Attenuation of Airway Hyperreactivity by Gram- Negative Lipopolysaccharide in a Murine Model of Asthma

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ABSTRACT

ATTENUATION OF AIRWAY HYPERREACTIVITY BY GRAM- NEGATIVE LIPOPOLYSACCHARIDE IN A MURINE MODEL OF ASTHMA

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Asthma exacerbations due to exposures to air pollution and environmental allergens are common, leading to diminished responses to treatment and increased hospitalizations. Endotoxin or lipopolysaccharide (LPS) is a component of the cell wall of Gram- negative bacteria found throughout the environment. Individually, asthma and inhalation of LPS increases lung inflammation and airway hyperresponsiveness (AHR). Previous studies have shown that LPS exposure modulates both inflammatory and physiological responses in the lungs of asthmatic individuals. The primary focus of this work was to investigate the effects of inhaled LPS exposure on the pathology and physiological responses in the lung of a murine model of ovalbumin (OVA) - induced allergic asthma. Additionally, I investigated potential mechanisms that may underlie the dissociation between airway inflammation and AHR present in allergic mice exposed to LPS (OVA-LPS). I hypothesized that the attenuation of AHR in OVA-LPS mice was dependent on the upregulation of the gene responsible for nitric oxide production, *Nos2*, induced by recruited neutrophils. Four specific aims were generated. In aim 1, I studied the relationships between the cellular character and distribution of lung lesions with components of AHR in OVA-LPS mice. To do so, naïve and OVA- induced allergic mice were exposed to LPS by intranasal instillation 48 hours following the final saline/OVA challenge. AHR was determined and animals were euthanized to collect

tissue samples 24 hours after LPS exposure. There was significant pulmonary inflammation consisting of eosinophils distributed within the central airway compartment composed of perivascular and peribronchiolar regions (OVA group), neutrophils located in central airway and peripheral lung tissue compartments composed of alveolar septa and airspaces (LPS group), and OVA-LPS mice had more severe inflammation combining both cell types distributed in both compartments as well. AHR was increased in both central airways and the peripheral lung tissue in OVA and LPS mice. By comparison, AHR was attenuated in OVA-LPS mice. In aim 2, I hypothesized that recruited airway neutrophils contributed to the attenuation of AHR in OVA-LPS mice. Systemic depletion of neutrophils prior to LPS exposure significantly lowered BALF neutrophils. Compared to neutrophil sufficient mice, neutrophil depletion did not alter AHR in OVA-LPS mice. In aim 3, I hypothesized that LPS activation of the transcription factor, NF-κB, attenuated AHR in OVA-LPS mice. Following treatment with a novel NF-κB inhibitor, OVA-LPS mice had significantly decreased BALF macrophages, eosinophils, and lymphocytes but not neutrophils compared to non-treated mice. In spite of the decrease in cellularity, AHR significantly increased suggesting that NF-κB activation contributes to the attenuation of AHR in OVA-LPS mice. In aim 4, I evaluated the relationship between expression of the genes nitric oxide synthase-2 (*Nos2*) and arginase-1 (*Arg1*) and the attenuation of AHR in OVA-LPS mice. Following LPS exposure, *Nos2* and *Arg1* were increased in OVA-LPS mice. Treatment with a NF-κB inhibitor significantly blunted the expression of both genes, suggesting that NF-κB mediated increased expression of *Nos2* potentially contributes to attenuation in AHR, which is reversed with downregulation of *NOS2* gene expression.

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KEY TO ABBREVIATIONS

OVA	Ovalbumin
LPS	Lipopolysaccharide
IgE	Immunoglobulin E
Th2	T helper-2
AHR	Airway hyperresponsiveness
IL	Interleukin
IFNγ	Interferon gamma
NO	Nitric oxide
Arg-1	Arginase-1
NOS	Nitric oxide synthase
nNOS	Neuronal Nitric oxide synthase
eNOS	Endothelial Nitric oxide synthase
iNOS	Inducible Nitric oxide synthase
cNOS	Constitutive Nitric oxide synthase
PM	Particulate matter
EPA	Environmental Protection Agency
TLR	Toll-like receptor
MCh	Methacholine
cGMP	Cyclic guanosine monophophate
$ONOO^-$	Peroxynitrite
FeNO	Fractional exhaled nitric oxide

HDM	House dust mite
FOT	Forced oscillation technique
MSU	Michigan State University
LPS	Lipopolysaccharide
i.p.	Intraperitoneal
IN	Intranasal
BALF	Bronchoalveolar lavage fluid
H&E	Hematoxylin and eosin
AB/PAS	Alcian Blue /Periodic Acid-Schiff
PAMPs	Pathogen- associated molecular patterns
DAMPs	Damage associated molecular patterns
PRRs	Pattern recognition receptors
NF-κB	Nuclear factor- kappa B
TGF-β	Tissue growth factor β
FEV	Forced expired volume

CHAPTER 1

GENERAL INTRODUCTION AND SPECIFIC AIMS

1.1 ALLERGIC ASTHMA

Asthma affects over 25 million people in the United States with annual costs of $50 billion (Akinbami et al., 2012; AAAAI, 2013). Asthma is a multifaceted, chronic inflammatory disease of the airways that is associated with reversible airway obstruction, mucus hyperproduction/hypersecretion in airway epithelium, airway smooth muscle hyperplasia, and subepithelial fibrosis (Busse and Lemanske, 2001; GINA, 2012). Due to its complexity, there has been a movement by researchers within the field to classify asthma as several diseases with associated reversible airflow obstruction, rather than a single disease entity with variation in clinical and pathological features (Lancet, 2006; Wenzel, 2006). To this end, a recent consensus report produced by experts from the European Academy of Allergy and Clinical Immunology and the American Academy of Allergy, Asthma & Immunology recommended that asthma be categorized by 'endotypes', which focused not only on clinical features as is done with asthma phenotypes, but also on distinctions of pathology, physiology, immunology, environmental influences, and response to treatment (Lötvall et al., 2011). The focus of my research is allergic asthma, an endotype of eosinophilic asthma, which is one of the most commonly studied asthma phenotypes.

The development of allergic asthma involves first the sensitization to a specific antigen (allergen) and production of allergen-specific immunoglobulin E (IgE). Exposure of sensitized subjects to an allergen results in a T helper-2 (Th2) lymphocyte-mediated inflammatory response, with histamine release, airway infiltration of eosinophils, and allergen-induced bronchoconstriction (Lötvall et al., 2011). Common allergens

associated with allergic asthma include those antigens derived from house dust mites, cockroaches, animal dander, and pollens from trees and grasses (Baxi and Phipatanakul, 2010). Individuals with genetic predispositions to develop atopy have an increased sensitivity for IgE production directed against these aeroallergens (Mukherjee and Zhang, 2011).

1.2 PATHOPHYSIOLOGY OF ASTHMA

Clinically, allergic asthma is characterized by variable airway obstruction and airway hyperresponsiveness (AHR) that is accompanied by airway inflammation and remodeling. Asthma pathogenesis consists of multiple components, such as structural changes and inflammation that interact to determine the severity of disease.

1.2.1 Structural Components

Studies have shown that asthmatics have extensive epithelial damage and loss along with defects in tight junctions (Wan et al., 2000; de Boer et al., 2008; Xiao et al., 2011). These defects allow allergens, pollutants, toxins, and pathogens to have better access to the subepithelial airway tissues (Holgate, 2007; Al-Muhsen et al., 2011). Also, there is goblet cell metaplasia in the central/conducting and peripheral airways resulting in increased production and secretion of large amounts of mucus (Evans et al., 2009). Lungs from individuals with fatal asthma have an increased percentage of epithelial goblets cells (20-25%) compared to non-asthmatics (5%) and a 20-fold increase in goblet cells in peripheral airways which are normally not present (Pawankar et al., 2009). The increased mucus in the airway lumen contributes to AHR by decreasing airway lumen size or caliber (Rogers, 2004). A study by Short and

colleagues showed a significant relationship between AHR in asthmatics and airway caliber or distensibility (Short et al., 2011). A decrease in airway caliber may further promote airway closure. In addition to changes in airway smooth muscle and epithelium, Holgate and Davies also noted that alterations in neural and vascular networks along with matrix deposition in airway wall promote the development of AHR (Holgate and Davies, 2009).

1.2.2 Cellular Mediators

Eosinophils are a common in allergic asthma and recruitment is dependent on Interleukin (IL)- 5 (Cohn et al., 2004). Eosinophils may play an important role in asthma pathogenesis by causing damage to airway epithelium and cholinergic nerve receptors. Also, eosinophils can mediate airway remodeling, mucus hypersecretion, and AHR by the degranulation and release of toxic enzymes, such as major basic protein, production of pro-inflammatory cytokines (IL-4, -5, and -13), chemokines (eotaxin), generation of reactive oxygen species and leukotrienes (Liu et al., 2006; Kudo et al., 2013).

Neutrophils are increased with severe, corticosteroid resistant forms of asthma, as well as during exacerbation and fatal exacerbation (Jatakanon et al., 1999; Goleva et al., 2008; Fahy, 2009). The role of neutrophils in the pathogenesis of asthma is not clear. Nabe and colleagues showed that neutrophils were inducers of the late asthmatic response, which occurred several hours following allergen challenge, in mice (Nabe et al., 2011). In a review by Foley and Hamid, they suggest that neutrophils may activate epithelial cells and eosinophils along, with increasing goblet cell mucus secretion and vascular permeability in acute asthma (Foley and Hamid, 2007).

Lymphocytes are crucial for the development of asthma because naïve CD4+ T lymphocytes differentiate into (Th2) cells when stimulated during the sensitization process. These T cells produce Th2 cytokines such as IL-4, -5, and -13 that promote the expansion of Th2 cells. These T cells activate B lymphocytes to produce allergen specific immunoglobulins (usually IgE) that bind to and activate mast cells (Cohn et al., 2004; Shum et al., 2008; Holgate and Sly, 2014).

Mast cells are important in the development of acute asthmatic response. They can cause mucus secretion, edema, and bronchoconstriction through the release of mediators, such as histamine and prostaglandins. Also, mast cells promote the Th2 response of IgE production and eosinophilic inflammation by secreting Th2 cytokines, as well as promoting antigen and Th17 cell neutrophilia (Murphy and O'Byrne, 2010). Mast cells can induce airway remodeling and has been associated with AHR (Mauad et al., 2011; Holgate and Sly, 2014).

1.2.3 Soluble Mediators

Th2 cytokines: As mentioned above, IL-4, -5, and -13 are important to maintain Th2 lymphocyte activation. IL-4 also promotes Th2 pathways by decreasing the production of Th1 cells, IFN-γ and IL-12, and induces alternative activation of macrophages into M2 cells and inhibits classical activation of macrophages into M1 cells (Gordon and Martinez, 2010). As previously stated, IL-5 is a potent stimulus for eosinophils, leading to their expansion, recruitment and activation during allergy. IL-13 has similar sequence homolog to IL-4, but appears to be a more important mediator of

airway hyperresponsiveness, goblet cell metaplasia and mucus hypersecretion, which all contribute to airway obstruction.

Nitric Oxide (NO) in exhaled breath has been used over the past decade as a biomarker of airway inflammation in asthmatics. However, NO is a readily soluble, ubiquitous messenger molecule that mediates various biological functions in several organs in the body. At low concentrations it functions as a physiological signal, such as to maintain blood flow as a vasodilator and is a neurotransmitter, while at high concentrations it is cytotoxic to defend against pathogens (Ricciardolo et al. 2004). In the lung, notable functions of NO include maintaining airway and vascular tone in addition to regulating mucus secretion (Reynaert et al. 2005; Zuo et al. 2013). Within the lung, NO is produced by many cells including nerves, endothelial cells, smooth muscle cells, respiratory epithelium, type II alveolar cells along with inflammatory cells, such as macrophages, mast cells, and neutrophils (Gaston et al. 1994; Ricciardolo et al. 2004). In vivo NO has a short half-life of less than 5 seconds due to its reactivity with biological compounds, such as hemoglobin (Ricciardolo et al. 2004).

NO is produced by the enzyme nitric oxide synthase (NOS), which consists of three isoforms: constitutively expressed neuronal NOS (nNOS; NOS1) and endothelial NOS (eNOS; NOS3) isoforms; and inducible NOS (iNOS; NOS2) isoform expressed in cells. Constitutive NOS enzymes depend on calcium and calmodulin to promote the release of small amounts of NO (Ricciardolo et al. 2004). While iNOS is calcium independent and production of large amounts of NO is induced by pro-inflammatory cytokines (Aktan 2004; Ricciardolo et al. 2004). Under normal physiologic conditions in

the lung, NO causes dilation of blood vessels and dilation of airways by increasing production of cyclic guanosine monophosphate (cGMP) causing smooth muscle relaxation (Dweik et al. 1998). However during disease states, cytotoxic effects are seen when NO interacts with superoxide to produce peroxynitrite ($ONOO^-$), which has been associated with the induction of AHR (SadeghiHashjin et al., 1996; Pacher et al., 2007).

1.3 NITRIC OXIDE AND ASTHMA

The functional role of NO in the pathogenesis of asthma is complex and not completely understood. Its effects have been considered beneficial by promoting bronchodilation and harmful due to edema formation from vasodilation, increased mucus secretion, and enhancement of Th2 inflammation (Prado et al., 2011). It has been well documented that increased NO is present in exhaled air (FeNO) and bronchoalveolar lavage fluid (BALF) from asthmatics (Kharitonov et al. 1994; Barnes and Liew 1995; Khatri et al. 2003; Anderson et al. 2011). It has been shown that elevated FeNO is associated with inflammation, particularly eosinophils, and its concentration decreases with corticosteroids (Dweik et al. 2010). Also, high FeNO has been correlated with asthma exacerbation (Guo et al. 2000). Prado and colleagues showed that NO derived from inflammatory cells can enhance the inflammatory response (Prado et al. 2006). FeNO is considered an indicator of airway inflammation (Aytekin and Dweik 2012). Despite its association with inflammation, no definitive link has been shown between high FeNO and asthma severity (Dweik et al. 2010). Recent studies suggest that FeNO levels may indicate a subtype of asthma rather than represent a clinical manifestation of asthma severity (Aytekin and Dweik 2012). For

example asthmatics with high FeNO generally were atopic with greater airway eosinophils, airway reactivity, and obstruction (Dweik et al. 2010).

In animal models of allergic asthma, increase NO levels are present within BALF in allergic subjects compared to saline control (Yang et al., 2010). Many studies have been conducted examining the roles of NO production by the different isoforms of NOS. Decreased bronchodilation due to the deficiency of NO derived from constitutive NOS (cNOS) has been implicated in development of asthma (de Boer et al. 1999; Meurs et al. 2003; Maarsingh et al. 2006; Prado et al. 2006). Conversely increased NO production by inducible NOS (iNOS) has been correlated with increased inflammation, injury of airway epithelium, and asthma severity (Prado et al. 2006; Anderson et al. 2011; Zuo et al. 2013).

1.4 NF-κB

Nuclear Factor- kappa B (NF-κB) is a transcription factor involved in the regulation of a variety of genes involved in cellular processes, immunity, and inflammation (Hayden and Ghosh, 2008). Inactive NF-κB is bound to inhibitory proteins of κB family (IκB) and sequestered in the cellular cytoplasm. Activation of NF-κB requires the degradation of IκB proteins by IκB kinase (IKK) complex. Activated IKK phosphorylates IκB proteins which targets them for ubiquitination. The ubiquitinated IκB proteins are then degraded by the 26S proteasome, and NF-κB enter the cell's nucleus to turn on expression of target genes, including its inhibitor IκB (Hayden and Ghosh, 2008; Hayden and Ghosh, 2011; Liu and Chen, 2011).

Downstream consequences of NF-κB activation are as diverse as the cells in which it resides, but are usually associated with activating, or increasing cellular functions. For example its activation is required for the development and proliferation of lymphocytes (Kumar et al., 2004; Hayden and Ghosh, 2011). NF-κB regulates the transcription of proinflammatory cytokines, chemokines, growth factors and cell adhesion molecules (Kumar et al., 2004; Verma, 2004). Because of these roles, investigations into its effects in disease have been widely studied. Persistent NF-κB activation has been noted with chronic inflammatory conditions such as diabetes, rheumatoid arthritis, and asthma (Gagliardo et al., 2003; Verma, 2004; Simmonds and Foxwell, 2008; Clarke et al., 2009; Rial et al., 2012).

NF-κB's role in inflammation is complex, as it has been shown to modulate both proinflammatory and anti-inflammatory functions and is tissue specific (Lawrence, 2009). It has been shown to induce proinflammatory genes during the initiation of inflammation, but is then associated with the expression of anti-inflammatory genes during the resolution (Lawrence et al., 2001). In intestinal epithelium activation of NF-κB is cytoprotective and anti-inflammatory during acute colitis, but is pro-inflammatory during chronic colitis (Eckmann et al., 2008). In studies by Poynter and colleagues, NF-κB activation in lung epithelium promotes inflammation in an acute model of allergic airway disease in BALB/c mice, and inhibition of NF-κB in the epithelium attenuated inflammation, cytokines, chemokines, and IgE (Poynter et al., 2002; Poynter et al., 2004). In addition to its influence on inflammation in asthma, NF-κB has also been implicated with AHR in experimental asthma depending on the mouse strain and the presence of inflammation (Pantano et al., 2008; Sheller et al., 2009; Alcorn et al., 2010).

Other pulmonary inflammatory diseases are associated with NF-κB activation. LPS is a common inducer of the canonical pathway of activation, and causes lung injury through sepsis or by inhalational exposure. Using mice with a transgene for NF-κB inhibition in airway epithelium, Poynter and colleagues demonstrated that LPS-induced airway inflammation can be modulated by epithelial NF-κB, and is associated with increased neutrophilic inflammation and AHR. However, NF-κB activation not only occurs in the epithelium, but also in inflammatory cells (Vargaftig, 1997; Poynter et al., 2003).

1.5 ANIMAL MODELS

Over one hundred years ago researchers at the Rockefeller Institute described edematous airways and bronchoconstriction in guinea pigs after multiple injections of horse serum (Auer and Lewis, 1910). By the 1970s, the guinea pig continued to be an important model for experimental hypersensitivity responses, but other animal models were developed such as the dog and monkey to understand different aspects of allergic airways diseases (Patterson n and Kelly, 1974). However, over the last 20 years *Mus musculus domesticus* (the laboratory mouse) has become the most frequently used laboratory animal due to the availability to perform detailed analysis of cellular and molecular responses, in addition to many transgenic and gene knockout strains that can target specific pathways involved in asthma (Holmes et al., 2011). For example, allergic airway disease induced by sensitization and challenge to an antigen induces specific IgE production; Th2 mediated inflammation, airway hyperreactivity and increased mucus production and secretion, all key features in human asthma (Fuchs and Braun, 2008; Kumar et al., 2008). Furthermore, like humans, these changes can be alleviated

by glucocorticoids and β_2-adrenergic receptor agonists treatments commonly used to control asthma symptoms (Takeda and Gelfand, 2009; Stevenson and Birrell, 2010).

Despite the critical role murine models have played in asthma research, they do have their limitations. Unlike humans, mice do not naturally develop asthma (Takeda and Gelfand, 2009). As well, there are differences in lung structure, such as the lack of intrapulmonary airway cartilage, dissimilar branching patterns, and differences in smooth muscle mass of airways (Hyde et al., 2009). Furthermore, the inflammatory pattern in mice with acute allergic airway disease is more similar to allergic alveolitis than the more focal, less intense inflammation present in human asthmatics (Cohn, 2001; Kumar and Foster, 2002; Holmes et al., 2011). As such, caution has been advised when interpreting and translating data from mouse studies (Wenzel and Holgate, 2006).

Ovalbumin (OVA), an antigen that is derived from chicken egg, is a common allergen used to develop allergic airway disease in animal models. It is inexpensive and highly purified. Its purity has allowed immunodominant epitopes that elicit immune responses to be identified (Lloyd et al., 2014). OVA-sensitized and challenged BALB/c mice display a robust Th2-type response with IL-4, -5, and -13 induction, IgE production, eosinophilic airway inflammation, and AHR (Gueders et al., 2009). Recently the use of environmental antigens with a human relevance has been introduced in experimental asthma, most notably cockroach- and house dust mite (HDM)-derived proteins. Whether these antigens will lead to superior animal models of asthma is a current subject of debate (Phillips et al., 2013).

Variations in the pathology produced in OVA-induced allergic airway disease are due to differences in the strain and gender of mouse (Takeda et al., 2001; Hayashi et al., 2003; Gueders et al., 2009; Alcorn et al., 2010), the source of OVA, such as endotoxin-free versus OVA with LPS contamination, (Watanabe et al., 2003; Tsuchiya et al.,2012) and route and frequency of OVA administrations (Zhang et al., 1997).

1.6 DEVELOPMENT OF EXPERIMENTAL ASTHMA

One of the most commonly utilized protocols for the development of allergic airway disease in mice is sensitization with ovalbumin (OVA) by intraperitoneal (i.p.) injection containing an adjuvant, such as aluminum potassium sulfate (Alum). Alum works by activating the cellular inflammasome (Nalp3) and IL-1β and IL-18 production (Eisenbarth et al., 2008). Naïve T lymphocytes (CD+ T cells) are primed by dendritic cell presentation of processed OVA peptides, and differentiate into IL-4 and -13 producing Th2 cells. IL-4 augments the Th2 response by stimulating IgE production and eosinophilic inflammation. Additionally, Th2 cell differentiation occurs by the transcription factor, STAT-6, in the presence of IL-4 (de Lafaille et al., 2010). A second i.p. injection of OVA amplifies the Th2 response and production of the immunoglobulins IgG and IgE (Kumar et al., 2008). Once the immune response is generated, the mice are re-exposed (challenged) with OVA directly into the airways via inhalation (aerosols) or instillation into the nose or trachea to produce an inflammatory response, pathological changes to airways, and airway hyperreactivity (AHR) (Bates et al., 2009).

1.7 AIR POLLUTION

With the increasing prevalence of asthma world-wide, there has been much interest in assessing the link between allergens and exposure to additional environmental agents, such as indoor and outdoor air pollution with the development and modulation of asthma. (Peden and Reed, 2010; Mukherjee and Zhang, 2011; Sly and Holt, 2011). Tobacco smoke has been well recognized to worsen airway symptoms in asthmatics (Weiss et al., 1999). Outdoor air pollutants such as particulate matter (PM), ozone, traffic exhaust, and biogenic and agricultural dusts are also important factors that can worsen asthma symptoms (Zeldin et al., 2006; Jacquemin et al., 2012; Laumbach and Kipen, 2012).

Particulate matter (PM) is a mixture of solid particles and liquid droplets in the air. The US Environmental Protection Agency (EPA) has set regulatory standards for PM in the size ranges of less than 10 μm aerodynamic size (PM10; coarse PM), and less than 2.5 μm aerodynamic size (PM2.5; fine PM) (USEPA, 2013). PM2.5 is associated with chronic diseases of the cardiovascular system, as well as compromised lung function, asthma exacerbation, and obstructive disease. Increases in ambient PM2.5 are related to allergic airway symptoms and emergency room visits for asthma (Millstein et al., 2004; Nikasinovic et al., 2006; Li et al., 2011). Cellular and molecular mechanisms of PM2.5 exacerbation of asthma has been successfully studied in rats (Harkema et al., 2004; Wagner et al., 2012).

Diesel Exhaust: A substantial component of traffic PM, diesel exhaust emissions are commonly used in clinical and preclinical studies to act as an adjuvant by promoting

allergic responses in humans and mice (Diaz-Sanchez et al., 1997; Diaz-Sanchez et al., 1999; Li et al., 2009; Acciani et al., 2013). In animal studies diesel exhaust can worsen responses during allergen challenge (Dong et al., 2005). Epidemiological studies in children suggest that traffic-related PM2.5 increases asthma and other respiratory disease (Searing and Rabinovitch, 2011). However, only few studies which have assessed pollution and sensitization suggest that pollution induces sensitization in children (Braback and Forsberg, 2009).

Ozone is a gaseous component of photochemical smog that is regulated by EPA due to its health effects on the cardiorespiratory system (USEPA, 2013). Airway inflammation is exacerbated in atopic asthmatics exposed to ozone (Peden et al., 1995; Michelson et al., 1999; Hernandez et al., 2010), and ozone increases intraepithelial mucus and Th2 cytokines in nose and lung of allergic rats (Wagner et al., 2002; Wagner et al., 2007). Studies in human volunteers suggest that ozone may modify asthma through the same toll-like receptor (TLR4) as endotoxin (Peden, 2011).

Endotoxin or lipopolysaccharide (LPS) is a component of the cell wall of Gram negative bacteria found throughout the environment. It is found in dust along with solid and liquid waste produced in various occupational settings ranging from animal husbandry, cotton industry, agricultural settings, and waste treatment facilities (Charavaryamath et al., 2005; Heederik et al., 2007; Sigsgaard et al., 2010).

In healthy individuals, inhalation of LPS increases sputum neutrophils and causes airway obstruction (Kline et al., 1999; Doyen et al., 2012; Möller et al., 2012); however, this response varies between subjects (Kline et al., 1999). Inhalation of LPS

in mice and guinea pigs increases AHR and neutrophilic inflammation in BALF and lung and decreases lung function (Toward and Broadley, 2000; Card et al., 2006; Hakansson et al., 2012). In rats, airway endotoxin has been well characterized to induce goblet cell hypertrophy and increased mucus production (Harkema and Hotchkiss, 1992).

1.8 ASTHMA AND ENDOTOXIN

Despite many years of research, pathways by which endotoxin modifies asthma have not been definitively elucidated. Evidence for both exacerbation and inhibition of allergic airway responses by endotoxin has been documented in both laboratory rodents and humans. The complexities of the endotoxin response in asthmatics are a consequence of not only the contrasting immunologic activities (i.e., Th1 vs. Th2 pathways), but also the pleiotropic effects endotoxin has on airway epithelium and inflammatory cells (Doreswamy and Peden, 2011; Peden, 2011). These effects are a result of the timing and dose of endotoxin exposure in asthmatics. The protective effects of endotoxin have generally been seen during asthma development in early life which has generated the “hygiene hypothesis” which states that childhood infections prevent the development of allergic disease by enhancing the Th1 immune response (Von et al., 2000; Wills-Karp et al., 2001; Vercelli, 2006). In a study by Braun-Fahrlander and colleagues, they found an inverse relationship between endotoxin present in mattresses of children and their incidence of having atopic asthma (Braun-Fahrlander et al., 2002). In mice George et al. (George et al., 2006) found that mice exposed to corn dust bedding containing endotoxin had decreased pulmonary inflammation. They also reported that mice exposed to the corn dust bedding in early life and then removed from the bedding also had attenuated inflammation when

exposed to ovalbumin in adult life. Another study in mice showed a decrease in eosinophilic inflammation, IgE production, and BALF Th2 cytokines (Delayre-Orthez et al., 2004). In rats LPS exposure before or early during sensitization prevented OVA sensitization (Tulic et al., 2000). In summary, most studies suggest that LPS can interrupt sensitization processes, but the definitive mechanism has yet to be identified.

Studies assessing the effect of endotoxin during allergen challenge have yielded conflicting results depicting exacerbation or attenuation of the allergic response. These variable responses have been attributed to differences in the amount of LPS given, timing of exposure, and other factors, such as gender and genetic variation (Liu, 2002). Eldrige and Peden reviewed several studies regarding the effect of the timing of LPS exposure on the modulation of the asthmatic response in people. They found that asthmatics are more sensitive to LPS based on studies showing that concurrent allergen and LPS exposure, or LPS exposure following allergen challenge, caused an enhanced response to endotoxin by airway neutrophilic inflammation, whereas prior exposure to LPS promoted the allergic response by increasing eosinophilic inflammation and AHR (Eldridge and Peden, 2000). Other studies in asthmatic individuals produced have also had variable results. For example, following allergen challenge in asthmatics there is an increase in CD14, a cell surface receptor for LPS binding protein, which is associated with increased neutrophilic inflammation and may account for increased sensitivity to LPS in asthmatics (Alexis et al., 2001). Inversely, it has been shown that intranasal challenge with LPS heightened the presence of eosinophils in the nasal tissue of allergic subjects (Peden et al., 1999). However, a recent study by Hernandez et al. reported that there was an attenuation in the

inflammatory response to inhaled LPS in atopic asthmatics, and was related to the expression of TLR4 (Hernandez et al., 2012). In addition to disease-dependent receptor expression, modulation of allergic responses by LPS may be dependent on polymorphisms to endotoxin receptors CD14 and TLR4 (Simpson and Martinez, 2010).

Studies have shown that the amount of LPS exposure dictates the response in asthmatics. Exposure to low doses of LPS is associated with exacerbation of the allergic inflammation in both asthmatics and allergic mice (Eisenbarth et al., 2002; Alexis et al., 2004; Alexis et al., 2008; Dong L; Li H, 2009; Bennett et al., 2013). Alexis and colleagues have shown that inhalation of low doses of LPS in asthmatics increases genes associated with antigen presentation, innate immunity, and inflammation in airway cells (Alexis et al., 2008), and they hypothesized that LPS promotes Th2 inflammation in the airways (Alexis et al., 2004). Also, they showed decrease phagocytosis by airway phagocytes (Alexis et al., 2003). Bennett et al. performed a particle deposition study and showed that low dose LPS exposure increased deposition of inhaled particles in asthmatics (Bennett et al., 2013). In addition, low LPS doses increased AHR in asthmatics (Boehlecke et al., 2003). In OVA-induced allergic mice, Dong et al. found that low dose LPS increased recruitment of eosinophils and neutrophils into the lung, mucus secretion, and Th2 cytokines (Dong L; Li H, 2009). Conversely, several investigations describe high doses of LPS as protective against allergy and atopy. Simpson and Martinez showed that in asthmatics with certain genetic traits were protected against the development of atopy by exposure with high doses of LPS (Simpson and Martinez, 2010). In allergic mice, high doses of inhaled LPS induced a Th1 response characterized by increased neutrophils and IFN-γ

production and decreased mucus secretion (Eisenbarth et al., 2002; Kim et al., 2007; Dong L; Li H, 2009). Reduced AHR after high LPS treatment was associated with variants of toll-like receptor 4 (TLR-4), (Senthilselvan et al., 2009). Given the dose-dependent effect of LPS on the allergic inflammation, it is not surprising that conflicting data is present in the epidemiological literature.

1.9 AIRWAY HYPERRESPONSIVENESS

Airway hyperresponsiveness (AHR) is the exaggerated response of airways to a contractile stimulus (Berend et al., 2008). In 1921 it was first described in asthmatics following systemic administration of pilocarpine, a cholinergic agonist, (O'Byrne and Inman, 2003). AHR is one of the main symptoms of asthma, but it can be present in other airway diseases as well. It is a measure of the relationship between the shortening of airway smooth muscle tissue and airway remodeling (Walker et al., 2013). AHR is used clinically to diagnose asthma because airway narrowing is easily induced in asthmatics; it can be reliably measured, and is often the cause of asthma mortality (O'Byrne, 2010; Bossé et al., 2013). AHR can predict the development of asthma in atopic individuals (Berend et al., 2008), asthma severity (Busse, 2010), and the effectiveness inhaled corticosteroid treatment in asthmatics (Walker et al., 2013).

Similar to asthma the mechanism of AHR is not completely known. AHR can be categorized as either persistent or variable based its occurrence (early vs. late phase response), and severity of histopathology (Cockcroft and Davis, 2006; Meurs et al., 2008; Busse, 2010). Persistent or chronic AHR is constantly present even in the absence of a stimulus. It is thought to be associated with chronic inflammation and

changes in airway structure, such as subepithelial membrane thickening, smooth muscle hypertrophy, and fibrosis. As a result airways are less compliant and have exaggerated response to bronchoconstrictors. Chronic AHR responds poorly to inhaled corticosteroids, and the association with specific inflammatory cells is inconsistent. Variable AHR develops and resolves within hours following exposure to allergens or stimuli. It has been linked to airway inflammation and is believed to reflect current asthma severity. Variable AHR can be affected by external exposures to allergens, respiratory infections, and corticosteroid treatment.

1.9.1 Airway Smooth Muscle (ASM) and AHR

There has been much debate regarding the role of airway smooth muscle (ASM) in AHR (Gunst and Panettieri, 2012; Paré and Mitzner, 2012). The basis of this argument lies in whether the alterations of ASM are due to intrinsic phenotypic changes or are a response by normal tissue to the modification of the surrounding milieu. There are many studies that support the theory that the AHR in asthmatics is caused by inherent abnormalities in ASM that alter its phenotype, such as increases in excitation-contraction coupling mechanisms, hyperplasia, extracellular matrix proteins, inflammatory chemokine and cytokine production (Tagaya and Tamaoki, 2007; Gunst and Panettieri, 2012). Wagers et al. found that intrinsic AHR in A/J mice was attributed to the exaggerated response of ASM (Wagers et al., 2007). Plant and colleagues demonstrated that acute OVA mouse models displayed active ASM proliferation that correlated with AHR in the peripheral airways, while chronic OVA mice developed hyperplastic and hypertrophic ASM which correlated with increased AHR in central airways (Plant et al., 2012). Other studies have focused on additional changes within

the lung, such as inflammatory mediators as the actual mediators of AHR that amplify normal ASM contraction in asthmatics (Wagers et al., 2004; Bosse et al., 2010; Paré and Mitzner, 2012).

1.9.2 Inflammation and AHR

Inflammation is one of the hallmarks of asthma, and its effects on AHR have been extensively studied. Inflammatory cells, such as mast cells, eosinophils, T lymphocytes, neutrophils, alveolar macrophages, and dendritic cells, have been associated with AHR (Wills-Karp, 1999; Short et al., 2011; Fuchs et al., 2012; Hakansson et al., 2012; Janssen-Heininger et al., 2012). A study by Elwood et al. found significant correlations between AHR and the presence of eosinophils during both early and late responses following OVA challenge and with neutrophils in the late response only (Elwood et al., 1992). Additional studies have shown that neutrophils can induce late asthmatic responses in mice (Nabe et al., 2011). Also, neutrophils have been associated with more severe forms of asthma, such as steroid resistant phenotype (Jatakanon et al., 1999; Ito et al., 2008; Choi et al., 2012).

1.9.3 Measuring AHR

The detection of AHR can be accomplished through indirect and direct stimulation. Indirect stimulation, which is not as widely performed, is produced by the inhalation of agents such as hypertonic saline or mannitol, or performing exercise to indirectly stimulate AHR via activation of inflammatory cells and the release of inflammatory mediators (Busse, 2010). This method indicates the level of current airway inflammation. Direct stimulation with the use of inhaled agents, such as

methacholine (MCh), is the most commonly used method of AHR detection. Methacholine is a cholinergic agonist that acts on the muscarinic receptors on the airway smooth muscle causing its contraction. This method best reflects persistent AHR (Cockcroft and Davis, 2006). In people, the extent of methacholine-induced AHR is believed to be correlated with asthma severity and has a high association with other indirect and direct stimuli of AHR, the presence of exhaled nitric oxide, and sputum eosinophils (Porsbjerg et al., 2008; Tepper et al., 2012).

Our current understanding of lung mechanics in normal and diseased states can be attributed to studies utilizing animal models. Previously, this data was generated using unrestrained plethysmography in which pressure changes within a closed chamber containing an awake, freely roaming animal were measured. Recently, the use of these pressure changes, calculated as enhanced pause (Penh), as a measurement of lung function has been shown to be misleading and can only accurately represent breathing patterns (Bates and Irvin, 2003; Lundblad et al., 2007). Input impedance utilizing the forced oscillation technique (FOT) is the most accepted technique for measuring lung function in small laboratory animals (Vanoirbeek et al., 2010). Using a small animal ventilator, a computer controlled piston determines pressure and flow within the trachea by measuring volume displacement of the piston within the pump cylinder (Schuessler and Bates, 1995; Bates and Irvin, 2003).

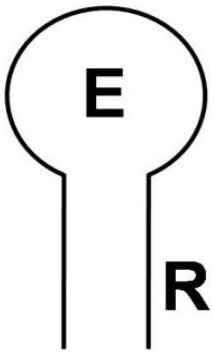
Measuring lung mechanics in mice is generally accomplished by using two mathematical models, single compartment and constant-phase models. The single compartment model represents the lung as a linear structure composed of a flow-

resistive pipe or airway supplying a single elastic compartment or alveolar tissue (Bates and Irvin, 2003; Irvin and Bates, 2003) as seen in figure 1. In the presence of an oscillatory flow, the single compartment model is mathematically described as

$$P = R\dot{V} + EV + P_o \qquad \textit{Equ.1}$$

where P is pressure at the opening of the pipe, $\dot{V}$ is flow of gas, V is volume of gas in the elastic compartment (E), P_o is resting applied pressure or positive end-expiratory pressure.

Figure 1. Diagram of single compartment model. Classical method of determining lung mechanics using single compartment model consisting of resistance (R) of conducting airways and elastance (E) of parenchymal tissue (Bates and Irvin, 2003).

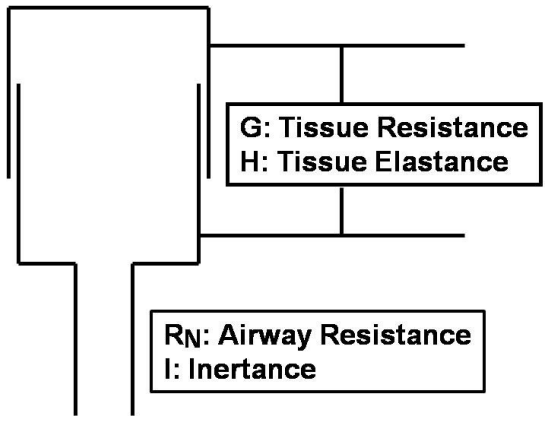


Resistance (R) and elastance (E) are determined by fitting recorded values of the other parameters into the equation using multiple linear regressions (Bates and Suki, 2008). In this model, lung resistance is a measurement of narrowing of the central airways and changes within the lung, while lung elastance measures peripheral lung alterations.

The constant-phase model established by Hantos et al. (Hantos et al., 1992) measures R and E over a range of frequencies and provides a better separation of central and peripheral lung changes (Irvin and Bates, 2003). Broad-band flow signals are used to determine respiratory system input impedance (Z_{rs}), which is a ratio of pressure to flow as a function of superimposed oscillation frequency (Kaczka and Dellaca, 2011). The Fourier Transform of *Eq. 1* produces input impedance; Eq. *2* describes the constant phase model:

$$Z_{rs} = R_N + i2\pi f I \frac{G - iH}{(2\pi f)^{\alpha}} \qquad \textit{Equ.2} \qquad \alpha = \frac{2}{\pi} \tan^{-1} \frac{H}{G}$$

Figure 2. Single compartment model with constant phase lung impedance. R_N is Newtonian resistance; i is the positive square root of -1 or imaginary unit; f is frequency; G is tissue damping, H is tissue elastance, and I is inertance or gas in the central airways (Irvin and Bates, 2003; Bates, 2009).



Newtonian resistance is the approximation of resistance within the central airways, and along with inertance composes impedance in airways. However, inertance is negligible in mice at frequencies fewer than 20 Hertz (Bates and Irvin, 2003; Bates, 2009). Tissue damping is the dissipative energy in the lung tissue and is related to tissue resistance. Alterations in tissue damping occur with distortion of peripheral tissue due to airway constriction, and increases indicate heterogeneity in airflow in the lung. Tissue elastance is the stored energy in the lung and is related to tissue stiffness. Increases in tissue elastance can indicate airway closure or increased tissue stiffness due to distortion of parenchymal tissue (Bates and Suki, 2008; Bates, 2009).

Applying a sine-wave oscillatory flow signal to the lungs measures dynamic lung mechanics, and the data is analyzed using the single compartment model, while a broadband oscillatory flow signal is applied to the lungs to measure respiratory impedance analyzed by the constant phase model (Bates, 2009). By comparison most assessments of lung function in asthmatics are evaluated by spirometric tests, such as forced expired volume in 1 second (FEV1). The magnitude of airway obstruction can be assessed by measuring FEV1 following the administration of bronchodilator agents and AHR is assessed following bronchoconstriction (Reddel et al., 2009). However, FEV1 is not a measurement of direct lung or airway resistance. To directly measure resistance in people, FOT has recently become more commonly employed (Walker et al., 2013).

1.10 RESEARCH GOALS

LPS is a component of gram-negative bacteria ubiquitously present in particulate matter of indoor and outdoor air, and has been demonstrated to modulate different aspects of allergic airway disease. While early life exposure has been linked to

decreased atopy in childhood, results from clinical studies suggest asthmatics can demonstrate either enhanced (Boehlecke et al., 2003) *or* blunted responses to endotoxin exposure (Hernandez et al., 2012). Allergic inflammation is driven by Th2 cytokines (e.g., IL-4, -5,-13) and eosinophil granulocytes, whereas LPS activates Th1 pathways (e.g., IFNγ, TNF-α) and produces neutrophilic inflammation. In general, the actions of these disparate immunity pathways oppose the action of the other, but the mechanisms for LPS to either exacerbate or attenuate symptoms in asthmatics are unknown. Furthermore, the fundamental knowledge of the interaction of LPS and allergen to modulate airway inflammation and hyperreactivity is lacking. In my murine model of allergic airway disease I demonstrated the attenuation in airway hyperresponsiveness to methacholine despite an increase in the severity of inflammation following airway LPS exposure. Therefore, I used this model of allergic airway disease to identify the cellular and molecular mechanisms whereby airway exposure to LPS modulates asthma.

