increased in response to HDM (Fig. 7A+B), demonstrating that in addition to the known ability of HDM to activate the classical NF- κ B pathway, it also induces activation of the alternative NF- κ B pathway in lung epithelial cells.

Discussion

NF- κ B is a regulator of inflammation and immunity, and its role in the pathogenesis in asthma has been suggested based upon evidence of its activation in the bronchiolar epithelium from asthmatics (Zhao et al., 2001) and from studies in mouse models of allergic airways disease (Poynter et al., 2002). Notably, studies previously performed in our laboratory have revealed a crucial role for lung epithelial NF- κ B in the Alum/Ova model of allergic airways disease (Poynter et al., 2004). Moreover, transgenic expression of constitutively active IKK β in lung epithelial cells was sufficient to cause neutrophilic inflammation and airways hyperresponsiveness, and enhanced sensitization to an inhaled antigen (Ather et al., 2011; Pantano et al., 2008). Despite these prior observations, the more generalized importance of epithelial NF- κ B in allergic airways disease following sensitization/exposure to a relevant allergen via the airways has yet to be determined. Results presented herein describe a critical role for non-ciliated airway epithelial NF- κ B in promoting inflammation, AHR, and fibrotic remodeling following extended challenges of HDM, while mucus metaplasia differences did not appear affected.

Results of the present study somewhat contrast our previous work using the Alum/Ova model of intraperitoneal sensitization followed by challenges of aerosolized

Ova. Notably, CC10-I κ B α_{SR} mice subjected to the Alum/Ova protocol were strongly protected against the development of eosinophilic inflammation and mucus metaplasia, but were not protected against Ova-induced AHR (Poynter et al., 2004). The reasons for these discrepant findings remain unclear. It is likely that the route of sensitization of an allergen dictates the nature of the subsequent immunological and pathophysiological response. In support of the latter is a previous report demonstrating that the IP sensitization regimen with Alum/Ova triggers eosinophilic, Th2-driven inflammation (Kung et al., 1994). In contrast, sensitization with Ova via the airways, along lipopolysaccharide as the adjuvant, led to neutrophilic-dependent AHR and Th17-associated inflammation (Wilson et al., 2009).

In the present study we demonstrated that CC10-I κ B α_{SR} mice did not display increases in airway neutrophils following 15 challenges with HDM compared to PBS controls, while neutrophils were robustly increased in BAL of WT mice. In contrast, HDM-induced eosinophilia was only partially attenuated in CC10-I κ B α_{SR} mice, while BAL lymphocytes were not significantly affected. IL-5, a potent eosinophil chemoattractant in mice and humans, was not detectable at any experimental time points (data not shown); however, we did demonstrate a strong decrease in KC (human IL-8) in CC10-I κ B α_{SR} mice in comparison to WT mice exposed to HDM. These findings demonstrate that following inhibition of epithelial NF- κ B in the setting of HDM-induced disease, the inflammatory process is not uniformly inhibited, but preferentially affects neutrophils. This potential differential effect of NF- κ B inhibition on the inflammatory process could explain the impact of airway remodeling and AHR. Consistent with the

continued presence of eosinophils, HDM-specific IgG1 and IgE, and IL-13 in CC10-I κ B α _{SR} mice exposed to HDM, mucus metaplasia and Muc5AC expression remained elevated in these animals. Previous studies demonstrated that IL-13 can activate epithelial cells to produce Muc5ac in an NF- κ B independent mechanism (Whittaker et al., 2002). In light of those observations, the lack of an impact of CC10-I- κ B α SR mice in HDM-induced mucus metaplasia is therefore, perhaps, not surprising.

The role of neutrophils in the pathogenesis of asthma remains unclear. HDM exposure in mice has been associated with mixed neutrophilic/eosinophilic, Th2/Th17-linked inflammation, and production of IL-13 and IL-17A (Kudo et al., 2012). In this study, the preferential diminution of neutrophils following inhibition of epithelial NF- κ B was associated with normalization of tissue damping and elastance parameters towards values observed in controls, while in contrast, Newtonian resistance, reflective of the central airways, was unaffected. The disparate effects of the CC10-I κ B α _{SR} transgene on tissue damping and elastance, as compared to Newtonian resistance, are puzzling. Previous studies have suggested that inflammation contributed to enhanced leakage of fibrin to the airway surface, leading to decreased stability of surfactant proteins and increased surface tension, and facilitated contractility of smooth muscle. These mechanisms have been linked to increases in the closure of distal airways (Hohlfeld, 2002; Wagers et al., 2004; Yager, Kamm, & Drazen, 1995). It is plausible that decreases in inflammation, along with decreased α -smooth muscle actin in bronchioles, account for decreases in tissue damping and elastance, which were observed in CC10-I κ B α _{SR} transgenic mice. This putative explanation would need to be addressed with additional

studies. Our data also demonstrate that KC, a potent neutrophil chemokine, was not increased in CC10-I κ B α_{SR} HDM-exposed mice in comparison to PBS controls, indicating a potential mechanism whereby neutrophils are decreased in CC10-I κ B α_{SR} mice. All together, these findings are suggestive of a role for neutrophils in promoting increases in peri-bronchiolar collagen deposition and associated changes in tissue damping and elastance. Alternatively, it is also plausible that other mediators control neutrophil trafficking to the airways, promote peri-bronchiolar remodeling, and changes in AHR. In this regard, IL-17A, which we also demonstrate to be decreased in the tissue of CC10-I κ B α_{SR} mice, has been shown to stimulate production of chemokines important in neutrophil recruitment in a KC-dependent manner (Laan et al., 1999) and has been implicated in the pathogenesis of pulmonary fibrosis (Hasan et al., 2013), yet its functional contribution here remains to be determined. Additionally, eosinophils expressing TGF- β 1 have been shown to be important in allergen-induced peribronchial fibrosis (Cho et al., 2004), and hence it is possible that TGF- β 1 also plays a role in peri-bronchiolar fibrosis downstream of activation of NF- κ B. Additional studies will be required to formally address these putative scenarios.

In addition to demonstrating a role for epithelial NF- κ B in promoting inflammation, AHR, and fibrosis following 15 challenges of HDM, we also established a contributing role for epithelial NF- κ B in regulating pro-inflammatory gene expression following a single exposure of HDM. Numerous pro-inflammatory mediators have been implicated in the development of allergic airways disease (Swamy et al., 2010), and our studies demonstrate a putative role for epithelial NF- κ B in regulating expression of

several of these molecules in response to HDM. GM-CSF and CCL20 have been shown to be important in the recruitment/activation of dendritic cells, which are required for T cell activation and recognition of antigens. Notably, exposure of human asthmatic bronchial epithelial cells to the HDM component, Derp1, was shown to be important in dendritic cell recruitment in a CCL20 dependent manner (Pichavant et al., 2005). Additionally, IL-33 has been shown to be important in the activation of a variety of cell types crucial to the development of asthma, such as T helper type 2 cells, eosinophils, dendritic cells, and mast cells (Borish & Steinke, 2011). As previously mentioned, the activation and infiltration of neutrophils is emerging as a potential phenotype in severe, steroid resistant asthma. Cytokines KC and MIP-2 have both been shown to be important in the recruitment of neutrophils and production of HDM-specific IgE (McKinley, Kim, Bolgos, Siddiqui, & Remick, 2005). Altogether, our data indicate a likely role for epithelial NF- κ B in the recruitment/activation of several cell types important for the development of HDM-induced asthma. This suggests a mechanism whereby epithelial NF- κ B activation is the crucial step between contact with an allergen and downstream manifestations of asthma.

In addition to the role of NF- κ B in airway epithelium demonstrated herein, it is plausible that activation NF- κ B in other cell types contributes to the pathophysiology of allergic airways disease. Notably our findings demonstrate increased immunofluorescence of nuclear RelA and RelB in parenchymal regions following exposure to HDM (Fig. 3). Unraveling the cell types in which NF- κ B is activated and their functional contribution to allergic airways disease would require additional cell specific labeling and targeting

strategies, which were beyond the scope of the present study. For example, neutrophil elastase-induced secretion of Transforming Growth Factor β -1 from smooth muscle cells was shown to be dependent on NF- κ B activation (Lee et al., 2006), and the smooth muscle contractile force in response to IL-17A was dependent on NF- κ B activation (Kudo et al., 2012), suggesting a putative role of NF- κ B activation in smooth muscle cells in airways hyperresponsiveness and remodeling. Secretion of eotaxin, a potent eosinophil activating factor, by fibroblasts was also shown to be dependent on NF- κ B activation (Rokudai et al., 2006). One notable finding of the present study is that HDM activates both the classical and alternative NF- κ B pathway within the bronchiolar epithelium, evidenced by the increases in nuclear presence of both RelA as well as RelB in mice exposed to HDM. Similar to observations herein, we recently demonstrated increases in nuclear RelA and RelB in the parenchymal regions following administration of lipopolysaccharide, a component of HDM (Tully et al., 2012), suggesting that TLR4 activation by HDM may be contributing to the observed increases in RelA and RelB observed. Increases in nuclear RelA and RelB within the epithelium occurred rapidly and were sustained at least for 24 h after 3 challenges. However, 72 h post 5, 10 or 15 challenges, there was no clear evidence of increased RelA or RelB in the bronchiolar epithelium, possibly due to the timing of tissue analysis post the last administration of HDM, which was 72 h, a time when NF- κ B activation may have resolved. However, we did observe sustained increases in nuclear RelB in lung tissue homogenates 72 hr post 15 challenges with HDM in WT mice, suggestive of NF- κ B activation in other cell types. Increases in nuclear RelB were attenuated in CC10-I κ B α _{SR} mice, suggesting a putative

role for RelB in the orchestration of HDM-mediated AHR and fibrotic remodeling. In addition to the demonstration that RelB was increased in the bronchiolar epithelium following HDM exposure, we also demonstrated that HDM directly activated both NF- κ B pathways in both human bronchial and nasal epithelial cells. Although extensive studies have been conducted to unravel the molecular regulation and pathophysiological relevance of the classical NF- κ B pathway, far less information is available for the alternative pathway. The latter pathway was originally thought to play a role in adaptive immune responses, development of lymphocytes and lymphoid organs. However, emerging studies have pointed to a coordinate function of both classical and alternative NF- κ B in the orchestration of pro-inflammatory responses. For example, exposure of lung epithelial cells with Tumor Necrosis Factor- α , Polyinosinic acid, LPS, IL-17A, lipoteichoic acid, and CD40L, agonists that signal through distinct families of receptors, led to a coordinate activation of classical and alternative NF- κ B pathways and subsequent pro-inflammatory responses (Tully et al., 2012). In contrast, adenovirus-mediated delivery of RelB afforded protection against cigarette smoke-induced neutrophilic inflammation (McMillan et al., 2011), suggesting potentially complex roles of the alternative NF- κ B pathway in the regulation of pro-inflammatory and immune responses. The classical NF- κ B pathway previously has been shown to be important in HDM-mediated pro-inflammatory responses in human bronchial epithelial cells *in vitro* (Osterlund et al., 2009), and Derp1, a component of HDM, was shown to be important in the activation of NF- κ B in human asthmatic bronchial epithelial cells (Stacey et al., 1997). Patients with allergic asthma demonstrate increased classical NF- κ B activation in

nasal epithelial cells in response to HDM in comparison to healthy controls (Vroling et al., 2008). Intriguingly, the HDM component, β -glucan, was shown to be crucial for activation of allergic rhinitis in nasal epithelial cells via TLR2, in contrast to another HDM component, LPS, which is important in promoting allergic airways disease via activation of TLR4 in bronchial epithelial cells (Ryu et al., 2013). Despite these previous studies and data presented herein, additional studies are needed to better understand the timing and locale of activation of classical and alternative NF- κ B pathways, the components of HDM that are required to activate either pathway, as well as the relative contributions of the classical and alternative NF- κ B pathways in eliciting HDM-triggered allergic airways disease.

In summary, we demonstrate in the present study the importance of NF- κ B activation within the bronchiolar epithelium in HDM-induced inflammation, AHR and fibrotic airway remodeling. We also demonstrate that both classical and alternative NF- κ B pathways are activated by HDM. Data presented herein showed that in the setting of HDM-induced allergic airway disease, inhibition of epithelial NF- κ B plays a more prominent role in attenuating neutrophilia, AHR, and remodeling, in comparison to eosinophilia, IgE, and mucus metaplasia. Therefore, it is plausible that therapeutic approaches which target NF- κ B via interference with degradation of I κ B α may have a stronger impact on asthmatic patients with predominant neutrophilia in contrast to patients with predominant eosinophilia. Corticosteroids, the most common therapy for asthma, inhibit NF- κ B, but have many off target effects. Current therapies that are being developed for asthma are aimed at inhibiting the pro-inflammatory effects of NF- κ B

signalling in the lung (Edwards et al., 2009), and are focused on inhibition of IKK β , the dominant kinase in the classical NF- κ B pathway (Edwards et al., 2009). The small molecule IKK β inhibitor, IMD-0354, attenuated HDM- induced eosinophilia, goblet cell hyperplasia, subepithelial fibrosis, smooth muscle cell hypertrophy, and lung resistance using an intraperitoneal sensitization model (Ogawa et al., 2011). The disparate findings of the latter study with the present findings may relate to the different sensitization route, mechanism of inhibition of NF- κ B, and the cell types wherein NF- κ B inhibition occurred. Furthermore, intravenous administration of RelA antisense oligonucleotides prior to ovalbumin challenge resulted in dampened responses of inflammation, AHR, and TH2 responses in mice (Choi, Kim, Ko, & Lee, 2004). Based upon these collective findings, therapeutics designed to inhibit both facets of the NF- κ B pathway may hold larger therapeutic potential for allergic asthma and other allergic diseases of the lung.