1.11 HYPOTHESIS AND AIMS

My central hypothesis is that attenuation of airway hyperresponsiveness (AHR) by LPS in asthmatic mice is dependent on upregulation of nitric oxide synthase 2 (*Nos2*) gene that is associated with the recruitment of neutrophils. The specific aims used to test this hypothesis are:

Aim 1: to determine the relationships between the cellular character and anatomical distribution of lung lesions with specific components of AHR in allergic mice exposed to airway LPS.

Aim 2: to determine the role of neutrophils in allergen-induced AHR following LPS exposure in allergic mice.

Aim 3: to determine the role of nuclear transcription factor kappa beta (NF-κB), a central modulator of inflammation, in the attenuation of allergen-induced AHR following LPS exposure in allergic mice.

Aim 4: to determine the role of nitric oxide synthase in allergen-induced AHR in LPS exposed allergic mice.

In aims 1-3, I used male BALB/c mice as a model of allergic airway disease by injecting 20 μg of ovalbumin, an allergen, with 1 mg Alum, an adjuvant, into the peritoneum of each allergic mouse. Ten days following this injection, mice were injected again in the peritoneum with 20 μg of ovalbumin, and in addition a 0.5% ovalbumin solution was instilled through the nares. Control mice received intranasal instillation of saline. A week later OVA-treated mice were intranasally (IN) challenged with 0.5% ovalbumin for two consecutive days and control mice received saline. Two days following last ovalbumin challenge, allergic and non-allergic mice received either 0 or 3 μg of lipopolysaccharide by intranasal instillation. The next day airway hyperresponsiveness data was collected and/or animal were euthanized to collect samples to characterize and assess pulmonary alterations and injury by histopathological; histochemical (intraepithelial mucosubstances staining by periodic acid Schiff); immunohistochemical (eosinophils and neutrophils); molecular (gene expression by real time PCR); and immunological (evaluation of cytokines by flow cytometry) methods.

I found that the attenuation in AHR to methacholine in allergic mice exposed to LPS was not dependent on the location or severity of inflammation along the airways or in parenchymal tissue. For aim 2, neutrophil recruitment by LPS exposure was prevented by depleting neutrophils using intraperitoneal injection of a neutrophil depleting antibody for 2 consecutive days prior to LPS exposure. This study demonstrated that the presence of neutrophils was not associated with an increase in AHR in allergic mice. Aim 3 examined the effects of NF-κB activation on the modulation of allergic airway disease by endotoxin. A novel NF-κB inhibitor was injected i.p. 2 days following the last OVA challenge and 1 hour prior to LPS or saline exposure. In this study, I found that NF-κB inactivation does not affect the inflammatory response in late phase asthma but modified the inflammatory cell mix of the acute inflammatory response to inhaled LPS in this model. However, its inhibition reversed the attention of AHR associated with LPS exposure in allergic mice. Specific aim 4 used lung tissues collected from studies in aims 1 and 3 to analyze the expression of NOS 2 gene expression. I observed that NOS2 gene expression was increased in the lungs of allergic mice exposed to LPS compare to mice with ovalbumin or LPS alone. There was also a decrease in NOS 2 in the lungs of allergic mice exposed to LPS that were given the NF-κB inhibitor compared to similar mice which only received the vehicle control. The results of these studies demonstrate that LPS can attenuate AHR in established asthma by increasing gene expression of iNOS.

REFERENCES

REFERENCES

AAAAI. 2013. Asthma Statistics. Retrieved 10/21/2013, 2013, from http://www.aaaai.org/about-the-aaaai/newsroom/asthma-statistics.aspx.

Acciani T H, Brandt E B, Khurana Hershey G K and Le Cras T D. 2013. Diesel Exhaust Particle Exposure Increases Severity of Allergic Asthma in Young Mice. Clinical & Experimental Allergy **43**(12): 1406-1418.

Akinbami L J, Moorman M S, Bailey C, Zahran H S, King M, Johnson C A and Liu X. 2012. Trends in Asthma Prevalence, Health Care Use, and
Mortality in the United States, 2001–2010, Hyattsville, MD: National Center for Health Statistics.

Al-Muhsen S, Johnson J R and Hamid Q. 2011. Remodeling in Asthma. Journal of Allergy and Clinical Immunology **128**(3): 451-462.

Alcorn J F, Ckless K, Brown A L, Guala A S, Kolls J K, Poynter M E, Irvin C G, van der Vliet A and Janssen-Heininger Y M W. 2010. Strain-Dependent Activation of Nf-Kappa B in the Airway Epithelium and Its Role in Allergic Airway Inflammation. American Journal of Physiology-Lung Cellular and Molecular Physiology **298**(1): L57-L66.

Alexis N, Eldridge M, Reed W, Bromberg P and Peden D B. 2001. Cd14-Dependent Airway Neutrophil Response to Inhaled Lps: Role of Atopy. Journal of Allergy and Clinical Immunology **107**(1): 31-35.

Alexis N E, Eldridge M W and Peden D B. 2003. Effect of Inhaled Endotoxin on Airway and Circulating Inflammatory Cell Phagocytosis and Cd11b Expression in Atopic Asthmatic Subjects. Journal of Allergy and Clinical Immunology **112**(2): 353-361.

Alexis N E, Lay J C, Almond M and Peden D B. 2004. Inhalation of Low-Dose Endotoxin Favors Local T(H)2 Response and Primes Airway Phagocytes in Vivo. Journal of Allergy and Clinical Immunology **114**(6): 1325-1331.

Alexis N E, Brickey W J, Lay J C, Wang Y, Roubey R A S, Ting J P Y and Peden D B. 2008. Development of an Inhaled Endotoxin Challenge Protocol for Characterizing Evoked Cell Surface Phenotype and Genomic Responses of Airway Cells in Allergic Individuals. Annals of Allergy Asthma & Immunology **100**(3): 206-215.

Anderson J T, Zeng M Q, Li Q, Stapley R, Moore D R, Chenna B, Fineberg N, Zmijewski J, Eltoum I E, Siegal G P, Gaggar A, Barnes S, Velu S E, Thannickal V J, Abraham E, Patel R P, Lancaster J R, Chaplin D D, Dransfield M T and Deshane J S. 2011. Elevated Levels of No Are Localized to Distal Airways in Asthma. Free Radical Biology and Medicine **50**(11): 1679-1688.

Auer J and Lewis P A. 1910. The Physiology of the Immediate Reaction of Anaphylaxis in the Guinea-Pig. Journal of Experimental Medicine **12**(2): 151-175.

Bates J H T and Irvin C G. 2003. Measuring Lung Function in Mice: The Phenotyping Uncertainty Principle. Journal of Applied Physiology **94**(4): 1297-1306.

Bates J H T and Suki B I. 2008. Assessment of Peripheral Lung Mechanics. Respiratory Physiology & Neurobiology **163**(1): 54-63.

Bates J H T. 2009. Lung Mechanics: An Inverse Modeling Approach, Cambridge University Press.

Bates J H T, Rincon M and Irvin C G. 2009. Animal Models of Asthma. American Journal of Physiology-Lung Cellular and Molecular Physiology **297**(3): L401-L410.

Baxi S N and Phipatanakul W. 2010. The Role of Allergen Exposure and Avoidance in Asthma. Adolescent medicine: state of the art reviews **21**(1): 57.

Bennett W D, Herbst M, Zeman K L, Wu J, Hernandez M L and Peden D B. 2013. Effect of Inhaled Endotoxin on Regional Particle Deposition in Patients with Mild Asthma. Journal of Allergy and Clinical Immunology **131**(3): 912-913.

Berend N, Salome C M and King G G. 2008. Mechanisms of Airway Hyperresponsiveness in Asthma. Respirology **13**(5): 624-631.

Boehlecke B, Hazucha M, Alexis N E, Jacobs R, Reist P, Bromberg P A and Peden D B. 2003. Low-Dose Airborne Endotoxin Exposure Enhances Bronchial Responsiveness to Inhaled Allergen in Atopic Asthmatics. Journal of Allergy and Clinical Immunology **112**(6): 1241-1243.

Bosse Y, Riesenfeld E P, Pare P D and Irvin C G. 2010. It's Not All Smooth Muscle: Non-Smooth-Muscle Elements in Control of Resistance to Airflow. Annual Review of Physiology **72**: 437-462.

Bossé Y, Lee-Gosselin A, Boulet L P and King G G. 2013. Airway Hyperresponsiveness in Asthma: A Better Understanding yet to Yield Clinical Benefit. J Allergy Ther **4**(150): 2.

Braback L and Forsberg B. 2009. Does Traffic Exhaust Contribute to the Development of Asthma and Allergic Sensitization in Children: Findings from Recent Cohort Studies. Environmental Health **8**(1): 17.

Braun-Fahrlander C, Riedler J, Herz U, Eder W, Waser M, Grize L, Maisch S, Carr D, Gerlach F, Bufe A, Lauener R P, Schierl R, Renz H, Nowak D and von Mutius E. 2002. Environmental Exposure to Endotoxin and Its Relation to Asthma in School-Age Children. New England Journal of Medicine **347**(12): 869-877.

Busse W W and Lemanske R F. 2001. Advances in Immunology - Asthma. New England Journal of Medicine **344**(5): 350-362.

Busse W W. 2010. The Relationship of Airway Hyperresponsiveness and Airway Airway Hyperresponsiveness in Asthma: Its Measurement and Clinical Significanceinflammation. Chest **138**(2): 4S-10S.

Card J W, Carey M A, Bradbury J A, DeGraff L M, Morgan D L, Moorman M P, Flake G P and Zeldin D C. 2006. Gender Differences in Murine Airway Responsiveness and Lipopolysaccharide-Induced Inflammation. Journal of Immunology **177**(1): 621-630.

Charavaryamath C, Janardhan K S, Townsend H G, Willson P and Singh B. 2005. Multiple Exposures to Swine Barn Air Induce Lung Inflammation and Airway Hyper-Responsiveness. Respiratory Research **6**(1): 50.

Choi J S, Jang A S, Park J S, Park S W, Paik S H, Uh S T, Kim Y H and Park C S. 2012. Role of Neutrophils in Persistent Airway Obstruction Due to Refractory Asthma. Respirology **17**(2): 322-329.

Clarke D, Damera G, Sukkar M B and Tliba O. 2009. Transcriptional Regulation of Cytokine Function in Airway Smooth Muscle Cells. Pulmonary Pharmacology & Therapeutics **22**(5, Sp. Iss. SI): 436-445.

Cockcroft D W and Davis B E. 2006. Mechanisms of Airway Hyperresponsiveness. Journal of Allergy and Clinical Immunology **118**(3): 551-559.

Cohn L. 2001. Food for Thought. American Journal of Respiratory Cell and Molecular Biology **24**(5): 509-512.

Cohn L, Elias J A and Chupp G L. 2004. Asthma: Mechanisms of Disease Persistence and Progression. Annual Review of Immunology **22**(1): 789-815.

de Boer J, Duyvendak M, Schuurman F E, Pouw F M H, Zaagsma J and Meurs H. 1999. Role of L-Arginine in the Deficiency of Nitric Oxide and Airway Hyperreactivity after the Allergen-Induced Early Asthmatic Reaction in Guinea-Pigs. British Journal of Pharmacology **128**(5): 1114-1120.

de Boer W I, Sharma H S, Baelemans S M I, Hoogsteden H C, Lambrecht B N and Braunstahl G J. 2008. Altered Expression of Epithelial Junctional Proteins in Atopic Asthma: Possible Role in Inflammation. Canadian Journal of Physiology and Pharmacology **86**(3): 105-112.

de Lafaille M A C, Lafaille J J and Graca L. 2010. Mechanisms of Tolerance and Allergic Sensitization in the Airways and the Lungs. Current Opinion in Immunology **22**(5): 616-622.

Delayre-Orthez C, de Blay F, Frossard N and Pons F. 2004. Dose-Dependent Effects of Endotoxins on Allergen Sensitization and Challenge in the Mouse. Clinical and Experimental Allergy **34**(11): 1789-1795.

Diaz-Sanchez D, Tsien A, Fleming J and Saxon A. 1997. Combined Diesel Exhaust Particulate and Ragweed Allergen Challenge Markedly Enhances Human in Vivo Nasal Ragweed-Specific Ige and Skews Cytokine Production to a T Helper Cell 2-Type Pattern. The Journal of Immunology **158**(5): 2406-2413.

Diaz-Sanchez D, Garcia M P, Wang M, Jyrala M and Saxon A. 1999. Nasal Challenge with Diesel Exhaust Particles Can Induce Sensitization to a Neoallergen in the Human Mucosa. Journal of Allergy and Clinical Immunology **104**(6): 1183-1188.

Dong C C, Yin X J J, Ma J Y C, Millecchia L, Wu Z X, Barger M W, Roberts J R, Antonini J M, Dey R D and Ma J K H. 2005. Effect of Diesel Exhaust Particles on Allergic Reactions and Airway Responsiveness in Ovalbumin-Sensitized Brown Norway Rats. Toxicological Sciences **88**(1): 202-212.

Dong L; Li H W S, Li Y. 2009. Different Doses of Lipolysaccharides Regulate the Lung Inflammation of Asthmatic Mice Via Tlr4 Pathway in Alveolar Macrophages. Journal of Asthma **46**: 229-233.

Doreswamy V and Peden D B. 2011. Modulation of Asthma by Endotoxin. Clinical and Experimental Allergy **41**(1): 9-19.

Doyen V, Kassengera Z, Dinh D and Michel O. 2012. Time Course of Endotoxin-Induced Airwaysâ€™ Inflammation in Healthy Subjects. Inflammation **35**(1): 33-38.

Eckmann L, Nebelsiek T, Fingerle A A, Dann S M, Mages J, Lang R, Robine S, Kagnoff M F, Schmid R M, Karin M, Arkan M C and Greten F R. 2008. Opposing Functions of Ikkβ During Acute and Chronic Intestinal Inflammation. Proceedings of the National Academy of Sciences **105**(39): 15058-15063.

Eisenbarth S C, Piggott D A, Huleatt J W, Visintin I, Herrick C A and Bottomly K. 2002. Lipopolysaccharide-Enhanced, Toll-Like Receptor 4-Dependent T Helper Cell Type 2 Responses to Inhaled Antigen. Journal of Experimental Medicine **196**(12): 1645-1651.

Eisenbarth S C, Colegio O R, Oâ€™Connor W, Sutterwala F S and Flavell R A. 2008. Crucial Role for the Nalp3 Inflammasome in the Immunostimulatory Properties of Aluminium Adjuvants. Nature **453**(7198): 1122-1126.

Eldridge M W and Peden D B. 2000. Airway Response to Concomitant Exposure with Endotoxin and Allergen in Atopic Asthmatics. Journal of Toxicology and Environmental Health Part A **61**(1): 27-37.

Elwood W, Barnes P J and Chung K F. 1992. Airway Hyperresponsiveness Is Associated with Inflammatory Cell Infiltration in Allergic Brown-Norway Rats. International Archives of Allergy and Immunology **99**(1): 91-97.

Evans C M, Kim K, Tuvim M J and Dickey B F. 2009. Mucus Hypersecretion in Asthma: Causes and Effects. Current Opinion in Pulmonary Medicine **15**(1): 4.

Fahy J V. 2009. Eosinophilic and Neutrophilic Inflammation in Asthma. Proceedings of the American Thoracic Society **6**(3): 256-259.

Foley S C and Hamid Q. 2007. Images in Allergy and Immunology: Neutrophils in Asthma. The Journal of allergy and clinical immunology **119**(5): 1282-1286.

Fuchs B and Braun A. 2008. Improved Mouse Models of Allergy and Allergic Asthma - Chances Beyond Ovalbumin. Current Drug Targets **9**(6): 495-502.

Fuchs B, Sjöberg L, Westerberg C M, Ekoff M, Swedin L, Dahlén S-E, Adner M and Nilsson G P. 2012. Mast Cell Engraftment of the Peripheral Lung Enhances Airway Hyperresponsiveness in a Mouse Asthma Model. American Journal of Physiology - Lung Cellular and Molecular Physiology **303**(12): L1027-L1036.

Gagliardo R, Chanez P, Mathieu M, Bruno A, Costanzo G, Gougat C, Vachier I, Bousquet L, Bonsignore G and Vignola A M. 2003. Persistent Activation of Nuclear Factor-Kappa B Signaling Pathway in Severe Uncontrolled Asthma. American Journal of Respiratory and Critical Care Medicine **168**(10): 1190-1198.

George C L S, White M L, Kulhankova K, Mahajan A, Thorne P S, Snyder J M and Kline J N. 2006. Early Exposure to a Nonhygienic Environment Alters Pulmonary Immunity and Allergic Responses. American Journal of Physiology-Lung Cellular and Molecular Physiology **291**(3): L512-L522.

GINA. 2012. From the Global Strategy for Asthma Management and Prevention, Global Initiative for Asthma (Gina) 2012, Available from: http://www,ginasthma.org/.

Goleva E, Hauk P J, Hall C F, Liu A H, Riches D W H, Martin R J and Leung D Y M. 2008. Corticosteroid-Resistant Asthma Is Associated with Classical Antimicrobial Activation of Airway Macrophages. Journal of Allergy and Clinical Immunology **122**(3): 550-559.

Gordon S and Martinez F O. 2010. Alternative Activation of Macrophages: Mechanism and Functions. Immunity **32**(5): 593-604.

Gueders M M, Paulissen G, Crahay C, Quesada-Calvo F, Hacha J, Van Hove C, Tournoy K, Louis R, Foidart J-M and NoÃ«l A. 2009. Mouse Models of Asthma: A Comparison between C57bl/6 and Balb/C Strains Regarding Bronchial Responsiveness, Inflammation, and Cytokine Production. Inflammation Research **58**(12): 845-854.

Gunst S J and Panettieri R A. 2012. Point:Counterpoint: Alterations in Airway Smooth Muscle Phenotype Do/Do Not Cause Airway Hyperresponsiveness in Asthma. Journal of Applied Physiology **113**(5): 837-839.

Hakansson H F, Smailagic A, Brunmark C, Miller-Larsson A and Lal H. 2012. Altered Lung Function Relates to Inflammation in an Acute Lps Mouse Model. Pulmonary Pharmacology & Therapeutics **25**(5): 399-406.

Hantos Z, Daroczy B, Suki B, Nagy S and Fredberg J J. 1992. Input Impedance and Peripheral Inhomogeneity of Dog Lungs. Journal of Applied Physiology **72**(1): 168-178.

Harkema J R and Hotchkiss J A. 1992. In Vivo Effects of Endotoxin on Intraepithelial Mucosubstances in Rat Pulmonary Airways. Quantitative Histochemistry. Am J Pathol. 1992 Aug;141(2):307-17.

Harkema J R, Keeler G, Wagner J, Morishita M, Timm E, Hotchkiss J, Marsik F, Dvonch T, Kaminski N and Barr E. 2004. Effects of Concentrated Ambient Particles on Normal and Hypersecretory Airways in Rats. Research report (Health Effects Institute)(120): 1-68; discussion 69-79.

Hayashi T, Adachi Y, Hasegawa K and Morimoto M. 2003. Less Sensitivity for Late Airway Inflammation in Males Than Females in Balb/C Mice. Scandinavian Journal of Immunology **57**(6): 562-567.

Hayden M S and Ghosh S. 2008. Shared Principles in Nf-ºb Signaling. Cell **132**(3): 344-362.

Hayden M S and Ghosh S. 2011. Nf-Kb in Immunobiology. Cell Res **21**(2): 223-244.

Heederik D, Sigsgaard T, Thorne P S, Kline J N, Avery R, BÃ¸nlÃ¸kke J H, Chrischilles E A, Dosman J A, Duchaine C and Kirkhorn S R. 2007. Health Effects of Airborne Exposures from Concentrated Animal Feeding Operations. Environmental Health Perspectives **115**(2): 298.

Hernandez M L, Lay J C, Harris B, Esther C R, Jr., Brickey W J, Bromberg P A, Diaz-Sanchez D, Devlin R B, Kleeberger S R, Alexis N E and Peden D B. 2010. Atopic Asthmatic Subjects but Not Atopic Subjects without Asthma Have Enhanced Inflammatory Response to Ozone. Journal of Allergy and Clinical Immunology **126**(3): 537-U228.

Hernandez M L, Herbst M, Lay J C, Alexis N E, Brickey W J, Ting J P Y, Zhou H and Peden D B. 2012. Atopic Asthmatic Patients Have Reduced Airway Inflammatory Cell

Recruitment after Inhaled Endotoxin Challenge Compared with Healthy Volunteers. Journal of Allergy and Clinical Immunology **130**(4): 869-876.e862.

Holgate S T. 2007. Epithelium Dysfunction in Asthma. Journal of Allergy and Clinical Immunology **120**(6): 1233-1246.

Holgate S T and Davies D E. 2009. Rethinking the Pathogenesis of Asthma. Immunity **31**(3): 362-367.

Holgate S T and Sly P D. 2014. Asthma Pathogenesis. Middleton's Allergy: Principles and Practice (Expert Consult-Online). N F Adkinson Jr, B S Bochner, A W Burks et al. Philadelphia, PA, Elsevier Health Sciences. **1:** 812-841.

Holmes A M, Solari R and Holgate S T. 2011. Animal Models of Asthma: Value, Limitations and Opportunities for Alternative Approaches. Drug Discovery Today **16**(15-16): 659-670.

Hyde D M, Hamid Q and Irvin C G. 2009. Anatomy, Pathology, and Physiology of the Tracheobronchial Tree: Emphasis on the Distal Airways. Journal of Allergy and Clinical Immunology **124**(6): S72-S77.

Irvin C G and Bates J H. 2003. Measuring the Lung Function in the Mouse: The Challenge of Size. Respiratory Research **4**(4).

Ito K, Herbert C, Siegle J S, Vuppusetty C, Hansbro N, Thomas P S, Foster P S, Barnes P J and Kumar R K. 2008. Steroid-Resistant Neutrophilic Inflammation in a Mouse Model of an Acute Exacerbation of Asthma. American Journal of Respiratory Cell and Molecular Biology **39**(5): 543-550.

Jacquemin B, Schikowski T, Carsin A E, Hansell A, Kramer U, Sunyer J, Probst-Hensch N, Kauffmann F and Kunzli N. 2012. The Role of Air Pollution in Adult-Onset Asthma: A Review of the Current Evidence. Seminars in Respiratory and Critical Care Medicine **33**(6): 606-619.

Janssen-Heininger Y M, Irvin C G, Scheller E V, Brown A L, Kolls J K and Alcorn J F. 2012. Airway Hyperresponsiveness and Inflammation: Causation, Correlation, or No Relation? Journal of allergy & therapy **2012**(Suppl 1).

Jatakanon A, Uasuf C, Maziak W, Lim S, Chung K F and Barnes P J. 1999. Neutrophilic Inflammation in Severe Persistent Asthma. American Journal of Respiratory and Critical Care Medicine **160**(5): 1532-1539.

Kaczka D W and Dellaca R L. 2011. Oscillation Mechanics of the Respiratory System: Applications to Lung Disease. Critical reviews in biomedical engineering **39**(4): 337-359.

Kim Y K, Oh S Y, Jeon S G, Park H W, Lee S Y, Chun E Y, Bang B, Lee H S, Oh M H, Kim Y S, Kim J H, Gho Y S, Cho S H, Min K U, Kim Y Y and Zhu Z. 2007. Airway Exposure Levels of Lipopolysaccharide Determine Type 1 Versus Type 2 Experimental Asthma. Journal of Immunology **178**(8): 5375-5382.

Kline J N, Cowden J D, Hunninghake G W, Schutte B C, Watt J L, Wohlford-Lenane C L, Powers L S, Jones M P and Schwartz D A. 1999. Variable Airway Responsiveness to Inhaled Lipopolysaccharide. American Journal of Respiratory and Critical Care Medicine **160**(1): 297-303.

Kudo M, Ishigatsubo Y and Aoki I. 2013. Pathology of Asthma. Frontiers in Microbiology **4**.

Kumar A, Takada Y, Boriek A M and Aggarwal B B. 2004. Nuclear Factor-Kappa B: Its Role in Health and Disease. Journal of Molecular Medicine-Jmm **82**(7): 434-448.

Kumar R K and Foster P S. 2002. Modeling Allergic Asthma in Mice: Pitfalls and Opportunities. American Journal of Respiratory Cell and Molecular Biology **27**(3): 267-272.

Kumar R K, Herbert C and Foster P S. 2008. The "Classical" Ovalbumin Challenge Model of Asthma in Mice. Current Drug Targets **9**(6): 485-494.

Lancet T. 2006. A Plea to Abandon Asthma as a Disease Concept. The Lancet **368**(9537): 705.

Laumbach R J and Kipen H M. 2012. Respiratory Health Effects of Air Pollution: Update on Biomass Smoke and Traffic Pollution. Journal of Allergy and Clinical Immunology **129**(1): 3-13.

Lawrence T, Gilroy D W, Colville-Nash P R and Willoughby D A. 2001. Possible New Role for Nf-Kappa B in the Resolution of Inflammation. Nature Medicine **7**(12): 1291-1297.

Lawrence T. 2009. The Nuclear Factor Nf-Kb Pathway in Inflammation. Cold Spring Harbor Perspectives in Biology **1**(6).

Li N, Wang M, Bramble L A, Schmitz D A, Schauer J J, Sioutas C, Harkema J R and Nel A E. 2009. The Adjuvant Effect of Ambient Particulate Matter Is Closely Reflected by the Particulate Oxidant Potential. Environmental Health Perspectives **117**(7): 1116.

Li S, Batterman S, Wasilevich E, Wahl R, Wirth J, Su F C and Mukherjee B. 2011. Association of Daily Asthma Emergency Department Visits and Hospital Admissions with Ambient Air Pollutants among the Pediatric Medicaid Population in Detroit: Time-Series and Time-Stratified Case-Crossover Analyses with Threshold Effects. Environmental Research **111**(8): 1137-1147.

Liu A H. 2002. Endotoxin Exposure in Allergy and Asthma: Reconciling a Paradox. Journal of Allergy and Clinical Immunology **109**(3): 379-392.

Liu L Y, Mathur S K, Sedgwick J B, Jarjour N N, Busse W W and Kelly E A B. 2006. Human Airway and Peripheral Blood Eosinophils Enhance Th1 and Th2 Cytokine Secretion. Allergy **61**(5): 589-597.

Liu S and Chen Z J. 2011. Expanding Role of Ubiquitination in Nf-Kappa B Signaling. Cell Research **21**(1): 6-21.

Lloyd C, Lewkowich I P, WillsKarp M and Saglani S. 2014. Mouse Models of Allergic Airways Disease. Middleton's Allergy: Principles and Practice
N F Adkinson Jr, B S Bochner, A W Burks et al. Philadelphia, PA, Elsevier Health Sciences. **1:** 842-860.

Lötvall J, Akdis C A, Bacharier L B, Bjermer L, Casale T B, Custovic A, Lemanske Jr R F, Wardlaw A J, Wenzel S E and Greenberger P A. 2011. Asthma Endotypes: A new Approach to Classification of Disease Entities within the Asthma Syndrome. Journal of Allergy and Clinical Immunology **127**(2): 355-360.

Lundblad L K A, Irvin C G, Hantos Z, Sly P, Mitzner W and Bates J H T. 2007. Penh Is Not a Measure of Airway Resistance! European Respiratory Journal **30**: 805-805.

Maarsingh H, Leusink J, Bos I S T, Zaagsma J and Meurs H. 2006. Arginase Strongly Impairs Neuronal Nitric Oxide-Mediated Airway Smooth Muscle Relaxation in Allergic Asthma. Respiratory Research **7**.

Mauad T, Poon A H and Hamid Q. 2011. Pathology, Inflammation and Cytokines of Severe Asthma. Eur Respir Mon **51**.

Meurs H, Maarsingh H and Zaagsma J. 2003. Arginase and Asthma: Novel Insights into Nitric Oxide Homeostasis and Airway Hyperresponsiveness. Trends in Pharmacological Sciences **24**(9): 450-455.

Meurs H, Gosens R and Zaagsma J. 2008. Airway Hyperresponsiveness in Asthma: Lessons from in Vitro Model Systems and Animal Models. European Respiratory Journal **32**(2): 487-502.

Michelson P H, Dailey L, Devlin R B and Peden D B. 1999. Ozone Effects on the Immediate-Phase Response to Allergen in the Nasal Airways of Allergic Asthmatic Subjects. Otolaryngology-Head and Neck Surgery **120**(2): 225-232.

Millstein J, Gilliland F, Berhane K, Gauderman W J, McConnell R, Avol E, Rappaport E B and Peters J M. 2004. Effects of Ambient Air Pollutants on Asthma Medication Use and Wheezing among Fourth-Grade School Children from 12 Southern California Communities Enrolled in the Children's Health Study. Archives of Environmental Health **59**(10): 505-514.

Möller W, Heimbeck I, Hofer T P J, Saba G I K, Neiswirth M, Frankenberger M and Ziegler-Heitbrock L m. 2012. Differential Inflammatory Response to Inhaled Lipopolysaccharide Targeted Either to the Airways or the Alveoli in Man. Plos One **7**(4): e33505.

Mukherjee A B and Zhang Z. 2011. Allergic Asthma: Influence of Genetic and Environmental Factors. Journal of Biological Chemistry **286**(38): 32883-32889.

Murphy D M and O'Byrne P M. 2010. Recent Advances in the Pathophysiology of Asthma. CHEST Journal **137**(6): 1417-1426.

Nabe T, Hosokawa F, Matsuya K, Morishita T, Ikedo A, Fujii M, Mizutani N, Yoshino S and Chaplin D D. 2011. Important Role of Neutrophils in the Late Asthmatic Response in Mice. Life Sciences **88**(25-26): 1127-1135.

Nikasinovic L, Just J, Sahraoui F, Seta N, Grimfeld A and Momas I. 2006. Nasal Inflammation and Personal Exposure to Fine Particles Pm2.5 in Asthmatic Children. Journal of Allergy and Clinical Immunology **117**(6): 1382-1388.

O'Byrne P M and Inman M D. 2003. Airway Hyperresponsiveness*. CHEST Journal **123**(3_suppl): 411S-416S.

O'Byrne P M. 2010. Conclusion: Airway Hyperresponsiveness in Asthma: Its Measurement and Clinical Significance. CHEST Journal **138**(2_suppl): 44S-45S.

Pacher P, Beckman J S and Liaudet L. 2007. Nitric Oxide and Peroxynitrite in Health and Disease. Physiological Reviews **87**(1): 315-424.

Pantano C, Ather J L, Alcorn J F, Poynter M E, Brown A L, Guala A S, Beuschel S L, Allen G B, Whittaker L A, Bevelander M, Irvin C G and Janssen-Heininger Y M W. 2008. Nuclear Factor-Kappa B Activation in Airway Epithelium Induces Inflammation and Hyperresponsiveness. American Journal of Respiratory and Critical Care Medicine **177**(9): 959-969.

Paré P D and Mitzner W. 2012. Counterpoint: Alterations in Airway Smooth Muscle Phenotype Do Not Cause Airway Hyperresponsiveness in Asthma. Journal of Applied Physiology **113**(5): 839-842.

Patterso.R and Kelly J F. 1974. Animal-Models of Asthmatic State. Annual Review of Medicine **25**: 53-68.

Pawankar R, Holgate S, Rosenwasser L and Cohn L. 2009. Mechanisms of Mucus Induction in Asthma. Allergy Frontiers: Clinical Manifestations, Springer Japan. **3:** 173-185.

Peden D and Reed C E. 2010. Environmental and Occupational Allergies. Journal of Allergy and Clinical Immunology **125**(2): S150-S160.

Peden D B, Setzer R W and Devlin R B. 1995. Ozone Exposure Has Both a Priming Effect on Allergen-Induced Responses and an Intrinsic Inflammatory Action in the Nasal Airways of Perennially Allergic Asthmatics. American Journal of Respiratory and Critical Care Medicine **151**(5): 1336-1345.

Peden D B, Tucker K, Murphy P, Newlin-Clapp L, Boehlecke B, Hazucha M, Bromberg P and Reed W. 1999. Eosinophil Influx to the Nasal Airway after Local, Low-Level Lps Challenge in Humans. Journal of Allergy and Clinical Immunology **104**(2): 388-394.

Peden D B. 2011. The Role of Oxidative Stress and Innate Immunity in O3 and Endotoxin-Induced Human Allergic Airway Disease. Immunological Reviews **242**(1): 91-105.

Phillips J E, Peng R Q, Harris P, Burns L, Renteria L, Lundblad L K A, Fine J S, Bauer C M T and Stevenson C S. 2013. House Dust Mite Models: Will They Translate Clinically as a Superior Model of Asthma? Journal of Allergy and Clinical Immunology **132**(1): 242-244.

Plant P J, North M L, Ward A, Wardy M, Khanna N, Correa J, Scott J A and Batt J. 2012. Hypertrophic Airway Smooth Muscle Mass Correlates with Increased Airway Responsiveness in a Murine Model of Asthma. American Journal of Respiratory Cell and Molecular Biology **46**(4): 532-540.

Porsbjerg C, Brannan J D, Anderson S D and Backer V. 2008. Relationship between Airway Responsiveness to Mannitol and to Methacholine and Markers of Airway Inflammation, Peak Flow Variability and Quality of Life in Asthma Patients. Clinical & Experimental Allergy **38**(1): 43-50.

Poynter M E, Irvin C G and Janssen-Heininger Y M W. 2002. Rapid Activation of Nuclear Factor-Kappa B in Airway Epithelium in a Murine Model of Allergic Airway Inflammation. American Journal of Pathology **160**(4): 1325-1334.

Poynter M E, Irvin C G and Janssen-Heininger Y M W. 2003. A Prominent Role for Airway Epithelial Nf-Kappa B Activation in Lipopolysaccharide-Induced Airway Inflammation. Journal of Immunology **170**(12): 6257-6265.

Poynter M E, Cloots R, van Woerkom T, Butnor K J, Vacek P, Taatjes D J, Irvin C G and Janssen-Heininger Y M W. 2004. Nf-Kappa B Activation in Airways Modulates Allergic Inflammation but Not Hyperresponsiveness. Journal of Immunology **173**(11): 7003-7009.

Prado C M, Leick-Maldonado E A, Yano L, Leme A S, Capelozzi V L, Martins M A and Tiberio I F L C. 2006. Effects of Nitric Oxide Synthases in Chronic Allergic Airway Inflammation and Remodeling. American Journal of Respiratory Cell and Molecular Biology **35**(4): 457-465.

Prado C M, Martins M A and Tibério I F L C. 2011. Nitric Oxide in Asthma Physiopathology. ISRN allergy **2011**.

Reddel H K, Taylor D R, Bateman E D, Boulet L P, Boushey H A, Busse W W, Casale T B, Chanez P, Enright P L, Gibson P G, de Jongste J C, Kerstjens H A M, Lazarus S C, Levy M L, O'Byrne P M, Partridge M R, Pavord I D, Sears M R, Sterk P J, Stoloff S W, Sullivan S D, Szefler S J, Thomas M D, Wenzel S E, Amer Thoracic S and European Resp Soc Task Force A. 2009. An Official American Thoracic Society/European Respiratory Society Statement: Asthma Control and Exacerbations Standardising Endpoints for Clinical Asthma Trials and Clinical Practice. American Journal of Respiratory and Critical Care Medicine **180**(1): 59-99.

Rial N S, Choi K, Nguyen T, Snyder B and Slepian M J. 2012. Nuclear Factor Kappa B (Nf-Kb): A Novel Cause for Diabetes, Coronary Artery Disease and Cancer Initiation and Promotion? Medical Hypotheses **78**(1): 29-32.

Rogers D F. 2004. Airway Mucus Hypersecretion in Asthma: An Undervalued Pathology? Current Opinion in Pharmacology **4**(3): 241-250.

SadeghiHashjin G, Folkerts G, Henricks P A J, Verheyen A, vanderLinde H J, vanArk I, Coene A and Nijkamp F P. 1996. Peroxynitrite Induces Airway Hyperresponsiveness in Guinea Pigs in Vitro and in Vivo. American Journal of Respiratory and Critical Care Medicine **153**(5): 1697-1701.

Schuessler T F and Bates J H T. 1995. A Computer-Controlled Research Ventilator for Small Animals - Design and Evaluation. Ieee Transactions on Biomedical Engineering **42**(9): 860-866.

Searing D A and Rabinovitch N. 2011. Environmental Pollution and Lung Effects in Children. Current Opinion in Pediatrics **23**(3): 314-318.

Senthilselvan A, Dosman J A, Chenard L, Burch L H, Predicala B Z, Sorowski R, Schneberger D, Hurst T, Kirychuk S, Gerdts V, Cormier Y, Rennie D C and Schwartz D A. 2009. Toll-Like Receptor 4 Variants Reduce Airway Response in Human Subjects at High Endotoxin Levels in a Swine Facility. Journal of Allergy and Clinical Immunology **123**(5): 1034-1040.

Sheller J R, Polosukhin V V, Mitchell D, Cheng D S, Peebles R S and Blackwell T S. 2009. Nuclear Factor Kappa B Induction in Airway Epithelium Increases Lung

Inflammation in Allergen-Challenged Mice. Experimental Lung Research **35**(10): 883-895.

Short P M, Lipworth S I W and Lipworth B J. 2011. Relationships between Airway Hyperresponsiveness, Inflammation, and Calibre in Asthma. Lung **189**(6): 493-497.

Shum B O, Rolph M S and Sewell W A. 2008. Mechanisms in Allergic Airway Inflammation-Lessons from Studies in the Mouse. Expert Rev Mol Med **10**: e15.

Sigsgaard T, Heederik D, Omland Ø and Thorne P. 2010. Asthma-Like Diseases in Agriculture. Occupational Asthma, BirkhÃ¤user Basel**:** 163-183.

Simmonds R E and Foxwell B M. 2008. Signalling, Inflammation and Arthritis: Nf-Kb and Its Relevance to Arthritis and Inflammation. Rheumatology **47**(5): 584-590.

Simpson A and Martinez F D. 2010. The Role of Lipopolysaccharide in the Development of Atopy in Humans. Clinical and Experimental Allergy **40**(2): 209-223.

Sly P D and Holt P G. 2011. Role of Innate Immunity in the Development of Allergy and Asthma. Current Opinion in Allergy and Clinical Immunology **11**(2): 127-131 110.1097/ACI.1090b1013e32834487c32834486.

Stevenson C S and Birrell M A. 2010. Moving Towards a New Generation of Animal Models for Asthma and Copd with Improved Clinical Relevance. Pharmacology & Therapeutics **130**(2): 93-105.

Tagaya E and Tamaoki J. 2007. Mechanisms of Airway Remodeling in Asthma. Allergol Int **56**(4): 331-340.

Takeda K, Haczku A, Lee J J, Irvin C G and Gelfand E W. 2001. Strain Dependence of Airway Hyperresponsiveness Reflects Differences in Eosinophil Localization in the Lung. American Journal of Physiology-Lung Cellular and Molecular Physiology **281**(2): L394-L402.

Takeda K and Gelfand E W. 2009. Mouse Models of Allergic Diseases. Current Opinion in Immunology **21**(6): 660-665.

Tepper R S, Wise R S, Covar R, Irvin C G, Kercsmar C M, Kraft M, Liu M C, O'Connor G T, Peters S P, Sorkness R and Togias A. 2012. Asthma Outcomes: Pulmonary Physiology. Journal of Allergy and Clinical Immunology **129**(3): S65-S87.

Toward T J and Broadley K J. 2000. Airway Reactivity, Inflammatory Cell Influx and Nitric Oxide in Guinea-Pig Airways after Lipopolysaccharide Inhalation. British Journal of Pharmacology **131**(2): 271-281.

Tsuchiya K, Siddiqui S, Risse P-A, Hirota N and Martin J G. 2012. The Presence of Lps in Ova Inhalations Affects Airway Inflammation and Ahr but Not Remodeling in a Rodent Model of Asthma. American Journal of Physiology - Lung Cellular and Molecular Physiology.

Tulic M K, Wale J L, Holt P G and Sly P D. 2000. Modification of the Inflammatory Response to Allergen Challenge after Exposure to Bacterial Lipopolysaccharide. American Journal of Respiratory Cell and Molecular Biology **22**(5): 604-612.

USEPA. 2013. Technology Transfer Network National Ambient Air Quality Standards., from http://www.epa.gov/ttn/naaqs/pm/pm25_index.html.

Vanoirbeek J A J, Rinaldi M, De Vooght V, Haenen S, Bobic S, Gayan-Ramirez G, Hoet P H M, Verbeken E, Decramer M, Nemery B and Janssens W. 2010. Noninvasive and Invasive Pulmonary Function in Mouse Models of Obstructive and Restrictive Respiratory Diseases. American Journal of Respiratory Cell and Molecular Biology **42**(1): 96-104.

Vargaftig B B. 1997. Modifications of Experimental Bronchopulmonary Hyperresponsiveness. American Journal of Respiratory and Critical Care Medicine **156**(4): S97-S102.

Vercelli D. 2006. Mechanisms of the Hygiene Hypothesis Â€" Molecular and Otherwise. Current Opinion in Immunology **18**(6): 733-737.

Verma I M. 2004. Nuclear Factor (Nf)-Kappa B Proteins: Therapeutic Targets. Annals of the Rheumatic Diseases **63**: 57-61.

Von M, Braun F, Schierl, Riedler, Ehlermann, Maisch, Waser and Nowak. 2000. Exposure to Endotoxin or Other Bacterial Components Might Protect against the Development of Atopy. Clinical & Experimental Allergy **30**(9): 1230-1234.

Wagers S, Lundblad L K A, Ekman M, Irvin C G and Bates J H T. 2004. The Allergic Mouse Model of Asthma: Normal Smooth Muscle in an Abnormal Lung? Journal of Applied Physiology **96**(6): 2019-2027.

Wagers S S, Haverkamp H C, Bates J H T, Norton R J, Thompson-Figueroa J A, Sullivan M J and Irvin C G. 2007. Intrinsic and Antigen-Induced Airway Hyperresponsiveness Are the Result of Diverse Physiological Mechanisms. Journal of Applied Physiology **102**(1): 221-230.

Wagner J G, Hotchkiss J A and Harkema J R. 2002. Enhancement of Nasal Inflammatory and Epithelial Responses after Ozone and Allergen Coexposure in Brown Norway Rats. Toxicological Sciences **67**(2): 284-294.

Wagner J G, Jiang Q, Harkema J R, Illek B, Patel D D, Ames B N and Peden D B. 2007. Ozone Enhancement of Lower Airway Allergic Inflammation Is Prevented by Γ-Tocopherol. Free Radical Biology and Medicine **43**(8): 1176-1188.

Wagner J G, Morishita M, Keeler G J and Harkema J R. 2012. Divergent Effects of Urban Particulate Air Pollution on Allergic Airway Responses in Experimental Asthma: A Comparison of Field Exposure Studies. Environmental Health **11**.

Walker J K L, Kraft M and Fisher J T. 2013. Assessment of Murine Lung Mechanics Outcome Measures: Alignment with Those Made in Asthmatics. Frontiers in physiology **3**: 491-491.

Wan, Winton, Soeller, Gruenert, Thompson, Cannell, Stewart, Garrod and Robinson. 2000. Quantitative Structural and Biochemical Analyses of Tight Junction Dynamics Following Exposure of Epithelial Cells to House Dust Mite Allergen Der P 1. Clinical & Experimental Allergy **30**(5): 685-698.

Watanabe J, Miyazaki Y, Zimmerman G A, Albertine K H and McIntyre T M. 2003. Endotoxin Contamination of Ovalbumin Suppresses Murine Immunologic Responses and Development of Airway Hyper-Reactivity. Journal of Biological Chemistry **278**(43): 42361-42368.

Weiss S T, Utell M J and Samet J M. 1999. Environmental Tobacco Smoke Exposure and Asthma in Adults. Environmental Health Perspectives **107**: 891-895.

Wenzel S and Holgate S T. 2006. The Mouse Trap - It Still Yields Few Answers in Asthma. American Journal of Respiratory and Critical Care Medicine **174**(11): 1173-1176.

Wenzel S E. 2006. Asthma: Defining of the Persistent Adult Phenotypes. The Lancet **368**(9537): 804-813.

Wills-Karp M. 1999. Immunologic Basis of Antigen-Induced Airway Hyperresponsiveness. Annual Review of Immunology **17**(1): 255-281.

Wills-Karp M, Santeliz J and Karp C L. 2001. The Germless Theory of Allergic Disease: Revisiting the Hygiene Hypothesis. Nature Reviews Immunology **1**(1): 69-75.

Xiao C, Puddicombe S M, Field S, Haywood J, Broughton-Head V, Puxeddu I, Haitchi H M, Vernon-Wilson E, Sammut D, Bedke N, Cremin C, Sones J, DjukanoviÄ‡ R, Howarth P H, Collins J E, Holgate S T, Monk P and Davies D E. 2011. Defective Epithelial Barrier Function in Asthma. The Journal of allergy and clinical immunology **128**(3): 549-556.e512.

Yang X D, Sun Q Z, Asim M B R, Jiang X G, Zhong B, Shahzad M, Zhang F J, Han Y and Lu S M. 2010. Nitric Oxide in Both Bronchoalveolar Lavage Fluid and Serum Is Associated with Pathogenesis and Severity of Antigen-Induced Pulmonary Inflammation in Rats. Journal of Asthma **47**(2): 135-144.

Zeldin D C, Eggleston P, Chapman M, Piedimonte G, Renz H and Peden D. 2006. How Exposures to Biologics Influence the Induction and Incidence of Asthma. Environmental Health Perspectives **114**(4): 620-626.

Zhang Y, Lamm W J, Albert R K, Chi E Y, Henderson W R and Lewis D B. 1997. Influence of the Route of Allergen Administration and Genetic Background on the Murine Allergic Pulmonary Response. American Journal of Respiratory and Critical Care Medicine **155**(2): 661-669.

Zuo L, Koozechian M S and Chen L L. 2013. Characterization of Reactive Nitrogen Species in Allergic Asthma. Annals of Allergy, Asthma & Immunology.

CHAPTER 2

EFFECTS OF INHALED LIPOPOLYSACCHARIDE ON AIRWAY INFLAMMATION AND AIRWAY HYPERRESPONSIVENESS IN ALLERGIC MICE

2.1 ABSTRACT

Lung inflammation is a major characteristic of asthma, and is often used as an indicator of severity. Nonetheless, inflammation severity has not correlated with asthma control, and there are conflicting data determining whether increased inflammation is associated with functional changes in the lung. In this study, I examined the relationship of pulmonary inflammation with changes in AHR by using a mouse model of LPS-induced exacerbation of established OVA- induced allergic airway disease. Male BALB/c mice were sensitized and challenged with saline or OVA. Two days following the last challenge, allergic and non-allergic mice received either 0 or 3 μg LPS by IN. Twenty-four hours later AHR was evaluated and animals were euthanized to collect tissue samples. A significant inflammatory response consisting of infiltrations of eosinophils in OVA alone mice and neutrophils in LPS only mice was present in the BALF and lung. OVA-LPS mice produced a more severe inflammatory cell response combining both eosinophils and neutrophils. OVA-associated expression of *Il13*, *Muc5ac*, and *Gob5 genes* in lung tissue was decreased following LPS exposure in OVA-LPS mice, while expression of genes for Th1 cytokine *Ifng* and the regulatory cytokine *Il10* was increased. AHR to methacholine challenge was increased in the central airways and the peripheral lung tissue of both OVA and LPS alone mice, although the magnitude was greater in LPS mice. Despite an increase in inflammation, AHR was attenuated in OVA-LPS mice compared to both OVA and LPS only. Morphometric analyses of eosinophils and neutrophils demonstrated that eosinophils in allergic animals were localized within the central airway compartments (perivascular

and peribronchiolar regions). With LPS exposure, neutrophils were present throughout the central airway and peripheral lung tissue. In OVA-LPS mice, both eosinophils and neutrophils were distributed in the central and peripheral compartments. OVA and LPS mice had increase AHR in the central airways and peripheral tissue. However, in OVA-LPS mice AHR was attenuated in both of these compartments even with increased inflammation present in those regions. These findings suggest that AHR is not linked to inflammation severity.

2.2 INTRODUCTION

Asthma is characterized by lung inflammation, reversible airway obstruction, decreased lung function, and airway hyperresponsiveness (AHR) (Busse and Lemanske, 2001; Fitzpatrick et al., 2008). Increased inflammation is often used as an indicator of asthma severity but the relation to functional changes is inconsistent. For example, elevations of induced sputum biomarkers or exhaled nitric oxide are used to estimate the severity of airway inflammation but thus far have not been effectively correlated with asthma control (Louis et al., 2000; Busse, 2010; Walker et al., 2013). Furthermore, elevations in pulmonary eosinophils and neutrophils that occur in severe asthmatics are not closely related with AHR (Wenzel et al., 1999; Holgate, 2008; Busse, 2010). These inconsistencies may be due to the sampling site of inflammation versus the site of AHR. Decreased airway function in some asthmatics may have critical contributions from small airways, a region that is difficult and unreliable to sample (Tulic and Hamid, 2006; Ueda et al., 2006; Burgel, 2011; Sterk and Bel, 2011; Farah et al., 2012). As such, efforts to specifically target small and/or large airways with therapeutic interventions would benefit from a deeper understanding of the relationship between regional inflammatory status and functional changes within specific airways.

Experimental asthma in laboratory rodents can reproduce many of the acute features of asthmatic airways, including inflammation, epithelial remodeling, and airway obstruction and reactivity (Nials and Uddin, 2008; Bates et al., 2009; Hyde et al., 2009). These models allow the complete examination of the tracheobronchial tree that is

difficult to obtain by bronchial biopsy in humans. Importantly, it is also possible to conduct invasive respiratory maneuvers that provide a detailed analysis of functional changes that occur during the development of allergic airways disease. Although the forced oscillation technique (FOT) is currently considered an emerging technique to measure pulmonary function in asthmatics (Tepper et al., 2012), it is commonly used to assess experimental asthma in animals and provides greater specificity in determining changes the airways (ie. central vs. peripheral airways) (Bates et al., 2009; Vanoirbeek et al., 2010).

We have previously used allergic Brown Norway rats to show that environmental agents such as ozone and particulate matter (PM2.5) can exacerbate airway inflammation and mucus cell metaplasia (Wagner et al., 2007; Wagner et al., 2012). We also found that ozone-induced epithelial lesions can be enhanced by another airborne contaminant, lipopolysaccharide (LPS), a component of the cell wall of Gram negative bacteria (Wagner et al., 2003) that has been shown to be present in particulate matter in ambient air and occupational settings (Becker et al., 2002; Mueller-Anneling et al., 2004). Despite eliciting a neutrophilic, innate immune, Th1 type response, inhaled LPS can exacerbate asthma symptoms in humans (Boehlecke et al., 2003), as well as worsen allergic airway disease in rats (Tulic et al., 2000). However, the histopathological changes induced by LPS in asthmatic airways have not been characterized in detail, nor has the influence of LPS-induced neutrophil recruitment on reactivity of asthmatic airways been described.

In the present study we developed a murine model of LPS-induced exacerbation of asthma to test the hypotheses that 1) LPS induces site-specific neutrophilic lesions in the allergic lung and that 2) the severity and location of lesions would be correlates with treatment-related change in airway resistance. We combined a detailed morphological assessment of lung tissue with rigorous pulmonary function testing to explore associations between changes in central airways and peripheral resistance with anatomical lesions induced by allergen and LPS.

2.3 MATERIALS AND METHODS

2.3.1 Laboratory Animals

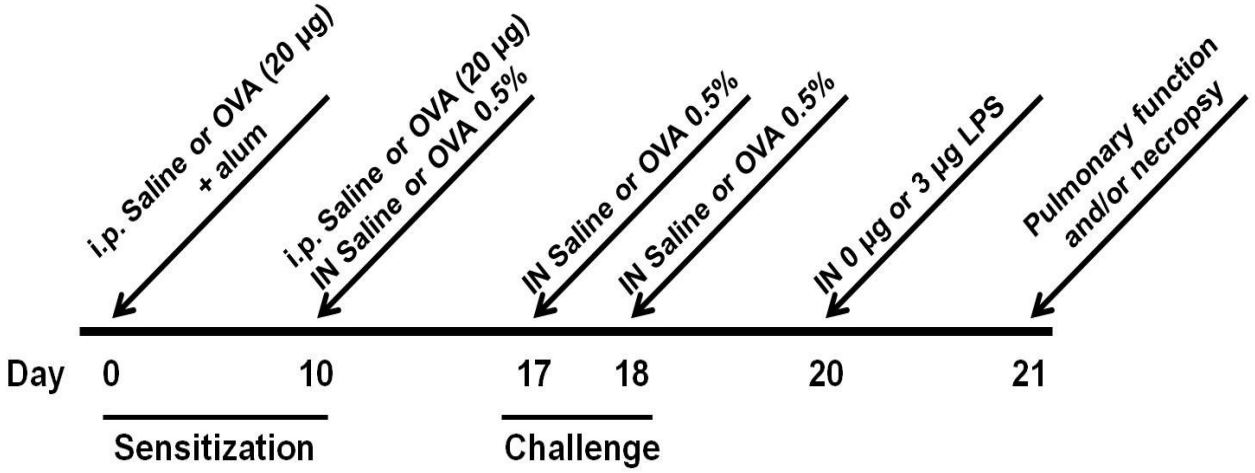
Male BALB/c mice (Charles River Laboratories, Portage, MI), 6-8 weeks of age were housed in individual ventilated cages (IVC, Innocage; Innovive, San Diego, CA) that contained heat-treated aspen hardwood bedding (Northeastern Product Corp-NEPCO, Warrensburg, NY). Cages were placed in a rodent ventilated housing system (Innorack; Innovive, San Diego, CA). Animals were placed 3-4 per cage with ad libitum food (Harlan Teklad Laboratory Rodents 22/5 diet, Madison, WI) and laboratory grade acidified water (Aquavive; Innovive, San Diego, CA). Mice were maintained at Michigan State University (MSU) animal facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Animal rooms were kept at a temperature of 21-24°C and relative humidity of 45-70%, with a 12 hour light/dark cycle. All protocols and animal procedures were approved by MSU Institutional Animal Care and Use Committee.

2.3.2 Experimental protocol: Development of allergic airway disease and LPS exposure

Mice were randomly assigned to one of four experimental groups consisting of 6 animals. Mice designated to receive the allergen were sensitized to ovalbumin (OVA, Sigma-Aldrich, St. Louis, MO) by intraperitoneal (i.p.) injection with 0.25 ml saline containing 20 µg of OVA with 1 mg alum adjuvant (aluminum potassium sulfate, Sigma-Aldrich) on Day 0. On Day 10, mice were administered a boost with i.p. injection of

alum free OVA (20 µg) in 0.25 ml saline along with an intranasal (IN) instillation of 30 µl 0.5% OVA. Delivery of OVA by IN instillation was chosen to produce a robust inflammatory response in the lung and induce AHR (Swedin et al., 2010) Seven days later, the mice were challenged IN with 30 µl 0.5% OVA for two consecutive days (Days 17 & 18). All remaining mice received only saline during sensitization and challenge. Two days following the last OVA or saline challenge (Day 20), mice in allergic and non-allergic groups were divided in half and received IN either 0 µg or 3 µg lipopolysaccharide (LPS; *P.aeruginosa*, Sigma-Aldrich) in 30 µl saline. Twenty-four hours following LPS exposure (Day 21) pulmonary function testing and/or necropsy of mice was performed (Fig 3).

Figure 3. Allergen sensitization, challenge, and LPS exposure protocol



2.3.3 Necropsy, Lavage Collection, and Tissue Preparation

Mice were anesthetized with an i.p. injection of with sodium pentobarbital (10 mg; Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI). Blood was collected from the caudal vena cava for separation of plasma, and animals were euthanized by transection of the abdominal aorta. The lungs and heart were harvested en bloc. The lungs were instilled twice with 800 µL saline via the cannulated trachea to collect BALF. After lavage the right lung lobes were ligated, separated and stored in RNAlater (Qiagen) for RNA isolation or snap frozen in liquid nitrogen for storage. The left lobe was inflated with 10% neutral buffered formalin to a pressure of 30 cm H_2O for 1 hour and then stored in a large volume of the same fixative.

2.3.4 Bronchoalveolar Lavage Analysis

Total BALF leukocytes were counted with a hemocytometer and cytological slides were prepared using a Cytospin centrifuge (Shandon). Slides were stained with Diff-Quick (Dade Behring, Newark, DE) and cell differential (macrophages, eosinophils, neutrophils, and lymphocytes) were counted. The remaining BAL fluid was centrifuged, and the supernatants were analyzed for inflammatory cytokine concentrations of interferon-gamma (IFN-γ), interleukin (IL)-17, IL-6, and tumor necrosis factor-alpha (TNF-α). All cytokine kits were purchased as either Flex Set reagents or as preconfigured cytometric bead array kits (BD Biosciences, San Jose, CA). Cytokine analysis was performed using a FACSCalibur flow cytometer (BD Biosciences). 50 µl of BALF was added to the antibody-coated bead complexes and incubation buffer. Phycoerythrin-conjugated secondary antibodies were added to form sandwich

complexes. After acquisition of sample data using the flow cytometer, cytokine concentrations were calculated based on standard curve data using FCAP Array software (BD Biosciences).

2.3.5 Histopathology

Following fixation, two traverse sections of the left lung were taken along the axial airway at the levels of the 5^{th} and 11^{th} generation (G5 and G11) to examine the proximal and distal airways (Harkema and Hotchkiss, 1992). The tissues were embedded in paraffin and stained with hematoxylin and eosin (H&E) for routine histological examination, Alcian blue periodic-acid-Schiff stain (AB/PAS) to identify intraepithelial mucosubstances, and immunohistochemical stains to detect eosinophils (major basic protein, MBP; Mayo Clinic, AZ) and neutrophils (NIMP-R14; Serotec, Raleigh, NC).

2.3.6 Morphometry

Estimation of the amount of the intraepithelial mucosubstances in epithelium lining axial airways (G5 and G11) was performed as specified in previous studies (Wagner et al., 2003). Using a computerized image analysis system, the volume density (Vs) of AB/PAS-stained mucosubstances was quantified by calculating the area of positive stained mucosubstances from the automatically circumscribed perimeter of stained material using a Dell XPS 400 computer and Scion Image (Scion Corporation). The length of the basal lamina beneath the surface epithelium was calculated from the contour length on the digitized image. The volume of stored mucosubstances per unit of

surface area of epithelial basal lamina was estimated using the method described in detail by (Harkema et al., 1987). The Vs of intraepithelial mucosubstances is expressed as nanoliters of intraepithelial mucosubstances per mm square of basal lamina.

Inflammatory cells were quantified by using digitalized histological slides captured by a slide scanner (VS110, Olympus). At 20x magnification, lung tissue images were captured by systematic random sampling. Digital images were uploaded to the web based Stepanizer program, a computer based software tool for stereological evaluation of digital images (Tschanz et al., 2011). A point grid was superimposed over random images of sampled lung to count the numbers of points positively stained for eosinophils or neutrophils within the regions of interest. The data was expressed as percent of positive staining points within a tissue region / total points counted in the tissue region. The lung (left lobe) was classified into regions consisting of bronchioles with adjacent blood vessels (bronchovascular), terminal bronchioles, alveolar walls or interstitium, and the airspaces.

2.3.7 Real –time PCR of Lung

Total RNA was isolated from the right caudal lung lobe using Rneasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Tissues were homogenized using Qiagen's TissueLyser II Bead Beater and three 2.8mm Zirconium Oxide beads in 600µl buffer RLT containing β -Mercaptoethanol. Homogenate was then centrifuged at 12,000g for 3 minutes and RNA was purified from the supernatant using the RNeasy capture column. Eluted RNA was diluted 1:5 with Rnase free water and quantified using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific,

Waltman, MA). Reverse transcription was accomplished by using High Capacity cDNA Reverse Transcription Kit reagents (Applied Biosystems). Each RT reaction was run in a 50 μl reaction volume containing 5 μg of total RNA with cDNA Master Mix prepared according to the manufacturer's protocol. The reaction mixture was incubated in a in GeneAmp PCR System 9700 Thermocycler PE (Applied Biosystems, Foster City, CA) at 25ºC for 10 Minutes, 37ºC for 2 hours, then held at 4º.

Relative quantitative mRNA expression analysis of targeted genes for molecular analysis included *Gob5*, *Muc5ac*, *TNF*α, *IFN*γ, *IL-4*,*-5,-6*, *-10 -13*s was conducted on an ABI PRISM 7900 HT Sequence Detection System at Michigan State University's Research Technology Support Facility using Taqman Gene Expression Assay reagents (Applied Biosystems). 2μl cDNA and 8μl reagents were dispensed (in duplicate) into a 384-well reaction plate. The cycling parameters were 48 °C for 2 minutes, 95 °C for10 minutes, and 50 cycles of 95 °C for 15 seconds followed by 60 °C for 1 minute

Gene expression levels were reported as fold-change (FC) of mRNA in experimental samples compared to a control sample. Real-time PCR amplifications were relatively quantified using the $\Delta\Delta$Ct method. Following the PCR, amplification plots (change in dye fluorescence versus cycle number) were examined and a dye fluorescence threshold within the exponential phase of the reaction was set for the target gene and the endogenous references. The cycle number at which each amplified product crosses the set threshold represents the C_T value. The amount of target gene normalized to its endogenous reference was calculated by subtracting the geometric mean of the C_Ts from endogenous controls (*Arbp, Gusb, and Gapdh*) from the target

gene C_T (delta Ct (ΔCt)) This normalization strategy has been utilized for accurate RT-PCR expression profiling in biological samples with small expression differences (Vandesompele et al., 2002). The ΔCt value for the experimental sample is subtracted from the ΔCt value of the corresponding control sample ($\Delta\Delta$Ct). The FC in experimental samples relative to control samples is then calculated as: FC= $2^{-\Delta\Delta Ct}$.

2.3.8 Airway Hyperresponsiveness (AHR) Measurements

On day 21, some of the mice were anesthetized by i.p. sodium pentobarbital (100 mg/kg). A tracheostomy was performed to insert an 18 gauge cannula which was attached to a mechanical ventilator with a computer controlled piston pump (flexiVent; Scireq, Montreal, Canada). Mice were ventilated at a respiratory rate of 150 breaths / minute, tidal volume of 10 ml/kg, and positive end expiratory pressure (PEEP) of 2-3 cm H_2O. Incremental concentrations (0, 1.25, 2.5, 5, 10, and 20 mg/ml) of a bronchoconstrictor, acetyl-β-methacholine (MCh, Sigma-Aldrich) were delivered into the trachea via an attached nebulizer (Aeroneb; Aerogen, Galaway, Ireland). Prior to each MCh response curve, two deep inspirations were given. Following MCh administration, 12 perturbation maneuvers consisting of alternating measurements of sinusoidal, single frequency, oscillations (SnapShot) and broadband, multi-frequency, oscillations (Quick-prime) were performed. From the collected data, the flexiVent software calculated total respiratory system resistance using the single compartment model, as well as airway resistance, tissue damping, and tissue elastance using the constant phase model (Hantos et al., 1992). The mean of the responses for each concentration of methacholine was determined. A dose- response curve was generated. The lowest

dose of MCh with the greatest variation in response between groups was at 10 mg/ml. Therefore, the data were expressed as the percent change at 10 mg/ml compared to baseline for each group.

2.3.9 Statistical Analysis

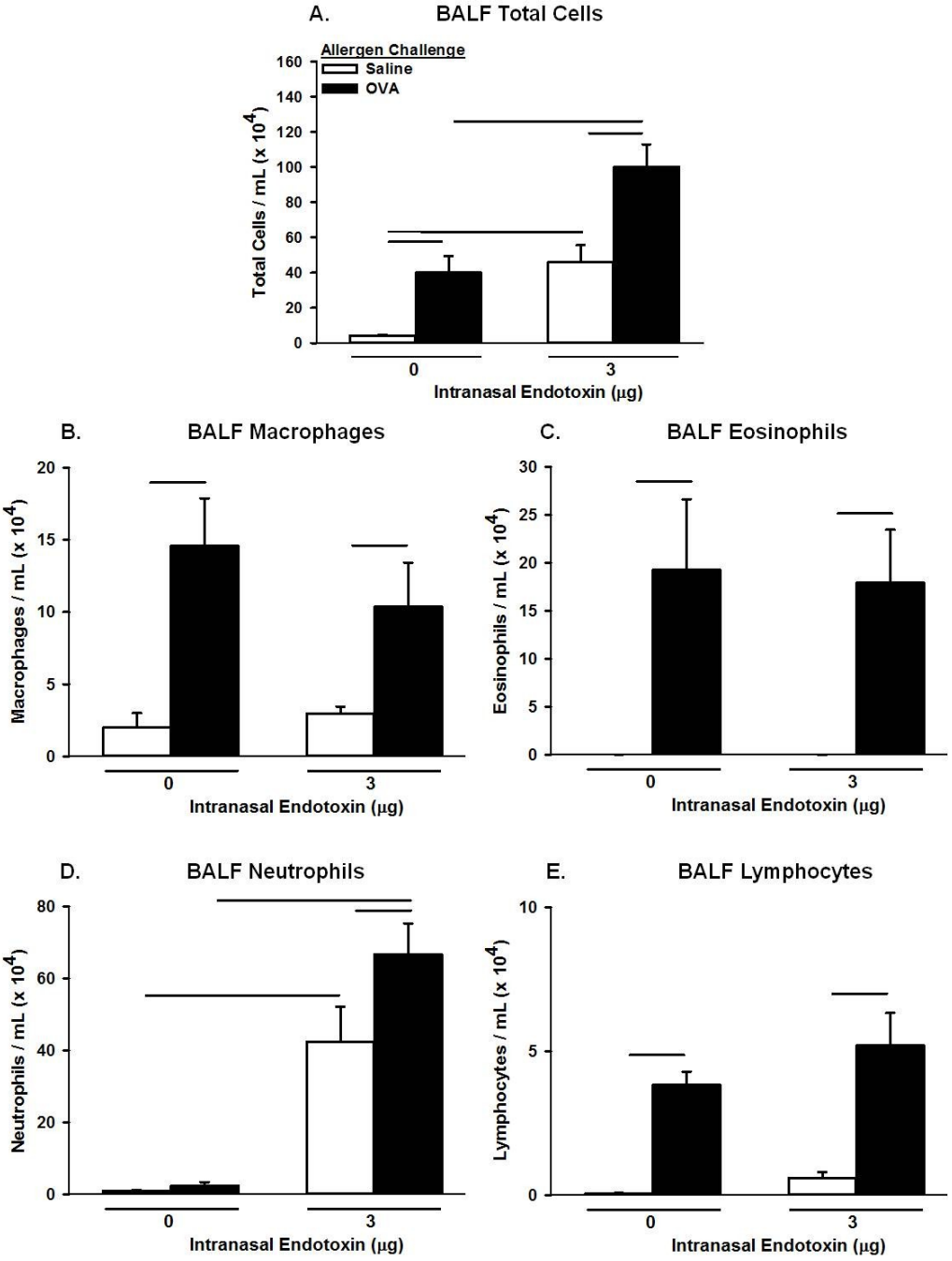
For BALF and AHR analysis, data were expressed as group means $\pm$ the standard error of the mean (mean $\pm$ SEM). Grubb's outlier test was used to determine and remove outliers. A two-way analysis of variance (ANOVA) was performed to determine statistical differences. Significant results were further analyzed with Student-Newman-Keuls post hoc test to make direct comparison between groups. Significant differences between group means were based on p values set at $p < 0.05$. For RT-PCR, statistical differences of ΔCt values between groups were determined with two-way ANOVA with Student-Newman-Keuls post hoc test to make direct comparison between groups; $p \leq 0.05$. SigmaPlot statistical software was used for data analysis (Systat Software Inc, San Jose, CA).

2.4 RESULTS

2.4.1 Bronchoalveolar lavage fluid (BALF).

Seventy-two hours following the final allergen challenge, OVA elicited a significant accumulation of inflammatory cells in BALF (Fig.4), consisting of predominately of eosinophils and macrophages. Twenty-four hours following exposure, intranasal LPS caused an increase in BALF cellularity due primarily to neutrophils in non-allergic (LPS) mice. In allergic mice given ovalbumin and then followed with LPS (OVA-LPS; black bars), total BALF inflammatory cells were two-fold greater than that elicited by either ovalbumin or endotoxin alone, with the increase due to neutrophils.

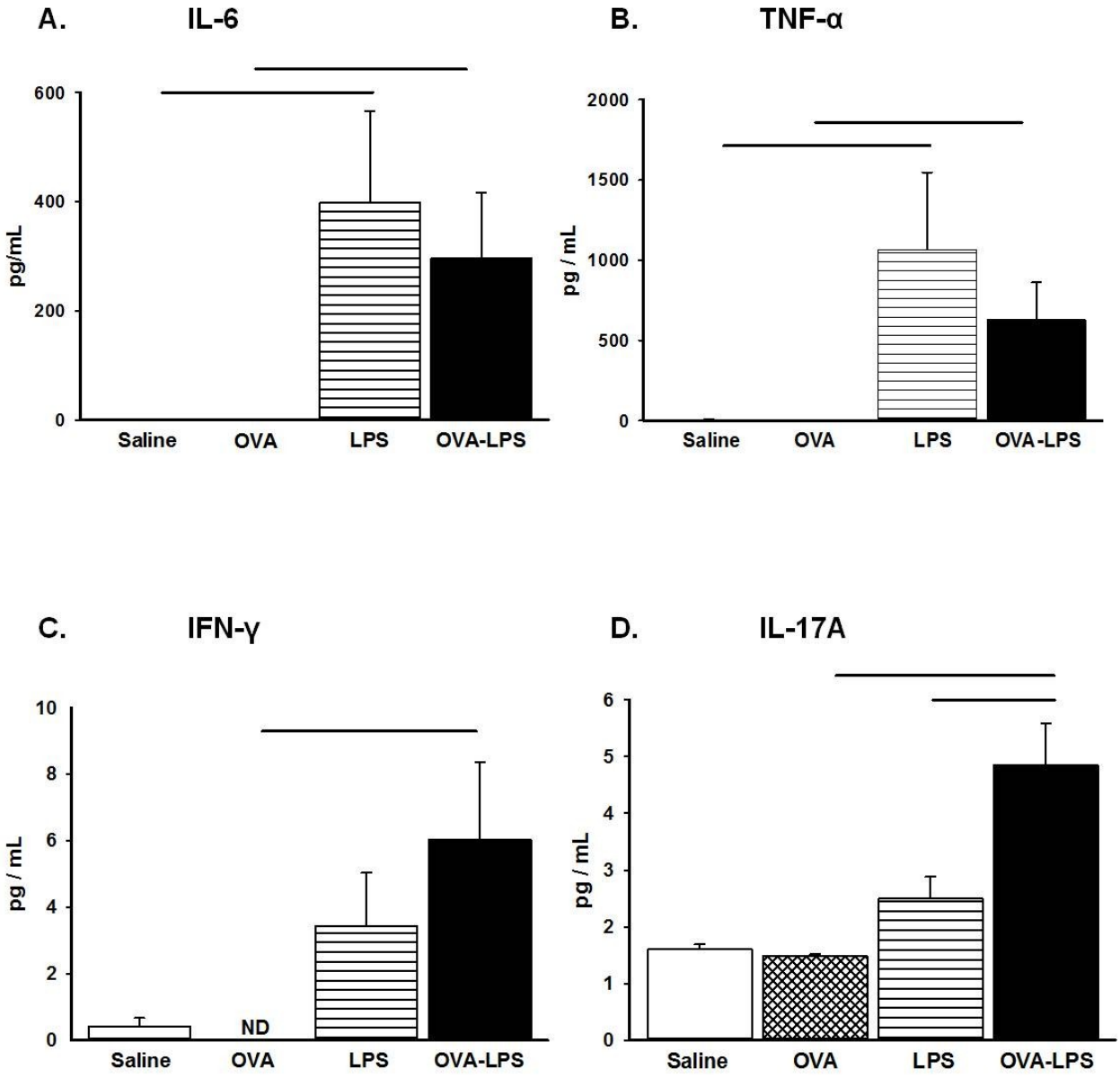
Figure 4. BALF of non-allergic and allergic mice exposed to LPS. Total cells, macrophages, eosinophils, neutrophils, and lymphocytes were determined in BALF collected from non-allergic BALB/c mice which received saline during sensitization and challenge (open bars); and allergic mice sensitized and challenged to OVA (solid bars) grouped by exposure to LPS (0 or 3 µg) as described in Materials and Methods. Values are expressed as mean ± SE (n=5-6). Horizontal lines indicate significant difference; $p < 0.05$.



2.4.2 BALF Cytokines

At 72 hours after the last allergen challenge, there were no significant changes in any cytokine evaluated in OVA mice compared to saline control mice. LPS treatment induced a significant accumulation of TNFα and IL-6 in BAL fluid that was similar in non-allergic (LPS alone) (Fig. 5 A-B). By comparison, in allergic mice given LPS (OVA-LPS), concentrations in IFN-γ, and IL-17 were increased compared to either OVA or LPS mice.

Figure 5. BALF cytokines in non-allergic and allergic mice exposed to inhaled LPS. Concentrations in BAL fluid of IFNγ, IL-17, TNFα and IL-6 were determined by cytometric bead assays as described in Materials and Methods. Values are expressed as mean± SE (n=5-6). Horizontal lines indicate significant difference; $p < 0.05$.



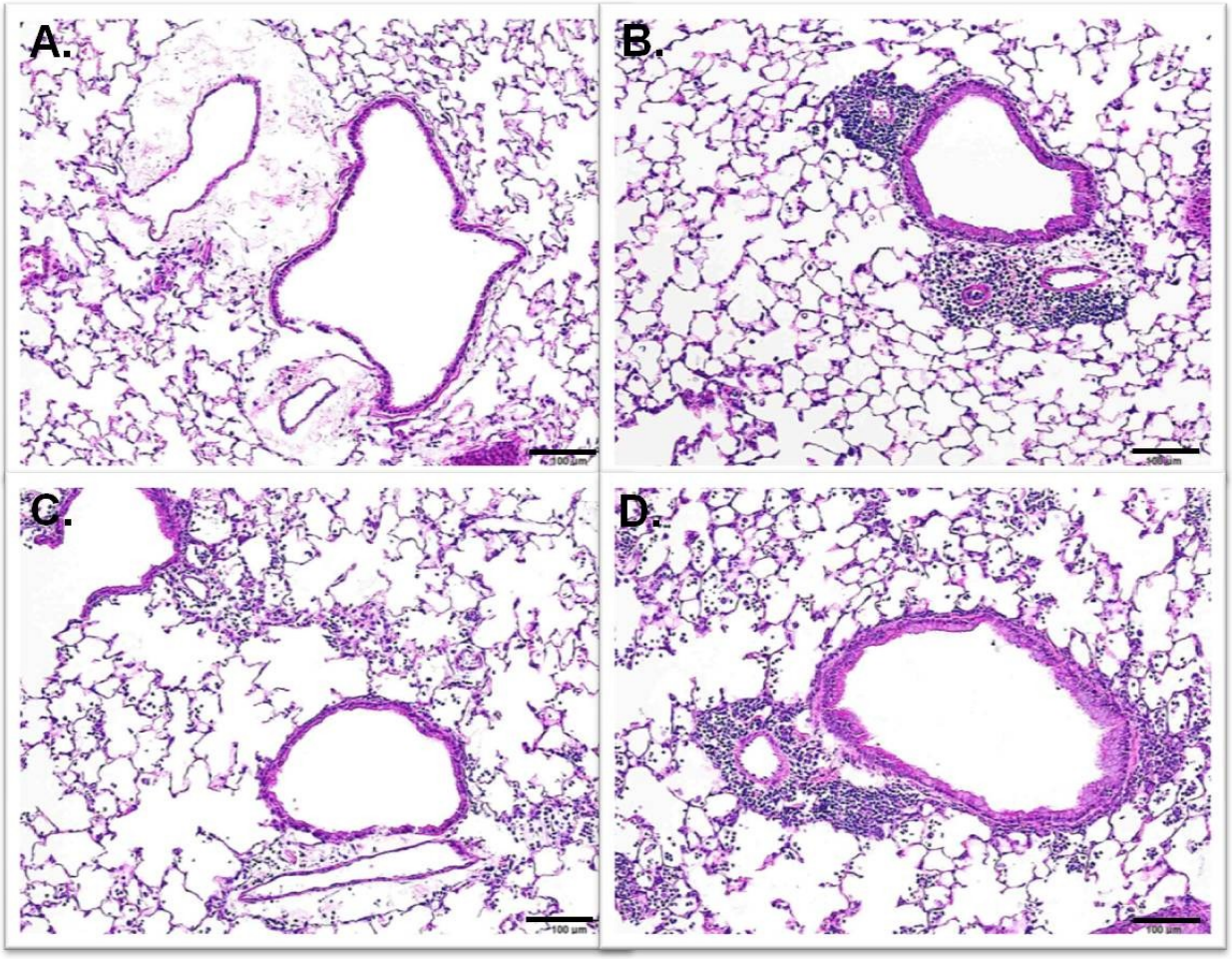
2.4.3 Histopathology

The treatment-related responses I observed in BALF cellularity was confirmed with microscopic examination of the lung. Qualitative evaluation of inflammation in peribronchial, perivascular, and lung interstitial regions was determined by scoring histological slides based on the following criteria: 0 – no / minimal inflammation; 1 – occasional airways and adjacent blood vessels with aggregate of cells one layer thick and occasional inflammatory cells present in alveolar spaces and interstitium; 2 – airways / blood vessels with cellular cuffing of 2-5 layers thick and inflammation in 10-30% of the interstitium and airspace; and 3 - airways / blood vessels with cellular cuffing of >5 cell layers thick and inflammation in > 30% of the interstitium and airspace (Table 1). Representative lesions are depicted histologically in Figure 6. Non-sensitized, saline mice did not show any histopathology in the lung (Fig 6A). OVA caused a moderate to severe inflammation consisting of predominately lymphocytes, eosinophils, macrophages, and lesser numbers of neutrophils in peribronchiolar and perivascular regions. Inflammation occasionally extended into adjacent alveolar attachments (Fig 6B). LPS elicited a mild neutrophilic influx and edema surrounding bronchioles and blood vessels which extended into adjacent alveolar attachments and airspaces (Fig 6C). OVA-LPS also induced peribronchiolar and perivascular inflammation similar to OVA alone. However, inflammation was more severe and characterized by increased neutrophils along with lymphocytes, eosinophils, and macrophages in these mice. Inflammatory cells were present throughout the lung interstitial tissue and within alveolar spaces (Fig 6D).

Table 1. Qualitative evaluation of Inflammation in the lung. Numerical scores are defined as follows: 0 – no / minimal inflammation; 1 – airways / adjacent blood vessels with aggregate of cells one layer thick and occasional inflammatory cells present in alveolar spaces and interstitium; 2 – airways / blood vessels with cellular cuffing of 2-5 layers thick and inflammation in 10-30% of the interstitium and airspace; and 3 - airways / blood vessels with cellular cuffing of >5 cell layers thick and inflammation in > 30% of the interstitium and airspace.

Inflammation Score			
Group	**Peribronchiolar**	**Perivascular**	**Alveoli / Alveolar Space**
Saline	**0**	**0**	**0**
OVA	**2**	**3**	**1**
LPS	**1**	**1**	**1**
OVA-LPS	**3**	**3**	**3**

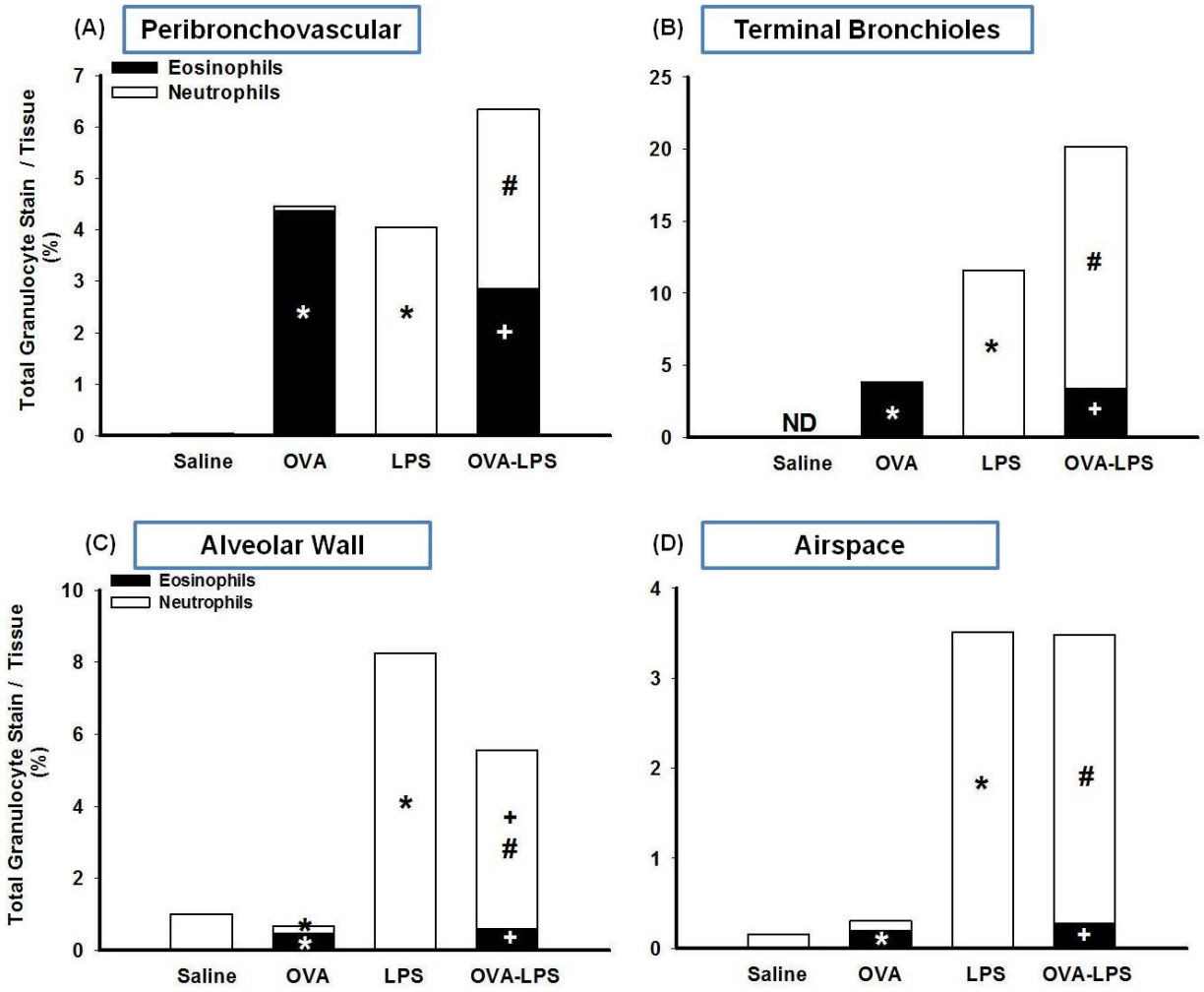
Figure 6. Pulmonary histology of non-allergic and allergic mice exposed to inhaled LPS. Light photomicrographs (H&E) of lungs showing no inflammation (saline, A) and increased inflammation surrounding airways and blood vessels in mice sensitized and challenged with OVA along with mucous cells metaplasia within airway epithelium (OVA, B). LPS alone produced a milder inflammatory response (LPS, C). OVA-LPS mice had a inflammatory pattern similar to both OVA and LPS with peribronchiolar and perivascular inflammation and thickening of airway epithelium due to mucus metaplasia and extension of inflammation into the alveolar tissue and spaces (OVA-LPS, D). Scale bar = 100 μm



2.4.4 Morphometric inflammatory cell analysis

To better characterize the location and distribution of eosinophils and neutrophils associated with different treatments morphometric analysis was performed on the proximal sections of lungs (Fig. 7). Percentages of positive-staining cells for eosinophils and neutrophils was determined by point counting in multiple reference sites which included bronchioles and adjacent blood vessels (peribronchovascular), terminal bronchioles, lung interstitial tissue, and the alveolar spaces. OVA mice had a significant influx of eosinophils which were predominately located in peribronchovascular regions and around terminal bronchioles (Figs 7A, B), with scant accumulation in alveolar region (Fig 7C, D). Accumulation of neutrophils was not significant in these mice compared to saline mice. In comparison, LPS mice had neutrophils accumulations located in all studied compartments. In OVA-LPS mice, there were larger percentages of inflammatory cells within the peribronchovascular region and terminal bronchioles. The peribronchovascular region contained a nearly equal distribution of eosinophils and neutrophils, while the terminal bronchioles had larger numbers of neutrophils. There was a significant decrease in the percentage of neutrophils in the alveolar wall of OVA-LPS mice compared to LPS mice.