Figure Legends

Figure 1: Analysis of cell totals and differentials in BAL of mice exposed to HDM. (A) Schematic depicting the time course of HDM exposure and euthanization. Instill: 50 μ g HDM, or PBS as the vehicle control were administered intranasally once at the days indicated via downward arrows. Mice were euthanized 2 h, 6 h or 24 h post a single challenge, 24 h after 3 challenges or 72 h after 5, 10, or 15 challenges, indicated by the upward arrows. (B) Mice (n=5/group/time point) were exposed to PBS or HDM according to the schematic in A, and BAL was assessed for neutrophils, eosinophils,

lymphocytes, and macrophages. * $p < 0.05$ (ANOVA) compared to the PBS group at the same time point.

Figure 2: Assessment of nuclear localization of RelA and RelB in airway epithelium in response to HDM: BALB/c mice were exposed to PBS and HDM and harvested as indicated. De-paraffinized lung sections were incubated with antibodies directed against RelA or RelB, followed by fluorophore-conjugated secondary antibodies, and the nuclei were counterstained with Sytox Green. Images were captured by laser scanning confocal microscopy using identical instrument settings in all groups. Images are representative results of two independent experiments, $n=5$ /group/time. RelA/RelB: **red**, DNA: **green**, Nuclear RelA/RelB: yellow. scale bar= $50\mu\text{m}$.

Figure 3: Nuclear RelA and RelB, and pro-inflammatory cytokine mRNA expression in CC10-NF- $\kappa\text{B}_{\text{SR}}$ mice following exposure to HDM: CC10-NF- $\kappa\text{B}_{\text{SR}}$ mice, or WT littermate controls were exposed to PBS or HDM for 2 h. (A) Nuclear localization of RelA and RelB was assessed in lung tissues as described in Fig. 2. Images are representative results of two independent experiments with 5 mice/group, RelA/RelB, **red**; DNA, **green**; Nuclear RelA/RelB, yellow, scale bar= $50\mu\text{m}$. (B) Assessment of mRNA expression for IL-33, GM-CSF, CCL20, KC, MIP-2, and IL-6 in homogenized lung tissue. Data were normalized to cyclophilin and are presented as relative expression. Values reflect 6 mice/group/time point. * $p < 0.05$ (ANOVA) compared to the PBS group at the same time point. † $p < 0.05$ (ANOVA) compared to WT mice exposed to HDM

Figure 4: Impact of RelB knockdown on pro-inflammatory cytokine expression following exposure to HDM: (A) WT BALB/c mice were oropharyngeally instilled with either scrambled siRNA or RelB siRNA 2 and 4 days prior to 1 dose HDM and harvested 2 h post challenge. RelB content was assessed via immunofluorescence. (B) mRNA expression was analyzed from lung extracts and assessed for IL-33, GM-CSF, CCL20, KC, MIP-2, and IL-6. Statistical significance between PBS and HDM exposed groups were indicated by * and $p < 0.05$ were considered significant. Statistical significance between scrambled siRNA and RelB siRNA HDM exposed groups were indicated by † and $p < 0.05$ were considered significant.

Figure 5: Assessment of HDM-induced inflammation, immunoglobins, T-cell cytokines, and nuclear RelB in WT or CC10-NF- κ B_{SR} mice following 15 challenges with HDM: Assessment of (A) total cells and differential cell counts (B) and KC levels (C) in BAL. (D) Total IgG1 and IgE in serum. (E) IFN γ , IL-13, and IL-17A in homogenized tissue by ELISA. (F) Nuclear content of RelB in homogenized lung tissue. Histone H3 is shown as a loading control. Data represents 8 mice/group/time point. * $p < 0.05$ (ANOVA) compared to the PBS group at the same time point. † $p < 0.05$ (ANOVA) compared to WT mice exposed to HDM.

Figure 6: Assessment of HDM-induced alterations in respiratory mechanics and airway remodeling in WT or CC10-NF- κ B_{SR} mice following 15 challenges with HDM: (A) Evaluation of airway hyperresponsiveness via forced oscillation mechanics in response to 3.125, 12.5, and 25 mg methacholine. Shown are the parameters Newtonian resistance (Rn), Tissue damping (G) and Elastance (H) assessed via forced oscillation (B)

Histopathological evaluation of tissue inflammation (H&E), mucus metaplasia (PAS) and peri-bronchiolar collagen deposition (M.T.). scale bar=50 μ m. (C) Analysis of Muc5ac mRNA expression in homogenized lung tissue. Data were normalized to cyclophilin and are presented as relative expression. (D) Quantification of mucus metaplasia. Bronchioles of similar size (n=3) were analyzed/mouse by two blinded scorers, and average scores presented as average units. (E) Assessment of collagen from the upper right lobe. (F) Quantification of peri-bronchiolar collagen deposition. Bronchioles of similar size (n=3) were analyzed/mouse by two blinded scorers, and average scores presented as average units. (G) Assessment of α -SMA immunohistochemistry, red= α -SMA, scale bar=1 μ m. (F) Quantification of α -SMA immunoreactivity. Bronchioles of similar size (n=3/mouse) were analyzed by two independent blinded scorers, and average scores presented as average units. Data represent 8 mice/group/time point. * p< 0.05 (ANOVA) compared to the PBS group at the same time point. † p<0.05 (ANOVA, Kruskal Wallis) compared to WT mice exposed to HDM.

Figure 7: Assessment of activation of classical and alternative NF- κ B pathways in human lung epithelial cells exposed to HDM. (A) Primary human nasal epithelial (PHNE) cells or (B) Human bronchial epithelial (HBE) cells were exposed to PBS or 25 μ g HDM once a day for consecutive days and harvested thereafter at either 72 h (PHNE) or 48 h (HBE). Cells were lysed for evaluation of NIK, p52, phospho RelA 536, by Western blot analyses. β -Actin (loading control). Right panels: Densitometric evaluation of Western blots shown in (A) PHNE (n=3 patients) or (B) HBE (n=3 experimental

repeats). Results are expressed as arbitrary density and were normalized to corresponding β -Actin bands.

Figure S1: BALB/c mice were exposed to a single dose of either PBS or HDM and harvested 2, 6, or 24 h following challenge. Additionally, mice were challenged for three consecutive days and harvested 24 h following the 3rd challenge. mRNA isolated from homogenized lung tissue was analyzed for expression of IL-33, GM-CSF, CCL20, KC, MIP-2, and IL-6. Data represent 5 mice/group/time point. * $p < 0.05$ (ANOVA) compared to the PBS group at the same time point.

Figure S2: Effects of Repeated HDM Exposure on HDM-specific IgG1 and IgE, AHR and airways remodeling: BALB/c mice were exposed to HDM and harvested at the indicated time points. (A) Assessment of total IgG1 and IgE in serum. (B) Assessment of airway hyperresponsiveness in response to increasing doses (3.125, 12.5, and 25 mg) of methacholine via forced oscillation mechanics. Parameters measured were Newtonian Resistance (Rn), Tissue Damping (G) and Tissue Elastance (H). (C) Histopathological analysis of inflammation (H&E), mucus metaplasia (PAS) and peri-bronchiolar collagen deposition (M.T.) in mice exposed to HDM for the indicated times. scale bar=50 μ m. (D) Analysis of Muc5ac mRNA expression in homogenized lung tissue. Data were normalized to cyclophilin and are presented as relative expression. (E) Quantification of collagen from the upper right lobe. Data represent 5 mice/group/time point. * $p < 0.05$ (ANOVA) compared to the PBS group at the same time point.

Figure 1: HDM-induced pulmonary inflammation

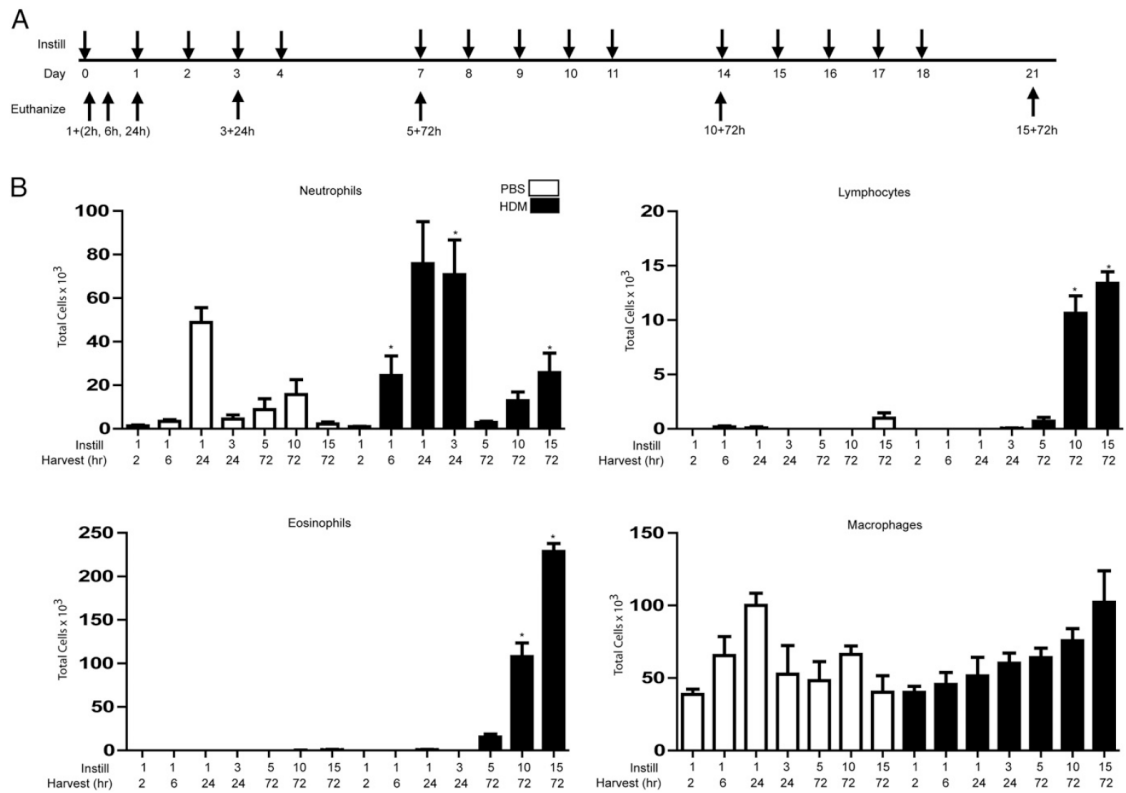


Figure 2: HDM-induced classical and alternative NF- κ B *in vivo*

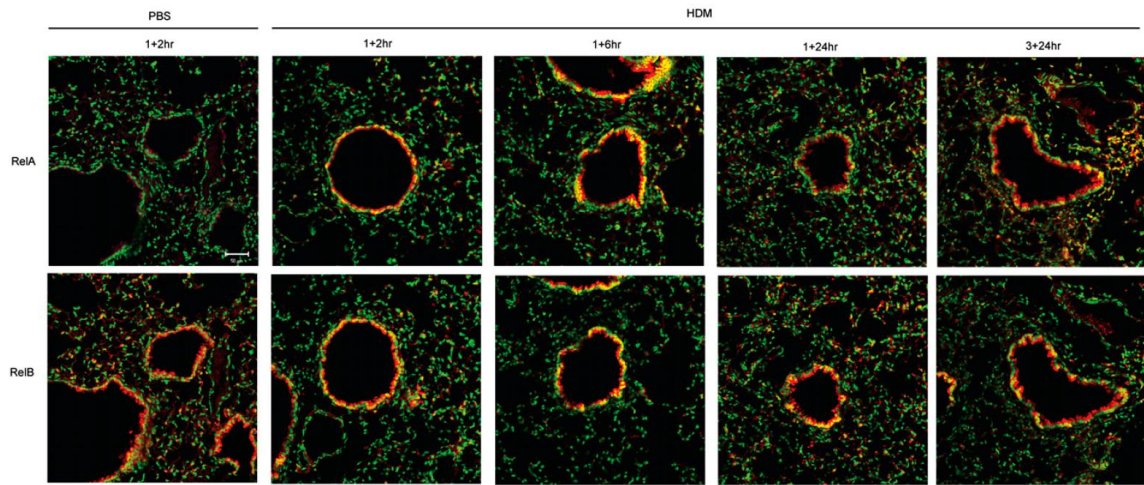


Figure 3: Impact of epithelial NF- κ B inhibition on pro-inflammatory cytokines

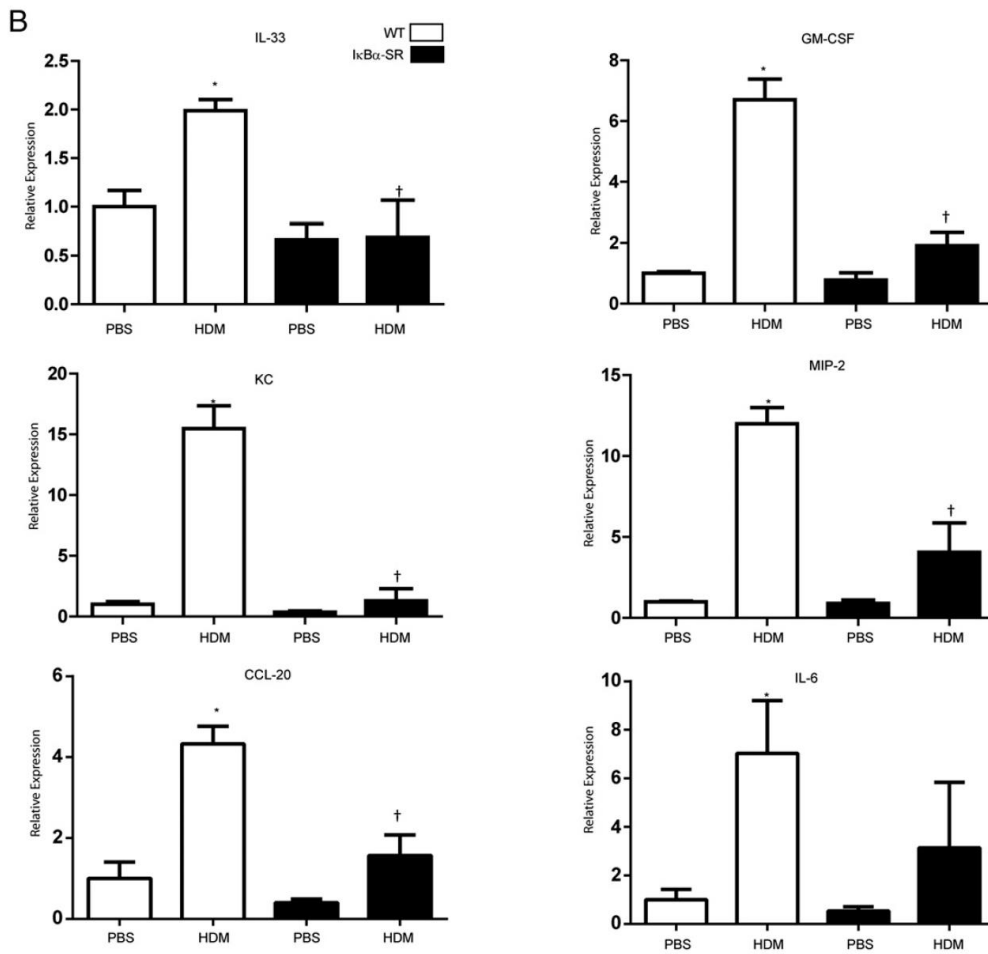
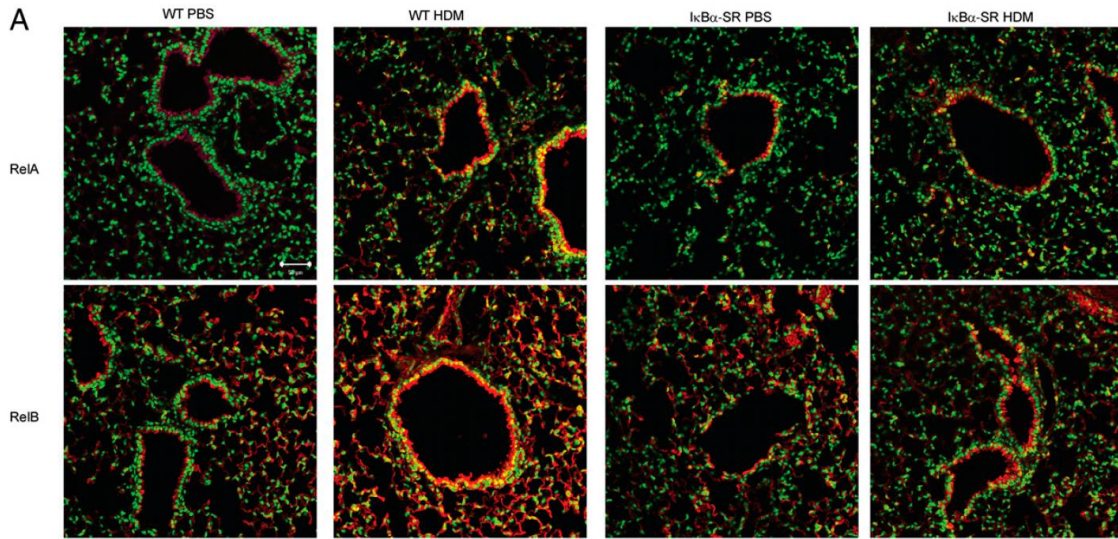


Figure 4: Impact of RelB knockdown on pro-inflammatory cytokines

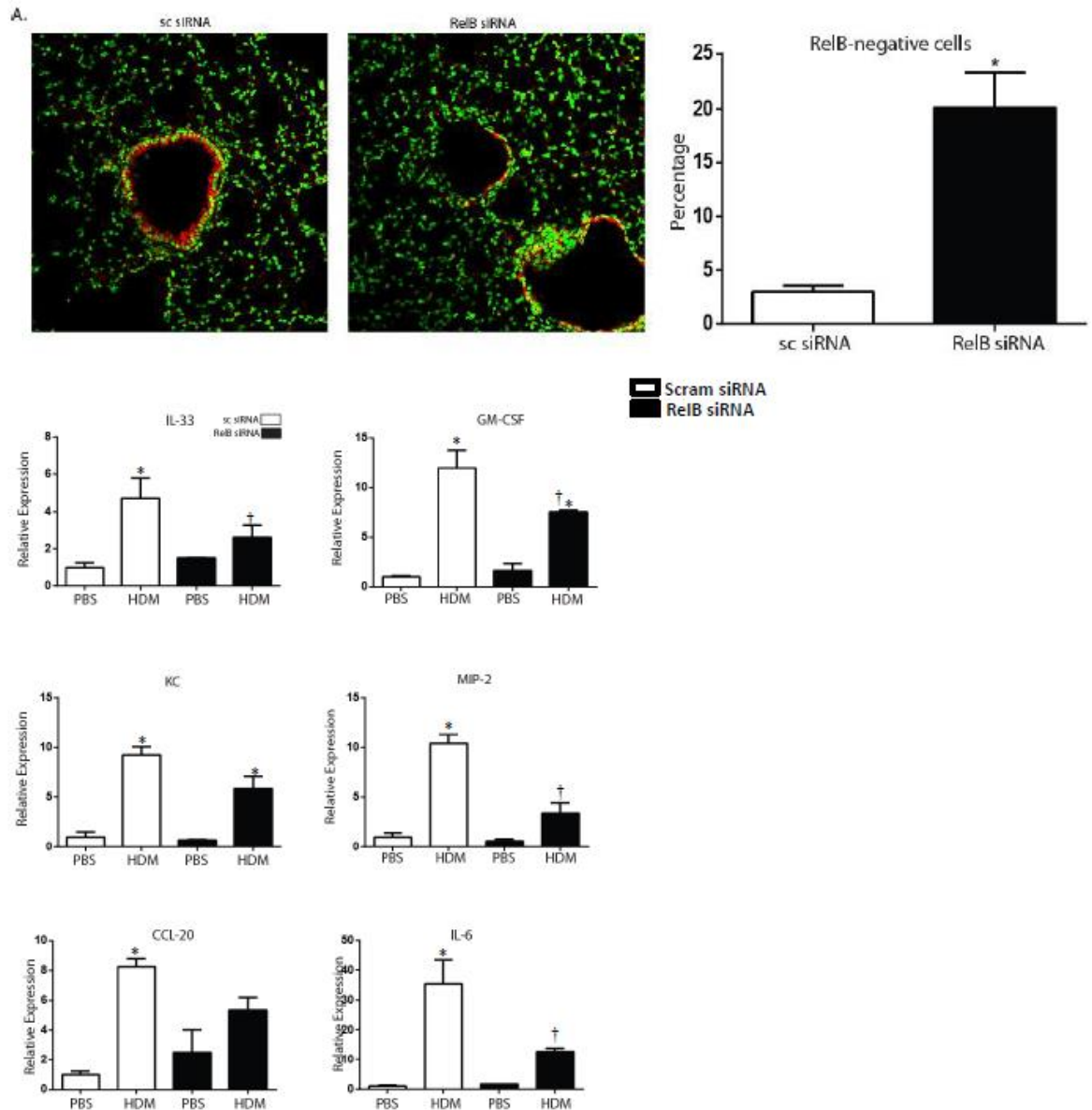


Figure 5: Impact of epithelial NF- κ B inhibition HDM-induced allergic inflammation

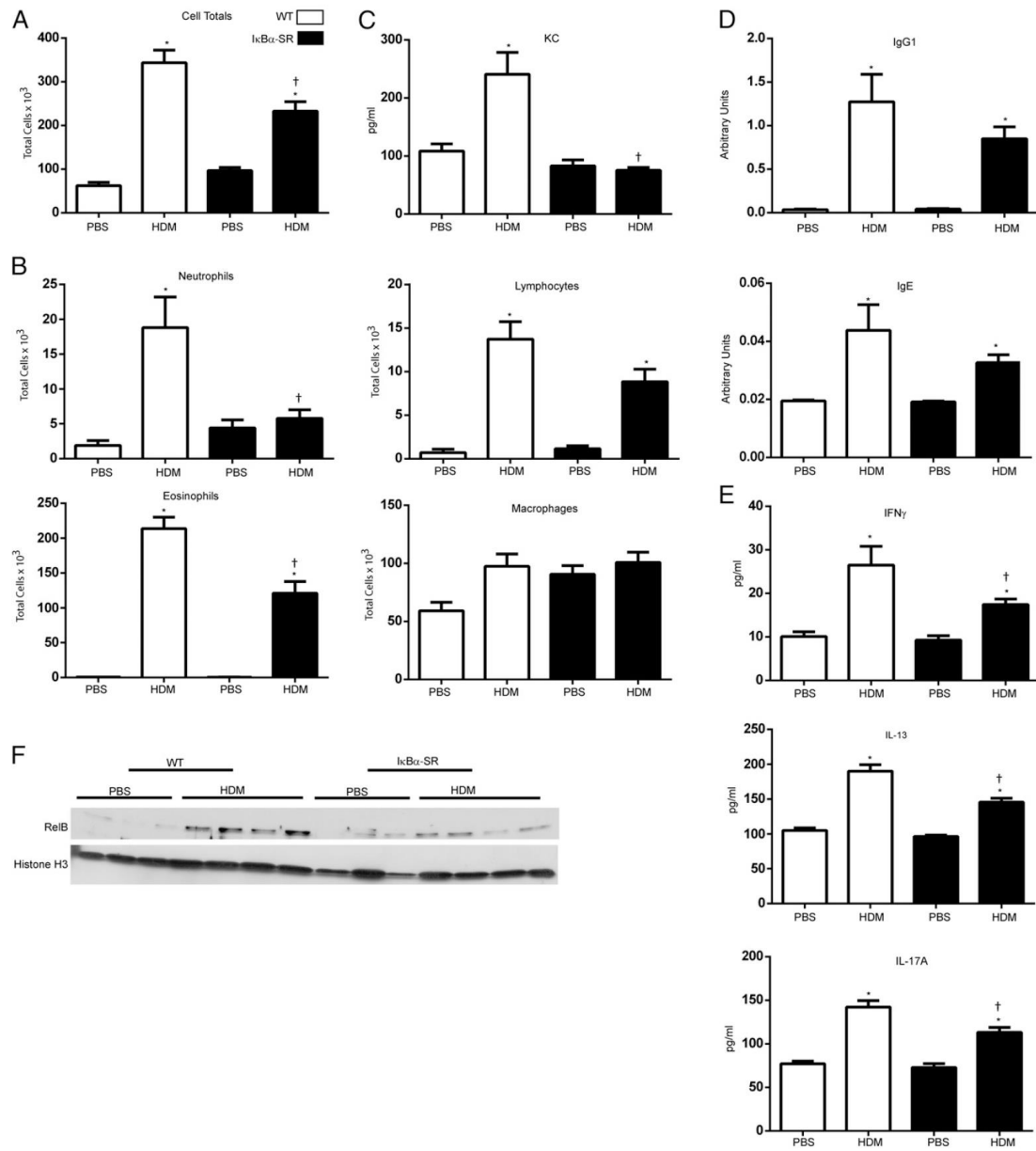


Figure 6: Impact of epithelial NF- κ B inhibition HDM-induced airway hyperresponsiveness and remodeling

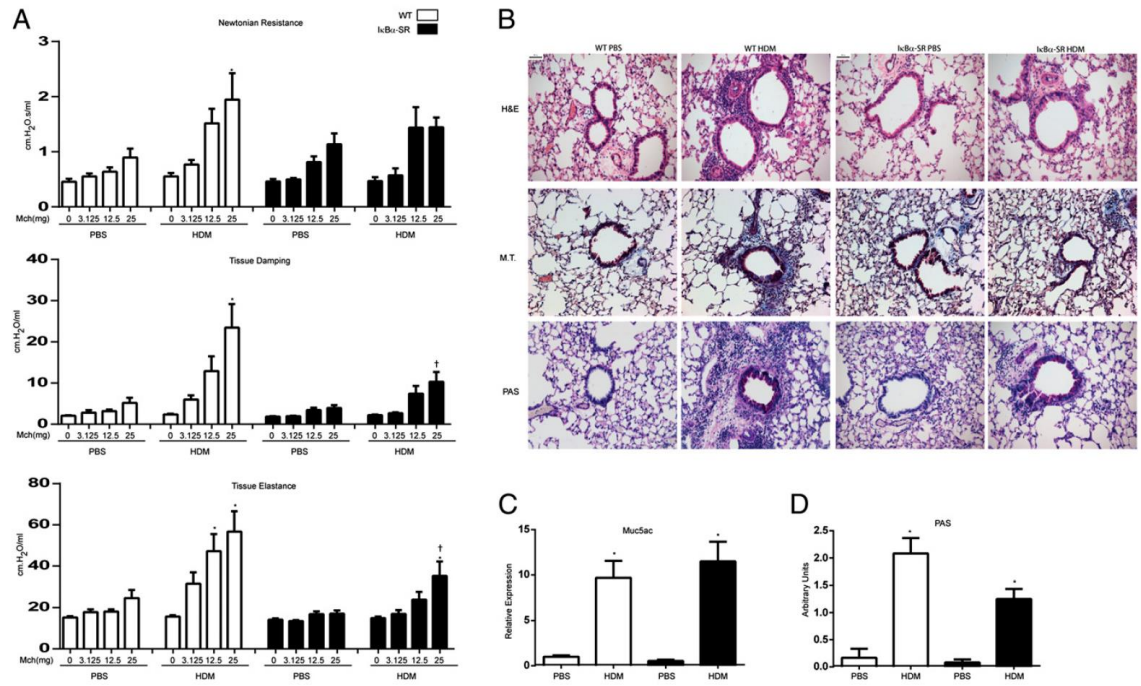


Figure 6 cont: Impact of epithelial NF- κ B inhibition HDM-induced airway hyperresponsiveness and remodeling