Figure 7. Eosinophil and neutrophil distribution in the lung. Lung sections were immunohistochemically stained for neutrophils or eosinophils as described in Methods. Data are expressed as the percent of positive-staining eosinophils or neutrophils per the total counted points in the reference tissue: (A) peribronchiolar and perivascular region, (B) terminal bronchioles; (c) alveolar wall; and (D) airspace. Significantly different from (*) saline group; (+) LPS group; (#) OVA group $p<0.05$; n=5-6.



2.4.5 Airway Epithelial Mucus Production

There was no AB/PAS positive staining in saline mice (Fig 8 A). OVA caused remodeling of the airway epithelium characterized by marked mucous cell metaplasia present in the axial airways, small bronchioles, and pre-terminal bronchioles as indicated by an increased staining of intraepithelial mucosubstances with AB/PAS (Fig 8 B). Minimal intraepithelial mucosubstances was present in LPS alone mice (Fig 8 C). Mucus was decreased in OVA-LPS compared to OVA mice (Fig. 8 D). Morphometric analysis determined that intraepithelial mucosubstances were significantly decreased in OVA-LPS mice when compared to OVA alone mice (Fig 8 E).

Figure 8. Airway epithelial mucus. Increased intraepithelial mucus in airways, a feature of asthma, was analyzed by staining with AB/PAS (A-D). Staining was increased in allergic mice (B, D), however, LPS blunted this increase in OVA-LPS mice as shown in (D). Also, quantification of mucus showed that LPS decreased OVA-induced intraepithelial airway mucosubstances (E). Horizontal lines indicate significant difference; $p < 0.05$. B, bronchiole; BV, blood vessel; arrowheads, intraepithelial mucus. Scale bar = 100 μm.

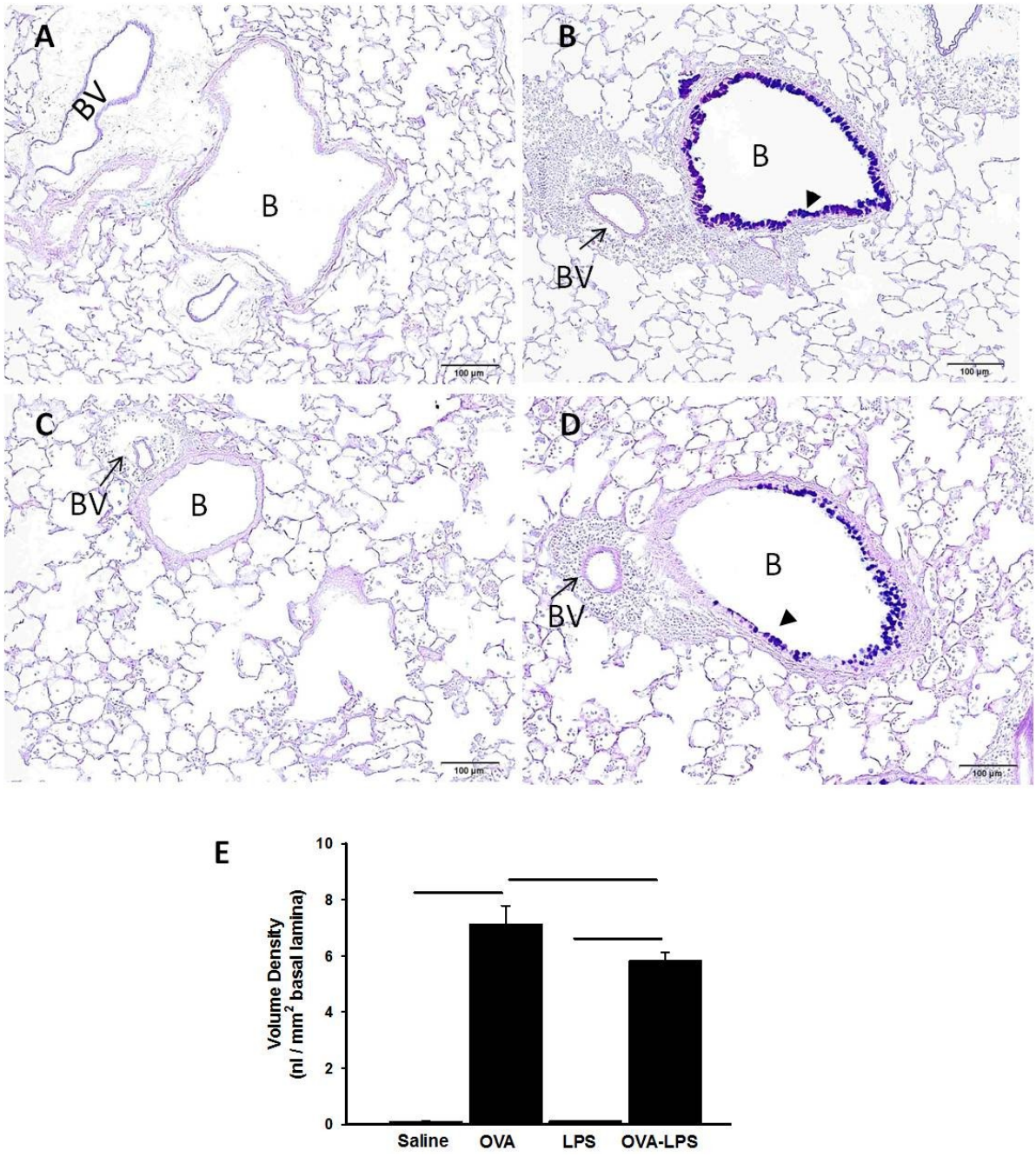
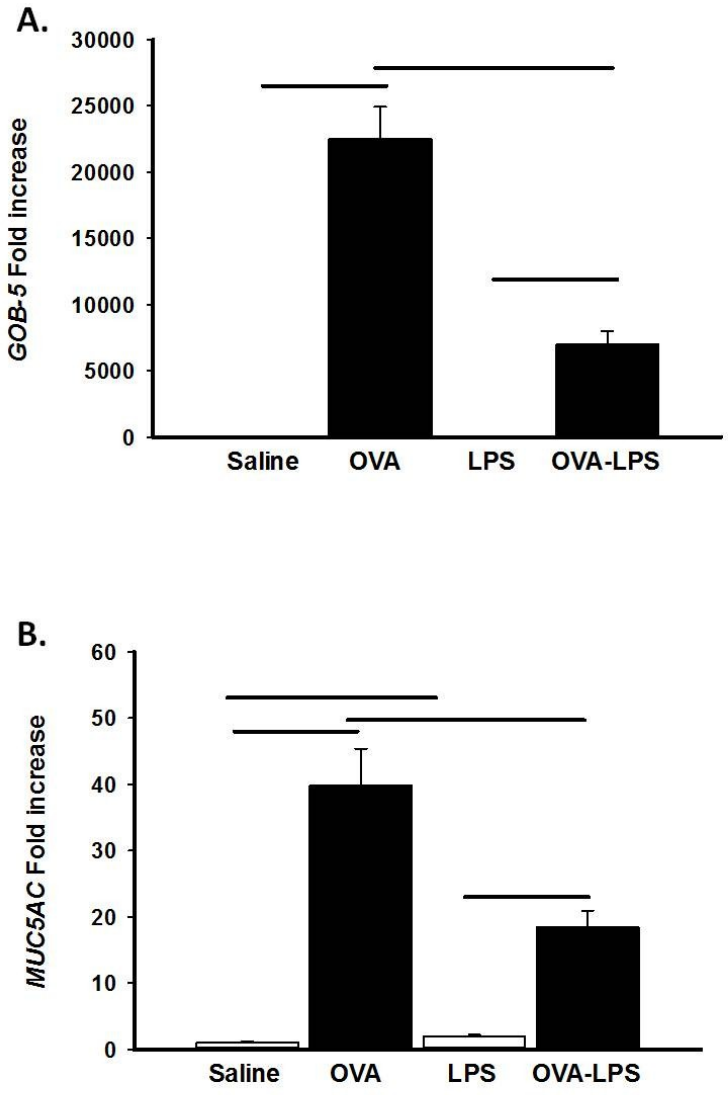


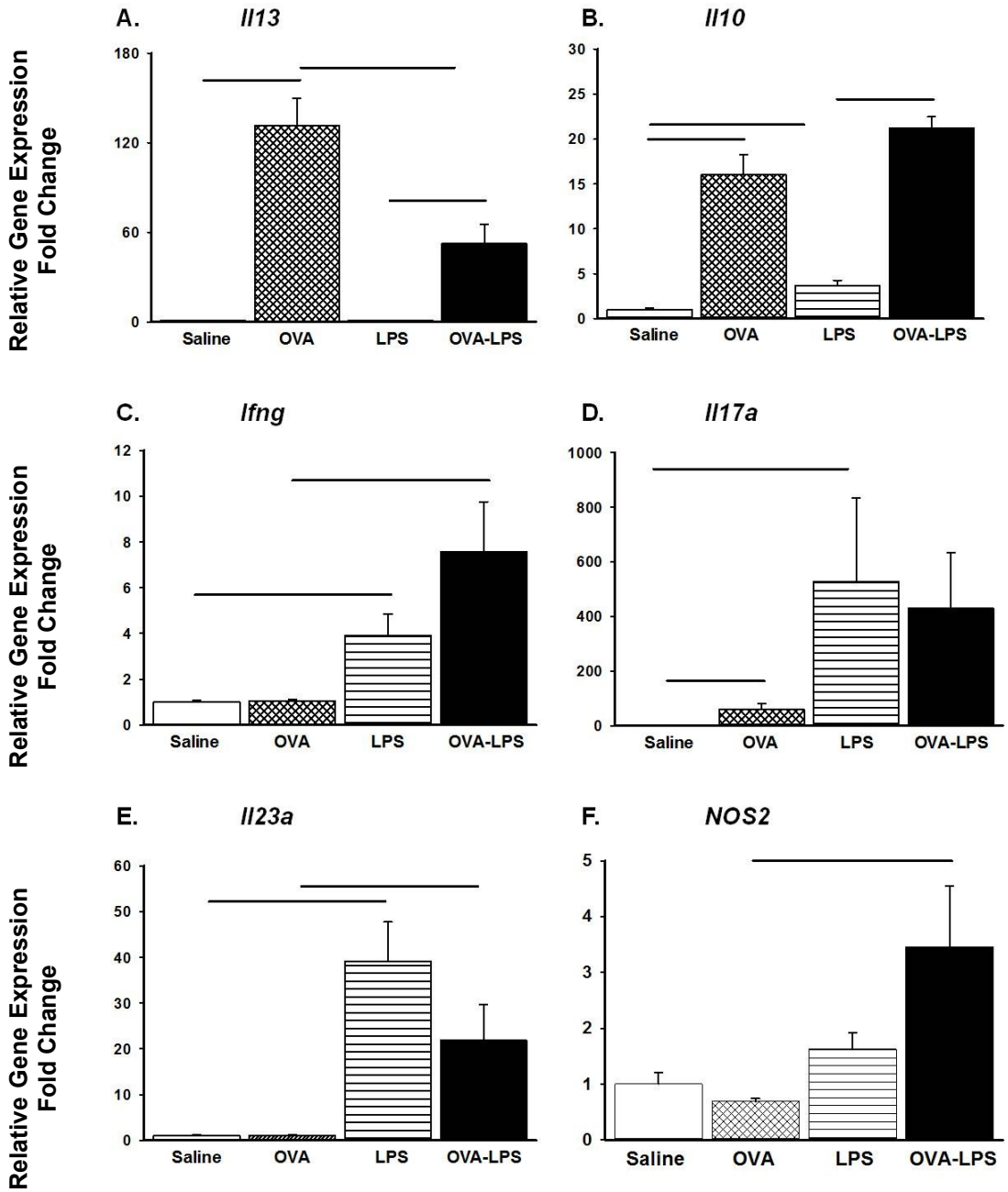
Figure 9. Airway mucus gene expression. (A) mRNA of mucus producing genes, Gob-5 and MUC5AC. Data are reported as the fold increase of the group mean ± SEM. (B) Volume density of intraepithelial mucosubstances in the proximal airway (G5). Significantly different from a- saline group; b- OVA group; c- LPS group; horizontal line indicates significant difference between specified groups, $p<0.05$; n=6/group.



2.4.6 Lung tissue mRNA expression

Expression of genes involved in mucus overproduction, *Gob-5*, and goblet cell metaplasia, *Muc5ac*, were both induced in OVA mice, but in OVA-LPS mice both genes had decreased expression when compared to OVA alone (Fig. 9). These changes are in alignment with the decreased presence of intraepithelial mucosubstances in OVA-LPS mice. OVA up-regulated *Il13*, but in comparison *Il13* was decreased in OVA-LPS mice (Fig 10, A). *Il10* was increased in both OVA and OVA-LPS mice (Fig 10, B). In both LPS and OVA-LPS mice there was an increase expression of *Ifng* (Fig 10, C). Both OVA and LPS have an increase in expression of *Il17a* (*Fig* 10, D). Both LPS and OVA-LPS had significant increases in *Il23a* mRNA expression compared to saline and ova alone animals, respectively (Fig 10, E). In addition, inducible nitric oxide synthase, *NOS2*, was elevated in only OVA-LPS mice (Fig 10, F).

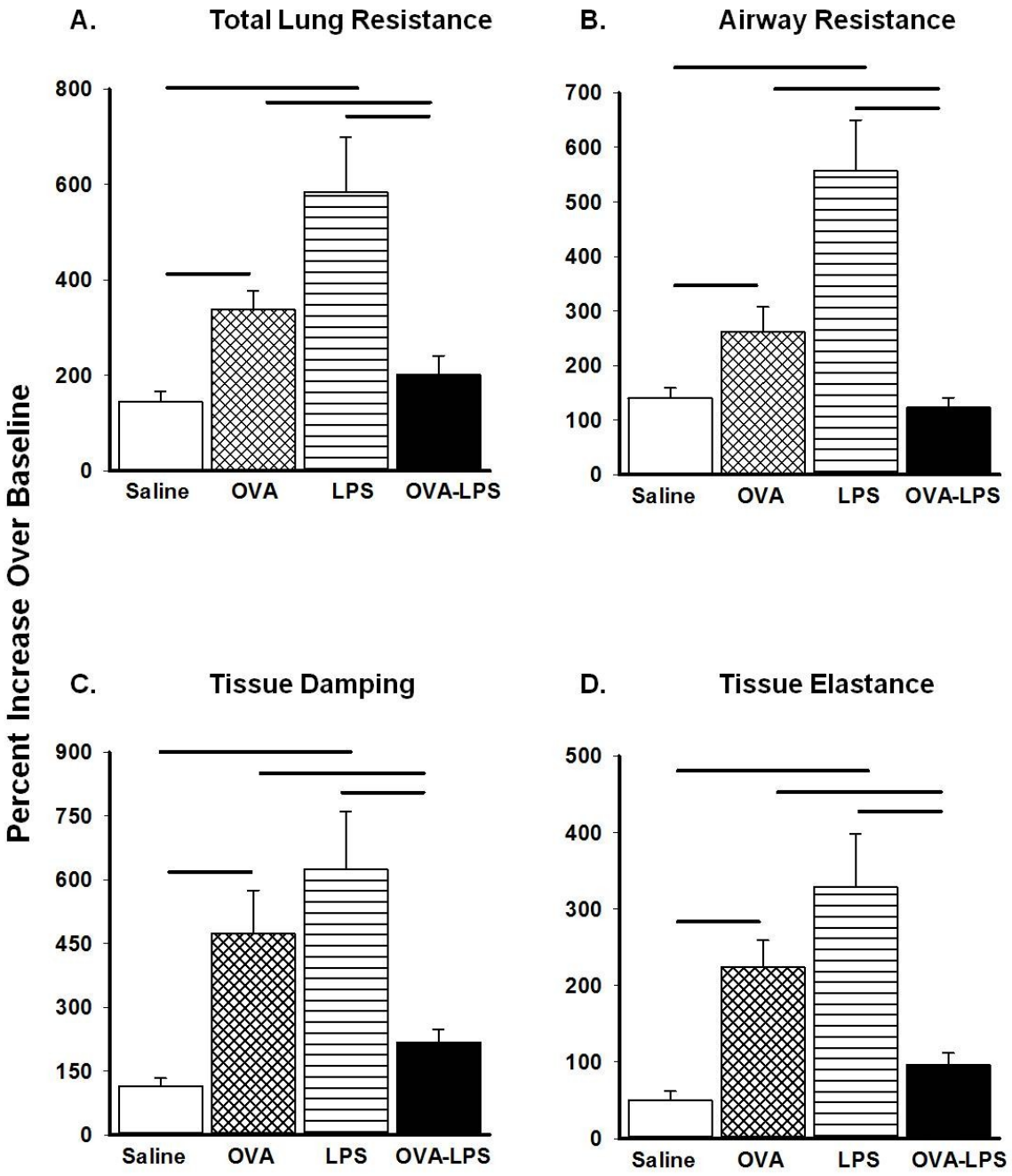
Figure 10. Pulmonary gene expression in non-allergic and allergic mice exposed to inhaled LPS. Relative expressions of mRNA encoding for cytokines IL-13, IL-10, IFN-γ, IL-17a and IL-23a and the enzyme, NOS2, were determined in right caudal lobes by RT-PCR and described in Materials and Methods. Values are expressed as fold increase of mRNA expression compared to control (Saline) animals (n=5-6). Horizontal lines indicate significant differences between specified groups, $p<0.05$.



2.4.7 Airway Hyperresponsiveness

Alterations in total lung resistance due to hyperresponsiveness elicited by aerosolized β-methacholine (10 mg/ml) were significantly increased by OVA alone and LPS alone compared to saline. However, the hyperreactivity present with OVA or LPS was significantly decreased in OVA mice exposed to LPS (Fig 11, A). Also, similar increases in OVA and LPS mice with significant decreases in OVA-LPS mice were present in airway resistance, tissue damping, and tissue elastance (Fig 11, B-D). OVA mice displayed prominent changes in tissue damping and tissue elastance with a greater than 4-fold increase over saline animals (Fig 11, C-D). While in LPS mice, the predominant change occurred in tissue elastance which displayed an almost 7-fold increase over saline mice (Fig 11, D).

Figure 11. Measurement of AHR in non-allergic and allergic mice exposed to LPS. Methacholine-induced changes in total lung resistance (A), airway resistance (B), tissue damping (C) and tissue elastance (D) were measured in intubated and ventilated mice using the Flexivent system as described in Materials and Methods. Values are expressed as the percent increase from baseline value after administration of 10 mg/ml of methacholine (n=12-14). Horizontal line indicates significant difference between specified groups, $p<0.05$.



2.5 DISCUSSION

In this study I sought to determine the relationship of pulmonary inflammation with changes in AHR by using a mouse model of LPS-induced exacerbation of established allergic airway disease. My approach was to match airway lesions with airway compartment-specific AHR, specifically by morphometric evaluation of the site, severity and types of inflammatory cells in lesions, and by partitioning airway resistance as arising from either lung tissue or conducting airways using forced oscillation techniques. My results demonstrate that LPS worsened the inflammation in allergic mice by eliciting a robust recruitment of neutrophils, but that AHR was attenuated in these mice. As such, rather than exacerbate AHR as I hypothesized, my data suggests that LPS-induced signaling for the recruitment and/or activation of neutrophils may contribute to a decrease in allergic airway reactivity.

Previous studies that have characterized the airway inflammatory responses to OVA or LPS in mice describe infiltration of eosinophils and neutrophils, respectively (Kung et al., 1994; Hakansson et al., 2012). In the current study I show that eosinophils and lymphocytes were primarily concentrated around bronchioles and blood vessels in OVA mice (i.e., conducting airways). By comparison in LPS mice, neutrophils were localized in these same perivascular and peribronchial regions, but lesions also extended to a mild to moderate alveolitis and extravasation of neutrophils into airspaces (i.e., both conducting airways and tissues). Others have described that airway challenge with OVA or LPS alone can cause AHR that is associated temporally with airway inflammation in mice (Gueders et al., 2009; Hakansson et al., 2012). My current results

are similar to these reports, as I measured AHR in mice 72 hours after OVA challenge or 24 hours after LPS exposure. Furthermore, LPS elicited a neutrophilic recruitment and AHR in both airway and tissue compartments, suggesting a link between inflammation and functional changes. However severity of airway inflammation and AHR were not associated in the same compartments in OVA mice, where eosinophilic involvement was concentrated around airways, while most of the measured resistance came from tissues.

While plausible hypotheses have been proposed for the contribution of granulocytes and their products to the development of AHR (Wardlaw et al., 2002), supporting evidence in experimental asthma models has been inconsistently reported. Some findings demonstrate critical roles for inflammatory cell influxes (Webb et al., 2001; Walsh et al., 2008), while others indicate that eosinophils and/or neutrophils are unnecessary to manifest AHR (Birrell et al., 2003; Shen et al., 2003; Cui et al., 2005; Siegle et al., 2006). In humans with fatal asthma, airway lesions are characterized by widespread but unequally distributed eosinophils throughout the lung, while neutrophils are localized along peribronchiolar-alveolar attachments (Simoes et al., 2005). Similar to my own findings, others researchers using allergic BALB/c mice describe eosinophil infiltration that is predominately in peribronchiolar regions, and is correlated with increases in total lung resistance and dynamic compliance (Takeda et al., 2001). In the present study, treatment of allergic mice with LPS did not change eosinophil localization in this region; however it did result in decreased AHR that putatively arises from obstruction in these conducting airways. As such, if eosinophils contribute to local AHR in my model, then LPS may act directly on these cells to modulate their production of

inflammatory or bronchodilatory mediators. Alternatively, LPS-induced co-localization of neutrophils along airways may be involved in the modulation of AHR in this region.

The primary pathological feature associated with reversal of AHR in LPS-treated allergic mice is widespread infiltration of neutrophils in all the compartments of the lung (i.e., both tissues and airways). Potential neutrophil-derived mediators associated with AHR include elastase (Koga et al., 2013) and IL-17 (Kudo et al., 2012), which had increased gene expression and presence in BAL fluid of OVA-LPS mice. Along with IL-23, IL-17 is associated in asthmatics with a more severe, neutrophil-associated phenotype (Cosmi et al., 2011), and at least one report suggests that IL-17 can activate asthmatic smooth muscle cells (Dragon et al., 2014). However I did not observe increased AHR with PMN infiltration in the current study. Alternatively, elaboration of the bronchodilator mediators from LPS-stimulated neutrophils such as nitric oxide (NO) (Prado et al., 2006) and prostaglandin E2 (Tanaka et al., 2005) may underlie the inhibition of AHR I observed in OVA-LPS mice. Localization of neutrophils in both the airways and tissues is consistent with their potential influence to reduce airway reactivity that I measured in both of these lung compartments.

Rodriquez and colleges have shown that LPS exposure during OVA challenge decreased BALF concentrations of IL-5 and -13 (Rodriguez et al., 2003). In my studies these Th2 cytokines were below the limit of detection in BALF in both OVA and OVA-LPS mice. However, the OVA-associated expression of the IL-13 gene in lung tissue was decreased following LPS exposure in OVA-LPS mice, suggesting that LPS may be downregulating allergic, Th2-type pathways. This change in immune pathways is supported by the pulmonary expression of the Th1 cytokine IFN-γ and the regulatory

cytokine IL-10 that coincide with the decrease in Il-13 expression in OVA-LPS mice. My results are similar to others, who report that inhaled LPS can induce a Th1 response characterized by increased neutrophils, IFN-γ production and decreased mucus secretion in allergic mice (Eisenbarth et al., 2002; Kim et al., 2007; Dong L; Li H, 2009). Simpson and Martinez showed that asthmatics with certain genetic traits were protected against the development of atopy by exposure with high doses of LPS (Simpson and Martinez, 2010). The effect of LPS on allergic airway responses may be dose-dependent in mice, as Dong et al. found that low dose LPS increased recruitment of eosinophils and neutrophils into the lung, mucus secretion, and Th2 cytokines (Dong L; Li H, 2009). Future studies that employ lower doses of LPS than those used in my protocol are needed to determine dose-sensitive pathways of LPS exposure in pre-existing asthma.

In addition to airway inflammation, goblet cell metaplasia and mucus hypersecretion are hallmarks of human asthma that are reproduced in the OVA BALB/c mouse model (Long et al., 2006; Pawankar et al., 2009). Inhibition of mucus secretion can protect from allergic AHR in mice (Agrawal et al., 2007), which suggests that luminal accumulation of mucus might contribute to airway obstruction. In the current study, I describe OVA-induced mucus cell metaplasia and hyperplasia, along with induction of genes associated with mucus production and secretion, Gob-5 and MUC5ac. In allergic mice treated with LPS these mucous responses were significantly reduced. Therefore, it is possible that the inhibition of AHR by LPS is due to changes in mucin secretion.

2.6 SUMMARY

Sensitization and challenge with OVA caused mucous cell metaplasia, airway eosinophil accumulation, and AHR in central airways and peripheral tissues in lungs of BALB/c mice. Introduction of LPS into airways with pre-existing allergic inflammation caused an accumulation of neutrophils into the lung, no change in eosinophils, but led to a reduction in AHR. Therefore my hypothesis that the contributions from the central airways and peripheral tissues to AHR in allergic mice exposed to endotoxin are dependent on the location and severity of neutrophilic lesions in the lung was not supported by the data. Indeed, the increased inflammation in LPS-treated allergic mice, specifically of neutrophils, was related to a decrease in AHR. As such my data suggests that neutrophils or a neutrophil product may contribute to the decrease in OVA-induced AHR. Approaches to test this possibility include blocking neutrophil migration, activation, or the activity of its potential mediators. Two potential neutrophil-derived mediators that have bronchodilatory effects are nitric oxide and the prostaglandin PGE2. Another possibility is that LPS had direct effects on cells responsible for AHR, such as eosinophils, airway smooth muscle cells, epithelial cells, or airway macrophages. However cell receptors such as CD14 or TLR4 are required for activation by LPS, and such activation usually results in NF-κB-mediated production of inflammatory cytokines. In general this response may oppose the Th2 responses induced by OVA, and my data of decreased IL-13 in OVA-LPS support this possibility. Taken together there are several reasonable explanations for the effects of LPS to reverse allergic AHR. In the next few chapters I will focus on the role of neutrophils,

which were the most obvious histological change that accompanied the reduction of AHR in allergic lungs.

REFERENCES

REFERENCES

Agrawal A, Rengarajan S, Adler K B, Ram A, Ghosh B, Fahim M and Dickey B F. 2007. Inhibition of Mucin Secretion with Marcks-Related Peptide Improves Airway Obstruction in a Mouse Model of Asthma. Journal of Applied Physiology **102**(1): 399-405.

Bates J H T, Rincon M and Irvin C G. 2009. Animal Models of Asthma. American Journal of Physiology-Lung Cellular and Molecular Physiology **297**(3): L401-L410.

Becker S, Fenton M J and Soukup J M. 2002. Involvement of Microbial Components and Toll-Like Receptors 2 and 4 in Cytokine Responses to Air Pollution Particles. American Journal of Respiratory Cell and Molecular Biology **27**(5): 611-618.

Birrell M A, Battram C H, Woodman P, McCluskie K and Belvisi M G. 2003. Dissociation by Steroids of Eosinophilic Inflammation from Airway Hyperresponsiveness in Murine Airways. Respir Res **4**(3).

Boehlecke B, Hazucha M, Alexis N E, Jacobs R, Reist P, Bromberg P A and Peden D B. 2003. Low-Dose Airborne Endotoxin Exposure Enhances Bronchial Responsiveness to Inhaled Allergen in Atopic Asthmatics. Journal of Allergy and Clinical Immunology **112**(6): 1241-1243.

Burgel P R. 2011. The Role of Small Airways in Obstructive Airway Diseases. European Respiratory Review **20**(119): 023-033.

Busse W W. 2010. The Relationship of Airway Hyperresponsiveness and Airway Hyperresponsiveness in Asthma: Its Measurement and Clinical Significance inflammation. Chest **138**(2): 4S-10S.

Busse W W and Lemanske R F. 2001. Advances in Immunology - Asthma. New England Journal of Medicine **344**(5): 350-362.

Cosmic L, Liotta F, Maggi E, Romagnani S and Annunziato F. 2011. Th17 Cells: New Players in Asthma Pathogenesis. Allergy **66**(8): 989-998.

Cui J Q, Pazdziorko S, Miyashiro J S, Thakker P, Pelker J W, DeClercq C, Jiao A P, Gunn J, Mason L, Leonard J P, Williams C M M and Marusic S. 2005. T(H)1-Mediated

Airway Hyperresponsiveness Independent of Neutrophilic Inflammation. Journal of Allergy and Clinical Immunology **115**(2): 309-315.

Dong L; Li H W S, Li Y. 2009. Different Doses of Lipolysaccharides Regulate the Lung Inflammation of Asthmatic Mice Via Tlr4 Pathway in Alveolar Macrophages. Journal of Asthma **46**: 229-233.

Dragon S, Hirst S J, Lee T H and Gounni A S. 2014. Interleukin-17a Mediates a Selective Gene Expression Profile in Asthmatic Human Airway Smooth Muscle Cells. American Journal of Respiratory Cell and Molecular Biology(ja).

Eisenbarth S C, Piggott D A, Huleatt J W, Visintin I, Herrick C A and Bottomly K. 2002. Lipopolysaccharide-Enhanced, Toll-Like Receptor 4-Dependent T Helper Cell Type 2 Responses to Inhaled Antigen. Journal of Experimental Medicine **196**(12): 1645-1651.

Farah C S, King G G, Brown N J, Downie S R, Kermode J A, Hardaker K M, Peters M J, Berend N and Salome C M. 2012. The Role of the Small Airways in the Clinical Expression of Asthma in Adults. Journal of Allergy and Clinical Immunology **129**(2): 381-U162.

Fitzpatrick A M, Holguin F, Teague W G and Brown L A S. 2008. Alveolar Macrophage Phagocytosis Is Impaired in Children with Poorly Controlled Asthma. Journal of Allergy and Clinical Immunology **121**(6): 1372-1378.

Gueders M M, Paulissen G, Crahay C, Quesada-Calvo F, Hacha J, Van Hove C, Tournoy K, Louis R, Foidart J-M and NoÃ«l A. 2009. Mouse Models of Asthma: A Comparison between C57bl/6 and Balb/C Strains Regarding Bronchial Responsiveness, Inflammation, and Cytokine Production. Inflammation Research **58**(12): 845-854.

Hakansson H F, Smailagic A, Brunmark C, Miller-Larsson A and Lal H. 2012. Altered Lung Function Relates to Inflammation in an Acute Lps Mouse Model. Pulmonary Pharmacology & Therapeutics **25**(5): 399-406.

Hantos Z, Daroczy B, Suki B, Nagy S and Fredberg J J. 1992. Input Impedance and Peripheral Inhomogeneity of Dog Lungs. Journal of Applied Physiology **72**(1): 168-178.

Harkema J R and Hotchkiss J A. 1992. In Vivo Effects of Endotoxin on Intraepithelial Mucosubstances in Rat Pulmonary Airways. Quantitative Histochemistry. Am J Pathol. 1992 Aug;141(2):307-17.

Harkema J R, Plopper C G, Hyde D M and St George J A. 1987. Regional Differences in Quantities of Histochemically Detectable Mucosubstances in Nasal, Paranasal, and Nasopharyngeal Epithelium of the Bonnet Monkey. J Histochem Cytochem. 1987 Mar;35(3):279-86.

Holgate S T. 2008. Pathogenesis of Asthma. Clinical and Experimental Allergy **38**(6): 872-897.

Hyde D M, Hamid Q and Irvin C G. 2009. Anatomy, Pathology, and Physiology of the Tracheobronchial Tree: Emphasis on the Distal Airways. Journal of Allergy and Clinical Immunology **124**(6): S72-S77.

Kim Y K, Oh S Y, Jeon S G, Park H W, Lee S Y, Chun E Y, Bang B, Lee H S, Oh M H, Kim Y S, Kim J H, Gho Y S, Cho S H, Min K U, Kim Y Y and Zhu Z. 2007. Airway Exposure Levels of Lipopolysaccharide Determine Type 1 Versus Type 2 Experimental Asthma. Journal of Immunology **178**(8): 5375-5382.

Koga H, Miyahara N, Fuchimoto Y, Ikeda G, Waseda K, Ono K, Tanimoto Y, Kataoka M, Gelfand E W, Tanimoto M and Kanehiro A. 2013. Inhibition of Neutrophil Elastase Attenuates Airway Hyperresponsiveness and Inflammation in a Mouse Model of Secondary Allergen Challenge: Neutrophil Elastase Inhibition Attenuates Allergic Airway Responses. Respiratory Research **14**.

Kudo M, Melton A C, Chen C, Engler M B, Huang K E, Ren X, Wang Y, Bernstein X, Li J T and Atabai K. 2012. Il-17a Produced by [Alpha][Beta] T Cells Drives Airway Hyper-Responsiveness in Mice and Enhances Mouse and Human Airway Smooth Muscle Contraction. Nature Medicine **18**(4): 547-554.

Kung T T, Jones H, Adams Iii G K, Umland S P, Kreutner W, Egan R W, Chapman R W and Watnick A S. 1994. Characterization of a Murine Model of Allergic Pulmonary Inflammation. International Archives of Allergy and Immunology **105**(1): 83-90.

Long A J, Sypek J P, Askew R, Fish S C, Mason L E, Williams C M M and Goldman S. 2006. Gob-5 Contributes to Goblet Cell Hyperplasia and Modulates Pulmonary Tissue Inflammation. American Journal of Respiratory Cell and Molecular Biology **35**(3).

Louis R, Lau L C K, Bron A O, Roldaan A C, Radermecker M and Djukanovic R. 2000. The Relationship between Airways Inflammation and Asthma Severity. American Journal of Respiratory and Critical Care Medicine **161**(1): 9-16.

Mueller-Anneling L, Avol E, Peters J M and Thorne P S. 2004. Ambient Endotoxin Concentrations in Pm10 from Southern California. Environ Health Perspect. **112**(5): 583-588.

Nials A T and Uddin S. 2008. Mouse Models of Allergic Asthma: Acute and Chronic Allergen Challenge. Disease Models & Mechanisms **1**(4-5): 213-220.

Pawankar R, Holgate S, Rosenwasser L and Cohn L. 2009. Mechanisms of Mucus Induction in Asthma. Allergy Frontiers: Clinical Manifestations, Springer Japan. **3:** 173-185.

Prado C M, Leick-Maldonado E A, Yano L, Leme A S, Capelozzi V L, Martins M A and Tiberio I F L C. 2006. Effects of Nitric Oxide Synthases in Chronic Allergic Airway Inflammation and Remodeling. American Journal of Respiratory Cell and Molecular Biology **35**(4): 457-465.

Rodriguez D, Keller A C, Faquim-Mauro E L, de Macedo M S, Cunha F Q, Lefort J, Vargaftig B B and Russo M. 2003. Bacterial Lipopolysaccharide Signaling through Toll-Like Receptor 4 Suppresses Asthma-Like Responses Via Nitric Oxide Synthase 2 Activity. Journal of Immunology **171**(2): 1001-1008.

Shen H H H, Ochkur S I, McGarry M P, Crosby J R, Hines E M, Borchers M T, Wang H Y, Biechelle T L, O'Neill K R, Ansay T L, Colbert D C, Cormier S A, Justice J P, Lee N A and Lee J J. 2003. A Causative Relationship Exists between Eosinophils and the Development of Allergic Pulmonary Pathologies in the Mouse. Journal of Immunology **170**(6): 3296-3305.

Siegle J S, Hansbro N, Herbert C, Yang M, Foster P S and Kumar R K. 2006. Airway Hyperreactivity in Exacerbation of Chronic Asthma Is Independent of Eosinophilic Inflammation. American Journal of Respiratory Cell and Molecular Biology **35**(5): 565-570.

Simoes S D, dos Santos M A, Oliveira M D, Fontes E S, Fernezlian S, Garippo A L, Castro I, Castro F F M, Martins M D, Saldiva P H N, Mauad T and Dolhnikoff M. 2005. Inflammatory Cell Mapping of the Respiratory Tract in Fatal Asthma. Clinical and Experimental Allergy **35**(5): 602-611.

Simpson A and Martinez F D. 2010. The Role of Lipopolysaccharide in the Development of Atopy in Humans. Clinical and Experimental Allergy **40**(2): 209-223.

Sterk P J and Bel E H. 2011. Small Airways, Big Challenge. European Respiratory Review **20**(119): 001-002.

Swedin L, Ellis R, Kemi C, Ryrfeldt Ã, Inman M, Dahlen S E and Adner M. 2010. Comparison of Aerosol and Intranasal Challenge in a Mouse Model of Allergic Airway Inflammation and Hyperresponsiveness. International Archives of Allergy and Immunology **153**(3): 249-258.

Takeda K, Haczku A, Lee J J, Irvin C G and Gelfand E W. 2001. Strain Dependence of Airway Hyperresponsiveness Reflects Differences in Eosinophil Localization in the Lung. American Journal of Physiology-Lung Cellular and Molecular Physiology **281**(2): L394-L402.

Tanaka H, Kanako S and Abe S. 2005. Prostaglandin E2 Receptor Selective Agonists E-Prostanoid 2 and E-Prostanoid 4 May Have Therapeutic Effects on Ovalbumin-Induced Bronchoconstriction*. CHEST Journal **128**(5): 3717-3723.

Tepper R S, Wise R S, Covar R, Irvin C G, Kercsmar C M, Kraft M, Liu M C, O'Connor G T, Peters S P, Sorkness R and Togias A. 2012. Asthma Outcomes: Pulmonary Physiology. Journal of Allergy and Clinical Immunology **129**(3): S65-S87.

Tschanz S A, Burri P H and Weibel E R. 2011. A Simple Tool for Stereological Assessment of Digital Images: The Stepanizer. Journal of Microscopy **243**(1): 47-59.

Tulic M K and Hamid Q. 2006. New Insights into the Pathophysiology of the Small Airways in Asthma. Clinics in Chest Medicine **27**(1): 41-52.

Tulic M K, Wale J L, Holt P G and Sly P D. 2000. Modification of the Inflammatory Response to Allergen Challenge after Exposure to Bacterial Lipopolysaccharide. American Journal of Respiratory Cell and Molecular Biology **22**(5): 604-612.

Ueda T, Niimi A, Matsumoto H, Takemura M, Hirai T, Yamaguchi M, Matsuoka H, Jinnai M, Muro S, Chin K and Mishima M. 2006. Role of Small Airways in Asthma: Investigation Using High-Resolution Computed Tomography. Journal of Allergy and Clinical Immunology **118**(5): 1019-1025.

Vanoirbeek J A J, Rinaldi M, De Vooght V, Haenen S, Bobic S, Gayan-Ramirez G, Hoet P H M, Verbeken E, Decramer M, Nemery B and Janssens W. 2010. Noninvasive and Invasive Pulmonary Function in Mouse Models of Obstructive and Restrictive Respiratory Diseases. American Journal of Respiratory Cell and Molecular Biology **42**(1): 96-104.