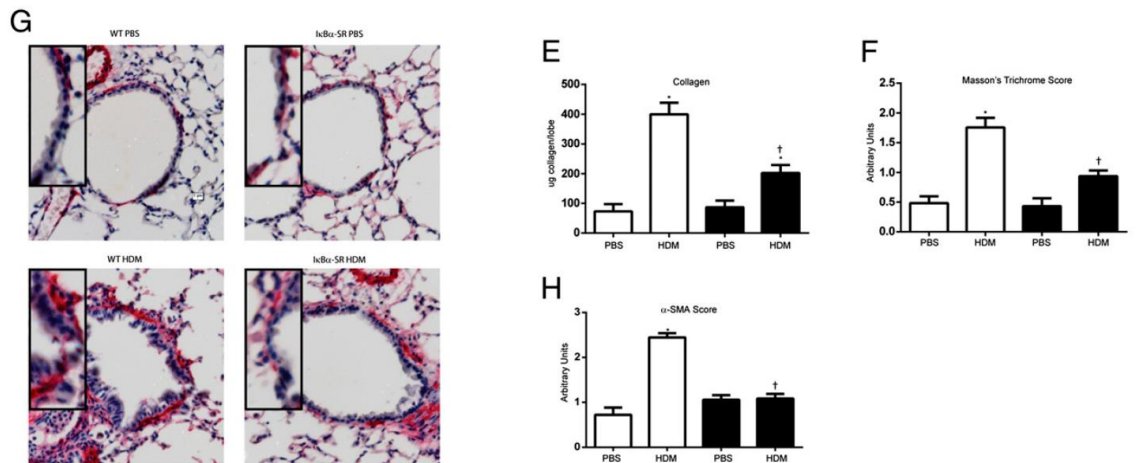


Figure 7: HDM-induced classical and alternative NF- κ B in human pulmonary epithelial cells

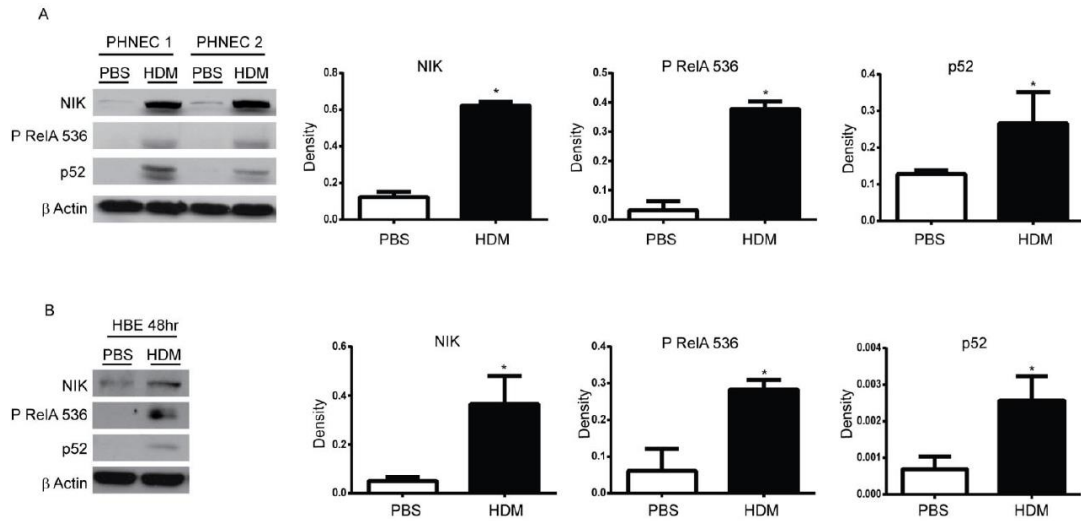


Figure S1: HDM-induced pro-inflammatory cytokine production

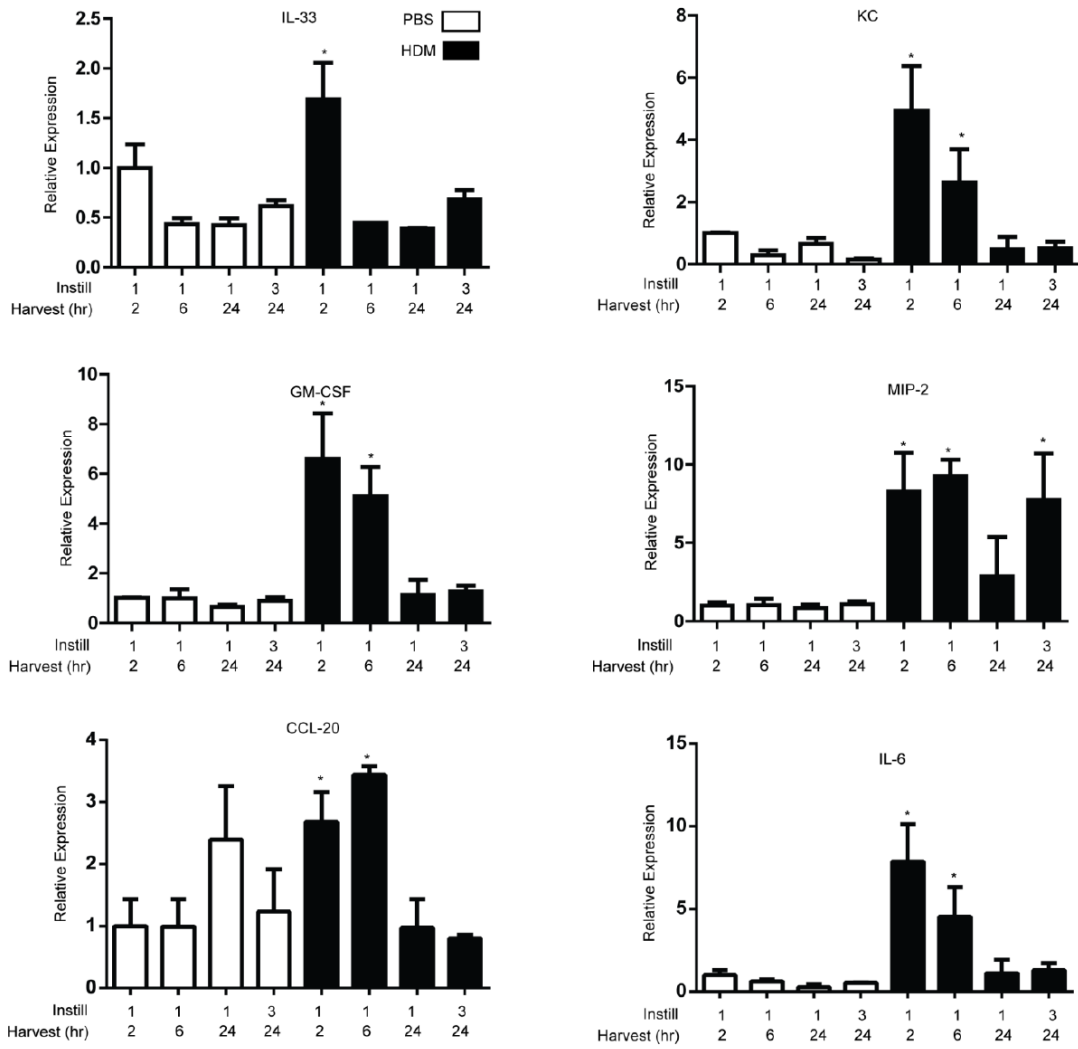
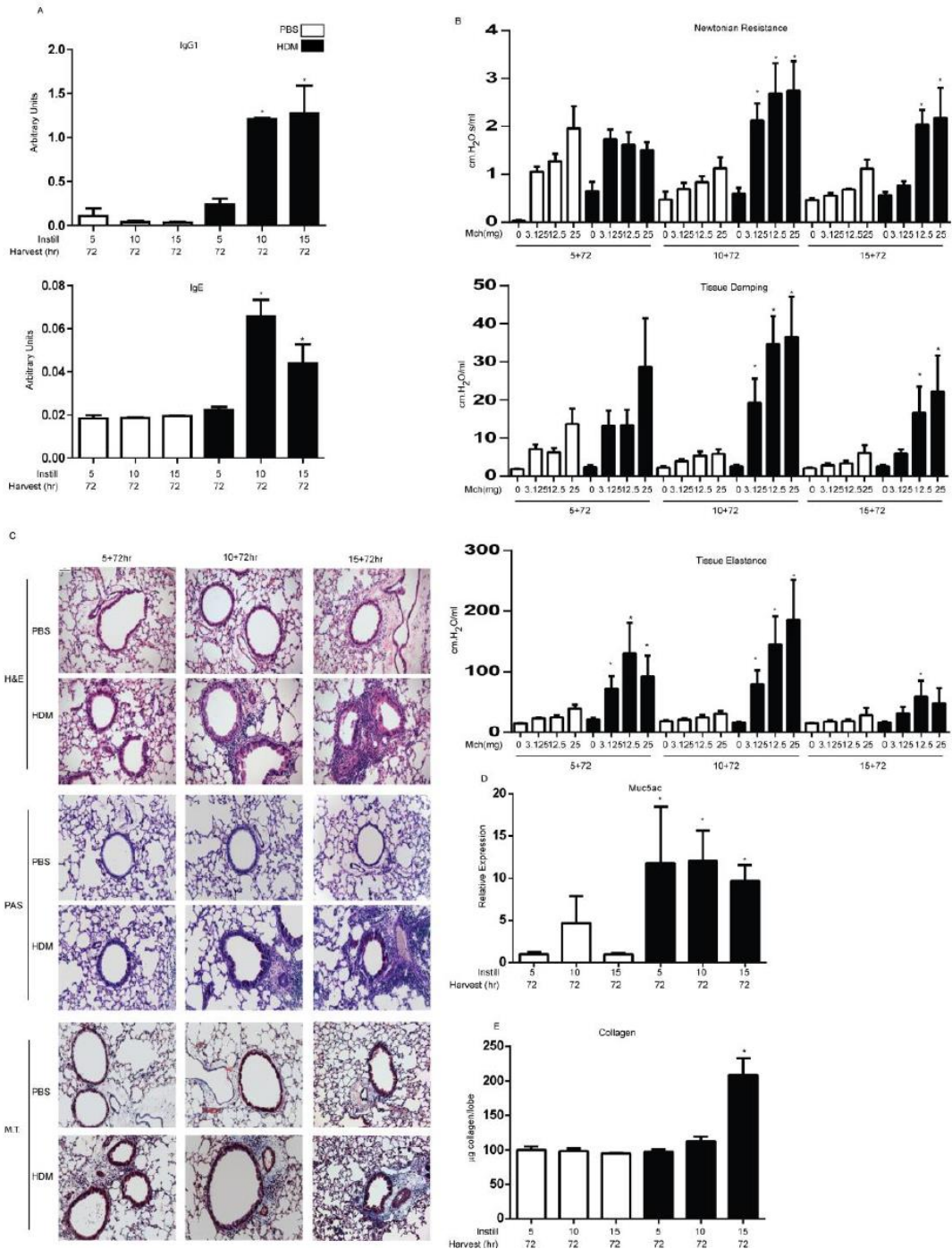


Figure S2: HDM-induced allergic airway disease



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Chapter 4

Glutathione S-Transferase pi modulates Nuclear Factor-kappaB activation and pro-inflammatory responses in lung epithelial cells

Abstract

Nuclear Factor kappaB (NF- κ B) is a transcription factor critical in regulating inflammation, proliferation, and cell survival pathways. The NF- κ B pathway is regulated by oxidant-induced post-translational modifications, which decrease the activity of numerous NF- κ B proteins. Protein S-glutathionylation, or the conjugation of the antioxidant molecule, glutathione, to reactive cysteines, inhibits the activity of NF- κ B kinase, IKK β . GSTP is an enzyme that has been shown to catalyze protein S-glutathionylation (PSSG) under conditions of oxidative stress. The present study aimed to determine whether GSTP regulates NF- κ B signaling, S-glutathionylation of IKK, and subsequent pro-inflammatory signaling. Results show that siRNA-mediated knockdown of GSTP modulates IKK α and IKK β -SSG, NF- κ B activation, NF- κ B transcriptional activity, and pro-inflammatory cytokine production in response to pro-inflammatory stimuli. Both WT GSTP and enzymatically inactive GSTP dampen IKK β -induced pro-inflammatory cytokines. Our results also show that GSTP associates with the inhibitor of NF- κ B, I κ B α , in unstimulated cells; however, exposure to LPS resulted in the rapid loss of association between I κ B α and GSTP. These studies indicate a dual role for GSTP in modulating NF- κ B, which may involve both the catalytic and ligandin functions of GSTP. Our results

collectively demonstrate that GSTP modulates NF- κ B signaling in lung epithelial cells, which may provide a novel target for control of NF- κ B.

Introduction

Nuclear Factor- κ B is a family of transcription factors involved in the regulation of pro-survival, pro-inflammatory, and immune pathways. Dysregulation of NF- κ B has been linked to a variety of chronic inflammatory diseases including cancer, sepsis, and asthma. NF- κ B activity is elevated in lung epithelial cells of asthmatic patients in comparison to healthy controls (Hart et al., 1998), and activation of classical NF- κ B in the lung epithelium is not only sufficient, but also necessary to regulate allergic airway inflammation in mice (Ather et al., 2011; Pantano et al., 2008; Poynter et al., 2004). Two parallel NF- κ B pathways exist: the classical and alternative, both of which contribute to inflammatory responses in lung epithelial cells (Tully et al., 2012). Classical NF- κ B is a transcription factor complex consisting of RelA and p50, and the Inhibitor of κ B kinase, I κ B α , sequesters RelA and p50 in the cytosol of an unstimulated cell. Following stimulation with a pro-inflammatory agonist, the IKK complex, containing the scaffolding protein, IKK γ , and catalytic subunits, IKK α and IKK β is activated. IKK β then phosphorylates I κ B α , which facilitates its K48-linked ubiquitination and degradation by the 26S proteasome. RelA and p50 translocate to the nucleus following degradation of I κ B α and initiates transcription of over 100 target genes (Gilmore, 2006). The alternative NF- κ B family involves RelB and p100 dimers. Upon activation, a distinct pool of an IKK α homodimer is phosphorylated by NF- κ B-inducing kinase (NIK) and subsequently phosphorylates p100, leading to its ubiquitination and partial processing to p52. These events facilitate the translocation of RelB and p52 to the nucleus and initiate transcription of distinct NF- κ B target genes (S. C. Sun, 2011).

NF- κ B is a crucial regulator of numerous biological functions, and as such, several mechanisms exist which tightly regulate its activation. Oxidants have been implicated in various diseases and can modify select proteins. Specifically, select cysteines with low pKa's are susceptible to oxidative modifications, such as S-nitrosylation, sulfenic acid formation, disulfide formation, and S-glutathionylation (Janssen-Heininger et al., 2009). NF- κ B is modified via S-glutathionylation, or the reversible conjugation of a glutathione molecule to reactive cysteine residues. S-glutathionylation of IKK β inhibits its kinase activity and downstream pro-inflammatory responses. Additionally, S-glutathionylation prevents the ubiquitination and subsequent degradation of I κ B α , as well as the DNA binding of RelA/p50 dimers (Kil et al., 2008; Pineda-Molina et al., 2001; Reynaert et al., 2006; Seidel et al., 2011), and, thus, is a crucial regulatory mechanism of NF- κ B.

S-glutathionylation is controlled by enzymes that regulate forward and reverse reactions, and under physiological conditions, glutaredoxins can deglutathionylate proteins. Hydrogen peroxide-induced S-glutathionylation of IKK β can be reversed by the oxidoreductase, Glutaredoxin-1 (Grx1) (Reynaert et al., 2006). Furthermore, overexpression of Grx1 enhances LPS-induced IKK β S-glutathionylation, NF- κ B activation, and pro-inflammatory cytokine production in lung epithelial cells (Aesif, Kuipers, et al., 2011). While it is known that Grx1 can de-glutathionylate IKK β under physiological conditions, the potential enzymatic mechanisms that catalyze IKK β S-glutathionylation have not been determined. Glutathione S-transferase pi 1 (GSTP) is an enzyme that was recently identified to be able to catalyze S-glutathionylation of proteins

by enhancing the nucleophilicity of bound glutathione moieties (Townsend et al., 2009). GSTP, traditionally known for its role in phase II drug metabolism, is located on the chromosome locus 11q3, which has been linked to asthma (McCunney, 2005). GSTP^{-/-} mice subjected to the Alum/OVA model of allergic airway disease demonstrate increased eosinophilia and remodeling (J. Zhou et al., 2008). GSTP also associates with TRAF2, a known regulator of NF- κ B (Y. Wu et al., 2006). Given the putative link between GSTP and asthma, the known role for epithelial NF- κ B in regulating allergic inflammation and the susceptibility of NF- κ B to redox regulation, we hypothesized that GSTP regulates the activity of NF- κ B via S-glutathionylation of IKK β , leading to decreases in NF- κ B-induced gene transcription and pro-inflammatory cytokine production. Our studies demonstrate that siRNA-mediated knockdown of GSTP increases NF- κ B activation, gene transcription, and pro-inflammatory responses, and that GSTP inhibits IKK β -induced proinflammatory cytokine production. These studies implicate a novel regulatory mechanism for modulation of the NF- κ B pathway.

Materials and Methods

Cell Culture and reagents: A spontaneously transformed type II mouse lung alveolar epithelial cell line (Malkinson et al., 1997) (C10) was cultured as described previously (Alcorn et al., 2008). A C10 cell line stably expressing NF- κ B luciferase was generated as previously described (Korn et al., 2001). Briefly, C10 cells were transfected with the plasmid, 6 κ B-tk-luc, in the presence of the plasmid, PSV-2Neo (Promega), conferring a resistance to neomycin. Following incubation with the antibiotic, geneticin,

antibiotic-resistant colonies were propagated. Luciferase units were corrected for total protein concentration. C10 cells were starved in medium containing 0.5% FBS for 16 hours prior to exposure of 1 μ g/mL Lipopolysaccharide (LPS, List Biological Laboratories, Inc).

Plasmids and siRNA: C10 cells were incubated with Dharmacon SMARTpool non-targeting small interfering (si)RNA or Dharmacon SMARTpool siRNA against murine GSTP1 (100nM) (Dharmacon, Lafayette, CO). Plasmid transfections were performed with Lipofectamine (Invitrogen, Carlsbad, CA). Murine Wild Type GSTP, murine GSTP Tyrosine 7 Phenylalanine (Y7F), and human GSTP variants A: Ile(105), Ala(114), B: Val(105), Ala(114), C: Val(105), Val(114), and D: Ile(105), Val(114) were cloned into PCMV. WT IKK β was cloned into pcDNA3. All transfections utilized a total of 1 μ g DNA/ml unless otherwise indicated.

Western Blotting and Antibodies: Protein concentration was determined by the Bio-Rad DC Protein Assay kit (Bio-Rad, Hercules, CA), and 20 μ g protein were used for Western Blot analysis. RelA, RelB, and β -Actin antibodies were purchased from Santa Cruz biotechnology, Santa Cruz, CA. Antibodies against phosphoserine I κ B α 32/36, I κ B α , IKK β , and phosphoserine RelA 536 were from Cell Signaling Technology, Danvers, MA. The IKK α antibody was purchased from Upstate, Lake Placid, NY and GSTP was purchased from MBL, Woburn, MA. Cytosolic and nuclear extracts were prepared as previously described (van der Velden et al., 2008).

Enzyme-Linked Immunosorbent Assay (ELISA): C10 cells were treated and/or transfected as indicated and medium was assessed for CCL-20 and GM-CSF cytokines by R & D Systems, Minneapolis, MN according to manufacturer's instructions.

GSTP activity assay: GSTP activity was determined as previously described (Anathy et al., 2012; Townsend et al., 2009). C10 cells were transfected with either WT-FLAG-GSTP or Y7F-FLAG-GSTP and harvested in ice cold PBS containing protease and phosphatase inhibitors. Immunoprecipitation for Flag (Sigma, St. Louis, MO) was performed in order to isolate exogenously expressed GSTP from lysate, and protein G beads (Invitrogen) were utilized for immunoprecipitation. Immunoprecipitated samples were incubated with 10.1mM GSH (Fluka, St. Louis, MO) and 10.1mM 1-Chloro-2,4-dinitrobenzene (CDNB) (Sigma) and vortexed. Following 5' incubation, samples were again vortexed and centrifuged 30'' 14,000 rpm and supernatant was measured at an absorbance of 340 nm in order to detect GSH-conjugated CDNB. Results are expressed as Δ OD/min/ mg of input protein.

Detection of protein S-glutathionylation: C10 cell lysates were subjected to immunoprecipitation of GSH-bound proteins as previously described (Anathy et al., 2009). Briefly, cells were harvested in the presence of 20mM N-ethyl maleimide (NEM) (Sigma) and 250 μ g protein was incubated with 1 μ g/ml GSH antibody (Virogen, Watertown, MA), immunoprecipitated with Protein G agarose beads, and subjected to Western Blot analysis. Prior to immunoprecipitation, select reagent control samples were incubated with 50mM DTT for 30' at 37°C, which was subsequently removed with

Microbiospin Chromatography Columns (Biorad) according to manufacturer's instructions.

Statistical Analysis: Data were evaluated by GraphPad Prism 5 Software using one-way analysis of variance (ANOVA) with Bonferroni to adjust for multiple comparisons. Results with $p < 0.05$ or smaller were considered statistically significant.

Results

LPS-induced associations of GSTP and NF- κ B proteins: In addition to its role in phase II drug metabolism, GSTP has been described as a ligandin, whose interactions typically inhibit the function of target proteins (Y. J. Kim et al., 2006; Y. Wu et al., 2006). We first investigated whether GSTP associated with IKK proteins and whether these associations altered in response to LPS. LPS exposure enhanced GSTP and IKK β interactions at 2h, which increased throughout the 24h exposure period (Figure 1). We were unable to detect associations between GSTP and IKK α in any of the time points assessed (Figure 1).

Function of GSTP in LPS-induced NF- κ B activation, transcriptional activity, and pro-inflammatory cytokine production: We next sought to examine a potential role for GSTP in regulating NF- κ B activation and downstream inflammatory responses. Nuclear RelA content increased in C10 cells transfected with GSTP siRNA in comparison to control siRNA cells following stimulation with LPS. However, only modest increases in RelB were detected (Figure 2A). NF- κ B luciferase, whose induction indicates transcriptional activity of NF- κ B, increased following LPS exposure in GSTP

siRNA-transfected cells as compared to control siRNA transfected cells (Figure 2B). Furthermore, CCL-20 and GM-CSF, cytokines transcriptionally controlled by NF- κ B in epithelial cells, also increased following LPS exposure in lung epithelial cells transfected with GSTP siRNA in comparison to control siRNA-treated cells (Figure 2C). These data demonstrate that GSTP plays a role in suppressing LPS-induced NF- κ B activation, transcriptional activity, and production of the pro-inflammatory mediators, CCL-20 and GM-CSF.

Role of GSTP in modulating IKK α and IKK β glutathionylation: Given the role for GSTP in regulating NF- κ B and pro-inflammatory cytokine production, we next determined whether S-glutathionylation of NF- κ B proteins was also dependent on GSTP. Following siRNA-mediated knockdown of GSTP, LPS-induced S-glutathionylation of IKK β decreased in comparison to control siRNA-transfected cells (Figure 3). Although we did not detect an interaction between GSTP and IKK α , S-glutathionylation of IKK α increased in response to LPS, and decreased in cells subjected to GSTP siRNA ablation (Figure 3). Decreases in S-glutathionylation of IKK α and IKK β were associated with increases in phospho-RelA and decreases in I κ B α content, indicative of protracted activation of IKK (Figure 3).

The role of GSTP in IKK β -dependent pro-inflammatory cytokine production: Based upon the observed interactions between GSTP and IKK β and the GSTP-dependent S-glutathionylation of IKK β , we next examined whether GSTP directly regulates IKK β -dependent pro-inflammatory responses and whether the catalytic activity of GSTP is important for regulation of IKK β . C10 cells were transfected with WT HA-IKK β in

conjunction with either WT FLAG-GSTP, Y7F FLAG-GSTP (catalytically inactive mutant of GSTP), or empty vector (PCMV) control (Figure 4A). Results in Figure 4B confirm that Y7F GSTP is catalytically inactive in comparison to WT GSTP. Following transfection with WT IKK β , GM-CSF content in the medium was significantly increased over PCMV controls 24 and 48h post transfection (Figure 4C), however, these increases were no longer observed following co-overexpression with either WT or Y7F FLAG-GSTP. These results indicate that GSTP-mediated suppression of IKK β -induced pro-inflammatory responses does not strictly require catalytic activity of GSTP. We therefore further investigated the potential ligandin function of GSTP, given that GSTP is known to inhibit protein function via protein/protein interactions (Adler et al., 1999). We performed co-immunoprecipitation experiments to detect potential interactions between FLAG-GSTP and HA-IKK β . Following transfection of C10 cells with HA-IKK β and FLAG-GSTP, surprisingly, no interaction between these two proteins was detected, based upon immunoprecipitation of HA-IKK β and subsequent detection of FLAG-GSTP (Figure 5A). These observations suggest that the association observed between endogenous GSTP and IKK β in Figure 1 may not reflect a direct association between IKK β and GSTP, and suggest that other proteins may be required for those associations to occur. We therefore examined whether GSTP interacted with I κ B α , as I κ B α is a substrate of IKK β in the NF- κ B pathway. In unstimulated cells, GSTP and I κ B α protein interactions were readily detectable, despite a lack of S-glutathionylation of I κ B α (data not shown). Upon stimulation of cells with LPS, the association of GSTP and I κ B α was no longer detectable over time periods up to 24 hours. Overall, these findings suggest a

potential role of GSTP in repression of the NF- κ B pathway by controlling the function of I κ B α .

Impact of human GSTP variants on IKK β -induced pro-inflammatory cytokine production: Finally, we examined the role of human GSTP polymorphic variants on IKK β -induced signaling. The presence of Val (105) (GSTP B and C) has been linked to decreases in association with atopic asthma and increases in lung function, and the presence of Ile (105) (GSTP A and D) has been associated with an increased enzymatic affinity for CDNB, a common substrate of GSTs (Watson, Stewart, Smith, Massey, & Bell, 1998). The presence of Val (114) (GSTP C and D) in comparison to Ala (114) (GSTP A and B) has not been strongly associated with changes in susceptibility to asthma nor affinity for CDNB, although it is important to note that multiple GST substrates exist. Following overexpression of WT IKK β (Figure 6A), GM-CSF content in the medium increased over EV controls (Figure 6B). Co-overexpression of variants A and D along with IKK β (Figure 6A) resulted in decreases in WT-IKK β -induced GM-CSF (Figure 6B). In contrast, GSTP B and C only partially suppressed GM-CSF content (Figure 6B).

Discussion

The airway epithelium plays a crucial role in allergic airway disease, based upon its dual roles in barrier function and its interface with cells of the immune system. GSTP is prominently expressed in lung epithelial cells (Reddy, Tu, & Wu, 1995), which is the location of NF- κ B activity in allergic airway disease (Hart et al., 1998; Pantano et al.,

2008; Poynter et al., 2002). S-glutathionylation of NF- κ B members is a known negative regulatory mechanism of NF- κ B; therefore, we aimed to investigate potential enzymes that catalyze this process. Given the prominent expression of GSTP in the epithelium, and its ability to catalyze S-glutathionylation, we explored the role of GSTP in regulating NF- κ B activation, S-glutathionylation, transcriptional activity, and pro-inflammatory cytokine production. Our results demonstrate that siRNA-mediated knockdown of GSTP enhanced NF- κ B activation and transcription, indicating a prominent role for GSTP in modulation of NF- κ B. GSTP associated with IKK β , but not IKK α in response to LPS, and played a causal role in the increases of IKK α and IKK β glutathionylation in response to LPS. Both WT GSTP and the catalytically inactive mutant of GSTP suppressed IKK β -induced GM-CSF production, suggesting that GSTP may regulate the NF- κ B pathway via multiple processes.