Wagner J, Morishita M, Keeler G and Harkema J. 2012. Divergent Effects of Urban Particulate Air Pollution on Allergic Airway Responses in Experimental Asthma: A Comparison of Field Exposure Studies. Environmental Health **11**(1): 45.

Wagner J G, Jiang Q, Harkema J R, Illek B, Patel D D, Ames B N and Peden D B. 2007. Ozone Enhancement of Lower Airway Allergic Inflammation Is Prevented by Γ-Tocopherol. Free Radical Biology and Medicine **43**(8): 1176-1188.

Wagner J G, Van Dyken S J, Wierenga J R, Hotchkiss J A and Harkema J R. 2003. Ozone Exposure Enhances Endotoxin-Induced Mucous Cell Metaplasia in Rat Pulmonary Airways. Toxicological Sciences **74**(2): 437-446.

Walker J K L, Kraft M and Fisher J T. 2013. Assessment of Murine Lung Mechanics Outcome Measures: Alignment with Those Made in Asthmatics. Frontiers in physiology **3**: 491-491.

Walsh E R, Sahu N, Kearley J, Benjamin E, Kang B H, Humbles A and August A. 2008. Strain-Specific Requirement for Eosinophils in the Recruitment of T Cells to the Lung During the Development of Allergic Asthma. The Journal of experimental medicine **205**(6): 1285-1292.

Wardlaw A J, Brightling C E, Green R, Woltmann G, Bradding P and Pavord I D. 2002. New Insights into the Relationship between Airway Inflammation and Asthma. Clinical Science **103**(2): 201-211.

Webb D C, McKenzie A N J, Matthaei K I, Rothenberg M E and Foster P S. 2001. Distinct Spatial Requirement for Eosinophil-Induced Airways Hyperreactivity. Immunology and Cell Biology **79**(2): 165-169.

Wenzel S E, Schwartz L B, Langmack E L, Halliday J L, Trudeau J B, Gibbs R L and Chu H W. 1999. Evidence That Severe Asthma Can Be Divided Pathologically into Two Inflammatory Subtypes with Distinct Physiologic and Clinical Characteristics. American Journal of Respiratory and Critical Care Medicine **160**(3): 1001-1008.

CHAPTER 3

THE EFFECT OF NEUTROPHIL DEPLETION ON AIRWAY HYPERRESPONSIVENESS IN ALLERGIC MICE EXPOSED TO INHALED LIPOPOLYSACCHARIDE

3.1 ABSTRACT

The innate immune response initiates pathways that lead to activation of adaptive immunity. Allergic asthma is an abnormal immune response involving adaptive immunity, and research has focused primarily on this response. Recent knowledge suggests that innate immunity may play a critical role in asthma. Therefore, examination into the role of the innate immune system in the development and exacerbation of allergic asthma is a topic of ongoing studies to identify novel targets for the treatment and prevention of asthma. Previously, I determined that endotoxin, a commonly used agent to study innate immunity, exacerbated the inflammatory response in the lungs of allergic mice by significantly increasing neutrophils (Chapter 2). In this study, I set forth to determine the role of neutrophils in allergen-induced AHR following LPS exposure in allergic mice. Mice were sensitized and challenged to OVA as previously described. Prior to LPS exposure, neutrophils were systemically depleted from the mice using an anti-Ly6g antibody. AHR was assessed 24 hours after LPS. Total BALF cells were significantly decreased in OVA mice, due to reductions in neutrophils along with eosinophils and lymphocytes. Also, BALF cellularity was significantly decreased in OVA-LPS mice as a result of large decreases in neutrophils along with lymphocytes. Neutrophil depletion did not affect AHR when comparing neutrophil sufficient and neutrophil deficient mice in OVA and OVA-LPS groups. These results suggest that neutrophils are not linked to the attenuation of AHR in OVA-LPS mice.

3.2 INTRODUCTION

Cells of the innate immune system provide immediate and general protection from pathogens by receptor-mediated identification of molecular structures present on microorganisms, known as pathogen-associated molecular patterns (PAMPs) or produced by damage cells, damage associated molecular patterns (DAMPs). These pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) are present on a variety of cells including respiratory epithelium, dendritic cells, macrophages, mast cells, and neutrophils (Condon et al., 2011; Minnicozzi et al., 2011; Parker and Prince, 2011; Liu et al., 2014). The innate immune response initiates pathways to lead to activation of the adaptive immune system which provides long lasting protection to a specific antigen presented by dendritic cells or other antigen presenting cells.

Allergic asthma is an aberrant immune response predominately associated with lymphocytes involved in the adaptive immunity. However, examination into the role of the innate immune system in the development and exacerbation of allergic asthma is a topic of ongoing studies. LPS, a prototypical PAMP recognized by TLR4, is often used to study innate immunity (Rajaiah et al., 2013). It has been shown that the dose and timing of LPS exposure can play a major role in asthma development and exacerbation (Delayre-Orthez, et al., 2004; Delayre-Orthez, et al., 2005; Kim et al., 2007). Studies have shown that during allergic sensitization exposure to high doses prevents asthma (Von et al., 2000). This finding has become the basis of the “hygiene hypothesis” which states that the decreased prevalence of childhood bacterial, viral, and parasitic infections in westernized countries contributes to the increase in asthma development in

those societies (Braun-Fahrlander et al., 2002). Additionally, it was demonstrated that exposure to low doses of LPS promote asthma development (Eisenbarth et al., 2002; Piggott et al., 2005). However, the stimulation of innate immunity in the response to endotoxin in asthmatics is controversial. Studies in humans and in experimental models have shown that asthmatic response can be either exacerbated or inhibited by LPS (Michel et al., 1989; Michel et al., 1991; Braun-Fahrlander et al., 2002; Liu, 2002).

Although asthma is affiliated with Th2 mediated adaptive immune response characterized predominately by eosinophilic inflammation, the function of neutrophils is critical to the pathogenesis in some forms of the disease. Neutrophils have been associated with severe, steroid resistant forms of asthma and asthma exacerbations (Jatakanon et al., 1999; Wenzel et al., 1999), in addition to being prominent in asthma in the elderly (Nyenhuis et al., 2010) and the obese (Scott et al., 2011).

In a rodent model of LPS modulation of asthma, we recently described the attenuation AHR that was associated with widespread infiltration of neutrophils into all compartments of the lung (Chapter 2). The contribution of neutrophils to AHR in asthmatic airways is unclear. Compared to eosinophilic asthma, a less severe AHR has been indentified in asthmatics with neutrophilic subtype (Baines et al., 2014). At least one report in a model of RSV-exacerbation of asthma suggests that neutrophils are associated with AHR inhibition (Aeffner and Davis, 2012). A similar role for neutrophil-mediated reversal of AHR may explain the attenuation by LPS in our endotoxin-asthma model. In the current study, I systemically depleted neutrophils prior to IN instillation of

LPS in allergic BALB/c mice to test the hypothesis that attenuation of AHR in LPS-exposed allergic mice is dependent on the recruitment of airway neutrophils..

3.3 MATERIALS AND METHODS

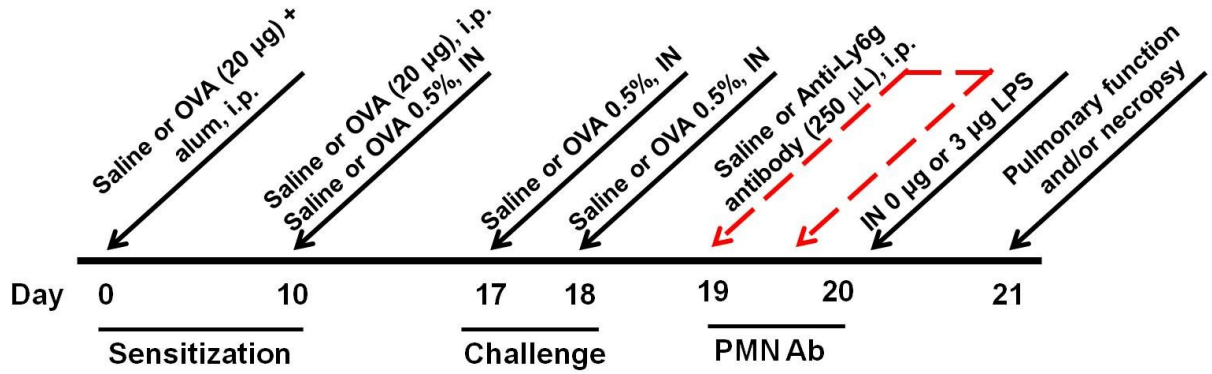
3.3.1 Laboratory Animals and Treatment protocols

Male BALB/c mice (Charles River Laboratories, Portage, MI), 6-8 weeks of age were housed and maintained as described in Chapter 2 MSU animal facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Mice were randomly assigned to one of six experimental groups consisting of 6 animals as follows: 1) animals were assigned to saline, OVA, or OVA-LPS groups and 2) half of each group were depleted of neutrophils prior to IN LPS (Fig 12).

3.3.2 Depletion of Neutrophils

Non-allergic and allergic groups were divided in half and on Days 19 and 20 mice received 0 or 250 μg i.p. of a neutrophil depleting antibody (anti-Ly6g [RB6-8C5], Abcam; Cambridge, MA). One hour following the last injection of antibody, the control and neutrophil-depleted mice groups were divided in half and received either IN saline or LPS (Fig 12). Neutrophil depletion was confirmed from peripheral blood smears prepared from blood collected at necropsy.

Figure 12. Experimental design for neutrophil depletion study in saline and OVA mice exposed to LPS.



3.3.3 Necropsy, Lavage Collection, and Tissue Preparation

Mice were anesthetized with an i.p. injection of with sodium pentobarbital (10 mg; Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI). Blood was collected from the caudal vena cava for separation of plasma, and animals were euthanized by transection of the abdominal aorta. The lungs and heart were harvested en bloc. The lungs were instilled twice with 800 µL saline via the cannulated trachea to collect BALF. After lavage the right lung lobes were ligated, separated and stored in RNAlater (Qiagen) for RNA isolation or snap frozen in liquid nitrogen for storage. The left lobe was inflated with 10% neutral buffered formalin to a pressure of 30 cm H_2O for 1 hour and then stored in a large volume of the same fixative.

3.3.4 Bronchoalveolar Lavage Analysis

Total BALF leukocytes were counted with a hemocytometer and cytological slides were prepared using a Cytospin centrifuge (Shandon). Slides were stained with Diff-Quick (Dade Behring, Newark, DE) and cell differential (macrophages, eosinophils, neutrophils, and lymphocytes) were counted. The remaining BAL fluid was centrifuged, and stored at -20°C.

3.3.5 Airway Hyperresponsiveness (AHR) Measurements

On day 21, some of the mice were anesthetized by i.p. sodium pentobarbital (100 mg/kg). A tracheostomy was performed to insert an 18 gauge cannula which was attached to a mechanical ventilator with a computer controlled piston pump (flexiVent; Scireq, Montreal, Canada). Mice were ventilated at a respiratory rate of 150 breaths / minute, tidal volume of 10 ml/kg, and positive end expiratory pressure (PEEP) of 2-3 cm H_2O. Incremental concentrations (0, 1.25, 2.5, 5, 10, and 20 mg/ml) of a bronchoconstrictor, acetyl-β-methacholine (MCh, Sigma-Aldrich) were delivered into the trachea via an attached nebulizer (Aeroneb; Aerogen, Galaway, Ireland). Prior to each MCh response curve, two deep inspirations were given. Following MCh administration, 12 perturbation maneuvers consisting of alternating measurements of sinusoidal, single frequency, oscillations (SnapShot) and broadband, multi-frequency, oscillations (Quick-prime) were performed. From the collected data, the flexiVent software calculated total respiratory system resistance using the single compartment model, as well as airway resistance, tissue damping, and tissue elastance using the constant phase model (Hantos et al., 1992). The mean of the responses for each concentration of methacholine was determined. A dose- response curve was

generated. The lowest dose of MCh with the greatest variation in response between groups was at 10 mg/ml. Therefore, the data were expressed as the percent change at 10 mg/ml compared to baseline for each group.

3.3.6 Statistical Analysis

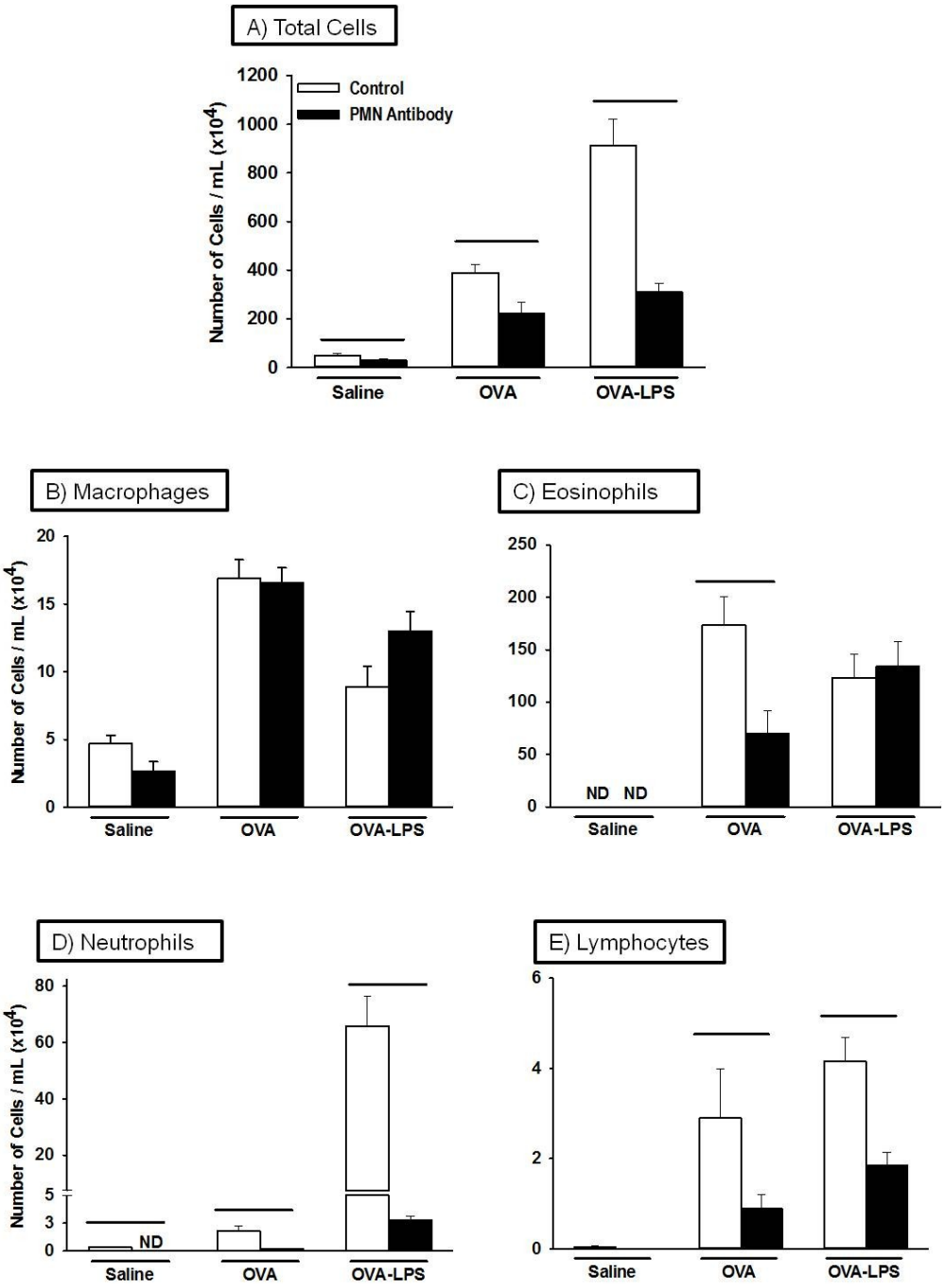
Data were expressed as group means $\pm$ the standard error of the mean (mean $\pm$ SEM). Grubb's outlier test was used to determine and remove statistical outliers. SigmaPlot statistical software (Systat Software Inc, San Jose, CA) was used to perform t-tests to make direct comparison between neutrophil- sufficient and depleted mice. Significant differences between group means were based on p values set at < 0.05.

3.4 RESULTS

3.4.1 Bronchoalveolar lavage fluid

Seventy-two hours following the final allergen challenge, ovalbumin elicited a significant accumulation of cells in BALF (Fig. 13), consisting predominately of eosinophils and macrophages, with lesser numbers of lymphocytes. Twenty-four hours following allergen exposure, instillation of intranasal LPS caused a two-fold increase in total cells that were due to a robust increase in neutrophils. Treatment with the neutrophil-depleting antibody decreased neutrophil numbers to control levels in OVA-LPS mice, but also caused reductions in eosinophils and lymphocytes in OVA mice, and in lymphocytes of OVA-LPS mice.

Figure 13. Comparison of BALF in neutrophil sufficient and neutrophil depleted mice. Total cells, macrophages, eosinophils, neutrophils, and lymphocytes were determined in BALF collected from BALB/c mice in the following groups: Saline (control), OVA, and OVA-LPS. Mice from each group were also treated i.p. with 250 μl saline vehicle (clear bars) or neutrophil-depleting antibody (PMN antibody; anti-Ly6g, solid bars) as described in Materials and Methods. Values are expressed as mean ± SE (n=5-6). Horizontal bars represent significant difference between indicated groups, $p < 0.05$.

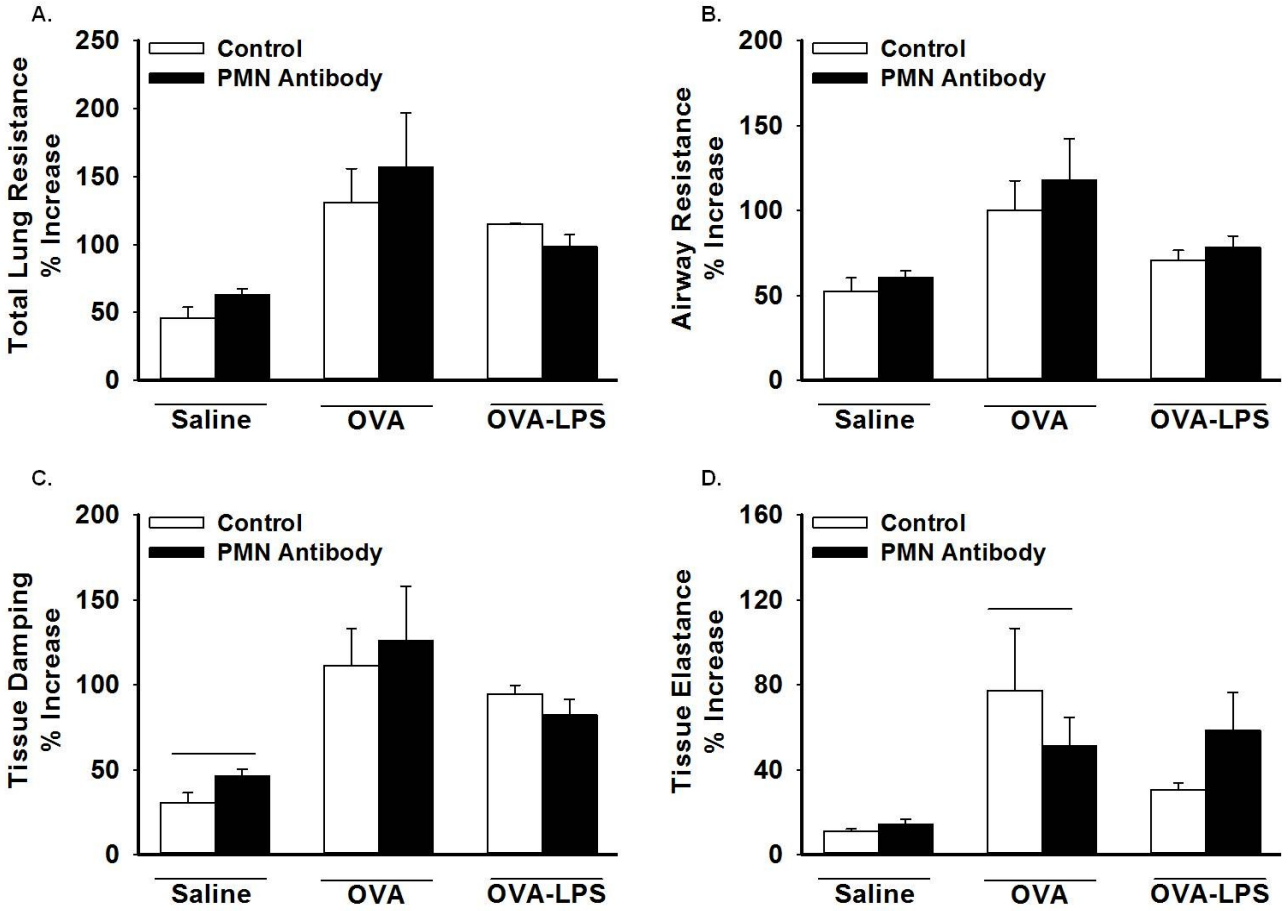


3.4.2 Airway Hyperresponsiveness

OVA sensitization and challenge caused airway hyperreactivity to methacholine as indicated by an increase in total lung resistance (Fig 14A), central airway resistance (Fig14B), tissue damping (Fig 14C), and elastance (Fig 14D). Instillation with LPS in allergic mice attenuated central airway resistance and elastance, and caused mild decreases in total resistance and tissue damping. Using the single compartment model to determine total lung resistance, there were no significant differences in AHR in mice that were depleted of neutrophils compared to neutrophil sufficient mice in saline, OVA, and OVA-LPS groups (Fig 14 A). Further analysis using the constant phase model to partition the airways showed no significant changes in airway resistance with neutrophil depletion in saline, OVA, and OVA-LPS mice (Fig 14B). Analyses of peripheral tissue changes show a significant increase in tissue damping in saline mice depleted of neutrophils compared saline neutrophil sufficient mice. However, there were no significant changes in OVA and OVA-LPS groups regardless of treatment (Fig 14C). Neutrophil depletion caused a significant decrease in tissue elastance in OVA mice, but no significant differences were notable with neutrophil depletion in OVA-LPS mice (Fig 14D).

Figure 14. Comparison of AHR in neutrophil sufficient and neutrophil depleted mice. Methacholine-induced changes in total lung resistance (A), airway resistance (B), tissue damping (C) and tissue elastance (D) were measured in intubated and ventilated mice using the FlexiVent system as described in Materials and Methods. Values are expressed as the percent increase from baseline value after administration of 10 mg/ml of methacholine (n=4-6). Horizontal bars represent significant difference between indicated groups, $p < 0.05$.

Figure 14. Comparison of AHR in neutrophil sufficient and neutrophil depleted mice. Methacholine-induced changes in total lung resistance (A), airway resistance (B), tissue damping (C) and tissue elastance (D) were measured in intubated and ventilated mice using the FlexiVent system as described in Materials and Methods. Values are expressed as the percent increase from baseline value after administration of 10 mg/ml of methacholine (n=4-6). Horizontal bars represent significant difference between indicated groups, $p < 0.05$.



3.5 Discussion

From my initial study (Chapter 2), I found that airway LPS induced a robust and widespread recruitment of neutrophils into lung tissue, and at the same time decreased allergic AHR in OVA-LPS mice. Activated neutrophils have the potential to make several bronchoactive mediators that may alter the hyperreactivity of the lung to methacholine-induced contraction of airway smooth muscle. In the current study I eliminated neutrophils prior to LPS treatment to test the hypothesis that neutrophils or a neutrophil product may contribute to the decrease in OVA-induced AHR. However depletion of neutrophils had no effect on AHR. Both OVA-induced increases, and LPS-induced decreases in airway resistance were similar in neutrophil-sufficient and -deficient mice. These results suggest that, despite their large presence in lung tissue, neutrophils do not affect AHR in OVA-LPS mice.

Other mouse models of AHR show strong associations between neutrophilic inflammation and increased airway resistance. Increased AHR has been associated with neutrophils and IL-17 production from Th17 lymphocytes in response to different immunologic stimuli (Wilson et al., 2009; Mizutani et al., 2012a). Although we did not observe a large neutrophil influx in our OVA model, neutrophils and neutrophil-derived products are actually required in some acute allergic AHR protocols using OVA-sensitized mice (Riesenfeld et al., 2010; Mizutani et al., 2012b; Koga et al., 2013). In the current study however, treatment with neutrophil-depleting antibody blocked the minor increase in BALF neutrophils in OVA mice, but it also reduced BALF eosinophils by more than 50% in these mice. AHR was unaffected in allergic mice, which suggests that

neither neutrophils nor eosinophils make a major contribution to allergic AHR in our OVA sensitization and challenge protocol. If AHR had been altered by treatment with the neutrophil antibody, further studies would be needed to determine if it were eosinophils or neutrophils were critical, since both were reduced by treatment.

Airway neutrophil infiltration has been linked to endotoxin-induced AHR in a number of animal models (Horie et al., 1988; Schwartz et al., 2001; Starkhammar et al., 2012). Like allergen-induced AHR, studies in laboratory animals suggest that endotoxin may elicit a biphasic response, with early and late AHR phases that are probably driven by different mechanisms (Cochran et al., 2002). Increased breathing frequency and bronchoconstriction 24 hours after LPS challenge in rats is dependent on neutrophils, whereas changes airway function at 2 hours was not (Spond et al., 2004). In allergic guinea pigs exposed to inhaled LPS, AHR occurred by 1 hour, but airways were hyporeactive 48 hours later when neutrophils in BALF were significantly elevated (Toward et al., 2005; Sharma et al., 2009). We observed no relation of neutrophils in LPS effects on AHR in the current study, but an important difference is that we exposed allergic airways that had ongoing inflammation and epithelial remodeling. In model of repeated pulmonary LPS exposure mice became tolerant to AHR with downregulation of muscarinic receptors in lung tissue, but maintained airway neutrophilia (Natarajan et al., 2010). A similar tolerance may occur in our allergic mice given a further inflammatory stimulus of endotoxin when, despite additional inflammatory cell recruitment, smooth muscle cells respond with downregulation in contractile pathways.

Reports of LPS-induced hyporesponsiveness are not unprecedented. Intratracheally instilled LPS in mice causes hyporesponsiveness to 5-HT-induced contraction that is dependent on TNFα and IL-6 (Brandolini et al., 2001). Airway hyporesponsiveness after LPS exposure has been linked to NO production that can be modulated by epithelial caveolin (Hsia et al., 2012) and by surfactant protein A (Pastva et al., 2011). Epithelial cell-dependent mechanisms that downregulate AHR are not limited to NO, but include potential mediators such as prostaglandins, acetylcholine, and inflammatory cytokines (Vanhoutte, 2013). An emerging hypothesis proposes that intermediate, subepithelial cells (e.g., fibroblasts) transduce signals from activated epithelium to smooth muscle via soluble mediators to promote airway dilation (Vanhoutte, 2013).

Lastly, intrinsic changes to the smooth muscle cells may mediate hyporesponsiveness in OVA-LPS mice. As previously mentioned, decreases in receptor numbers or receptor desensitization are possible explanations. Induction of nitric oxide synthase (iNOS) in smooth muscle by LPS would provide an immediate source of nitric oxide to promote relaxation. Both upregulation of iNOS and kinin receptors in smooth muscle are mediated by NF-κB (Zhang et al., 2013). Signal transduction from LPS binding to TLR4 or CD14 can lead to NF-κB activation in airway epithelium and smooth muscle to bypass neutrophils or other inflammatory cells (Safholm et al., 2011; Tully et al., 2013). As such, down modulation of smooth muscle cell responsiveness and/or activation of epithelial cells suggest plausible neutrophil-independent pathways by which LPS can inhibit AHR.

3.6 SUMMARY

Despite a robust infiltration of neutrophils into all lung compartments, their depletion had no effect on AHR in allergic mice given LPS. Therefore my hypothesis that attenuation of AHR in endotoxin-exposed allergic mice is dependent on the recruitment of airway neutrophils was not supported by the data. There is significant evidence that neutrophils produce both bronchoconstrictive and bronchodilatory mediators that are required for AHR in some models of allergen or LPS models of airway disease. However my results indicate that neutrophils are not involved in this particular model of LPS exposure to airways with ongoing allergic inflammation. The depleting antibody also reduced eosinophils and lymphocytes by 50%, but this decrease did not alter AHR when compared to mice that did not receive the antibody. Potential cellular mediators in my studies include lymphocytes, which have large infiltrations in allergic mice, airway macrophages, epithelial cells, and smooth muscle cells, among others. Epithelium and smooth muscle cells are intimately involved to control airway opening, and lymphocytes and macrophages can produce inflammatory mediators to modulate AHR. All these cell types should be responsive to LPS via the TLR4 or CD14 receptor. In the next chapter I will focus on the primary downstream transcription factor of LPS activation, NF-κB.

REFERENCES

REFERENCES

Aeffner F and Davis I C. 2012. Respiratory Syncytial Virus Reverses Airway Hyperresponsiveness to Methacholine in Ovalbumin-Sensitized Mice. Plos One **7**(10).

Baines K J, Simpson J L and Gibson P G. 2014. Biology of Neutrophils. Middleton's Allergy: Principles and Practice (Expert Consult-Online). N F Adkinson Jr, B S Bochner, A W Burks et al. Philadelphia, PA, Elsevier Health Sciences. **1:** 280-291.

Brandolini L, Intilangelo A, Caselli G and Bertini R. 2001. Role of Tumor Necrosis Factor-Alpha in Endotoxin-Induced Lung Parenchymal Hyporesponsiveness in Mice. Biochemical Pharmacology **62**(8): 1141-1144.

Braun-Fahrlander C, Riedler J, Herz U, Eder W, Waser M, Grize L, Maisch S, Carr D, Gerlach F, Bufe A, Lauener R P, Schierl R, Renz H, Nowak D and von Mutius E. 2002. Environmental Exposure to Endotoxin and Its Relation to Asthma in School-Age Children. New England Journal of Medicine **347**(12): 869-877.

Cochran J R, Khan A M, Elidemir O, Xue H S, Cua B, Fullmer J, Larsen G L and Colasurdo G N. 2002. Influence of Lipopolysaccharide Exposure on Airway Function and Allergic Responses in Developing Mice. Pediatric Pulmonology **34**(4): 267-277.

Condon T V, Sawyer R T, Fenton M J and Riches D W H. 2011. Lung Dendritic Cells at the Innate-Adaptive Immune Interface. Journal of Leukocyte Biology **90**(5): 883-895.

Eisenbarth S C, Piggott D A, Huleatt J W, Visintin I, Herrick C A and Bottomly K. 2002. Lipopolysaccharide-Enhanced, Toll-Like Receptor 4-Dependent T Helper Cell Type 2 Responses to Inhaled Antigen. Journal of Experimental Medicine **196**(12): 1645-1651.

Hantos Z, Daroczy B, Suki B, Nagy S and Fredberg J J. 1992. Input Impedance and Peripheral Inhomogeneity of Dog Lungs. Journal of Applied Physiology **72**(1): 168-178.

Horie T, Ohmori C, Koyama S, Saitoh O, Mutoh T, Okuma A and Okayasu M. 1988. Airway Hyperresponsiveness after Endotoxin Inhalation Depends on Leukocyte Infiltration. The Tohoku journal of experimental medicine **156**(2): 109-120.

Hsia B J, Pastva A M, Giamberardino C D, Potts-Kant E N, Foster W M, Que L G, Abraham S N, Wright J R and Zaas D W. 2012. Increased Nitric Oxide Production Prevents Airway Hyperresponsiveness in Caveolin-1 Deficient Mice Following Endotoxin Exposure. Journal of allergy & therapy(4).

Jatakanon A, Uasuf C, Maziak W, Lim S, Chung K F and Barnes P J. 1999. Neutrophilic Inflammation in Severe Persistent Asthma. American Journal of Respiratory and Critical Care Medicine **160**(5): 1532-1539.

Koga H, Miyahara N, Fuchimoto Y, Ikeda G, Waseda K, Ono K, Tanimoto Y, Kataoka M, Gelfand E W, Tanimoto M and Kanehiro A. 2013. Inhibition of Neutrophil Elastase Attenuates Airway Hyperresponsiveness and Inflammation in a Mouse Model of Secondary Allergen Challenge: Neutrophil Elastase Inhibition Attenuates Allergic Airway Responses. Respiratory Research **14**.

Liu A H. 2002. Endotoxin Exposure in Allergy and Asthma: Reconciling a Paradox. Journal of Allergy and Clinical Immunology **109**(3): 379-392.

Liu A H, johnston R B and Fenton M J. 2014. Innate Immunity. Middleton's Allergy: Principles and Practice (Expert Consult-Online). N F Adkinson Jr, B S Bochner, A W Burks et al. Philadelphia, PA, Elsevier Health Sciences. **1:** 2-19.

Michel O, Duchateau J and Sergysels R. 1989. Effect of Inhaled Endotoxin on Bronchial Reactivity in Asthmatic and Normal Subjects. Journal of Applied Physiology **66**(3): 1059-1064.

Michel O, Ginanni R, Duchateau J, Vertongen F, Le Bon B and Sergysels R. 1991. Domestic Endotoxin Exposure and Clinical Severity of Asthma. Clinical & Experimental Allergy **21**(4): 441-448.

Minnicozzi M, Sawyer R T and Fenton M J. 2011. Innate Immunity in Allergic Disease. Immunological Reviews **242**(1): 106-127.

Mizutani N, Goshima H, Nabe T and Yoshino S. 2012a. Complement C3a-Induced Il-17 Plays a Critical Role in an Ige-Mediated Late-Phase Asthmatic Response and Airway Hyperresponsiveness Via Neutrophilic Inflammation in Mice. The Journal of Immunology **188**(11): 5694-5705.

Mizutani N, Goshima H, Nabe T and Yoshino S. 2012b. Establishment and Characterization of a Murine Model for Allergic Asthma Using Allergen-Specific Ige Monoclonal Antibody to Study Pathological Roles of Ige. Immunology Letters **141**(2): 235-245.

Natarajan S, Kim J and Remick D G. 2010. Chronic Pulmonary Lps Tolerance Induces Selective Immunosuppression While Maintaining the Neutrophilic Response. Shock **33**(2): 162-169.

Nyenhuis S M, Schwantes E A, Evans M D and Mathur S K. 2010. Airway Neutrophil Inflammatory Phenotype in Older Subjects with Asthma. Journal of Allergy and Clinical Immunology **125**(5): 1163-1165.

Parker D and Prince A. 2011. Innate Immunity in the Respiratory Epithelium. American Journal of Respiratory Cell and Molecular Biology **45**(2): 189-201.

Pastva A M, Walker J K L, Maddox L A, Mukherjee S, Giamberardino C, Hsia B, Potts E, Zhu H M, Degan S, Sunday M E, Lawson B L, Korfhagen T R, Schwartz D A, Eu J P, Foster W M, McMahon T J, Que L and Wright J R. 2011. Nitric Oxide Mediates Relative Airway Hyporesponsiveness to Lipopolysaccharide in Surfactant Protein a-Deficient Mice. American Journal of Respiratory Cell and Molecular Biology **44**(2): 175-184.

Piggott D A, Eisenbarth S C, Xu L, Constant S L, Huleatt J W, Herrick C A and Bottomly K. 2005. Myd88-Dependent Induction of Allergic Th2 Responses to Intranasal Antigen. Journal of Clinical Investigation **115**(2): 459-467.

Rajaiah R, Perkins D J, Polumuri S K, Zhao A, Keegan A D and Vogel S N. 2013. Dissociation of Endotoxin Tolerance and Differentiation of Alternatively Activated Macrophages. The Journal of Immunology **190**(9): 4763-4772.

Riesenfeld E P, Sullivan M J, Thompson-Figueroa J A, Haverkamp H C, Lundblad L K, Bates J H T and Irvin C G. 2010. Inhaled Salmeterol and/or Fluticasone Alters Structure/Function in a Murine Model of Allergic Airways Disease. Respiratory Research **11**.

Safholm J, Lovdahl C, Swedin L, Boels P J M, Dahlen S E, Arner A and Adner M. 2011. Inflammation-Induced Airway Smooth Muscle Responsiveness Is Strain Dependent in Mice. Pulmonary Pharmacology & Therapeutics **24**(4): 361-366.

Schwartz D A, Christ W J, Kleeberger S R and Wohlford-Lenane C L. 2001. Inhibition of Lps-Induced Airway Hyperresponsiveness and Airway Inflammation by Lps Antagonists. American Journal of Physiology-Lung Cellular and Molecular Physiology **280**(4): L771-L778.

Scott H A, Gibson P G, Garg M L and Wood L G. 2011. Airway Inflammation Is Augmented by Obesity and Fatty Acids in Asthma. European Respiratory Journal **38**(3): 594-602.

Sharma R, Kaundal R K and Sharma S S. 2009. Amelioration of Pulmonary Dysfunction and Neutrophilic Inflammation by Pparî³ Agonist in Lps-Exposed Guinea Pigs. Pulmonary Pharmacology & Therapeutics **22**(3): 183-189.

Spond J, Billah M M, Chapman R W, Egan R W, Hey J A, House A, Kreutner W and Minnicozzi M. 2004. The Role of Neutrophils in Lps-Induced Changes in Pulmonary Function in Conscious Rats. Pulmonary Pharmacology & Therapeutics **17**(3): 133-140.

Starkhammar M, Georen S K, Swedin L, Dahlen S-E, Adner M and Cardell L O. 2012. Intranasal Administration of Poly(I:C) and Lps in Balb/C Mice Induces Airway Hyperresponsiveness and Inflammation Via Different Pathways. Plos One **7**(2).

Toward T J, Nials A T and Johnson F J. 2005. Guinea-Pig Lung Adenylyl and Guanylyl Cyclase and Pde Activities Associated with Airway Hyper-and Hypo-Reactivity Following Lps Inhalation. Life Sciences **76**(9): 997-1011.

Tully J E, Hoffman S M, Lahue K G, Nolin J D, Anathy V, Lundblad L K A, Daphtary N, Aliyeva M, Black K E and Dixon A E. 2013. Epithelial Nf-Kb Orchestrates House Dust Miteâ€“Induced Airway Inflammation, Hyperresponsiveness, and Fibrotic Remodeling. The Journal of Immunology **191**(12): 5811-5821.

Vanhoutte P M. 2013. Airway Epithelium-Derived Relaxing Factor: Myth, Reality, or Naivety? American Journal of Physiology-Cell Physiolog **304**(9): C813-C820.

Von M, Braun F, Schierl, Riedler, Ehlermann, Maisch, Waser and Nowak. 2000. Exposure to Endotoxin or Other Bacterial Components Might Protect against the Development of Atopy. Clinical & Experimental Allergy **30**(9): 1230-1234.

Wenzel S E, Schwartz L B, Langmack E L, Halliday J L, Trudeau J B, Gibbs R L and Chu H W. 1999. Evidence That Severe Asthma Can Be Divided Pathologically into Two

Inflammatory Subtypes with Distinct Physiologic and Clinical Characteristics. American Journal of Respiratory and Critical Care Medicine **160**(3): 1001-1008.

Wilson R H, Whitehead G S, Nakano H, Free M E, Kolls J K and Cook D N. 2009. Allergic Sensitization through the Airway Primes Th17-Dependent Neutrophilia and Airway Hyperresponsiveness. American Journal of Respiratory and Critical Care Medicine **180**(8): 720-730.

Zhang Y, Cardell L-O, Edvinsson L and Xu C-B. 2013. Mapk/Nf-Kb-Dependent Upregulation of Kinin Receptors Mediates Airway Hyperreactivity: A New Perspective for the Treatment. Pharmacological Research **71**: 9-18.

CHAPTER 4

EFFECTS OF NF-κB INHIBITION ON AIRWAY INFLAMMATION AND AIRWAY HYPERRESPONSIVENESS IN ALLERGIC MICE EXPOSED TO LIPOPOLYSACCHARIDE

4.1 ABSTRACT

Inhalation of particulate matter can exacerbate symptoms in asthmatics by increasing antigen induced inflammation, as well as, stimulate innate inflammatory responses. Recent knowledge suggests that induction of toll-like receptor-4 (TLR4), nuclear factor kappa-B (NF-κB), tumor growth factor-beta (TGFβ), and genes associated with airway remodeling are critical for PM-mediated responses in allergic airways. Current therapeutic strategies for asthma target the asthma- related adaptive immune response. Therefore, investigations into a broader set of inflammatory immune targets are needed to address exaggerated responses to air particulates in asthmatics. Endotoxin or LPS is ubiquitously present in the environment and is a component in PM of occupational, agricultural and urban air. LPS binds to TLR4 and activates both NF-κB and TGFβ pathways. NF-κB is an important mediator of the immune response and has recently been identified as a therapeutic target for multiple chronic inflammatory diseases. In chapter 2, I described the effects of airway LPS to enhance airway inflammation but attenuate AHR in mice with pre-existing allergic airways disease. I hypothesized that LPS activation of the transcription factor, NF-κB, attenuated AHR in OVA-LPS mice. In this study I sought to determine the role of NF-κB in the attenuation of AHR in OVA-LPS mice. To do this, I used OVA to establish allergic airway disease as described in Chapter 2. Prior to LPS exposure, mice were treated with a novel inhibitor of NF-κB. Comparisons between animals not treated and treated with the NF-κB inhibitor were made between IN instilled saline, OVA, and OVA-LPS groups. . There were no changes in inflammation or AHR in saline and OVA groups. In OVA-LPS mice, NF-κB inhibition did not affect the recruitment of neutrophils by LPS.