GST expression has been associated with the development of allergic airway disease in murine models. GST omega (GSTO), GST alpha (GSTA) and GSTP transcript and protein levels are increased in the lung epithelium of mice exposed to OVA-induced allergic airway disease (Dittrich et al., 2010; Sohn et al., 2013). Additionally, GST mu (GSTM) has been shown to have a protective role in epithelial cells exposed to ozone (W. Wu et al., 2011). Due to the unique association between GSTP polymorphisms and susceptibility to asthma, studies have been performed in order to address the role of GSTP in mouse models of allergic airway disease. GSTP^{-/-} mice display enhanced eosinophilia, remodeling, goblet cell hyperplasia, and lung resistance in comparison to Wild Type mice following subjection to Alum/OVA induced allergic airway disease (J.

Zhou et al., 2008). However, both increases and decreases in GSTP overall content have been observed in murine models of allergic airway disease. In one study, HDM exposure decreased GSTP expression, in association with decreased GST activity (Schroer et al., 2011). These results are in direct contrast to the aforementioned studies utilizing OVA (Sohn et al., 2013). Data from the present study suggest a protective role for GSTP in LPS-induced pro-inflammatory signaling in epithelial cells, and as such, future experiments aimed at modulating GSTP content specifically in the lung epithelium remain of great interest.

In addition to its ability to catalyze reduced glutathione to electrophilic substrates, GSTP is also a ligandin and can bind various protein targets. C-Jun N-terminal kinase (JNK), a kinase that regulates diverse biological pathways, is negatively regulated by GSTP via direct interaction. Following exposure to either radiation or treatment with hydrogen peroxide, GSTP oligomerizes and releases JNK, thus facilitating its activation (Adler et al., 1999). Additionally, GSTP associates with and inhibits the function of the adaptor protein, TRAF2. GSTP/TRAF2 associations prevent TRAF2/ASK-induced cell death. The association observed between GSTP and TRAF2 occurred independent of the catalytic activity of GSTP, demonstrating that GSTP can regulate cellular responses independently of its catalytic activity (Y. Wu et al., 2006). It is therefore plausible that GSTP can also physically associate with NF- κ B proteins and regulate their function, independent of its catalytic activity. In the present study, associations between exogenously expressed FLAG-GSTP and HA-IKK β were not detected, in contrast to results demonstrating an association between endogenous proteins. These data indicate

the lack of a direct association between IKK β and GSTP, and suggest that observed interactions in the immunoprecipitation may include a complex of proteins. In addition to the LPS-induced association between IKK β and GSTP, we also detected an association between GSTP and I κ B α in unstimulated cells. This interaction between GSTP and I κ B α could potentially prevent the phosphorylation and ubiquitination of I κ B α , thus preventing NF- κ B activation. Our data demonstrate that LPS-induced nuclear content of RelA was rapidly enhanced in GSTP siRNA cells in comparison to control siRNA transfected cells, and suggest a potential acute mechanism of GSTP in the regulation of I κ B α phosphorylation and/or degradation, independently of S-glutathionylation. As GSTP content does not affect IKK β/α -SSG until 6h post LPS exposure, S-glutathionylation of IKK proteins may reflect a catalytic mechanism to attenuate IKK's activity. This putative scenario wherein the ligandin function of GSTP prevents degradation of endogenous I κ B α and a catalytic mechanism of GSTP-mediated S-glutathionylation to shut down IKK reflects a versatile regulatory mechanism of regulation of NF- κ B, which will require further experiments.

GSTs catalyze incorporation of reduced glutathione to electrophilic substrates through thioether linkages and promote detoxification of oxidizing agents (Armstrong, 1997), a mechanism that counterbalances oxidative stress. GST presence has been linked to a variety of cancers and GSTP, specifically, is a prevalent protein in tumors, including ovarian, non-small cell lung, breast, colon, pancreas, and lymphomas (Tew, 2007). Due to its ability to detoxify chemicals, GST presence has been linked to resistance to therapeutics. In addition to extensive research performed elucidating the roles of GSTs in

cancer, GSTs are also hypothesized to contribute to multiple diseases, including allergic asthma. As mentioned, the GSTP gene is located on chromosome 11q13, a known locus for asthma-associated genes. Human polymorphisms of GSTP, particularly at amino acid 105, have been linked to atopic asthma; however, the functional contributions of polymorphic variants to asthma pathogenesis remain unclear. The frequency of GSTP Val (105) (GSTP B and C) is significantly lower in asthmatics, and has less of an association with asthma susceptibility than GSTP Ile (105) (GSTP A and D) (Aynacioglu, Nacak, Filiz, Ekinici, & Roots, 2004; Fryer et al., 2000). Furthermore, the presence of GSTP Val (105) is associated with decreased airway dysfunction (Fryer et al., 2000). In contrast to these studies, recent work demonstrated that the presence of the Ile (105) haplotype was associated with increased acute phase pro-inflammatory cytokines in humans (Hoskins et al., 2013). Interestingly, the GSTP Val (105) retains lower affinity for CDNB, a common substrate of GST's, in comparison to GSTP Ile (105) (Watson et al., 1998). We hypothesized that GSTP dampens NF- κ B activity via catalyzing S-glutathionylation, and our results demonstrate that GSTP WT Ile (105) (GSTP A) suppresses IKK β -induced GM-CSF induction. Additionally, we also demonstrate that GSTP Val (105) (GSTP C) suppresses IKK β -induced GM-CSF, although not to the extent of GSTP A. Lastly, we demonstrate that GSTP D, which also contains the Ile 105 allele, suppresses WT IKK β -induced GM-CSF nearly to the extent of GSTP A. Whether this suppression occurs through an altered binding capacity of GSTP to proteins that regulate NF- κ B, catalysis of S-glutathionylation, or both, remains an active area of investigation.

In the present study we demonstrate that GSTP modulates NF- κ B activation and pro-inflammatory cytokine production in lung epithelial cells. One potential mechanism by which this occurs is via GSTP-catalyzed S-glutathionylation of IKK β ; however, we also provide evidence that GSTP associates with NF- κ B proteins, notably IKK β and I κ B α , and suggests multiple mechanisms by which GSTP regulates NF- κ B. Given the reported relevance of GSTP polymorphisms in allergic asthma, these studies provide a potential mechanism by which GSTP can regulate pro-inflammatory signaling in allergic airway disease.

Figure Legends:

Figure 1: LPS-induced association of GSTP and IKK β : (A) C10 lung epithelial cells were exposed to LPS for the indicated times and lysates were subjected to immunoprecipitation (IP) for GSTP, followed by SDS-PAGE separation, and assessment of content of IKK β , IKK α , and GSTP via Western blot analysis. Whole cell lysates (WCL) under the same conditions were assessed for total IKK β and I κ B α . (β -Actin, loading control)

Figure 2: Role of GSTP in LPS-induced NF- κ B activation, transcriptional activity, and pro-inflammatory cytokine production. (A) C10 lung epithelial cells were transfected with either control siRNA or GSTP siRNA and exposed to LPS for the indicated times. Cytosolic and Nuclear fractions were separated by SDS PAGE. Cytosolic fractions were assessed for GSTP; nuclear fractions were assessed for RelA and RelB (β -Actin, loading control). (B) C10 cells stably transfected with NF- κ B

luciferase were subjected to either control siRNA or GSTP siRNA, and subsequently exposed to LPS for 6 h. Luciferase measurements were corrected for protein concentrations. (C) C10 cells were treated as in A and exposed to LPS as indicated. GM-CSF and CCL-20 content was assessed in the medium by ELISA. * $p < 0.05$ (ANOVA) compared LPS to the respective sham group, † $p < 0.05$ (ANOVA) compared respective siRNA groups at the same time points.

Figure 3: Role of GSTP in modulating IKK α and IKK β glutathionylation and NF- κ B activation: C10 lung epithelial cells were transfected with either control siRNA or GSTP siRNA and exposed to LPS for the indicated times, separated by SDS-PAGE, and whole cell lysates (WCL) were analyzed for content of phospho RelA, I κ B α , IKK α , IKK β , and GSTP (β -Actin, loading control). C10 cells were transfected and treated with LPS as indicated and subjected to Immunoprecipitation (IP) for GSH. Lysates were separated by SDS-PAGE and analyzed for protein content of IKK β and IKK α . +DTT indicated sample subjected to treatment with DTT to decrease PSSG prior to GSH IP.

Figure 4: Role of GSTP in IKK β -dependent pro-inflammatory cytokine production: (A) C10 lung epithelial cells were either transfected with WT HA-IKK β alone or co-overexpressed with either WT FLAG-GSTP or Y7F FLAG-GSTP. Protein content of IKK β and GSTP were assessed via Western Blot. (B) Assessment of WT FLAG-GSTP or Y7F FLAG-GSTP enzymatic activity was assessed by detection of GSH conjugated CDNB. (C) Amount of GM-CSF content in the medium of C10 cells was analyzed by ELISA following either WT HA-IKK β transfection alone or co-overexpression of WT HA-IKK β with either WT FLAG-GSTP or Y7F FLAG-GSTP 24

or 48h post transfection. * $p < 0.05$ (ANOVA) compared the EV group to the WT HA-IKK β -transfected groups, † $p < 0.05$ (ANOVA) compared WT HA-IKK β -transfected groups to either WT HA-IKK β /WT FLAG-GSTP or WT HA-IKK β /Y7F FLAG-GSTP-transfected groups.

Figure 5: GSTP associates with I κ B α : (A) C10 lung epithelial cells were co-transfected with WT FLAG-GSTP and WT HA-IKK β and exposed with LPS for times indicated. Lysates were subjected to Immunoprecipitation with HA antibody, followed by separation by SDS-PAGE and assessed for HA and FLAG. (B) C10 lung epithelial cells were exposed to the indicated times with LPS and lysates were subjected to immunoprecipitation (IP) with GSTP antibody, followed by SDS-PAGE separation, and assessed content of I κ B α and GSTP by Western Blot. IgG controls were subjected to IP by mouse IgG (A) or rabbit IgG (B) and assessed as in A or B.

Figure 6: Impact of human GSTP variants on pro-inflammatory cytokine production. (A) C10 lung epithelial cells were transfected with either PCMV, WT HA-IKK β , GSTP human haplotypes: A, B, C, or D or the indicated combinations. Lysates were separated by SDS-PAGE and assessed via Western Blot for analysis of IKK β , GSTP, and β -Actin (loading control). (B) C10 lung epithelial cells were transfected with either PCMV, WT HA-IKK β alone, or WT HA-IKK β with either GSTP haplotypes: A, B, C, or D and medium was assessed for content of GM-CSF via ELISA. * $p < 0.05$ (ANOVA) compared to the PCMV group (EV), † $p < 0.05$ (ANOVA) compared the HA-IKK β -transfected groups.

Figure 1: LPS-induced GSTP associations with IKK

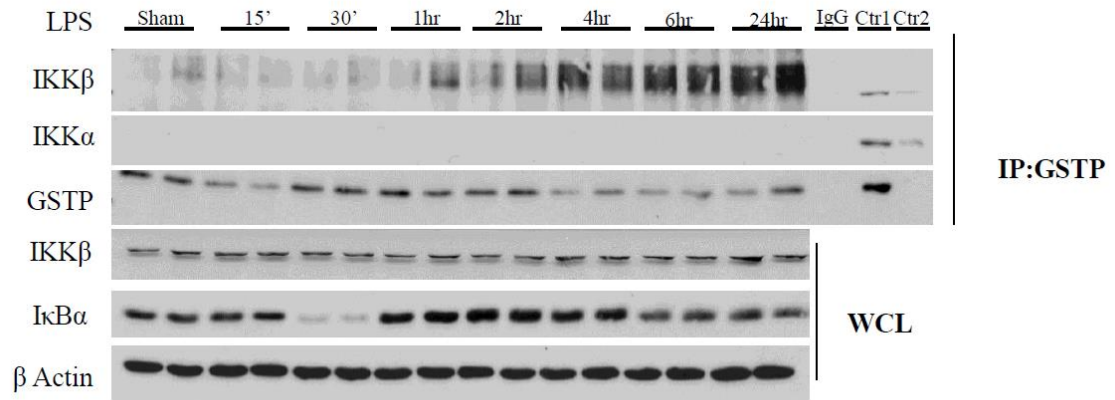


Figure 2: GSTP knockdown enhances LPS-induced NF- κ B activation, transcriptional activity, and cytokine production

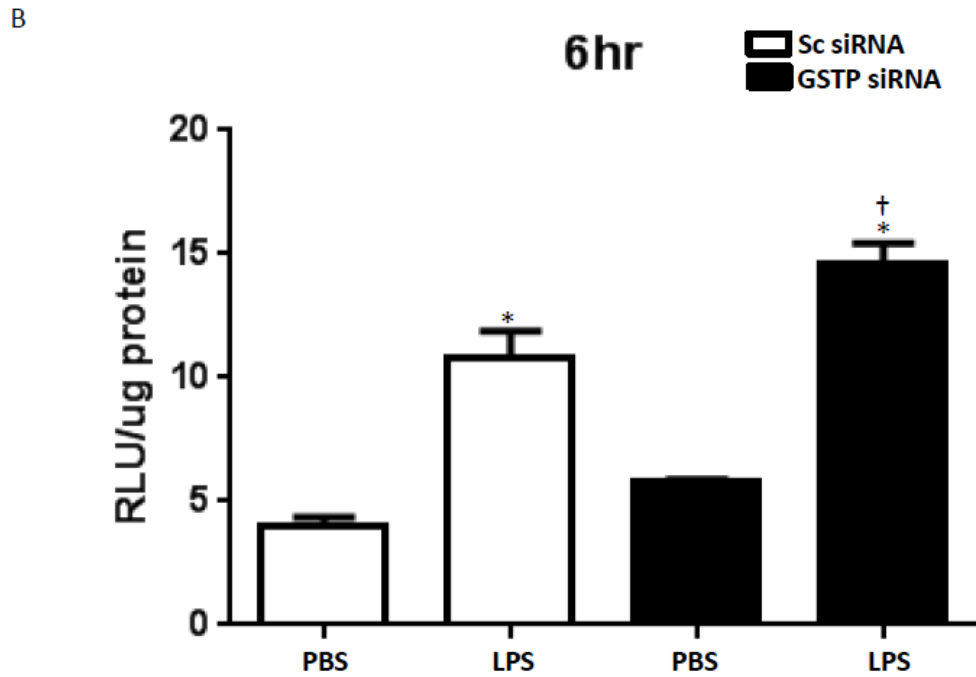
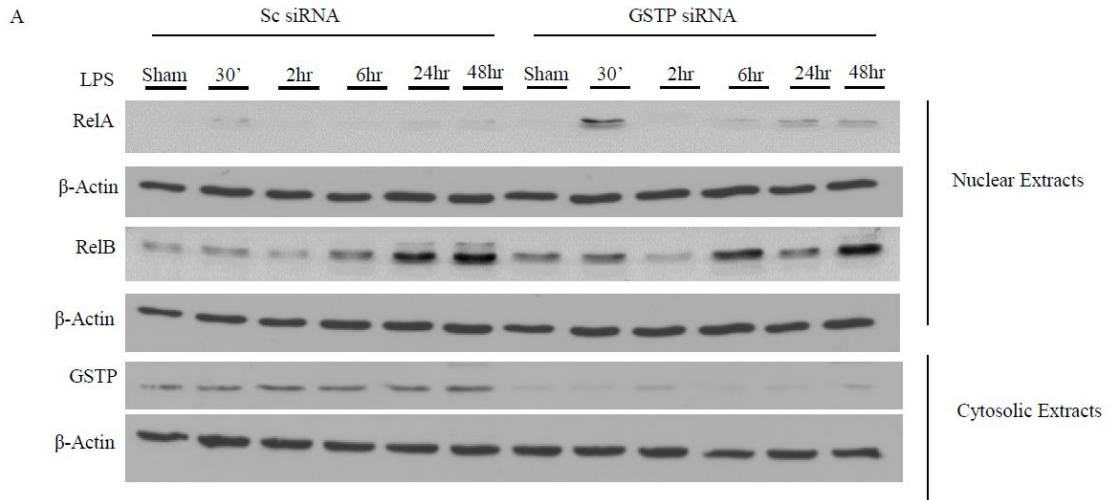


Figure 2 cont: GSTP knockdown enhances LPS-induced NF- κ B activation, transcriptional activity, and cytokine production

C

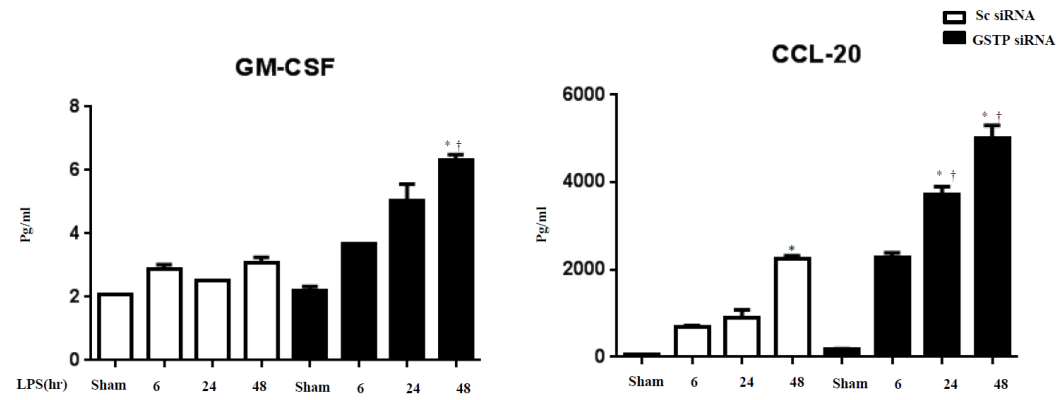


Figure 3: GSTP knockdown enhances IKK α and IKK β S-glutathionylation

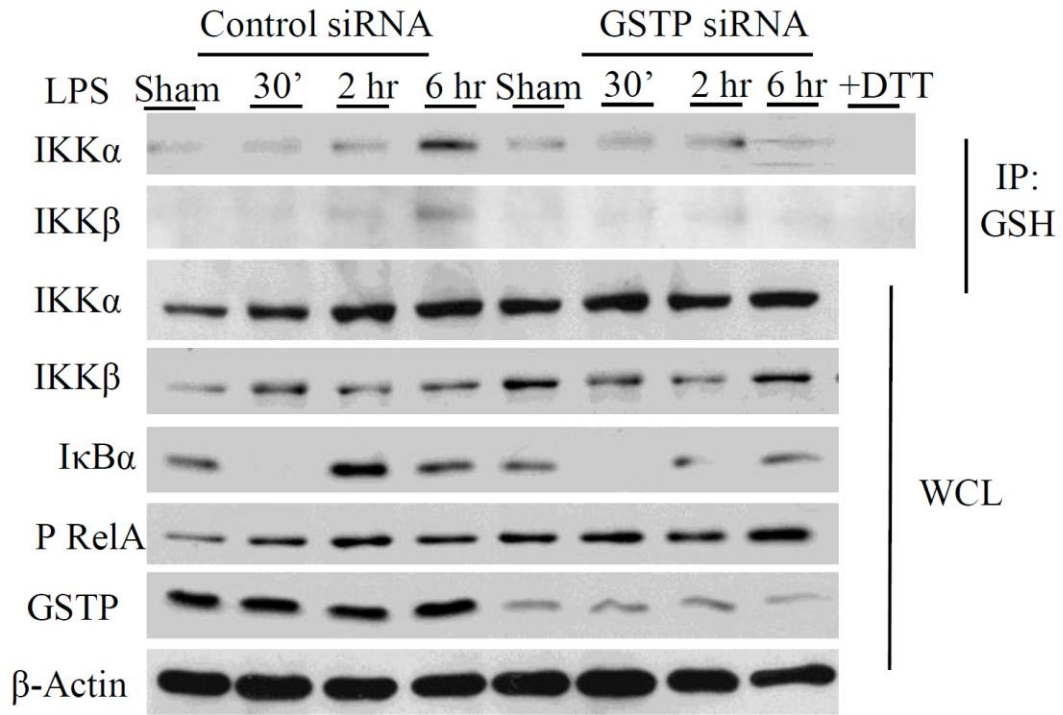


Figure 4: GSTP downregulates IKK β -induced pro-inflammatory response

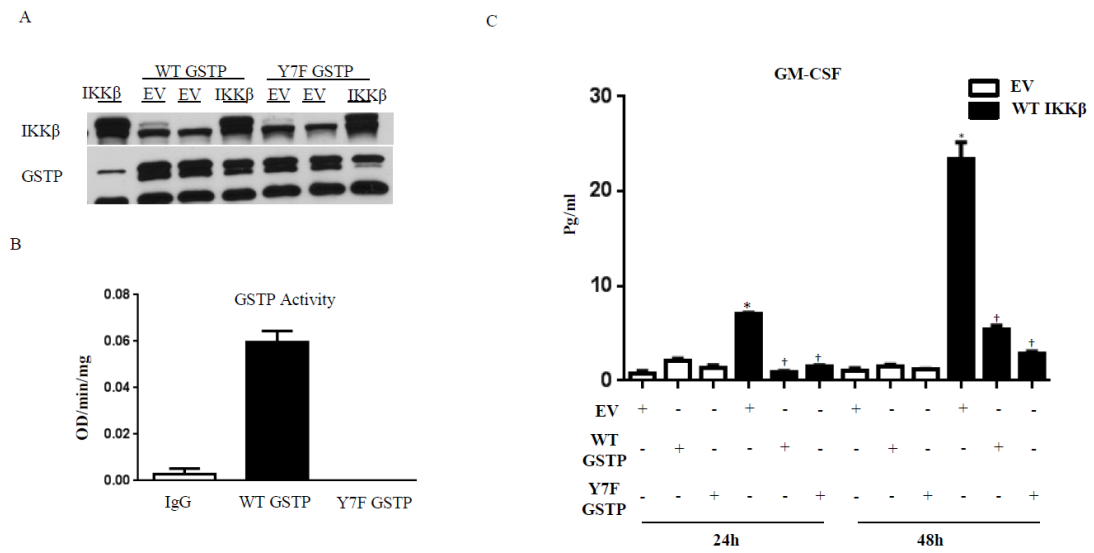
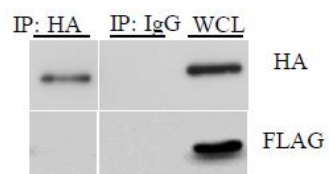


Figure 5: GSTP associates with I κ B α

A



B

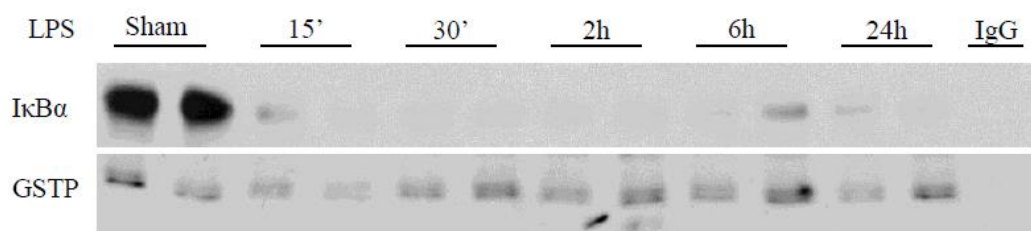
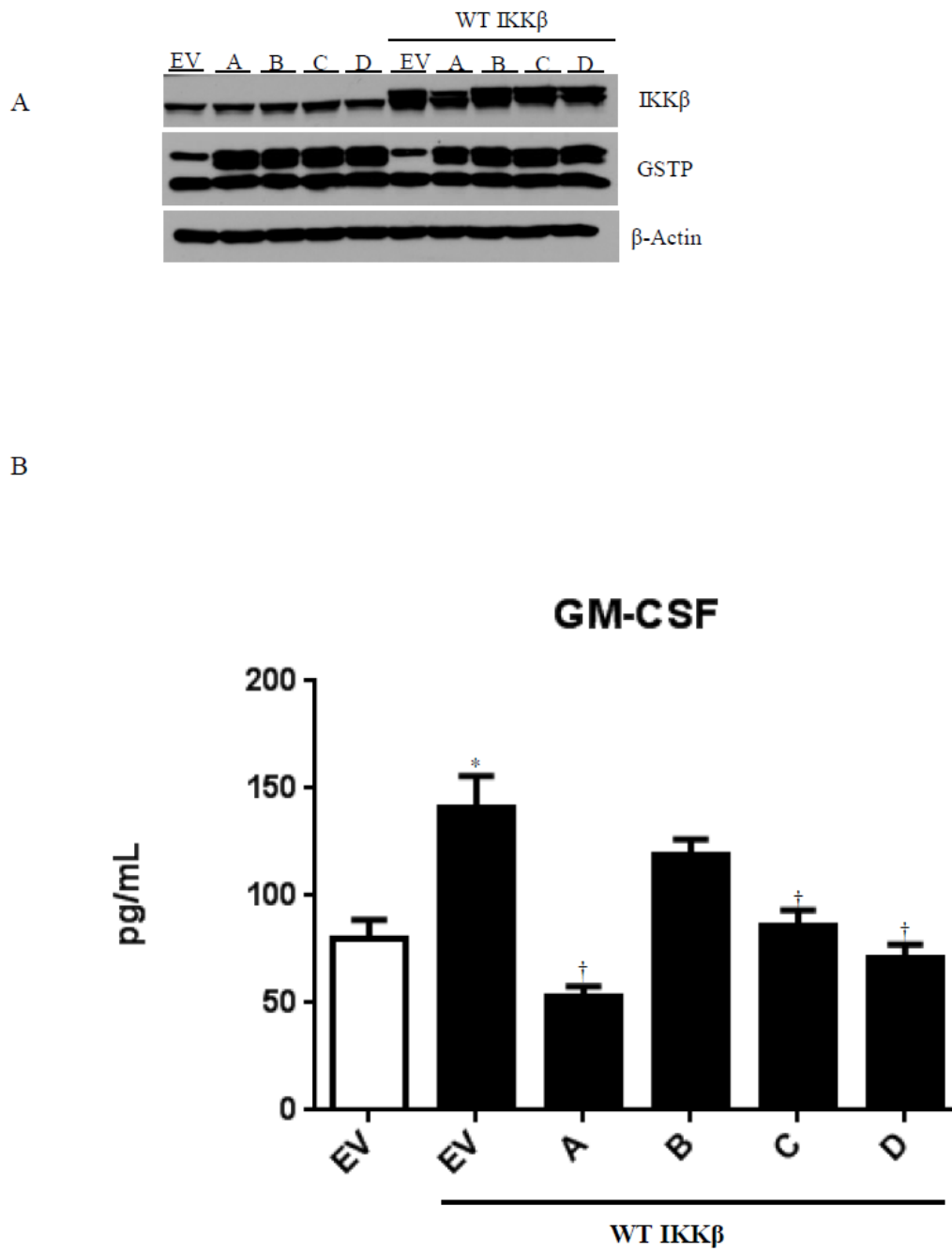


Figure 6: Human polymorphic GSTP variants modulate IKK β -induced responses



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Chapter 5

Concluding Remarks and Future Directions

Summary of Intent

Previous studies from our laboratory demonstrated a role for epithelial NF- κ B in regulating LPS and allergic-induced pulmonary inflammation. While activation of the classical pathway had been studied extensively in both *in vivo* and *in vitro* models of inflammation, the role of alternative NF- κ B in lung epithelial cells had remained unknown. A primary goal of this thesis, therefore, was to investigate activation of alternative NF- κ B in lung epithelial cells, as well as establish a potential role for alternative NF- κ B in regulating epithelial-derived inflammatory responses. Furthermore, we aimed to investigate crosstalk between classical and alternative signaling, and how they collectively contribute to epithelial-derived pro-inflammatory phenotypes (Chapter 2). Additionally, the role of epithelial NF- κ B in orchestrating allergic asthma had not been investigated in the context of a model utilizing an allergen relevant to human asthma. Therefore, a goal of this thesis was to evaluate the impact of inhibition of NF- κ B in the lung epithelium following exposure to House Dust Mite, a known asthma trigger in mice (Chapter 3). Finally, our laboratory previously demonstrated that IKK β kinase activity is inhibited through the post-translational modification, S-glutathionylation. Given the importance of S-glutathionylation in the regulation of NF- κ B, we aimed to investigate the enzyme, GSTP, as a potential regulator of NF- κ B, since GSTP was recently identified as a catalyst of S-glutathionylation reactions (Chapter 4).