However, in the BALF there were significant decreases in macrophages, eosinophils, and lymphocytes. Furthermore, NF-κB inhibition reversed the attenuation in AHR. These findings suggest that attenuation of AHR in OVA-LPS mice is mediated by NF-κB -dependent pathways.

4.2 INTRODUCTION

Inhalation of particulate matter (PM) from both indoor and outdoor air pollution can exacerbate airway inflammatory symptoms in asthmatics (Breysse et al., 2010; Kelly and Fussell, 2011). Pharmaceutical interventions that target allergic inflammation-specific pathways have been developed against IgE, histamines, and leukotrienes. More often prescribed are corticosteroids that target multiple, non-specific pathways in immune, inflammatory and epithelial cells, but that often cause undesirable side-effects. Results from both human and animal studies suggest that PM exposure can exacerbate antigen-induced, 'allergic pathways', as well as can induce additional 'innate' inflammatory responses to worsen airway pathology and function. Findings from genomics studies suggest that induction of toll-like receptor-4 (TLR4), nuclear factor kappa-B (NF-κB), tumor growth factor-beta (TGFβ), and genes associated with airway remodeling are critical for PM-mediated responses in allergic airways (Wang et al., 2008; Heidenfelder et al., 2009; Vawda et al., 2014). As such therapeutic strategies to prevent exaggerated responses to air particulates in asthmatics needs to encompass a broader set of inflammatory immune targets.

Endotoxin is a lipopolysaccharide (LPS) component of the cell wall of Gram negative bacteria and is found in PM of occupational, agricultural and urban air. LPS binds to TLR4 and activates both NF-κB and TGFβ pathways, and in both human and animals can exacerbate allergic airways responses (Kawai and Akira, 2007; Chen et al., 2008; Doreswamy and Peden, 2011). We have recently described novel effects of airway LPS to enhance airway inflammation but at the same time attenuate airway

hyperresponsiveness (AHR) in mice with pre-existing allergic airways disease (Chapter 2). While the underlying mechanism is unknown, the primary pathological feature associated with reversal of allergic AHR was widespread infiltration of neutrophils into all compartments of the lung and decreased storage of intraepithelial mucous. NF-κB activation in neutrophils can lead to downstream production of bronchoactive products including nitric oxide and prostaglandins that might modulate airway reactivity (Tanaka et al., 2005; Prado et al., 2006), and secretagogues such as elastase that may promote production and hypersecretion of mucus that contributes to airway obstruction (Koga et al., 2013).

Interrupting the NF-κB pathway may be an effective approach to mitigating the adverse effects of PM-induced responses in asthmatic airways. Activation of NF-κB has been shown in lungs of asthmatics (Hart et al., 2000), and allergic mice (Poynter et al., 2002; Sheller et al., 2009), and may be linked to AHR in experimental asthma (Donovan et al., 1999). Furthermore NF-κB also plays a role in promoting endotoxin-induced lung inflammation (Poynter et al., 2003). The potential role that NF-κB plays in endotoxin-induced modulation of allergic airway disease has not been examined.

Inactive NF-κB is sequestered in the cell cytoplasm by binding to the protein IκB-α (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha), and is released after inflammatory signal transduction pathways lead to the IκB kinase (IKK)-β - mediated phosphorylation and subsequent proteasomal degradation of IκB-α. As a result, specific NF-κB proteins can translocate to the nucleus where inflammatory gene transcription is activated. There are several types of inhibitors that target different

aspects of the NF-κB activation pathway (Edwards et al., 2009). A common approach to disrupt NF-κB function is to interfere with phosphorylation or proteasome degradation pathways. Members of our research team have used a novel imidazoline compound to selectively inhibit the 20S catalytic core of the proteasome and prevent IKK-α degradation, which in previous studies has blocked NF-κB-induced cytokine production in vitro (Kahlon et al., 2009; Lansdell et al., 2013). In the current study I used this novel proteasome inhibitor to test the hypothesis that LPS- mediated alterations of allergic AHR are dependent on NF-κB activation. By intervening with the inhibitor immediately prior to LPS administration, I hoped to limit the effects of NF-κB inhibition on allergic processes, and rather target the NF-κB-mediated responses to LPS exposure in pre-existing allergic airways.

4.3 MATERIALS AND METHODS

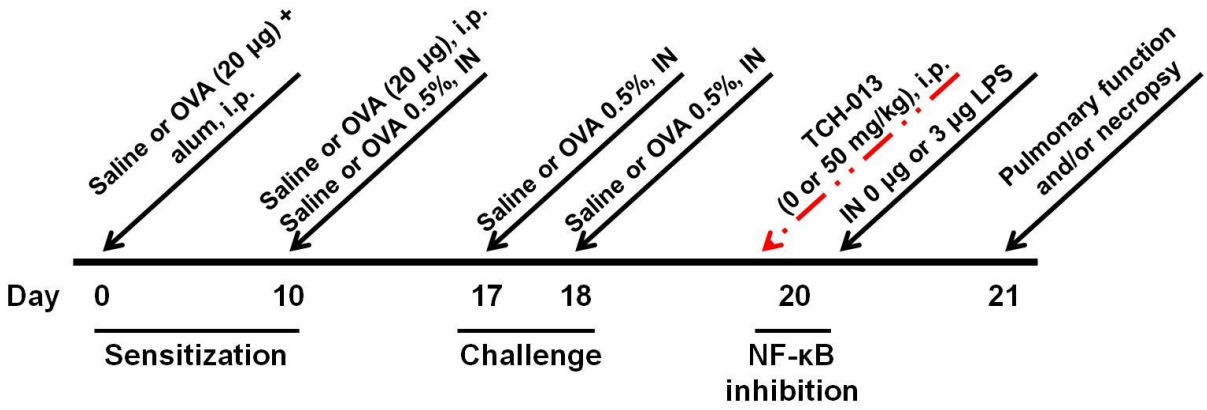
4.3.1 Laboratory Animals and Treatment protocols

Male BALB/c mice (Charles River Laboratories, Portage, MI), 6-8 weeks of age were housed and maintained as described in Chapter 2 MSU animal facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Mice were randomly assigned to one of six experimental groups consisting of 6 animals as follows: 1) animals were assigned to saline, OVA, or OVA-LPS groups and 2) each group was divided in half and were administered the vehicle or NF-κB inhibitor treatment prior to IN LPS (Fig 15).

4.3.2 NF-κB Inhibition

On Day 20, non-allergic and allergic animals were randomly divided into 2 groups to receive 0 or 50 mg/kg i.p. of the NF-κB proteasome inhibitor, TCH-013 in a vehicle comprised of 3:7 propylene glycol: 5% dextrose (gift from J. Tepe, MSU) 1 hour prior to IN LPS exposure (Fig 15).

Figure 15 Protocol for development of allergic airway disease and inhibition of NF-κB



4.3.3 Necropsy, Lavage Collection, and Tissue Preparation

Mice were anesthetized with an i.p. injection of with sodium pentobarbital (10 mg; Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI). Blood was collected from the caudal vena cava for separation of plasma, and animals were euthanized by transection of the abdominal aorta. The lungs and heart were harvested en bloc. The lungs were instilled twice with 800 µL saline via the cannulated trachea to collect BALF. After lavage the right lung lobes were ligated, separated and stored in RNAlater (Qiagen) for RNA isolation or snap frozen in liquid nitrogen for storage. The left lobe was inflated

with 10% neutral buffered formalin to a pressure of 30 cm H_2O for 1 hour and then stored in a large volume of the same fixative.

4.3.4 Bronchoalveolar Lavage Analysis

Total BALF leukocytes were counted with a hemocytometer and cytological slides were prepared using a Cytospin centrifuge (Shandon). Slides were stained with Diff-Quick (Dade Behring, Newark, DE) and cell differential (macrophages, eosinophils, neutrophils, and lymphocytes) were counted. The remaining BAL fluid was centrifuged, and the supernatants were stored at -20°C.

4.3.5 Histopathology

Following fixation, two traverse sections of the left lung were taken along the axial airway at the levels of the 5^{th} and 11^{th} generation (G5 and G11) to examine the proximal and distal airways (Harkema and Hotchkiss, 1992). The tissues were embedded in paraffin and stained with H&E for routine histological examination and AB/PAS to identify intraepithelial mucosubstances.

4.3.6 Morphometry

From the left lung lobe, estimation of the amount of the intraepithelial mucosubstances in epithelium lining the G5 axial airways was performed on histological slides stained with AB/PAS. Slides were scanned and digitalized with a slide scanner (VS110, Olympus). Using a specialized mucus quantification APP (Visopharm, Denmark), the G5 airway was manually selected as the region of interest in the scanned

images. The APP automatically highlights the basal lamina beneath the surface epithelium and the positive staining of mucusubstance to determine the area of mucus relative to the basal lamina expressed and $\mu m^2/\mu m$.

4.3.7 Real –time PCR of Lung

Total RNA was isolated from the right caudal lung lobe using Rneasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Briefly, reverse transcription was accomplished by using High Capacity cDNA Reverse Transcription Kit reagents (Applied Biosystems). Quantitative mRNA expression analysis was conducted on an ABI PRISM 7900 HT Sequence Detection System at Michigan State University's Research Technology Support Facility using Taqman Gene Expression Assay reagents (Applied Biosystems).

Targeted genes for molecular analysis included Resistin like alpha (*Retnla; Fizz1*), Chloride channel calcium activated 3 (*Clca3; Gob5*), Interferon gamma (*Ifng*), Interleukin 10 (*Il10*), *Il-12b*, *-13*, and *-17a* and chemokine ligand 2 (*Ccl2; MCP1*). Relative gene expression was normalized to endogenous controls (*Arbp, Gusb, and Gapdh*). Data was expressed as fold-increase in RNA expression compared to control animals, which were set at a value of 1.

4.3.8 Airway Hyperresponsiveness (AHR) Measurements

On day 21, mice designated for lung function measurement were anesthetized and evaluated for AHR with incremental doses of methacholine as outlined in Chapter 2. The mean of the responses for each concentration of methacholine was determined.

A dose- response curve was generated. The lowest dose of MCh with the greatest variation in response between groups was at 10 mg/ml. Therefore, the data were expressed as the percent change at 10 mg/ml compared to baseline for each group.

4.3.9 Statistical Analysis

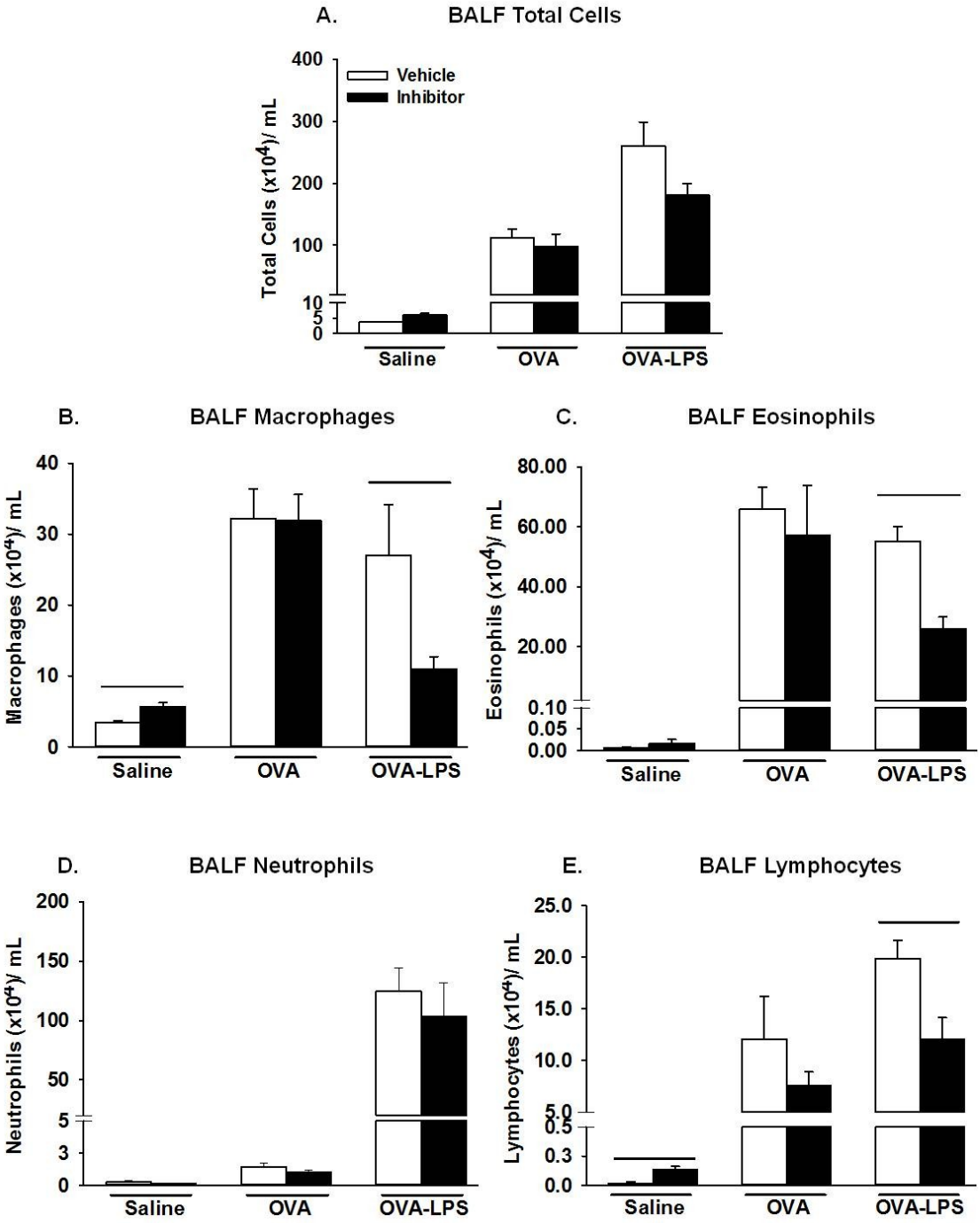
BALF data were expressed as group means $\pm$ the standard error of the mean (SEM). Grubb's outlier test was used to determine and remove outliers. Statistical differences were determined with two-way ANOVA with Student-Newman-Keuls post hoc test to make direct comparison between groups; $p \leq 0.05$. For RT-PCR, statistical differences of ΔCt values between groups were determined with t-tests to make direct comparison between groups (SigmaPlot, Systat Software Inc, San Jose, CA).

4.4 RESULTS

4.4.1 Bronchoalveolar lavage fluid

Consistent my previous results, sensitization and challenge to OVA induced a significant accumulation of total cells in BALF (Fig 16A). The increase consisted mostly of macrophages and eosinophils (Fig16 A, B, C). Allergic mice given IN LPS (OVA-LPS) had a significant increase in total cells compared to OVA mice, which was due to a robust infiltration of neutrophils and a minor increase in lymphocytes (Fig16 A, D). Treatment with the NF-κB inhibitor TCH-013 prior to LPS instillation reduced macrophage, eosinophil and lymphocytes in only OVA-LPS mice. Interestingly, there was no effect of NF-κB inhibition on neutrophil accumulation elicited by LPS. Lastly, treatment with TCH-013 resulted in small increases in macrophages and lymphocytes in saline sensitized and challenged mice (Fig 16 B, E).

Figure 16 Comparison of BALF in mice treated and not treated with NF-κB inhibitor. Total cells, macrophages, eosinophils, neutrophils, and lymphocytes were determined in BALF collected from Saline, OVA and OVA-LPS mice that received Vehicle (clear bars) or an Inhibitor (cross hatched bars) of NF-κB (50 µg of TCH-013, i.p.) as described in Methods. Values are expressed as mean ± SE (n=4-6). Horizontal bars represent significant difference between indicated groups, $p < 0.05$



4.4.2 Histological examination and morphometry

Following treatment with the NF-κB inhibitor, non-sensitized, mice did not show any histological lesions in the lung (Fig 17A). OVA- induced lesions were not altered by treatment with TCH-013. Lungs from OVA mice had focally extensive peribronchiolar and perivascular inflammation, which predominated around the axial airway and large diameter bronchioles with occasional extension into distal terminal bronchioles. Airway inflammation was predominately comprised of mononuclear cells (lymphocytes and macrophages) with lesser numbers of eosinophils (Fig 17B). OVA caused airway epithelial remodeling characterized by mucous cell metaplasia, identifiable by AB/PAS staining, affecting the axial airway and large diameter bronchioles (Fig 17D). Treatment with TCH-013 did not significantly alter the lesions in OVA-LPS mice. Exposure to LPS in OVA mice produced a mixed inflammatory influx in peribronchiolar and perivascular regions. Prominent inflammation surrounded the axial airway, large diameter bronchioles, and extended around many terminal bronchioles. Airway inflammation was characterized by mainly mononuclear cells (lymphocytes, macrophages and smaller numbers of multinucleate cells) and moderate numbers of granulocytes (eosinophils and neutrophils). Additionally, this mixed inflammation extended into the lung parenchyma and airspaces (Fig 17C). Morphometrically, the area of AB/PAS stained mucosubstances per basal lamina length in treated animals was not different from untreated animal (Fig 18A). Increases in stored mucous were accompanied by increased expression of *Gob5* in both OVA and OVA-LPS mice compared to saline mice (Fig 18B). Inhibition of NF-κB had no effect on Gob-5 expression.

Figure 17. Pulmonary histology of mice with NF-κB inhibition. Light photomicrographs (H&E) of lungs from mice treated with the NF-κB inhibitor, TCH-013 showing no inflammation (saline, A), peribronchiolar and perivascular inflammation and thickening of airway epithelium due to mucous cell metaplasia (OVA/OVA-LPS, B/C), extension of inflammation into the alveolar tissue and spaces (OVA-LPS, C). AB/PAS staining of intraepithelial mucus in axial airway (OVA, D). A, bronchiole; BV, blood vessel, and AA, axial airway. Scale bar = 100 μm

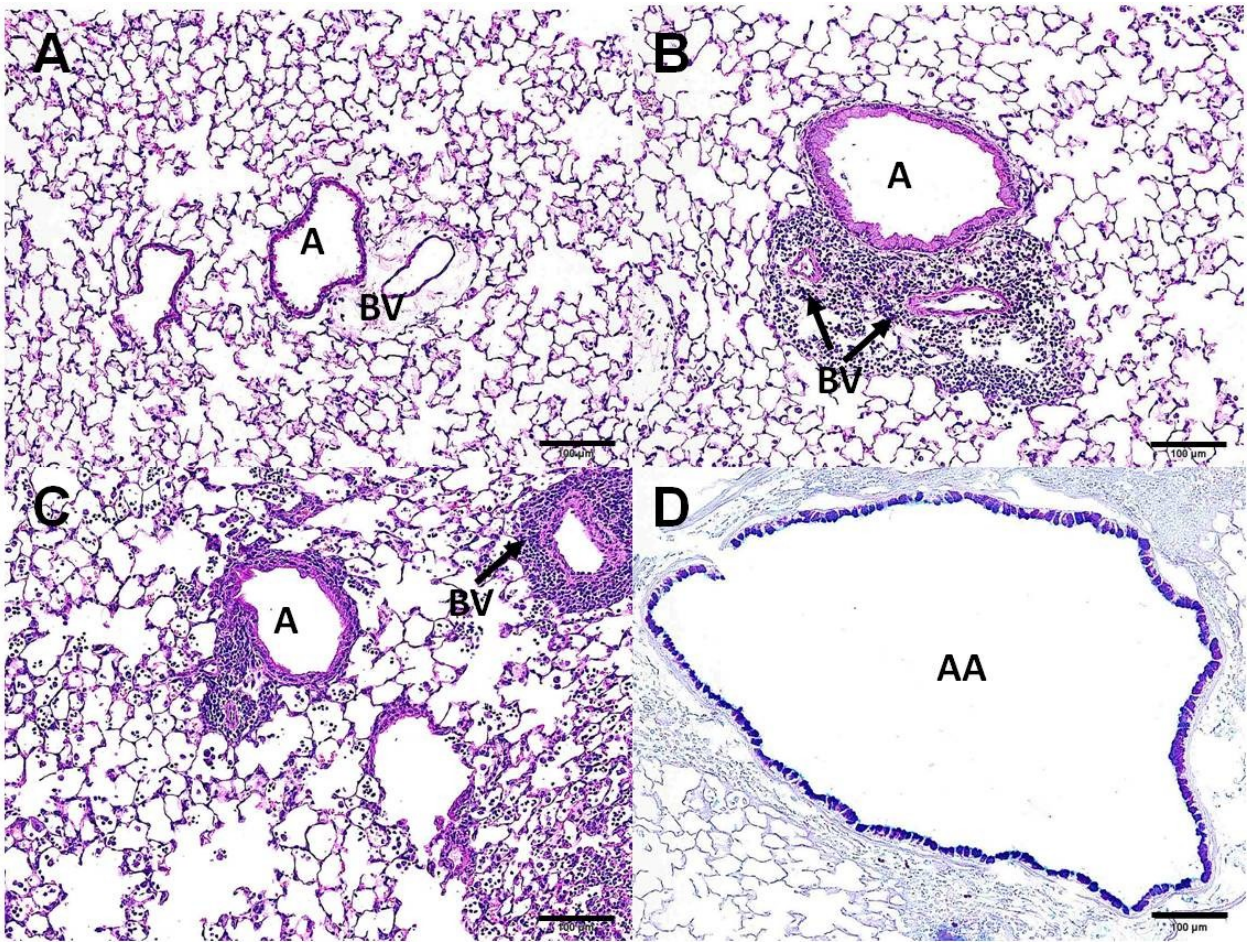
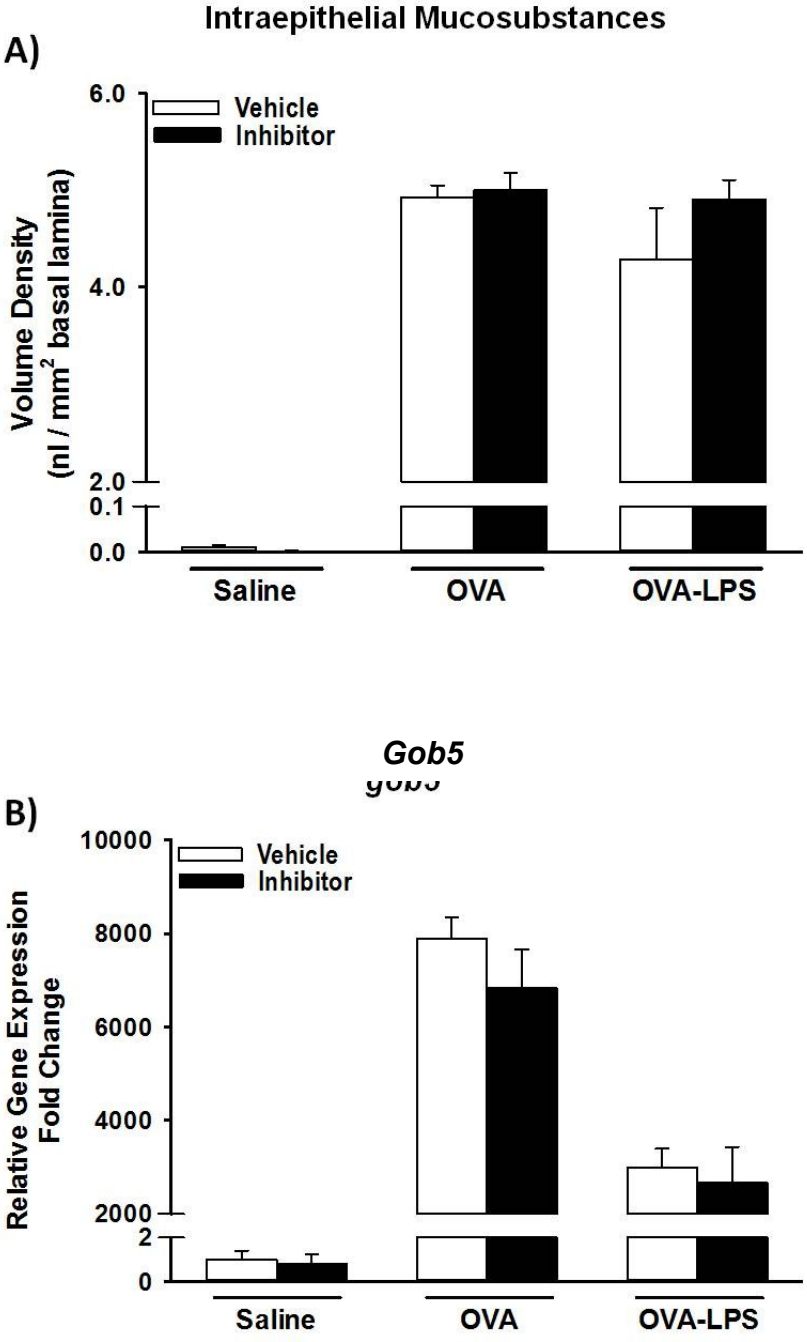


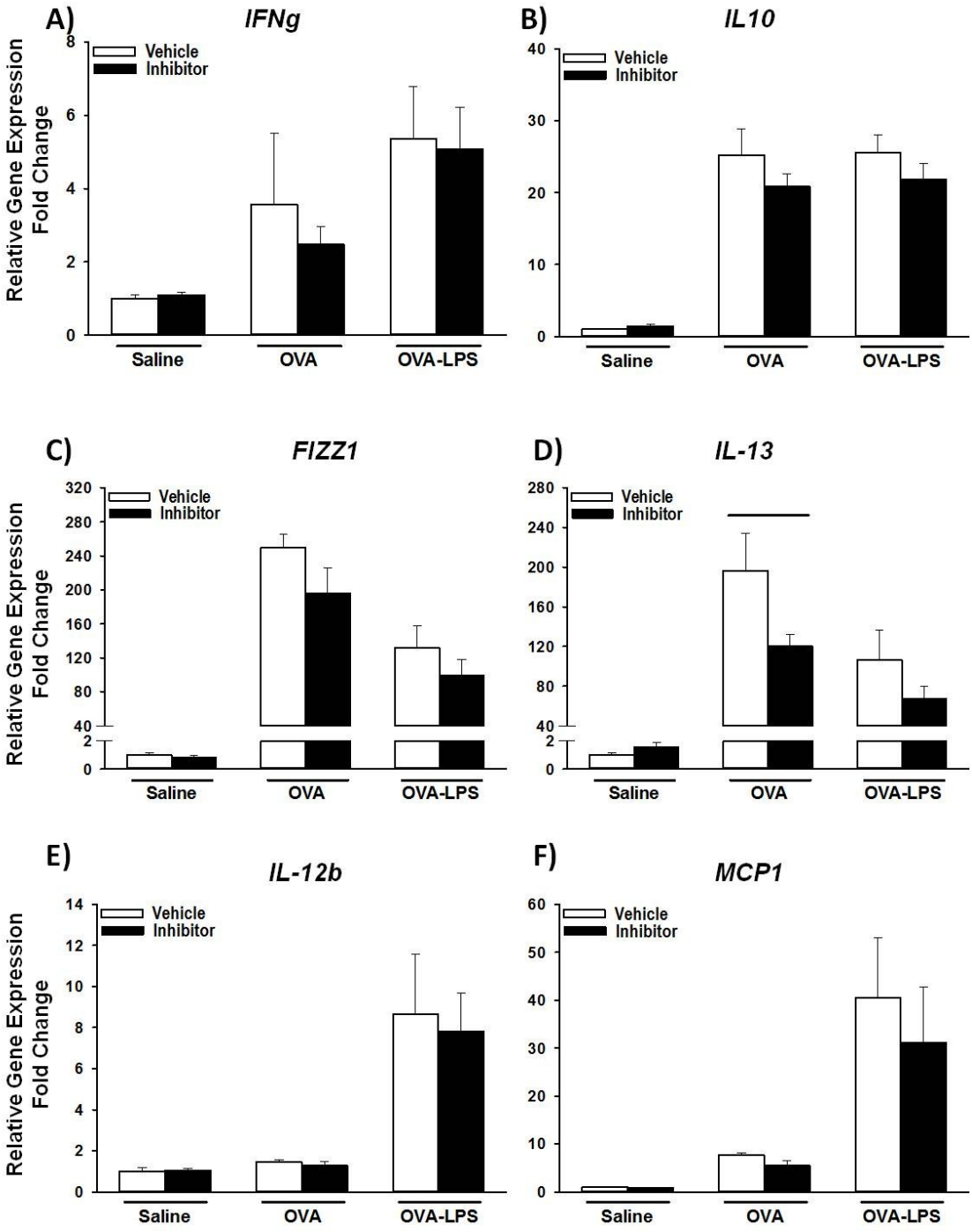
Figure 18. Airway mucosubstances and *Gob5* gene expression following NF-κB inhibition. (A) Area of mucosubstance per length of basal lamina of the proximal airway (G5). (B) mRNA Gob-*5,*involved in mucus secretion. Data were reported as the fold increase of the group mean ± SEM. Horizontal line indicates significant difference between specified groups, $p<0.05$; n=6/group.



4.4.3 Lung gene expression

Sensitization and challenge with OVA caused increased expression of *Ifng* and *Il10* (Fig 19, A-B). Also, OVA increased *Fizz1* and *Il13.* However, in OVA-LPS mice, induction of *Fizz1* and *Il13* were decreased compared to OVA mice (Fig 19, C-D), suggesting Th2 pathways were down-regulated with LPS treatment. Furthermore, induction of *IL12b* and *Mcp1* occurred only in OVA-LPS mice (Fig 19, E-F). The only effect of treatment with TCH-013 was the reduction of *Il13* expression in OVA mice.

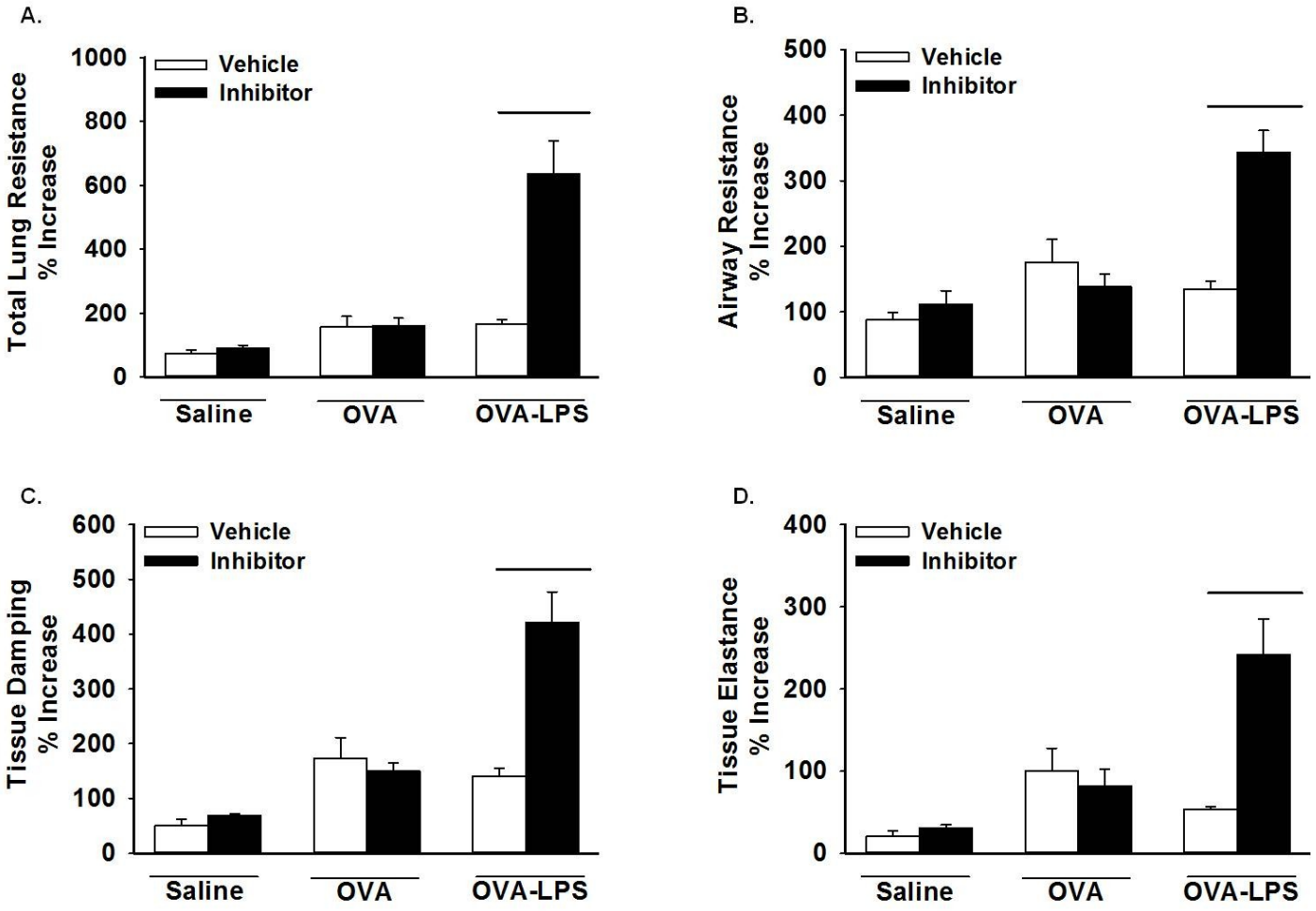
Figure 19. Pulmonary gene expression following NF-κB inhibition. Relative expressions of mRNA encoding for *Ifng, Il10, Fizz1, Il13, Il12b,* and *Mcp1* were determined in right caudal lobes by RT-PCR as described in Materials and Methods. Values are expressed as fold increase of mRNA expression compared to control (Saline) animals (n=5-6). Horizontal bars represent significant difference between indicated groups, $p < 0.05$.



4.4.4 Airway Hyperresponsiveness

Treatment with the NF-κB inhibitor, TCH-013, did not significantly alter airway or tissue resistance caused by methacholine challenge in either Saline or OVA mice (Fig 20, A-D). However, AHR was significantly increased in OVA-LPS mice treated with the inhibitor. Specifically, increases were measured in total lung resistance (200%, Fig20 A), central airways resistance (180%, Fig 20 B), tissue resistance (200%, Fig 20 C) and tissue elastance (400%, Fig 20 D).

Figure 20. Effects of NF-κB inhibition on AHR in OVA-LPS mice. Methacholine-induced changes in total lung resistance (A), airway resistance (B), tissue damping (C) and tissue elastance (D) were measured in intubated and ventilated mice using the FlexiVent system as described in Materials and Methods. Values are expressed as the percent increase from baseline value after administration of 10 mg/ml of methacholine (n=12-14). Horizontal bars represent significant difference between indicated groups, $p < 0.05$.



4.5 Discussion

We have previously described a LPS exacerbation model of asthma the co-existing effects of enhanced inflammatory cell recruitment and reduced airway AHR. The primary pathological feature associated with changes in AHR in LPS-treated allergic mice was widespread infiltration of neutrophils into all compartments of the lung. In the current study, inhibition of NF-κB had no effect on LPS-stimulated neutrophil recruitment, but rather led to a dramatic increase in AHR. Furthermore, these changes in airway physiology were associated with decreases in airway macrophages, eosinophils and lymphocytes. As such our results suggest that inflammation is inversely related to AHR in this model, and that this novel relationship is mediated by NF-κB-dependent pathways.

Activation of NFκB plays a central role in the adverse airway inflammatory and hyperreactivity responses to both allergen and LPS-stimulation in laboratory rodents. In BALB/c OVA models of asthma the inhibition of NF-κB is a common mechanism for therapeutic compounds to reduce eosinophil recruitment, Th2 cytokine production, mucous cell metaplasia and AHR (Desmet et al., 2004; Lee et al., 2005; Bao et al., 2007; Kim et al., 2007; Shimizu et al., 2012). Likewise, the neutrophil recruitment and airway injury caused by LPS can be blocked by NF-κB inhibition (Chuang et al., 2013; De Stefano et al., 2013). We designed the protocol to minimize the effects of NF-κB inhibition on allergic processes, by dosing with TCH-013 approximately 48h after the last OVA challenge. However we did see a small decrease in BALF lymphocytes in OVA mice, and a modest depression in resistance in their central airways and

peripheral tissues. These results suggest that ongoing allergic airway responses can be interrupted with an NF-κB inhibitor, and does not require interventions at the time of OVA challenge as was done in other mouse studies mentioned above.

Our protocol was designed to target the NF-κB-mediated responses to LPS exposure in pre-existing allergic airways. We expected that treatment with TCH-013 would reverse the marked recruitment of BALF neutrophils that were associated with LPS. Instead, eosinophils, macrophages, and lymphocytes were the inflammatory cells that were blocked by NF-κB inhibition in OVA-LPS mice. One explanation is that the dose of TCH-013 was insufficient to affect LPS-induced airway neutrophilia, but was capable of modulating chemokines specific for eosinophil and macrophages. Cytokines that promote eosinophil accumulation include eotaxin, IL-5 and GM-CSF, all of which activate NF-κB in eosinophils (Ip et al., 2005). Airway macrophages in asthmatic airways with LPS exposure may be a mixed population of M1 and M2 subtypes (classically or alternatively activated, respectively), where M1 cells may be more sensitive to NF-κB inhibition than M2 cells (Porta et al., 2009; Liao et al., 2011). Compared to OVA mice, both eosinophils and macrophages were trending toward reductions in allergic mice given LPS, and blocking NF-κB pathways may have further augmented these reductions. We did not determine if chemokine induction or production was changed by TCH-013 treatment, which might explain the reduced accumulation of inflammatory cells in BALF.

Binding and activation of TLR4 in airway macrophages and epithelial cells by endotoxin should stimulate neutrophil chemokine production (e.g., MIP-2, KC) (Schmal et al., 1996; Krakauer, 2002; Skerrett et al., 2004) by pathways that involve NF-κB

activation (Mizgerd et al., 2004). Therefore we were surprised that TCH-013 –induced reductions in BALF PMN accumulation were not significant. We used a dose of the inhibitor that was effective in reducing circulating TNFα in LPS-treated BALB/c mice (Lansdell et al., 2012), and that in dose ranging studies inhibited airway LPS-induced neutrophil recruitment by 60% (data not shown). It is possible that LPS-induced neutrophil accumulation in inflamed allergic airways involves activation of additional cell populations (epithelial cells, macrophages, eosinophils, Th17 cells) that do not participate in healthy, naïve airways. Higher doses of TCH-013 may be required to neutralize NF-κB in this enhanced inflammatory environment. Future studies are required to test this hypothesis. We did notice however, that treatment with TCH-013 resulted in a moderate decrease in the severity of peribronchial/perivascular inflammation, mostly by reducing the density of neutrophils in these regions. This finding supports the possibility that we have some effects on neutrophil recruitment and that higher doses of the inhibitor may further decrease neutrophil accumulations.

Despite the lack of significant effects on airway neutrophil accumulation, inhibition of NF-kB led to a dramatic increase in airway resistance in OVA-LPS mice. Inhaled methacholine binds to muscarinic receptors (Gq) on airway smooth muscle, which activates phospholipase C to release of inositol trisphosphate, and is followed by an increase in intracellular calcium that interacts with contractile proteins. Studies in allergic mice and guinea pigs demonstrate that enhanced airway contractility is due to an increase in Rho protein, which is critical to Rho-kinase mediated smooth muscle contraction (Schaafsma et al., 2004; Witzenrath et al., 2008). Results in vascular smooth muscle show that LPS-induced NF-kB activation leading to an increase in iNOS

expression and downregulation of Rho and Rho-associated kinase activity (Wei et al., 2006).

It is well established that upregulation of iNOS in smooth muscle by LPS is mediated by NF-kB activation (Hattori et al., 2003; Pingle et al., 2003; Gomez et al., 2005). Therefore, the large increase in AHR with NF-kB inhibition we observed in LPS-OVA mice may be explained by changes to smooth muscle cells, and not related to inflammatory lesions we described in lung tissues.

A limitation of this study is that we did not confirm that NF-kB was affected by our treatment with TCH-013. Although we used doses that were effective in other animal models of inflammation (Lansdell et al., 2012) and TCH-013 selectively inhibit the 20S catalytic core of the proteasome and prevents IKK-α degradation (Lansdell et al., 2013), we did not confirm the effects in pulmonary tissue from treated animals. TCH-013 clearly had effects on AHR and inflammatory cell infiltrations, but the effects on the proteasome need to be assessed.

4.6 Summary

Intervention with an NF-kB inhibitor in an OVA-LPS mouse model of asthma exacerbation produced some interesting and unexpected results. The attenuation of allergic AHR was reversed, with airway resistance in OVA-LPS mice being even greater than in OVA mice. However NF-kB had no effect on LPS induced neutrophil influx, the major histopathological difference from OVA mice. While results in Chapter 3 suggested no role for neutrophils in modulating AHR, the lack of effect by TCH-013 on their infiltration is puzzling, especially when eosinophil granulocytes were decreased in these same mice. Although the effects on inflammatory cell populations were unexpected, the results support the hypothesis that LPS-mediated alterations of allergic AHR were dependent on NF-κB activation.

Airway LPS activates many cell types in the lung that can influence AHR. Smooth muscle contraction induced by methacholine can be opposed by NO produced internally or by neighboring cells in the airway wall, including epithelial cells and fibroblasts. Among many inflammatory genes, LPS is well documented to upregulate iNOS by NF-kB pathways in both epithelial and smooth muscle cells. In the next chapter the role iNOS in LPS modulation of allergic AHR will be assessed.

REFERENCES

REFERENCES

Bao Z, Lim S, Liao W, Lin Y, Thiemermann C, Leung B P and Wong W S F. 2007. Glycogen Synthase Kinase-3b Inhibition Attenuates Asthma in Mice. American Journal of Respiratory and Critical Care Medicine **176**(5): 431-438.

Breysse P N, Diette G B, Matsui E C, Butz A M, Hansel N N and McCormack M C. 2010. Indoor Air Pollution and Asthma in Children. Proceedings of the American Thoracic Society **7**(2): 102.

Chen Y, Kam C S K, Liu F Q, Liu Y, Lui V C H, Lamb J R and Tam P K H. 2008. Lps-Induced up-Regulation of Tgf-B Receptor 1 Is Associated with Tnf-Î± Expression in Human Monocyte-Derived Macrophages. Journal of Leukocyte Biology **83**(5): 1165-1173.

Chuang K-H, Peng Y-C, Chien H-Y, Lu M-L, Du H-I and Wu Y-L. 2013. Attenuation of Lps-Induced Lung Inflammation by Glucosamine in Rats. American Journal of Respiratory Cell and Molecular Biology **49**(6): 1110-1119.

De Stefano D, Coletta C, Bianca R E d V, Falcone L, Angelo I, Ungaro F, Quaglia F, Carnuccio R and Sorrentino R. 2013. A Decoy Oligonucleotide to Nf-κb Delivered through Inhalable Particles Prevents Lps-Induced Rat Airway Inflammation. American Journal of Respiratory Cell and Molecular Biology **49**(2): 288-295.

Desmet C, Gosset P, Pajak B, Cataldo D, Bentires-Alj M, Lekeux P and Bureau F. 2004. Selective Blockade of Nf-Kappa B Activity in Airway Immune Cells Inhibits the Effector Phase of Experimental Asthma. Journal of Immunology **173**(9): 5766-5775.

Donovan C E, Mark D A, He H Z, Liou H C, Kobzik L, Wang Y S, De Sanctis G T, Perkins D L and Finn P W. 1999. Nf-Kappa B/Rel Transcription Factors: C-Rel Promotes Airway Hyperresponsiveness and Allergic Pulmonary Inflammation. Journal of Immunology **163**(12): 6827-6833.

Doreswamy V and Peden D B. 2011. Modulation of Asthma by Endotoxin. Clinical and Experimental Allergy **41**(1): 9-19.

Edwards M R, Bartlett N W, Clarke D, Birrell M, Belvisi M and Johnston S L. 2009. Targeting the Nf-Kappa B Pathway in Asthma and Chronic Obstructive Pulmonary Disease. Pharmacology & Therapeutics **121**(1): 1-13.

Gomez A B, MacKenzie C, Paul A and Plevin R. 2005. Selective Inhibition of Inhibitory Kappa B Kinase□Î² Abrogates Induction of Nitric Oxide Synthase in Lipopolysaccharidee□Stimulated Rat Aortic Smooth Muscle Cells. British Journal of Pharmacology **146**(2): 217-225.

Harkema J R and Hotchkiss J A. 1992. In Vivo Effects of Endotoxin on Intraepithelial Mucosubstances in Rat Pulmonary Airways. Quantitative Histochemistry. Am J Pathol. 1992 Aug;141(2):307-17.

Hart L, Lim S, Adcock I, Barnes P J and Chung K F. 2000. Effects of Inhaled Corticosteroid Therapy on Expression and DNA-Binding Activity of Nuclear Factor Kappa B in Asthma. American Journal of Respiratory and Critical Care Medicine **161**(1): 224-231.

Hattori Y, Hattori S and Kasai K. 2003. Lipopolysaccharide Activates Akt in Vascular Smooth Muscle Cells Resulting in Induction of Inducible Nitric Oxide Synthase through Nuclear Factor-Kappa B Activation. European Journal of Pharmacology **481**(2): 153-158.

Heidenfelder B L, Reif D M, Harkema J R, Hubal E A C, Hudgens E E, Bramble L A, Wagner J G, Morishita M, Keeler G J and Edwards S W. 2009. Comparative Microarray Analysis and Pulmonary Changes in Brown Norway Rats Exposed to Ovalbumin and Concentrated Air Particulates. Toxicological Sciences **108**(1): 207-221.

Ip W K, Wong C K, Wang C B, Tian Y P and Lam C W K. 2005. Interleukin-3, -5, and Granulocyte Macrophage Colony-Stimulating Factor Induce Adhesion and Chemotaxis of Human Eosinophils Via P38 Mitogen-Activated Protein Kinase and Nuclear Factor Îºb. Immunopharmacology and Immunotoxicology **27**(3): 371-393.

Kahlon D K, Lansdell T A, Fisk J S, Hupp C D, Friebe T L, Hovde S, Jones A D, Dyer R D, Henry R W and Tepe J J. 2009. Nuclear Factor-Kappa B Mediated Inhibition of Cytokine Production by Imidazoline Scaffolds. Journal of Medicinal Chemistry **52**(5): 1302-1309.

Kawai T and Akira S. 2007. Signaling to Nf-Kb by Toll-Like Receptors. Trends in molecular medicine **13**(11): 460-469.

Kelly F J and Fussell J C. 2011. Air Pollution and Airway Disease. Clinical & Experimental Allergy **41**(8): 1059-1071.

Kim D Y, Ryu S Y, Lim J E, Lee Y S and Ro J Y. 2007. Anti-Inflammatory Mechanism of Simvastatin in Mouse Allergic Asthma Model. European Journal of Pharmacology **557**(1): 76-86.

Koga H, Miyahara N, Fuchimoto Y, Ikeda G, Waseda K, Ono K, Tanimoto Y, Kataoka M, Gelfand E W, Tanimoto M and Kanehiro A. 2013. Inhibition of Neutrophil Elastase Attenuates Airway Hyperresponsiveness and Inflammation in a Mouse Model of Secondary Allergen Challenge: Neutrophil Elastase Inhibition Attenuates Allergic Airway Responses. Respiratory Research **14**.

Krakauer T. 2002. Stimulant-Dependent Modulation of Cytokines and Chemokines by Airway Epithelial Cells: Cross Talk between Pulmonary Epithelial and Peripheral Blood Mononuclear Cells. Clinical and diagnostic laboratory immunology **9**(1): 126-131.

Lansdell T A, Hurchla M A, Xiang J Y, Hovde S, Weilbaecher K N, Henry R W and Tepe J J. 2013. Noncompetitive Modulation of the Proteasome by Imidazoline Scaffolds Overcomes Bortezomib Resistance and Delays Mm Tumor Growth in Vivo. Acs Chemical Biology **8**(3): 578-587.

Lansdell T A, O'Reilly S, Woolliscroft T, Azevedo L M, Kahlon D K, Hovde S, McCormick J J, Henry R W, Cornicelli J A and Tepe J J. 2012. Attenuation of Collagen-Induced Arthritis by Orally Available Imidazoline-Based Nf-Kb Inhibitors. Bioorganic & Medicinal Chemistry Letters **22**(14): 4816-4819.

Lee S-H, Seo M, Choi S, Sohn Y, Kang K, Ahn B, Kwon J and Yoo M. 2005. Da-9201 Shows Anti-Asthmatic Effects by Suppressing Nf-κ Expression in an Ovalbumin-Induced Mouse Model of Asthma. Archives of Pharmacal Research **28**(12): 1350-1357.

Liao X, Sharma N, Kapadia F, Zhou G, Lu Y, Hong H, Paruchuri K, Mahabeleshwar G H, Dalmas E and Venteclef N. 2011. Kruppel-Like Factor 4 Regulates Macrophage Polarization. The Journal of clinical investigation **121**(7): 2736.

Mizgerd J, Lupa M and Spieker M. 2004. Nf-Kappab P50 Facilitates Neutrophil Accumulation During Lps-Induced Pulmonary Inflammation. BMC Immunology **5**(1): 10.

Pingle S C, Sanchez J F, Hallam D M, Williamson A L, Maggirwar S B and Ramkumar V. 2003. Hypertonicity Inhibits Lipopolysaccharide-Induced Nitric Oxide Synthase Expression in Smooth Muscle Cells by Inhibiting Nuclear Factor Îºb. Molecular Pharmacology **63**(6): 1238-1247.

Porta C, Rimoldi M, Raes G, Brys L, Ghezzi P, Di Liberto D, Dieli F, Ghisletti S, Natoli G and De Baetselier P. 2009. Tolerance and M2 (Alternative) Macrophage Polarization Are Related Processes Orchestrated by P50 Nuclear Factor Îºb. Proceedings of the National Academy of Sciences **106**(35): 14978-14983.

Poynter M E, Irvin C G and Janssen-Heininger Y M W. 2002. Rapid Activation of Nuclear Factor-Kappa B in Airway Epithelium in a Murine Model of Allergic Airway Inflammation. American Journal of Pathology **160**(4): 1325-1334.

Poynter M E, Irvin C G and Janssen-Heininger Y M W. 2003. A Prominent Role for Airway Epithelial Nf-Kappa B Activation in Lipopolysaccharide-Induced Airway Inflammation. Journal of Immunology **170**(12): 6257-6265.

Prado C M, Leick-Maldonado E A, Yano L, Leme A S, Capelozzi V L, Martins M A and Tiberio I F L C. 2006. Effects of Nitric Oxide Synthases in Chronic Allergic Airway Inflammation and Remodeling. American Journal of Respiratory Cell and Molecular Biology **35**(4): 457-465.

Schaafsma D, Gosens R, Bos I S T, Meurs H, Zaagsma J and Nelemans S A. 2004. Allergic Sensitization Enhances the Contribution of Rhoâ€□Kinase to Airway Smooth Muscle Contraction. British Journal of Pharmacology **143**(4): 477-484.

Schmal H, Shanley T P, Jones M L, Friedl H P and Ward P A. 1996. Role for Macrophage Inflammatory Protein-2 in Lipopolysaccharide-Induced Lung Injury in Rats. The Journal of Immunology **156**(5): 1963-1972.

Sheller J R, Polosukhin V V, Mitchell D, Cheng D S, Peebles R S and Blackwell T S. 2009. Nuclear Factor Kappa B Induction in Airway Epithelium Increases Lung Inflammation in Allergen-Challenged Mice. Experimental Lung Research **35**(10): 883-895.

Shimizu K, Konno S, Ozaki M, Umezawa K, Yamashita K, Todo S and Nishimura M. 2012. Dehydroxymethylepoxyquinomicin (Dhmeq), a Novel Nfâ€□Kappab Inhibitor, Inhibits Allergic Inflammation and Airway Remodelling in Murine Models of Asthma. Clinical & Experimental Allergy **42**(8): 1273-1281.

Skerrett S J, Liggitt H D, Hajjar A M, Ernst R K, Miller S I and Wilson C B. 2004. Respiratory Epithelial Cells Regulate Lung Inflammation in Response to Inhaled Endotoxin. American Journal of Physiology-Lung Cellular and Molecular Physiology **287**(1): L143-L152.

Tanaka H, Kanako S and Abe S. 2005. Prostaglandin E2 Receptor Selective Agonists E-Prostanoid 2 and E-Prostanoid 4 May Have Therapeutic Effects on Ovalbumin-Induced Bronchoconstriction*. CHEST Journal **128**(5): 3717-3723.

Vawda S, Mansour R, Takeda A, Funnell P, Kerry S, Mudway I, Jamaludin J, Shaheen S, Griffiths C and Walton R. 2014. Associations between Inflammatory and Immune Response Genes and Adverse Respiratory Outcomes Following Exposure to Outdoor Air Pollution: A Huge Systematic Review. American journal of epidemiology **179**(4): 432-442.

Wang T, Moreno-Vinasco L, Huang Y, Lang G D, Linares J D, Goonewardena S N, Grabavoy A, Samet J M, Geyh A S and Breysse P N. 2008. Murine Lung Responses to Ambient Particulate Matter: Genomic Analysis and Influence on Airway Hyperresponsiveness. Environmental Health Perspectives **116**(11): 1500-1508.

Wei C-Y, Huang K-C, Chou Y-H, Hsieh P-F, Lin K-H and Lin W-W. 2006. The Role of Rho-Associated Kinase in Differential Regulation by Statins of Interleukin-1î²- and Lipopolysaccharide-Mediated Nuclear Factor Îºb Activation and Inducible Nitric-Oxide Synthase Gene Expression in Vascular Smooth Muscle Cells. Molecular Pharmacology **69**(3): 960-967.

Witzenrath M, Ahrens B, Schmeck B, Kube S M, Hippenstiel S, Rosseau S, Hamelmann E, Suttorp N and SchÃ¼tte H. 2008. Rho-Kinase and Contractile Apparatus Proteins in Murine Airway Hyperresponsiveness. Experimental and Toxicologic Pathology **60**(1): 9-15.

CHAPTER 5

ROLE OF *Nos2* AND *Arg1* IN THE ATTENUATION OF AIRWAY HYPERRESPONSIVENESS IN ALLERGIC MICE EXPOSED TO INHALED LIPOPOLYSACCHARIDE

5.1 ABSTRACT

Nitric Oxide (NO) is thought to be both beneficial and deleterious to the asthmatic lung. This dual function appears to be both dose and cell specific. NO is a broncho- and vaso-dilator with anti-inflammatory properties. High NO concentration, commonly associated with iNOS induction, has been implicated in increased inflammation, promoting pathology, and AHR in asthma. In this study, I examined pulmonary expression of *Nos2* as well as *Arg1* mRNA, to determine if changes in these genes were associated with the attenuation of AHR to methacholine displayed in mice sensitized and challenged with OVA followed by inhalation exposure to LPS. In addition, I evaluated tissues from my studies of mice with established allergic airways disease treated with the vehicle or NF-κB inhibitor prior to LPS exposure to determine if the reversal of the attenuation in AHR present in OVA-LPS mice was associated with changes in *Nos2*. Lung tissues taken from BALB/c mice with OVA-induced allergic airways disease with and without LPS exposure and with or without treatment with a novel NF-κB inhibitor were analyzed by RT-PCR. Also, tissues were stained with an anti-iNOS antibody to determine protein expression within the lung. Compared to saline mice neither *Nos2* nor *Arg1* expression were upregulated in LPS mice and OVA mice had no change in *Nos2*, but had a significant increase in *Arg1* expression. OVA-LPS mice had significant increase in *Nos2* and *Arg1* expression compared to all other groups. Inhibition of NF-κB did not affect *Nos2* and *Arg1* expression in saline and OVA mice; however, there was a significant attenuation in expression of both genes in OVA-LPS mice. Furthermore, staining for the iNOS protein was not present in saline mice with or without NF-κB inhibition. In OVA mice, positive staining of iNOS was

predominately present in the epithelium of small pre-terminal bronchioles and terminal bronchioles, ciliated epithelial cells in the central conducting airway, and small numbers of positive staining inflammatory cells (macrophages) were in peribronchiolar and perivascular tissue. NF-κB inhibition did not affect positive iNOS staining in OVA mice. In OVA-LPS mice positive iNOS staining in the lung displayed a similar pattern as OVA mice, however, there was increased positive staining in inflammatory cells, consistent with macrophages and neutrophils, present in parenchymal tissue and alveolar spaces in addition to peribronchiolar and perivascular regions. Following NF-κB inhibition, positive staining was less intense and was present in fewer inflammatory cells compared to vehicle treated OVA-LPS mice. These results support a role for iNOS-mediated NO production in the attenuation of AHR to methacholine in my model of allergic mice exposed to LPS.

5.2 INTRODUCTION

Nitric oxide (NO) is a biological mediator of diverse physiological functions produced in a variety of cell types in many tissues (Ricciardolo et al., 2004). In the lung NO has been shown to be produced by neutrophils, macrophages, airway epithelium, fibroblasts, smooth muscle cells, and nonadrenergeric noncholinergic nerves (Gaston et al., 1994). Under normal physiological conditions, NO is a dilator of airways and blood vessels (Prado et al., 2011). The role(s) of NO in lung diseases, such as asthma, has not been definitively established. In spite of its known contribution to bronchodilation, NO measured in exhaled breath (FeNO) has been shown to be increased in asthmatics with severe asthma phenotypes, and has therefore been used to estimate airway inflammation (Dweik et al., 2010; Aytekin and Dweik, 2012; Yamamoto et al., 2012). High levels of NO may promote pro-inflammatory responses, such as an increase in neutrophils, eosinophils, and monocytes (Prado et al., 2006). I hypothesized that the upregulation of lung expression of *Nos2* was associated with attenuation of AHR in OVA-LPS mice.

NO is produced by the metabolism of L-arginine by the enzyme nitric oxide synthase (NOS), which exists as three isoforms: neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2), and endothelial NOS (eNOS or NOS3). Compared to constitutive isoforms (also referred to as cNOS), iNOS produces large amounts of NO, nM to μM respectively (Meurs et al., 2003; Prado et al., 2006). It has been proposed that increased NO production during oxidative stress in asthmatic lungs results in the formation of peroxynitrite ($ONOO^-$) from the reaction of NO with superoxide (O_2^-).

Further evidence demonstrates the importance of spatial and temporal production of NO to airway dilatation in asthma. For example impaired release of NO is associated with downregulation of eNOS in bronchial epithelium after allergen-induced AHR in asthmatics (Ricciardolo et al., 2001). Thus, the role of NO in asthma pathogenesis is isoform-and tissue-specific.

Alterations in NO production during asthma may be dictated by the competition for L-arginine, the common substrate of both NOS and arginase (Arg) enzymatic pathways (Maarsingh et al., 2009; Vonk et al., 2010). Increased arginase-1 (Arg-1) activity is associated with airway remodeling, decreased NO production by cNOS; and upregulation of iNOS and its increased production of peroxynitrite (Benson et al., 2011). Under normal physiological conditions, Arg-1 and NOS enzymes are counter-regulatory, with each producing intermediate metabolites that are inhibitory to the other enzyme (Meurs et al., 2003; Maarsingh et al., 2008).

Paradoxically, iNOS upregulation could potentially alleviate airway constriction by producing concentrations of NO that counteract the effects of Arg-1 (Hjoberg et al., 2004). In the study reported in chapter 2, I showed that while allergic (OVA) mice exposed to LPS had increased pulmonary inflammation, they also had attenuated allergen-associated AHR when induced by inhaled methacholine. Importantly, these mice demonstrated an upregulation in the NOS2 gene (i.e, iNOS). In the study reported in chapter 4, NF-κB inhibition reversed the inhibitory effect of LPS on allergen-associated AHR. The goal of the present investigation was to distinguish alterations in gene and protein expression of iNOS and Arg-1 in airway tissues in order to test the

hypothesis that attenuation of AHR by LPS in asthmatic mice is dependent on iNOS expression in the lungs. Previously, I have shown that LPS exposure increases iNOS gene expression in allergic mice (Chapter 2). In the current investigation I expect that OVA-LPS mice treated with a NF-κB inhibitor will have a decrease in iNOS gene expression that is associated with the increase in AHR.

5.3 MATERIALS AND METHODS

5.3.1 Animals and Treatment Protocols.

Evaluated tissues were taken from BALB/c mice sensitized, challenged, and exposed to LPS as previously described in Chapter 2. Some animals were treated with the TCH-013, an inhibitor of NF-κB, as described in detail in Chapter 4.

5.3.2 Relative Quantification Real-Time PCR

Right caudal lung lobes were removed and placed in RNAlater (Qiagen, Valencia, CA) where it was kept at 4°C for 24 hours and then stored at -20°C until processed and analyzed. Total RNA was isolated from the right caudal lung lobe using Rneasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Tissues were homogenized using Qiagen's TissueLyser II Bead Beater and three 2.8mm Zirconium Oxide beads in 600µl buffer RLT containing β -Mercaptoethanol. Homogenates were then centrifuged at 12,000g for 3 minutes and RNA was purified from the supernatant using the RNeasy capture column. Eluted RNA was diluted 1:5 with Rnase free water and quantified using the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltman, MA). Reverse transcription was accomplished by using High Capacity cDNA Reverse Transcription Kit reagents (Applied Biosystems). Each RT reaction was run in a 50 µl reaction volume containing 5 µg of total RNA with cDNA Master Mix prepared according to the manufacturer's protocol. The reaction mixture was incubated in a in GeneAmp PCR System 9700 Thermocycler PE (Applied Biosystems, Foster City, CA) at 25ºC for 10 Minutes, 37ºC for 2 hours, then held at 4º.

Relative quantitative mRNA expression analysis of *Nos2* and *Arg1* genes was conducted on an ABI PRISM 7900 HT Sequence Detection System at Michigan State University's Research Technology Support Facility using Taqman Gene Expression Assay reagents (Applied Biosystems). 2µl cDNA and 8µl reagents were dispensed (in duplicate) into a 384-well reaction plate. The cycling parameters were 48 °C for 2 minutes, 95 °C for10 minutes, and 50 cycles of 95 °C for 15 seconds followed by 60 °C for 1 minute

Gene expression levels were reported as fold-change (FC) of mRNA in experimental samples compared to a control sample. Real-time PCR amplifications were relatively quantified using the ΔΔCt method. Following the PCR, amplification plots (change in dye fluorescence versus cycle number) were examined and a dye fluorescence threshold within the exponential phase of the reaction was set for the target gene and the endogenous references. The cycle number at which each amplified product crosses the set threshold represents the C_T value. The amount of target gene normalized to its endogenous reference was calculated by subtracting the geometric mean of the C_Ts from endogenous controls (*Arbp, Gusb, and Gapdh*) from the target gene C_T (delta Ct (ΔCt)) This normalization strategy has been utilized for accurate RT-PCR expression profiling in biological samples with small expression differences (Vandesompele et al., 2002). The ΔCt value for the experimental sample is subtracted from the ΔCt value of the corresponding control sample (ΔΔCt). The FC in experimental samples relative to control samples is then calculated as: $FC= 2^{-\Delta\Delta Ct}$.

5.3.3 Western blot analysis of iNOS and arginase-1 proteins in the lung

The cranial lung lobes were homogenized as described previously (Kang et al., 2010). Briefly, frozen lungs were ground and sonicated in 7 M urea containing protease inhibitors for 10 seconds (3 times) and centrifuged at 13,000g for 15 minutes at 4°C. Following protein quantification, supernatants were pooled from the same group. 15 µg of total lysate protein from pooled samples were subject to electrophoresis on 10-20% SDS-polyacrylamide gels before transferring to PVDF membranes. HRP-conjugated secondary antibody (GE Life Sciences) followed by enhanced chemiluminescent ECL substrate was used to develop the blots according to the manufacturer's instructions. Primary antibodies were used to detect the expression of iNOS (AbCam, Cambridge, MA) and Arg-1 (Santa Cruz, CA) proteins.

5.3.4 Immunohistochemistry

Paraffin-embedded sections of the proximal lung (G5) were prepared from formalin-fixed tissues and prepare by routine immunohistochemical techniques to detect iNOS (sc-7271, Santa Cruz Biotechnology).

5.3.5 Statistical Analysis

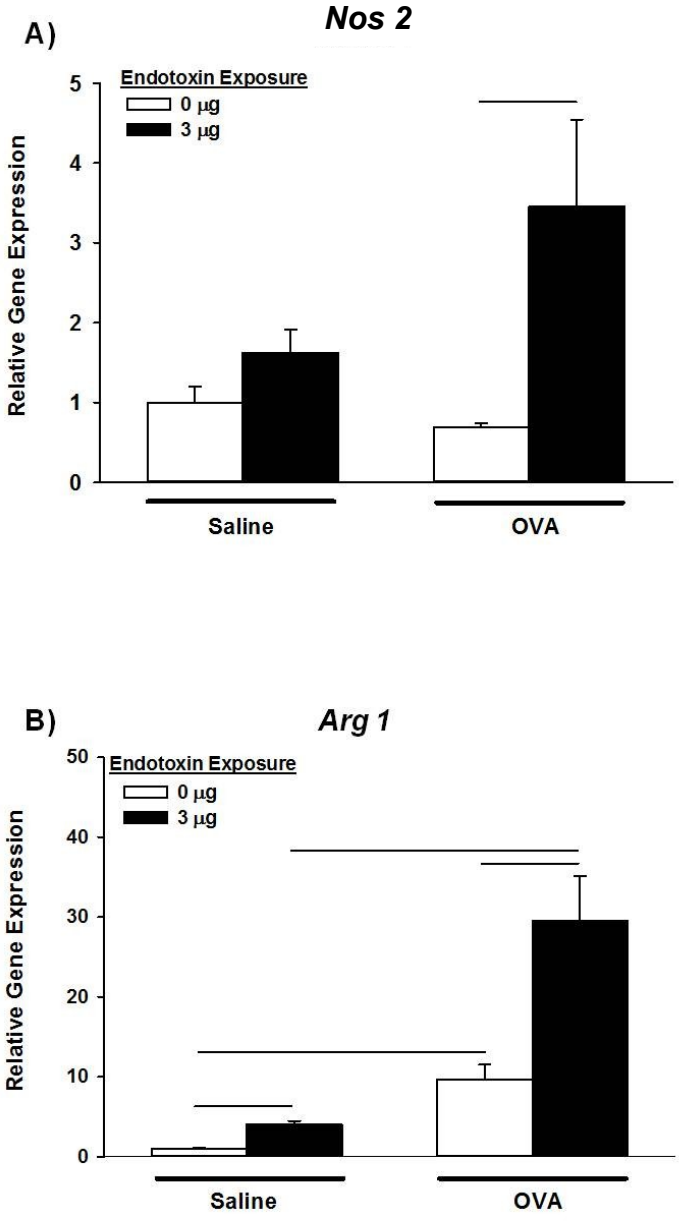
Data were expressed as group means $\pm$ the standard error of the mean (mean $\pm$ SEM). Grubb's outlier test was used to determine and remove statistical outliers. Statistical differences between ΔCt values between groups were statistically determined with two-way ANOVA with Student-Newman-Keuls post hoc test to make direct comparison between groups; $p \leq 0.05$ (SigmaPlot, Systat Software Inc, San Jose, CA).

5.4 RESULTS

5.4.1 Lung tissue *Nos2* and *Arg1* mRNA expression

To assess differences in the expression of genes involved in L-arginine metabolism, the pulmonary expression of the *Nos2* gene that produces the protein iNOS, and the *Arg1* gene that induces arginase-1, were evaluated by RT-PCR. Twenty-four hours following LPS exposure, *Nos2* expression was not significantly increased in LPS or OVA alone mice (Fig 21 A, saline, black bar; OVA, white bar). OVA-LPS mice had significant, 3-fold increase in *Nos2* expression compared to OVA alone (Fig 21 A; OVA, black bar). *Arg1* was upregulated by 3-fold in LPS mice (Fig 21 B; saline, black bar) in reference to control mice. There was a 9-fold increase in the expression of *Arg1* in OVA mice (Fig 21 B; OVA, white bar). Additionally, OVA-LPS mice had significant, 3-fold increases in *Arg1* expression compared to OVA alone (Fig 21 B; OVA, black bar).

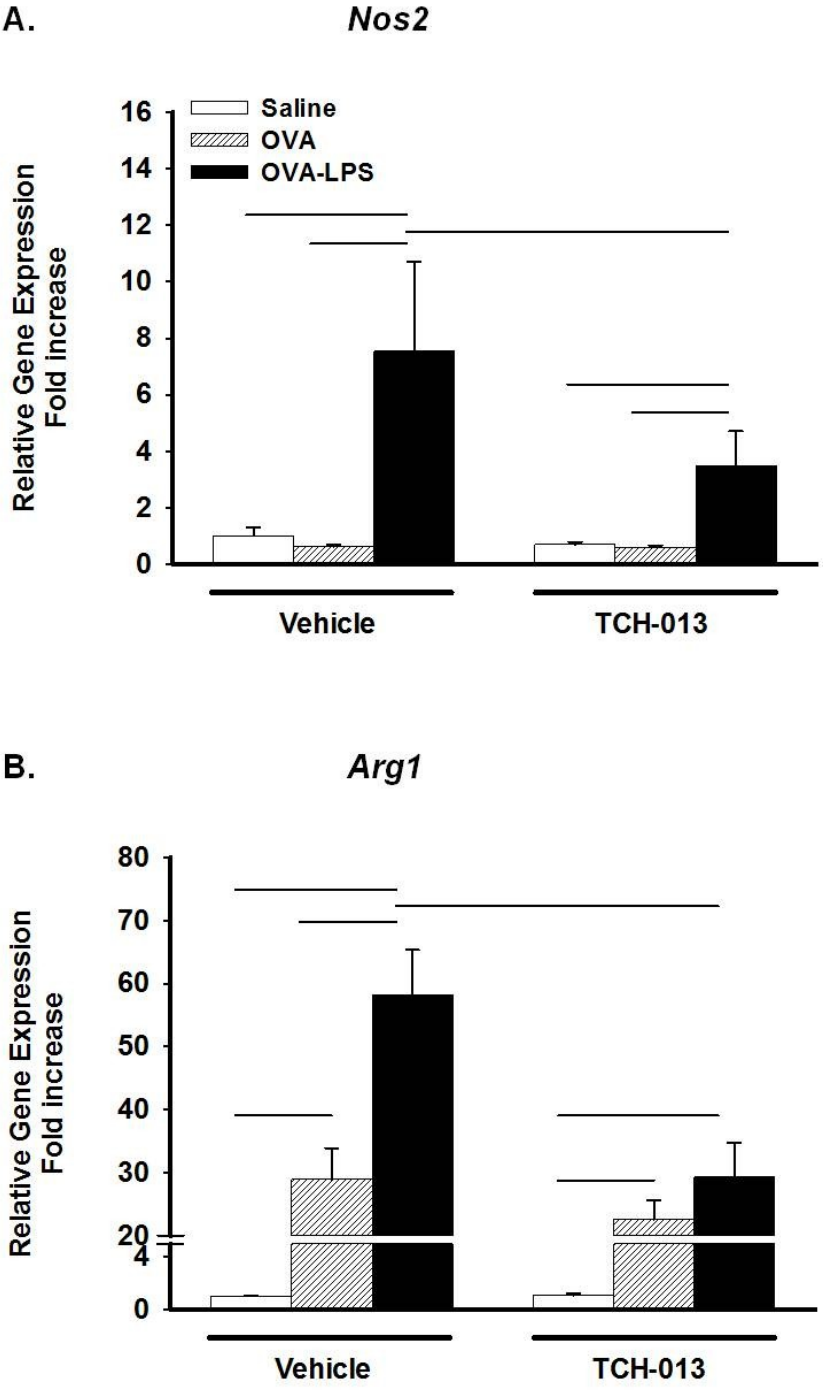
Figure 21. Pulmonary gene expression of *Nos2* and *Arg1* in allergic and non-allergic mice exposed to LPS. Relative expressions of mRNA for *Nos2* (A) and *Arg1* (B) were determined as described in Materials and Methods. Values are expressed as fold increase of mRNA expression compared to control (Saline) animals (n=5-6). Horizontal lines indicate significant difference; $p < 0.05$.



5.4.2 Effects of NF-κB inhibition on pulmonary *Nos2* and *Arg1* expression

Nos2 expression in OVA mice was not significantly different from control mice, regardless of treatment with NF-κB inhibitor (Fig 22 A; diagonal striped bar). In OVA-LPS mice, *Nos2* expression was increased almost 8-fold, however treatment with the NF-κB inhibitor TCH-013 blocked expression by 50%, such that relative increases were less than 4-fold compared to control mice treated with vehicle (Fig 22 A, black bars). OVA-LPS mice had significant increased expression of Nos2 compared to OVA alone mice regardless of treatment. Similarly, OVA mice had an almost 30-fold increase in expression compared to saline mice, which was not significantly affected with the NF-κB inhibitor (Fig 22 B; diagonal striped bar). There was a 60-fold and 2-fold increase in *Arg1* expression in OVA-LPS animals compared to saline mice and OVA alone mice respectively. This increase was significantly inhibit by 50% with the NF-κB inhibitor treatment when compared to untreated OVA-LPS mice resulting in expression levels similar to treated OVA mice (fig 5-2 B; black bars).

Figure 22. Effect of NF-κB inhibition on pulmonary gene expression of *Nos2* and *Arg1*. In lung lobes from control and NF-κB inhibitor treated (TCH-013) mice, relative expressions of mRNA for NOS2 and Arg1 were determined. Values are expressed as fold increase of mRNA expression compared to control (Saline) animals (n=5-6). Horizontal lines indicate significant difference; $p < 0.05$.



5.4.3 Protein expression of iNOS and Arg-1

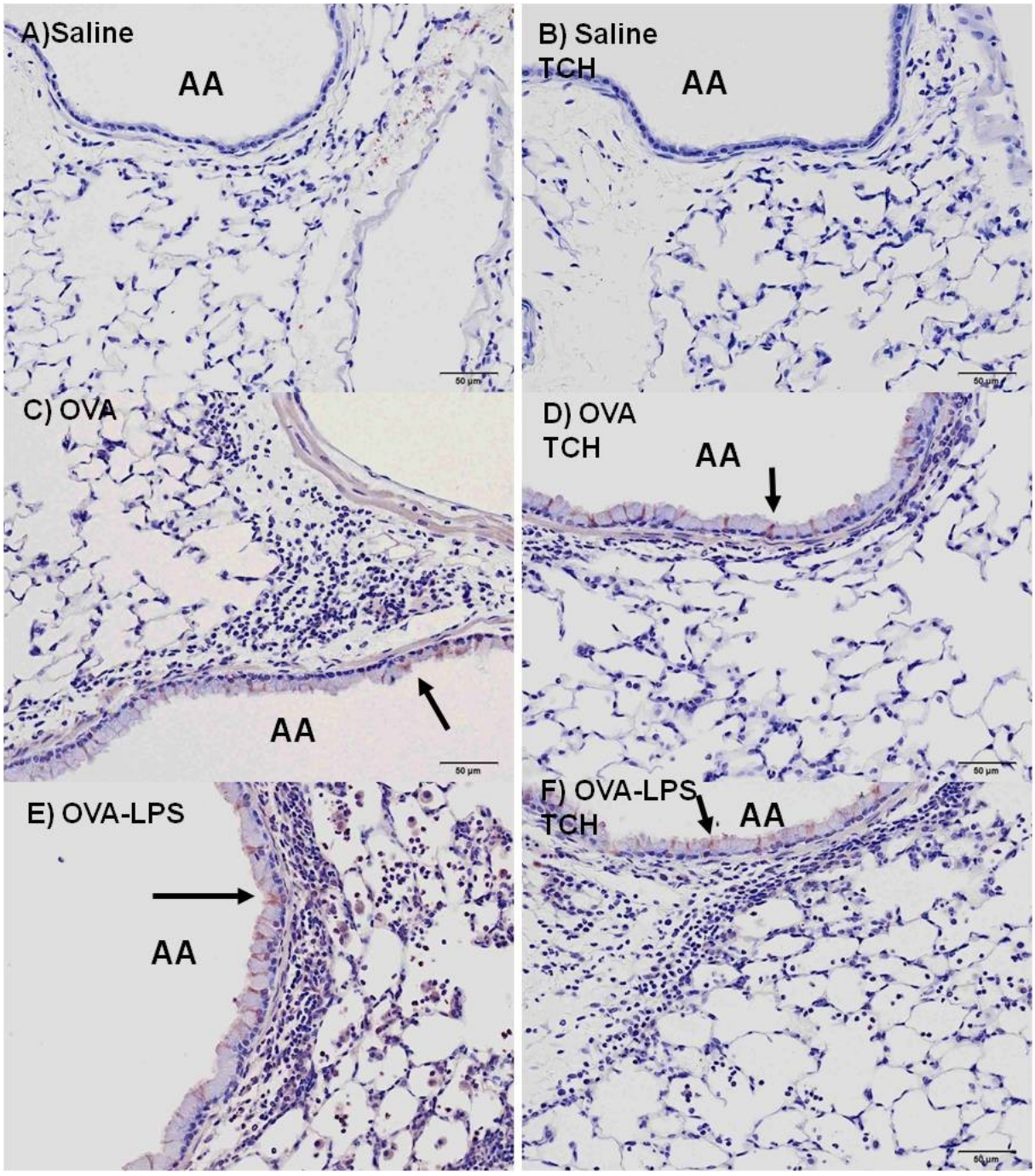
Using frozen right cranial lung lobes, protein was extracted and analyzed by western blot. After two separate runs iNOS protein could not be visualized in the tissue samples. Arg-1 protein was detected in the lung tissues of OVA and OVA-LPS mice, but not in LPS alone mice (data not shown). However, quantification of protein expression could not be obtained from the study samples. These results showed that western blot analysis of iNOS and Arg-1 proteins did not work in the desired manner. Several factors could have contributed to the inability of this approach to generate reliable data. The use of suboptimal antibodies for the tissues used in this study could be a major factor in ineffective data generation. Therefore, more stringent optimization of the antibodies used in this study to determine best dilutions and incubation times may have be needed. Also, the quantity and quality of the samples used may not have been adequate to generate data. For example, the protein of interest may not be abundantly present in the entire tissue. Therefore, maximizing the signal of the protein by only analyzing specific tissue, such as airway epithelium, may generate more protein than utilizing the entire lung tissue. This issue may be particularly true for iNOS protein expression.

5.4.4 Immunohistochemistry for iNOS

Proximal (G5) sections of lung tissue were stained with an anti-iNOS antibody to detect iNOS positive cells. Saline mice had occasional positive staining of airway epithelium in central and peripheral airways; however, there were no positive cells in mice treated with NF-κB (Fig 5-3 A-B). In OVA mice, the axial airways contained positive intracytoplasmic staining only in ciliated epithelial cells. More diffuse positive

staining was present in the epithelium of smaller peripheral airways, pre-terminal bronchioles, and distal terminal bronchioles. Additionally, there were occasional positive staining mononuclear cells morphologically consistent with macrophages present in peribronchiolar and perivascular tissue and alveolar spaces. NF-κB inhibition did not alter staining in OVA mice (Fig 23 C-D). Compared to OVA alone, lungs from OVA-LPS animals had similar positive staining in airway epithelium consisting of staining in the ciliated epithelium of the axial airways and more diffuse staining in pre-terminal and terminal bronchioles, however, there were increased numbers of positive staining inflammatory cells morphologically consistent with macrophages and neutrophils in peribronchiolar and perivascular tissue, alveolar septum, and alveolar spaces (Fig 23 E-F). Positive iNOS staining was less intense in the airway epithelium and inflammatory cells following NF-κB inhibition.

Figure 23. Immunohistochemical staining for iNOS. Lung sections without or with inhibition of NF-κB, respectively, were taken from saline (A and B), OVA (C and D), and OVA-LPS (E and F) mice and were probed with an anti-iNOS antibody. Positive staining is indicated by cytoplasmic brown staining. Arrows indicate positive staining in airway epithelium. AA, axial airway; BV, blood vessel; TB, terminal bronchioles; AD, alveolar duct. Original magnification, x20.



5.5 DISCUSSION

In this study my goal was to determine if changes in pulmonary iNOS following LPS exposure was related to the attenuation of AHR in asthmatic mice. I evaluated alterations in the expression of iNOS (*NOS2*) and arginase-1 (*Arg1*) genes in lungs from mice with or without OVA induced allergic airways disease exposed to intranasal saline or endotoxin, as well as in OVA and OVA-LPS mice treated with a novel NF-κB inhibitor. Results demonstrated that in OVA-LPS mice with the attenuated AHR phenotype, iNOS is upregulated compared to OVA mice, and NF-κB inhibition decreased iNOS expression in OVA-LPS mice, which is associated with a reversal of the attenuated AHR response. Additionally, *Arg1* expression was enhanced in OVA-LPS animals compared to OVA alone. This enhancement was also decreased with NF-κB inhibition. These results support my hypothesis that attenuation of airway hyperresponsiveness by LPS in asthmatic mice is dependent on *Nos2* expression in the lungs. Moreover, the data show increased *Arg1* expression is not necessary for the induction of AHR in asthma exacerbation. These findings could provide a better understanding of mechanisms by which environmental factors, such as endotoxin, can affect the asthmatic response which will lead to improved models of therapy for future studies.

Dysregulation of arginine metabolism has been associated with many diseases, such as vascular hypertension, sickle cell anemia, diabetes, and asthma (Morris et al., 2005; Benson et al., 2011; El-Bassossy et al., 2013). Current knowledge indicates that metabolism is controlled by opposing enzymes, particularly arginase and nitric oxide synthase. Arg-1 utilizes arginine to produce urea and ornithine, a precursor of proline, while, iNOS metabolizes arginine to produce citrulline and NO (Morris, 2008). Both

enzymes produce intermediate mediators that can effectively inhibit the opposing enzyme providing homeostasis in arginine metabolism.