Classical and Alternative NF- κ B in lung epithelial cells

Our results demonstrate that cooperation occurs between classical and alternative NF- κ B in lung epithelial cells. In other cell types, such as fibroblasts, alternative NF- κ B promotes an anti-inflammatory phenotype (Bagloli et al., 2008). However, we demonstrate herein that co-overexpression of constitutively active IKK α and IKK β cooperatively enhances production of pro-inflammatory cytokine content, in association with increases in nuclear content of both RelA and RelB. Mechanisms that govern this phenotype are not clear. It is possible that RelA/RelB heterodimers on target promoter regions may facilitate increased production of pro-inflammatory cytokines. Multiple reports have indicated a role for RelB in preventing RelA-induced transcription (Saccani et al., 2003), however other reports have contrasted these results. RelB occupancy on the GM-CSF promoter enhances RelA-induced transcription, illustrating a potential cooperative impact for RelA and RelB promoter occupancy (Sasaki et al., 2011). Future promoter studies investigating co-occupancy of RelA and RelB, in association with RNA PolIII, would take place in order to test this putative scenario. RelA/RelB-modulated promoter regulation may also be unique to each gene being evaluated. To address whether RelB does play a role in enhancing RelA-induced transcription, experiments in which NIK, IKK α , and RelB are ablated and RelA promoter associations assessed, can be performed.

One major aim of Chapter 2 of this thesis was to evaluate the role of the alternative NF- κ B pathway in lung epithelial cells; however, specific activation of the alternative NF- κ B pathway without activating classical NF- κ B proved challenging. This

observation demonstrates the complexity of interdependence between the two pathways. Despite this observation, alternative methods in the future may be employed in order to specifically activate alternative NF- κ B. Constitutively active IKK α increased nuclear content of RelA and RelB, while WT IKK α and RelB plasmid overexpression had no effect on either pathway (data not shown). Therefore, a future approach may directly target NIK, the kinase upstream of IKK α in the alternative pathway. TRAF3 constitutively ubiquitinates NIK, leading to its degradation and inactivation of the alternative NF- κ B pathway. However, upon degradation of TRAF3, NIK content stabilizes and activates alternative NF- κ B signaling (Liao et al., 2004). Recent work has demonstrated that a cleavage mutant of NIK, which retains the C terminus region of the protein, exhibits kinase activity, but is resistant to TRAF3-mediated degradation (Rosebeck et al., 2011). Potential future studies could utilize this construct in order to specifically activate alternative NF- κ B in lung epithelial cells and determine its role in regulation of inflammatory responses.

Despite the results obtained in Chapter 2, the role of alternative NF- κ B in the lung epithelium is not yet clear. We demonstrated that IL-6 content increased significantly following IKK α and RelB siRNA-mediated ablation, in contrast to RelA-mediated ablation. IL-6 is a pro-inflammatory cytokine derived primarily from epithelial cells, and is known for its ability to polarize CD4⁺ T cells. IL-6, in conjunction with TGF- β 1, is known to promote T cell polarization to a TH17 subtype and also promotes IL-4 release from TH2 cells (Dienz & Rincon, 2009). Interestingly, IL-6 is basally expressed in structural cells of the lung, as opposed to immune cells (Neveu et al., 2011). Therefore, it

is possible that the IL-6 derived from lung epithelial cells may have a role in TH17 polarization, and that this phenotype is mediated by attenuation of alternative NF- κ B. *In vivo* models of allergic inflammation would be required to address these putative scenarios.

Epithelial NF- κ B in HDM-induced allergic airway disease

Our results demonstrate that NF- κ B inhibition in the epithelium protects against HDM-induced inflammation, hyperresponsiveness, and remodeling. However, the exact mechanism by which this occurs is unknown. We demonstrate that inhibition of NF- κ B prevented the expression of IL-33, a cytokine known to promote the pathogenesis of asthma, among many other disorders (Borish & Steinke, 2011). It is still unknown whether activation of NF- κ B is important during the sensitization phase, challenge phase, or both, following HDM exposure, because of the constitutive expression of the transgene. Future studies for this project include elucidating whether the activation of NF- κ B is important in response to initial exposure of HDM itself, or whether it regulates adaptive immune responses following repeated exposure to HDM. Additional genetic models in which the NF- κ B_{SR} transgene is expressed conditionally could be developed in order to address these questions.

Both classical and alternative NF- κ B are inhibited in the epithelium following expression of the NF- κ B_{SR} transgene. This is not surprising, given the ability of NF- κ B Rel proteins to bind ankyrin repeat domains on I κ B's. It does, however, raise the question of whether classical, alternative, or both are important in HDM-induced allergic

airway disease. Following HDM administration, we observed activation of both classical and alternative NF- κ B, leading to the hypothesis that they both may play a role in the pathogenesis of disease. In order to begin to address the role of alternative NF- κ B alone, we administered RelB siRNA into the mouse lung prior to HDM exposure. While we were able to demonstrate that RelB ablation conveyed a partial protection against HDM-induced pro-inflammatory cytokine expression, the relevance RelB to HDM-induced allergic inflammation, hyperresponsiveness, and remodeling still remains unknown. These studies did not address the role of RelB specifically in the epithelium, as siRNA administration may target other resident cells in the lung. Therefore, in order to address the role of alternative NF- κ B specifically in the lung epithelium in HDM-induced allergic airway disease, additional genetic models should be generated. Crossing a NIK/IKK α /RelB^{flox/flox} mouse with the CC10-rtta tetOP/Cre mouse and exposing to HDM would have the potential to answer these questions.

Glutathione S-transferase-mediated regulation of NF- κ B

We demonstrate that IKK β -induced GM-CSF is decreased by the GSTP haplotypes; however, the mechanisms and overall impact on NF- κ B activity remain unclear. In future experiments, we could compare the ability of the different GSTP haplotypes to glutathionylate IKK β and modulate NF- κ B transcriptional activity. To date, despite the characterization of their ability to conjugate GSH to different electrophilic substrates, such as CDNB (Watson et al., 1998), nothing is known about the ability of different human GSTP variants to catalyze S-glutathionylation. Furthermore,

haplotype C is associated with protection against asthma in comparison to haplotype A (Aynacioglu et al., 2004). However, controversy still remains in the literature over this observation, as recent work demonstrated that patients with haplotype C demonstrated enhanced acute phase cytokine production (Hoskins et al., 2013). Therefore, understanding the molecular mechanisms that govern the ability of GSTP haplotypes to modulate NF- κ B, both through enzymatic and non-enzymatic mechanisms is crucial to understanding the role of GSTP in allergic asthma.

Our studies demonstrate that IKK β -SSG increases are dependent on GSTP content following exposure to LPS. Therefore, we hypothesized that GSTP specifically downregulates IKK β -induced cytokine production, and that this suppression is due to the catalytic activity of GSTP. Surprisingly, we observed that both WT and catalytically inactive GSTP equally suppressed IKK β -induced GM-CSF, indicating a mechanism distinct from the ability of GSTP to conjugate GSH to target proteins. GSTP is also a ligandin, whereby its association with a target protein inhibits its function. GSTP has already been reported to associate with TRAF2 and c-JUN N-terminal kinase (Adler et al., 1999; Y. J. Kim et al., 2006; Y. Wu et al., 2006), however, it is not known with which proteins GSTP may associate in the NF- κ B pathway. We demonstrate a possible association between GSTP and IKK β , as well as I κ B α . However, it is possible that GSTP has other binding partners that regulate the NF- κ B pathway. Future directions for this study would include mass spectrometry analysis to identify GSTP associated proteins.

It is also possible that GSTP associates with the IKK signalsome, given the indirect associations observed between IKK β and GSTP. Therefore, another future

direction of this project will include disrupting the IKK complex with NEMO binding domain peptides and evaluate LPS-induced GSTP associations with IKK β . As GSTP can also associate with TRAF2, it is additionally plausible that GSTP binds adaptor proteins upstream of the signalsome, which in turn leads to enhanced association with the IKK complex. In conjunction with these scenarios, it is also noteworthy that classical and alternative NF- κ B activation are both controlled through the IKK signalsome (Chapter 2). Current NF- κ B inhibitors target different components of the classical pathway, including IKK β , I κ B α degradation, and nuclear translocation of RelA (Horie, 2013). It is important to understand how these inhibitors effect both classical and alternative NF- κ B, as the results from this thesis clearly demonstrate that alternative NF- κ B also plays a role in epithelial-derived pro-inflammatory responses. Furthermore, indirectly targeting NF- κ B through known regulatory molecules, such as GSTP, may also impact both classical and alternative NF- κ B. Therefore, when considering methods by which to therapeutically target NF- κ B, one must take into account the potential impact on both classical and alternative NF- κ B.

Figure Legends:

Figure 1: Schematic depicting simultaneous activation and crosstalk of classical and alternative NF- κ B. Following stimulation of pro-inflammatory agonists, such as LPS, both classical and alternative NF- κ B are activated in lung epithelial cells. Pro-inflammatory phenotypes of both IKK α and IKK β each require the presence of both RelA and RelB. Furthermore, RelA and RelB nuclear translocation is dependent on the IKK signalsome.

Figure 2: Table summarizing differences between Wild Type and NF- κ B_{SR} mice following HDM administration. Wild Type and NF- κ B_{SR} BALB/c mice were exposed to 15 challenges (over 3 weeks) of HDM and assessed for the indicated parameters in the table. Arrows in WT column indicate impact of HDM, and arrows in NF- κ B_{SR} column indicate a comparison to WT mice.

Figure 3: Schematic depicting GSTP-induced regulation of NF- κ B pathway. Following stimulation with LPS, IKK phosphorylates I κ B α , thus activating NF- κ B. The presence of GSTP associates with I κ B α in unstimulated cells, and its release facilitates its degradation. Furthermore, following activation of IKK, GSTP catalyzes S-glutathionylation of IKK and dampens its kinase activity, thus shutting down NF- κ B dependent gene transcription.

Figures:

Figure 1:

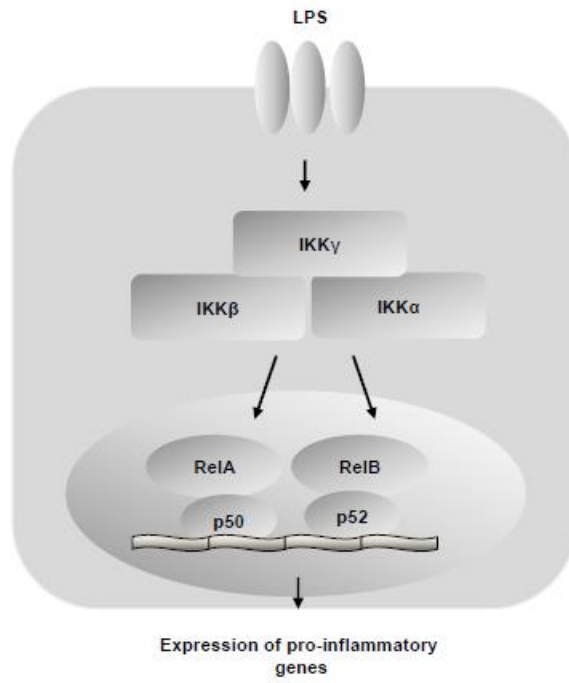
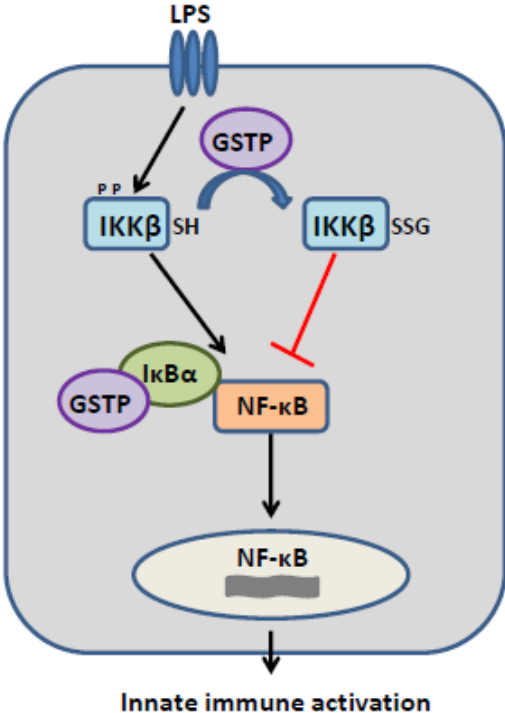


Figure 2:

| | WT HDM | NF- κ B _{SR} |
|--------------|--------|------------------------------|
| Eosinophilia | ↑ | ↓ |
| Neutrophilia | ↑ | ↓ |
| AHR | ↑ | ↓ |
| Mucus | ↑ | No Change |
| IgE | ↑ | No Change |
| Collagen | ↑ | ↓ |

Figure 3:



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