NO deficiency due to decreased arginine substrate that is diverted to increase arginase activity is a proposed mechanism of asthma pathogenesis (Meurs et al., 2002; Larsson et al., 2005). Th2 cytokines IL-4 and -13 that are produced during asthma promote arginase activity (Hesse et al., 2001). It has been shown that asthmatics have increased arginase activity in sputum and decreased plasma arginine (Morris et al., 2004). Similarly, in asthma animal models there is increased expression and activity of arginase-1 (Kenyon et al., 2008; North et al., 2009; Maarsingh et al., 2011). Increased arginase has been associated with airway remodeling and AHR. NO is a bronchodilator, however, there have been many reports of its detrimental effects in asthma. These effects appear to be dose and cell specific. High NO concentrations cause vasodilation with edema, mucus hypersecretion, and enhanced Th2 inflammation contributing to asthma pathology (Prado et al., 2011). Also, it has been shown that large concentrations of NO interact with reactive oxygen species to produce peroxynitrite, which causes tissue damage and is may contribute to AHR (SadeghiHashjin et al., 1996; Saleh et al., 1998). Similar to asthmatics and other models of asthma, OVA induced a significant increase in arginase-1 gene expression compared to saline control mice in my model of allergic airway disease. However, there was no increase in iNOS expression, and even a slight decrease in gene expression compared to control animals.

LPS is one of the major inducers of the iNOS in responsive cells such as macrophages, epithelial and smooth muscle (Beck et al., 1999). However, it has been shown that LPS coinduces Arg-1 and iNOS (Sonoki et al., 1997). Inhaled LPS causes increased inflammation, primarily neutrophils, and increases AHR to methacholine (Hakansson et al., 2012). The mechanism of AHR following LPS inhalation is not entirely known. It is thought to be mediated through toll like receptor 4 (TLR-4) (Hollingsworth et al., 2004). In my studies, LPS treatment induced increased pulmonary neutrophils and AHR (Chapter 2). Twenty-four hours following LPS exposure, these mice had a small increase in iNOS mRNA but it was not significantly different from control animals. However, there was a significant increase in Arg-1 mRNA. These findings may be due to the specific time point of analysis. Sonoki and colleagues identified that iNOS mRNA and protein began to decrease 12 hours after i.p. LPS administration in mice, while Arg-1 mRNA and protein began to increase at 12 hours following LPS (Sonoki et al., 1997).

Inhaled LPS produces variable responses indicating that some people are less sensitive to its effects than others (Kline et al., 1999). It has been shown that asthmatics can be more sensitive to LPS and require smaller doses to produce AHR (Michel et al., 1989). However, several studies of experimental asthma have shown an attenuation of AHR with LPS exposure in OVA allergic animals (Tulic et al., 2002; Rodriguez et al., 2003). In my studies, OVA-LPS mice had attenuated AHR to methacholine compared to OVA and LPS mice alone. This attenuation was accompanied by an increase in iNOS and Arg-1. In the study by Rodriguez et al, *NOS2* deficient animals did not have an attenuation in AHR when exposed to LPS (Rodriguez

et al., 2003). These data support a role for iNOS modulation of AHR in my study. Also, the data suggest a more novel role for arginase in the induction of AHR. In this study, AHR in OVA-LPS mice was not associated with increased *Arg1* expression, but rather decrease expression in the lung.

The many roles of NO in asthma pathogenesis are complex. NO has been associated with enhanced inflammation and promotion of the pathological features of asthma, such as mucus hypersecretion, edema, and increased Th2 mediated inflammation (Prado et al., 2011). However, NO is a biological mediator of bronchodilation and produces anti-inflammatory effects ((Maarsingh et al., 2011). It has been suggested that NO can inhibit the transcription factor NF-κB. In my investigations, NF-κB inhibition reversed the attenuation in AHR in OVA-LPS mice (Chapter 4) and attenuated the increase in iNOS and Arg-1 in these mice. The increase in iNOS suggests that LPS enhances the production of NO in allergic mice. These results are in accordance with previous studies that have shown NO to decrease methacholine induced AHR in asthmatics (Kacmarek et al., 1996) and reversed methacholine bronchoconstriction in dogs (Brown et al., 1994). These findings support my hypothesis that NO may contribute to the attenuation of AHR in OVA-LPS mice.

Since iNOS is regulated predominately at the transcriptional level by NF-κB, *de novo* synthesis is required for gene activation and protein production (Korhonen et al., 2005). Increased iNOS expression has been demonstrated in smooth muscle, airway epithelium and inflammatory cells such as macrophages and neutrophils. Evaluation of iNOS expression in lung tissue by immunohistochemistry revealed that OVA increase

expression of iNOS predominately in epithelial cells of smaller peripheral airways and terminal bronchioles, while only ciliated epithelial cells were positive in the axial airway. OVA-LPS mice displayed a similar pattern of iNOS expression as OVA in alone mice with an additional increase in iNOS positive staining inflammatory cells consistent with macrophages and neutrophils in alveolar parenchyma and airspaces. Previous studies have demonstrated similar staining patterns for iNOS with OVA sensitization and challenge (Trifilieff et al., 2000; Zimmermann et al., 2003). NF-κB inhibition did not alter positive iNOS staining in OVA or OVA-LPS mice.

5.6 SUMMARY

At 72 hours after OVA challenge, there was not an upregulation of Nos2 expression in the lung, however positive staining with anti-iNOS antibody was present in peripheral airway and terminal bronchial epithelium. Exposure to LPS augmented this iNOS expression in allergic mice with positive staining of cells in areas of inflammation that were predominately macrophages and polymorphonuclear cells. NF-κB inhibition attenuated the increase in *Nos2* expression in OVA-LPS mice and lessened staining intensity of iNOS. Evaluation of *Arg1* expression showed that OVA increased expression which was augmented with LPS exposure in OVA-LPS mice. NF-κB inhibition also attenuated this increase in OVA-LPS mice, but did not affect OVA mice alone. These results support my hypothesis that attenuation of airway hyperresponsiveness by LPS in asthmatic mice is dependent on *Nos2* expression. This increased *Nos2* may contribute to increase nitric oxide production in the lungs resulting in bronchodilation. This study contributes to body of research that supports the potential use of NO as pharmacological modulator of asthma. However, it is important to confirm these findings by quantifying iNOS protein and determining NO production in lung, which was not be determined in this study. Additional studies should be conducted to address the limitations in this study involving iNOS and Arg-1 protein quantification and confirming NO production in OVA-LPS mice. Although iNOS expression was reduced by NF-κB inhibition, further analysis of direct iNOS inhibition should be conducted to exclude the contribution of modification of cytokines or proteins, such as Arg-1, to the effects seen in my model.

REFERENCES

REFERENCES

Aytekin M and Dweik R A. 2012. Nitric Oxide and Asthma Severity: Towards a Better Understanding of Asthma Phenotypes. Clinical and Experimental Allergy **42**(5): 614-616.

Beck K-F, Eberhardt W, Frank S, Huwiler A, Messmer U K, Muhl H and Pfeilschifter J. 1999. Inducible No Synthase: Role in Cellular Signalling. Journal of Experimental Biology **202**(6): 645-653.

Benson R C, Hardy K A and Morris C R. 2011. Arginase and Arginine Dysregulation in Asthma. J Allergy (Cairo) **2011**: 736319.

Brown R H, Zerhouni E A and Hirshman C A. 1994. Reversal of Bronchoconstriction by Inhaled Nitric Oxide: Histamine Versus Methacholine. American Journal of Respiratory and Critical Care Medicine **150**(1): 233-237.

Dweik R A, Sorkness R L, Wenzel S, Hammel J, Curran-Everett D, Comhair S A A, Bleecker E, Busse W, Calhoun W J and Castro M. 2010. Use of Exhaled Nitric Oxide Measurement to Identify a Reactive, at-Risk Phenotype among Patients with Asthma. American Journal of Respiratory and Critical Care Medicine **181**(10): 1033.

El-Bassossy H M, El-Fawal R, Fahmy A and Watson M L. 2013. Arginase Inhibition Alleviates Hypertension in the Metabolic Syndrome. British Journal of Pharmacology **169**(3): 693-703.

Gaston B, Drazen J M, Loscalzo J and Stamler J S. 1994. The Biology of Nitrogen-Oxides in the Airways. American Journal of Respiratory and Critical Care Medicine **149**(2): 538-551.

Hakansson H F, Smailagic A, Brunmark C, Miller-Larsson A and Lal H. 2012. Altered Lung Function Relates to Inflammation in an Acute Lps Mouse Model. Pulmonary Pharmacology & Therapeutics **25**(5): 399-406.

Hesse M, Modolell M, La Flamme A C, Schito M, Fuentes J M, Cheever A W, Pearce E J and Wynn T A. 2001. Differential Regulation of Nitric Oxide Synthase-2 and Arginase-1 by Type 1/Type 2 Cytokines in Vivo: Granulomatous Pathology Is Shaped by the Pattern of L-Arginine Metabolism. The Journal of Immunology **167**(11): 6533-6544.

Hjoberg J, Shore S, Kobzik L, Okinaga S, Hallock A, Vallone J, Subramaniam V, De Sanctis G T, Elias J A, Drazen J M and Silverman E S. 2004. Expression of Nitric Oxide Synthase-2 in the Lungs Decreases Airway Resistance and Responsiveness. Journal of Applied Physiology **97**(1): 249-259.

Hollingsworth J W, Cook D N, Brass D M, Walker J K L, Morgan D L, Foster W M and Schwartz D A. 2004. The Role of Toll-Like Receptor 4 in Environmental Airway Injury in Mice. American Journal of Respiratory and Critical Care Medicine **170**(2): 126-132.

Jatakanon A, Lim S, Kharitonov S A, Chung K F and Barnes P J. 1998. Correlation between Exhaled Nitric Oxide, Sputum Eosinophils, and Methacholine Responsiveness in Patients with Mild Asthma. Thorax **53**(2): 91-95.

Kacmarek R M, Ripple R, Cockrill B A, Bloch K J, Zapol W M and Johnson D C. 1996. Inhaled Nitric Oxide. A Bronchodilator in Mild Asthmatics with Methacholine-Induced Bronchospasm. American Journal of Respiratory and Critical Care Medicine **153**(1): 128-135.

Kang X D, Li N, Wang M Y, Boontheung P, Sioutas C, Harkema J R, Bramble L A, Nel A E and Loo J A. 2010. Adjuvant Effects of Ambient Particulate Matter Monitored by Proteomics of Bronchoalveolar Lavage Fluid. Proteomics **10**(3): 520-531.

Kenyon N J, Bratt J M, Linderholm A L, Last M S and Last J A. 2008. Arginases I and Ii in Lungs of Ovalbumin-Sensitized Mice Exposed to Ovalbumin: Sources and Consequences. Toxicology and Applied Pharmacology **230**(3): 269-275.

Kline J N, Cowden J D, Hunninghake G W, Schutte B C, Watt J L, Wohlford-Lenane C L, Powers L S, Jones M P and Schwartz D A. 1999. Variable Airway Responsiveness to Inhaled Lipopolysaccharide. American Journal of Respiratory and Critical Care Medicine **160**(1): 297-303.

Korhonen R, Lahti A, Kankaanranta H and Moilanen E. 2005. Nitric Oxide Production and Signaling in Inflammation. Current Drug Targets-Inflammation & Allergy **4**(4): 471-479.

Larsson A K, Back M, Hjoberg J and Dahlen S E. 2005. Inhibition of Nitric-Oxide Synthase Enhances Antigen-Induced Contractions and Increases Release of Cysteinyl-Leukotrienes in Guinea Pig Lung Parenchyma: Nitric Oxide as a Protective Factor. Journal of Pharmacology and Experimental Therapeutics **315**(1): 458-465.

Maarsingh H, Dekkers B G J, Zuidhof A B, Bos I S T, Menzen M H, Klein T, Flik G, Zaagsma J and Meurs H. 2011. Increased Arginase Activity Contributes to Airway Remodelling in Chronic Allergic Asthma. European Respiratory Journal **38**(2): 318-328.

Maarsingh H, Zaagsma J and Meurs H. 2008. Arginine Homeostasis in Allergic Asthma. European Journal of Pharmacology **585**(2-3): 375-384.

Maarsingh H, Zaagsma J and Meurs H. 2009. Arginase: A Key Enzyme in the Pathophysiology of Allergic Asthma Opening Novel Therapeutic Perspectives. British Journal of Pharmacology **158**(3): 652-664.

Meurs H, Maarsingh H and Zaagsma J. 2003. Arginase and Asthma: Novel Insights into Nitric Oxide Homeostasis and Airway Hyperresponsiveness. Trends in Pharmacological Sciences **24**(9): 450-455.

Meurs H, McKay S, Maarsingh H, Hamer M A M, Macic L, Molendijk N and Zaagsma J. 2002. Increased Arginase Activity Underlies Allergen-Induced Deficiency of Cnos-Derived Nitric Oxide and Airway Hyperresponsiveness. British Journal of Pharmacology **136**(3): 391-398.

Michel O, Duchateau J and Sergysels R. 1989. Effect of Inhaled Endotoxin on Bronchial Reactivity in Asthmatic and Normal Subjects. Journal of Applied Physiology **66**(3): 1059-1064.

Morris C R, Kato G J, Poljakovic M and et al. 2005. Dysregulated Arginine Metabolism, Hemolysis-Associated Pulmonary Hypertension, and Mortality in Sickle Cell Disease. Jama **294**(1): 81-90.

Morris C R, Poljakovic M, Lavrisha L, Machado L, Kuypers F A and Morris S M. 2004. Decreased Arginine Bioavailability and Increased Serum Arginase Activity in Asthma. American Journal of Respiratory and Critical Care Medicine **170**(2): 148-153.

Morris S, Jr. 2008. Recent Advances in L-Arginine Metabolism. Fundamental & Clinical Pharmacology **22**: 14-14.

North M L, Khanna N, Marsden P A, Grasemann H and Scott J A. 2009. Functionally Important Role for Arginase 1 in the Airway Hyperresponsiveness of Asthma. American Journal of Physiology-Lung Cellular and Molecular Physiology **296**(6): L911-L920.

Prado C M, Leick-Maldonado E A, Yano L, Leme A S, Capelozzi V L, Martins M A and Tiberio I F L C. 2006. Effects of Nitric Oxide Synthases in Chronic Allergic Airway Inflammation and Remodeling. American Journal of Respiratory Cell and Molecular Biology **35**(4): 457-465.

Prado C M, Martins M A and Tibério I F L C. 2011. Nitric Oxide in Asthma Physiopathology. ISRN allergy **2011**.

Ricciardolo F L M, Sterk P J, Gaston B and Folkerts G. 2004. Nitric Oxide in Health and Disease of the Respiratory System. Physiological Reviews **84**(3): 731-765.

Ricciardolo F L M, Timmers M C, Geppetti P, van Schadewijk A, Brahim J J, Sont J K, de Gouw H, Hiemstra P S, van Krieken J and Sterk P J. 2001. Allergen-Induced Impairment of Bronchoprotective Nitric Oxide Synthesis in Asthma. Journal of Allergy and Clinical Immunology **108**(2): 198-204.

Rodriguez D, Keller A C, Faquim-Mauro E L, de Macedo M S, Cunha F Q, Lefort J, Vargaftig B B and Russo M. 2003. Bacterial Lipopolysaccharide Signaling through Toll-Like Receptor 4 Suppresses Asthma-Like Responses Via Nitric Oxide Synthase 2 Activity. Journal of Immunology **171**(2): 1001-1008.

SadeghiHashjin G, Folkerts G, Henricks P A J, Verheyen A, vanderLinde H J, vanArk I, Coene A and Nijkamp F P. 1996. Peroxynitrite Induces Airway Hyperresponsiveness in Guinea Pigs in Vitro and in Vivo. American Journal of Respiratory and Critical Care Medicine **153**(5): 1697-1701.

Saleh D, Ernst P, Lim S, Barnes P J and Giaid A. 1998. Increased Formation of the Potent Oxidant Peroxynitrite in the Airways of Asthmatic Patients Is Associated with Induction of Nitric Oxide Synthase: Effect of Inhaled Glucocorticoid. Faseb Journal **12**(11): 929-937.

Sonoki T, Nagasaki A, Gotoh T, Takiguchi M, Takeya M, Matsuzaki H and Mori M. 1997. Coinduction of Nitric-Oxide Synthase and Arginase I in Cultured Rat Peritoneal Macrophages and Rat Tissues in Vivo by Lipopolysaccharide. Journal of Biological Chemistry **272**(6): 3689-3693.

Trifilieff A, Fujitani Y, Mentz F, Dugas B, Fuentes M and Bertrand C. 2000. Inducible Nitric Oxide Synthase Inhibitors Suppress Airway Inflammation in Mice through Down-Regulation of Chemokine Expression. Journal of Immunology **165**(3): 1526-1533.

Tulic M K, Holt P G and Sly P D. 2002. Modification of Acute and Late-Phase Allergic Responses to Ovalbumin with Lipopolysaccharide. International Archives of Allergy and Immunology **129**(2): 119-128.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A and Speleman F. 2002. Accurate Normalization of Real-Time Quantitative Rt-Pcr Data by Geometric Averaging of Multiple Internal Control Genes. Genome Biology **3**(7): RESEARCH0034.

Vonk J M, Postma D S, Maarsingh H, Bruinenberg M, Koppelman G H and Meurs H. 2010. Arginase 1 and Arginase 2 Variations Associate with Asthma, Asthma Severity and Beta(2) Agonist and Steroid Response. Pharmacogenetics and Genomics **20**(3): 179-186.

Yamamoto M, Tochino Y, Chibana K, Trudeau J B, Holguin F and Wenzel S E. 2012. Nitric Oxide and Related Enzymes in Asthma: Relation to Severity, Enzyme Function and Inflammation. Clinical & Experimental Allergy **42**(5): 760-768.

Zimmermann N, King N E, Laporte J, Yang M, Mishra A, Pope S M, Muntel E E, Witte D P, Pegg A A, Foster P S, Hamid Q and Rothenberg M E. 2003. Dissection of Experimental Asthma with DNA Microarray Analysis Identifies Arginase in Asthma Pathogenesis. Journal of Clinical Investigation **111**(12): 1863-1874.

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CHAPTER 6

SUMMARY AND CONCLUSIONS

Effective treatment of asthma includes diminishing symptoms, improving lung function and preventing the number and severity of exacerbations (Johnston and Sears, 2006; Reddel et al., 2009). Asthma exacerbation increases health risks to asthmatics, places a significant burden on the healthcare system, and contributes to large financial costs; therefore, it is considered to be an important clinical outcome (Reddel et al., 2009). Recent research suggests that induction of TLR-4, NF-κB, TGF-β, and genes associated with airway remodeling are critical for PM-mediated responses in allergic airways (Wang et al., 2008; Heidenfelder et al., 2009; Vawda et al., 2014). As an activator of TLR-4 and NF-κB LPS is a potential model of exacerbation of an asthmatic lung by environmental triggers, such as PM and ozone. Epidemiological and experimental studies have shown that inhaled LPS can modulate the asthmatic response (Eldridge and Peden, 2000; Bennett et al., 2013). In this work, I explored the effects of inhaled LPS exposure on established allergic airways disease to identify the cellular and molecular mechanisms whereby airway exposure to LPS modulates asthma; and to test my hypothesis that attenuation of AHR by LPS in asthmatic mice is dependent on the induction of *Nos2* that is associated with the recruitment of neutrophils.

Following airway LPS exposure in non-allergic mice, I documented a significant increase in neutrophils in the BALF and histological examination of the lungs showed increased neutrophils infiltrating into peribronchiolar and perivascular regions with extension into alveolar septa and airspaces. Also, LPS significantly increased AHR to methacholine. By comparison, sensitization and challenge to OVA caused airway epithelial remodeling characterized by mucous cell metaplasia and hypertrophy and

produced peribronchiolar and perivascular inflammation comprised of predominately lymphocytes with large numbers of eosinophils and macrophages, and fewer neutrophils. Additionally, OVA increased AHR. In allergic mice, LPS exacerbated the inflammatory response by causing a significant increase in neutrophils along with elevations in eosinophils and lymphocytes. Histologically, OVA-LPS mice had increased peribronchiolar and perivascular inflammation along with increased infiltrations of inflammatory cells, predominately neutrophils, within the alveolar septa and in airspaces. Surprisingly, OVA-LPS mice had an attenuation of AHR compared to both LPS and OVA groups. In addition, OVA-LPS mice had increased mRNA expression of *Nos2*, a gene that induces iNOS protein expression to produce NO, a bronchodilator.

Reports have shown that airway challenge with OVA or LPS causes AHR that is associated with inflammation (Gueders et al., 2009; Hakansson et al., 2012). My results are similar in that AHR was detected at time points near maximal inflammatory cell infiltration, 72 hours after OVA challenge and 24 hours following LPS exposure (Faffe et al., 2000; Lommatzsch et al., 2006). Conversely, there are studies that indicate dissociation between inflammation and AHR (Crimi et al., 1998; Tournoy et al., 2000). Also, my findings are in accordance with these studies because OVA-LPS mice had greater lung inflammation but their airways exhibited a decrease in reactivity to methacholine compared to OVA and LPS mice. Inhaled LPS can induce a Th1 response characterized by increased neutrophils, IFN-γ production and decreased mucus secretion in allergic mice (Eisenbarth et al., 2002; Kim et al., 2007; Dong et al., 2009). Similar findings were present in my study in which there was increased

pulmonary expression of IFN-γ and IL-10 that coincided with decrease expression of IL-13 in OVA-LPS mice. This result suggests that LPS may down-regulate the Th2 inflammatory pathway.

Neutrophil derived mediators, such as elastase and IL-17, have been associated with AHR (Kudo et al., 2012; Koga et al., 2013). However, a recent study suggests that neutrophils contribute to the attenuation of AHR in allergic airways (Aeffner and Davis, 2012). The influx of neutrophils was a prominent change in the lungs of OVA-LPS mice in my study. Therefore, I proposed that that the influx of neutrophils potentially contributed to the attenuation of AHR. Depletion of neutrophils significantly reduced the numbers of BALF neutrophils, along with reducing eosinophils and lymphocytes. However, depleting neutrophils did not affect AHR in mice. This result suggests that neither neutrophils nor eosinophils are needed for the development of AHR. This lack of association between inflammation and AHR has been noted in prior works (Tournoy et al., 2000; Janssen-Heininger et al., 2012).

NF-κB is an important regulator of the immune response. Its activation has been associated with adverse airway inflammatory and hyperreactivity responses to both allergen and LPS-stimulation (Pantano et al., 2008; Sheller et al., 2009). Inhibition of NF-κB has been shown to reduce eosinophil recruitment, Th2 cytokine production, mucous cell metaplasia, AHR, and neutrophil recruitment in murine models (Desmet et al., 2004; Kim et al., 2007; Bao et al., 2009). Based on the implications that NF-κB activation occurs during both asthma and acute LPS exposure and my findings of increased *Nos2* expression, I hypothesized that NF-κB was involved in attenuation of AHR in OVA-LPS mice.

In my study, inhibition of NF- κB had no effect on LPS-stimulated neutrophil recruitment, and led to an increase in AHR. These changes in airway physiology were associated with decreases in airway macrophages, eosinophils and lymphocytes. The reduction in airway inflammation, particularly eosinophils, is consistent with other OVA models in which NF-κB has been inhibited (Poynter et al., 2004). In that study, NF- κB repression did not alter AHR in OVA mice. This finding is consistent with my results, as inhibition of NF-κB did not affect AHR in OVA mice. In my OVA-LPS model, there are two pathways of activation of NF-κB, which has been shown to play an important role in pathogenesis of asthma and development of AHR (Stacey et al., 1997). However, the data show a disparity between inflammation and AHR associated with asthma resulting in increased inflammation while decreasing AHR. These results demonstrate the complex nature of the immune system.

NO deficiency in the lung is a proposed mechanism of asthma pathogenesis (Meurs et al., 2002; Larsson et al., 2005). Biologically, NO is a bronchodilator, however, there have been many reports of its detrimental effects in asthma. These effects appear to be dose and cell specific. High NO concentrations cause vasodilation with edema, mucus hypersecretion, and enhanced Th2 inflammation contributing to asthma pathology. Additionally, high concentrations of NO can interact with reactive oxygen species to produce peroxynitrite causing tissue damage and potentially contributing to AHR (SadeghiHashjin et al., 1996; Saleh et al., 1998). Since OVA-LPS mice had increase expression of *Nos2*, I tested the hypothesis that increased *Nos2* expression contributed to the attenuation of AHR.

Analysis of mRNA expression for *Nos2* showed an upregulation of this gene in OVA-LPS mice but not OVA or LPS alone. However when NF-κB was inhibited, expression of *Nos2* was significantly decreased in OVA-LPS mice. This decreased expression was accompanied by the reversal of attenuation of AHR. These findings suggest that *Nos2* expression contributed to the attenuation of AHR. Additionally, I analyzed the expression of *Arg1* which induces arginase-1 enzyme that regulates the functional activity of iNOS enzyme. The Arg-1 competes with iNOS for substrate L-arginine, metabolism. *Arg1* expression was increased in OVA-LPS mice. It has been previously demonstrated that LPS can induce expression of both *Nos2* and *Arg1* (Sonoki et al., 1997). The mechanism and function of LPS-induced arginase induction is not completely known. In vitro studies have indicated that MAPK phosphatase 1 switches L-arginine metabolism from NO to ornithine, a metabolite of Arg-1, following LPS stimulation and may contribute to the down regulation of inflammation (Nelin et al., 2007). Increased expression of iNOS suggests an increase in NO production. Additional studies which focus on direct function of iNOS using NOS inhibition should be conducted to confirm that increased iNOS activity is needed for the attenuation of AHR. Also, quantification of NO should be measured to verify that increased NO production is the mediator of attenuation of AHR in OVA-LPS mice.

Although induction of iNOS appears to be a very promising mechanism of LPS-associated attenuation of AHR, there are other plausible pathways that can contribute to this phenomenon. The physical attributes of the allergic airways resulting in reduced caliber may contribute to this finding. Undoubtedly, LPS exposure can change the inflammatory milieu switching it from a Th2 to Th1 mediated response which could

attenuate AHR. Furthermore, LPS decreased IL-13 cytokine in BALF and expression in the lung, as well as, downregulating the expression of *Gob5* and *Muc5ac* which are all associated with mucin secretion. These findings are consistent with the decreased volume density of intraepithelial mucosubstances in OVA-LPS mice. Inhibition of mucus secretion was found to be protective against AHR allergic in mice (Agrawal et al., 2007). Another possibility is inflammation and airways of allergic mice may respond differently to LPS. TNF-α induced by LPS has associated with AHR development (Choi et al., 2005). It has been demonstrated that monocytes from asthmatics produce less TNF-α when stimulated by fungi than monocytes from healthy individuals (Johannessen et al., 2008). My results showed that OVA-LPS mice had less concentrations of TNF- α in BALF than LPS mice, and therefore, may contribute to diminished AHR.

These results clearly show that the attenuation of AHR in allergic mice is associated with the upregulation of both *Nos2* and *Arg1* following inhaled LPS exposure (24 hours), and that this expression is associated with activated NF-κB (Table 2). Increased expression of *Nos2* and the presence of iNOS protein suggest that NO production is increased in OVA-LPS mice; however, NO production was not determined in these studies nor was iNOS protein quantified. Therefore, increased NO production was not confirmed as the mediator of attenuation of AHR in OVA-LPS mice. Additional factors that could be addressed include a time course evaluation of LPS post exposure in allergic mice to determine if attenuation of AHR occurs prior to or extends past 24 hour post exposure to LPS. Also, lymphocytes are particularly important in the pathogenesis allergic asthma, for this reason it is important to determine if lymphocyte derived mediators could contribute to this attenuation in AHR. Other limiting factors

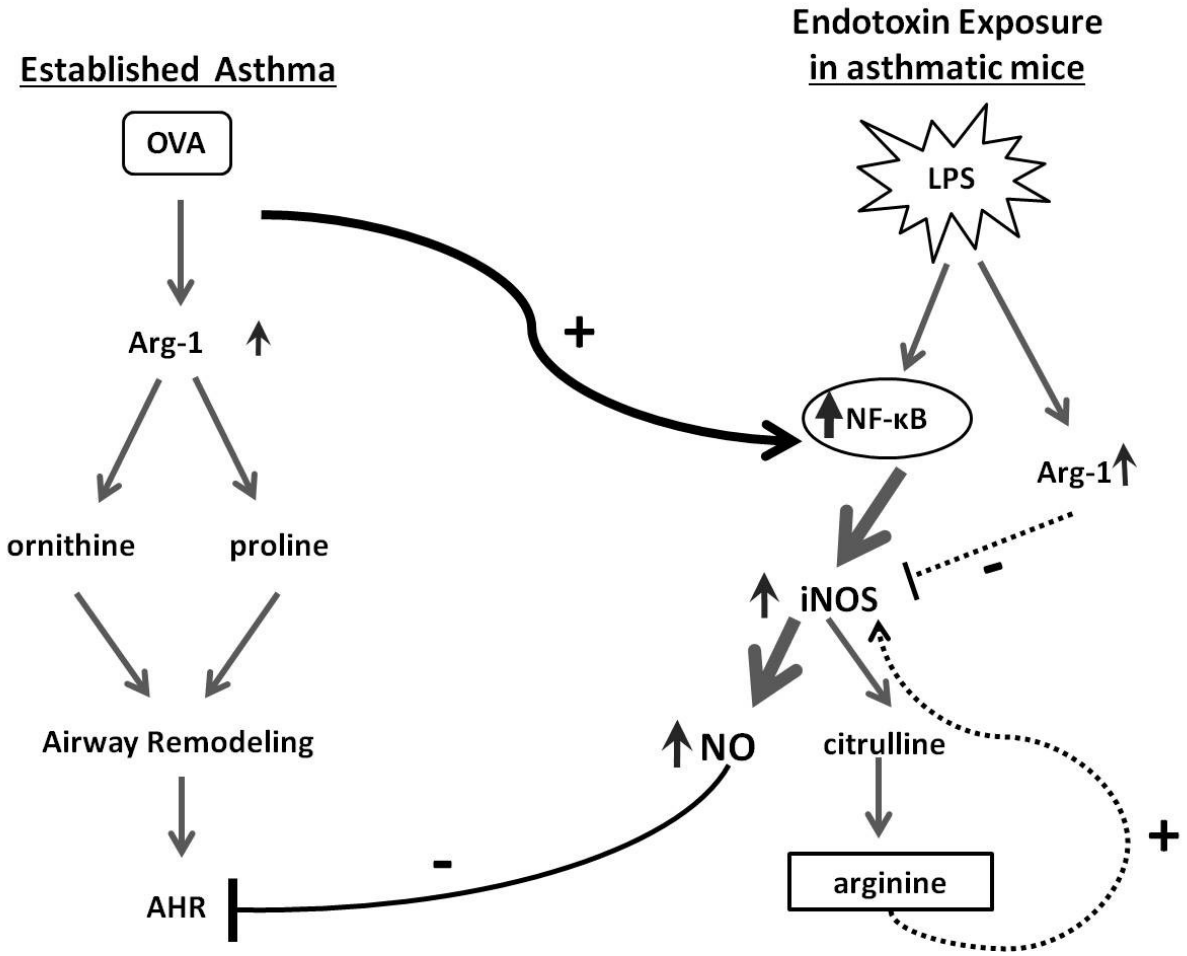
include the lack of specificity of the antibody used to deplete neutrophils. The loss of these non-neutrophil cells could affect AHR. Lastly, complete NF-κB inhibition could not be confirmed. Treatment with the inhibitor compound undoubtedly affected airway inflammation and AHR, however, its effects on proteasomal inhibition needs further assessment.

Results from this study demonstrate that severity of airway inflammation does not affect the severity of AHR. Additionally, neutrophils were not associated with the inhibition of AHR in OVA-LPS mice. NO can play multiple roles in pulmonary disease. My results suggest that NO can be beneficial to physiological function in allergic airway disease as depicted in Figure 24. To briefly summarize the mechanism of attenuation of AHR in OVA-LPS mice, I propose that 1) the effects of LPS could be enhanced by increased TLR4 signaling promoted by OVA (Rodriguez et al., 2003). 2) NF-κB activation is increased by both OVA- induced allergic airway disease and inhaled LPS which 3) promotes iNOS activity and 4) increases NO production resulting in the inhibition of AHR induced by allergic airway disease.

Table 2. Summary of changes in airway hyperresponsiveness in OVA-LPS mice. The attenuation of AHR in OVA-LPS mice was influence by several factors. LPS exposure produces an attenuation of AHR in allergic mice. Severe lung inflammation was present in central airways and peripheral tissue; however, this was associated with a less responsive phenotype to methacholine. There was no change in AHR when neutrophils were depleted. AHR significantly increased following NF-κB inhibition. Reversal of AHR attenuation was associated with decreased expression of *Nos2* and *Arg1*.

AHR EFFECTS IN OVA-LPS MICE	
PARAMETER	AHR EFFECT
↑ INFLAMMATION	↓
↓ NEUTROPHILS	NO EFFECT
↓ NF-κb	↑
↓ iNOS / ARG1	↑

Figure 24. Proposed mechanism for attenuation of AHR following LPS exposure in allergic mice. In allergic mice, OVA induce the expression of Arg-1 resulting in airway remodeling and AHR. Exposure of allergic airways to LPS leads to NF-κB activation, and a preferential increase of iNOS, and less expression of Arg-1. The role of Arg-1 is unknown, but may regulate iNOS activity through inhibition. NOS metabolism of arginine produces NO and citrulline. Arginine can be produced from recycling citrulline, increasing its bioavailability for NOS metabolism. Increased bronchoprotective NO is produced which inhibits AHR.



REFERENCES

REFERENCES

Aeffner F and Davis I C. 2012. Respiratory Syncytial Virus Reverses Airway Hyperresponsiveness to Methacholine in Ovalbumin-Sensitized Mice. Plos One **7**(10).

Agrawal A, Rengarajan S, Adler K B, Ram A, Ghosh B, Fahim M and Dickey B F. 2007. Inhibition of Mucin Secretion with Marcks-Related Peptide Improves Airway Obstruction in a Mouse Model of Asthma. Journal of Applied Physiology **102**(1).

Bao Z, Guan S P, Cheng C, Wu S L, Wong S H, Kemeny D M, Leung B P and Wong W S F. 2009. A Novel Antiinflammatory Role for Andrographolide in Asthma Via Inhibition of the Nuclear Factor-Kappa B Pathway. American Journal of Respiratory and Critical Care Medicine **179**(8): 657-665.

Bennett W D, Herbst M, Zeman K L, Wu J, Hernandez M L and Peden D B. 2013. Effect of Inhaled Endotoxin on Regional Particle Deposition in Patients with Mild Asthma. Journal of Allergy and Clinical Immunology **131**(3): 912-913.

Choi I W, Sun K, Kim Y S, Ko H M, Im S Y, Kim J H, You H J, Lee Y C, Lee J H, Park Y M and Lee H K. 2005. Tnf-Alpha Induces the Late-Phase Airway Hyperresponsiveness and Airway Inflammation through Cytosolic Phospholipase a(2) Activation. J Allergy Clin Immunol **116**(3): 537-543.

Crimi E, Spanevello A, Neri M, Ind P W, Rossi G A and Brusasco V. 1998. Dissociation between Airway Inflammation and Airway Hyperresponsiveness in Allergic Asthma. American Journal of Respiratory and Critical Care Medicine **157**(1): 4-9.

Desmet C, Gosset P, Pajak B, Cataldo D, Bentires-Alj M, Lekeux P and Bureau F. 2004. Selective Blockade of Nf-Kappa B Activity in Airway Immune Cells Inhibits the Effector Phase of Experimental Asthma. Journal of Immunology **173**(9): 5766-5775.

Dong L, Li H, Wang S and Li Y. 2009. Different Doses of Lipopolysaccharides Regulate the Lung Inflammation of Asthmatic Mice Via Tlr4 Pathway in Alveolar Macrophages. Journal of Asthma **46**(3): 229-233.

Eisenbarth S C, Piggott D A, Huleatt J W, Visintin I, Herrick C A and Bottomly K. 2002. Lipopolysaccharide-Enhanced, Toll-Like Receptor 4-Dependent T Helper Cell Type 2 Responses to Inhaled Antigen. Journal of Experimental Medicine **196**(12): 1645-1651.

Eldridge M W and Peden D B. 2000. Airway Response to Concomitant Exposure with Endotoxin and Allergen in Atopic Asthmatics. Journal of Toxicology and Environmental Health Part A **61**(1): 27-37.

Faffe D S, Seidl V R, Chagas P S, de Moraes V L G, Capelozzi V L, Rocco P R and Zin W A. 2000. Respiratory Effects of Lipopolysaccharide-Induced Inflammatory Lung Injury in Mice. European Respiratory Journal **15**(1): 85-91.

Gueders M M, Paulissen G, Crahay C, Quesada-Calvo F, Hacha J, Van Hove C, Tournoy K, Louis R, Foidart J-M and NoÃ«l A. 2009. Mouse Models of Asthma: A Comparison between C57bl/6 and Balb/C Strains Regarding Bronchial Responsiveness, Inflammation, and Cytokine Production. Inflammation Research **58**(12): 845-854.

Hakansson H F, Smailagic A, Brunmark C, Miller-Larsson A and Lal H. 2012. Altered Lung Function Relates to Inflammation in an Acute Lps Mouse Model. Pulmonary Pharmacology & Therapeutics **25**(5): 399-406.

Janssen-Heininger Y M, Irvin C G, Scheller E V, Brown A L, Kolls J K and Alcorn J F. 2012. Airway Hyperresponsiveness and Inflammation: Causation, Correlation, or No Relation? Journal of allergy & therapy **2012**(Suppl 1).

Johannessen L N, LÃ¸vik M, Steinshamn S and Nilsen A M. 2008. Monocyte Exposure to Saccharomyces Cerevisiae Cell Wall Mannan and Decreased Tnf alpha Production in Mild Asthma: A Role for Mannan□Binding Lectin? Scandinavian Journal of Immunology **68**(5): 511-515.

Johnston N W and Sears M R. 2006. Asthma Exacerbations· 1: Epidemiology. Thorax **61**(8): 722-728.

Kim Y K, Oh S Y, Jeon S G, Park H W, Lee S Y, Chun E Y, Bang B, Lee H S, Oh M H, Kim Y S, Kim J H, Gho Y S, Cho S H, Min K U, Kim Y Y and Zhu Z. 2007. Airway Exposure Levels of Lipopolysaccharide Determine Type 1 Versus Type 2 Experimental Asthma. Journal of Immunology **178**(8): 5375-5382.

Koga H, Miyahara N, Fuchimoto Y, Ikeda G, Waseda K, Ono K, Tanimoto Y, Kataoka M, Gelfand E W, Tanimoto M and Kanehiro A. 2013. Inhibition of Neutrophil Elastase Attenuates Airway Hyperresponsiveness and Inflammation in a Mouse Model of Secondary Allergen Challenge: Neutrophil Elastase Inhibition Attenuates Allergic Airway Responses. Respiratory Research **14**.

Kudo M, Melton A C, Chen C, Engler M B, Huang K E, Ren X, Wang Y, Bernstein X, Li J T and Atabai K. 2012. Il-17a Produced by [Alpha][Beta] T Cells Drives Airway Hyper-Responsiveness in Mice and Enhances Mouse and Human Airway Smooth Muscle Contraction. Nature Medicine **18**(4): 547-554.

Larsson A K, Back M, Hjoberg J and Dahlen S E. 2005. Inhibition of Nitric-Oxide Synthase Enhances Antigen-Induced Contractions and Increases Release of Cysteinyl-Leukotrienes in Guinea Pig Lung Parenchyma: Nitric Oxide as a Protective Factor. Journal of Pharmacology and Experimental Therapeutics **315**(1): 458-465.

Lommatzsch M, Julius P, Kuepper M, Garn H, Bratke K, Irmscher S, Luttmann W, Renz H, Braun A and Virchow J C. 2006. The Course of Allergen-Induced Leukocyte Infiltration in Human and Experimental Asthma. Journal of Allergy and Clinical Immunology **118**(1): 91-97.

Meurs H, McKay S, Maarsingh H, Hamer M A M, Macic L, Molendijk N and Zaagsma J. 2002. Increased Arginase Activity Underlies Allergen-Induced Deficiency of Cnos-Derived Nitric Oxide and Airway Hyperresponsiveness. British Journal of Pharmacology **136**(3): 391-398.

Nelin L D, Wang X, Zhao Q, Chicoine L G, Young T L, Hatch D M, English B K and Liu Y. 2007. Mkp-1 Switches Arginine Metabolism from Nitric Oxide Synthase to Arginase Following Endotoxin Challenge. American Journal of Physiology-Cell Physiology **293**(2): C632-C640.

Pantano C, Ather J L, Alcorn J F, Poynter M E, Brown A L, Guala A S, Beuschel S L, Allen G B, Whittaker L A, Bevelander M, Irvin C G and Janssen-Heininger Y M W. 2008. Nuclear Factor-Kappa B Activation in Airway Epithelium Induces Inflammation and Hyperresponsiveness. American Journal of Respiratory and Critical Care Medicine **177**(9): 959-969.

Poynter M E, Cloots R, van Woerkom T, Butnor K J, Vacek P, Taatjes D J, Irvin C G and Janssen-Heininger Y M W. 2004. Nf-Kappa B Activation in Airways Modulates Allergic Inflammation but Not Hyperresponsiveness. Journal of Immunology **173**(11): 7003-7009.

Reddel H K, Taylor D R, Bateman E D, Boulet L P, Boushey H A, Busse W W, Casale T B, Chanez P, Enright P L, Gibson P G, de Jongste J C, Kerstjens H A M, Lazarus S C, Levy M L, O'Byrne P M, Partridge M R, Pavord I D, Sears M R, Sterk P J, Stoloff S W, Sullivan S D, Szefler S J, Thomas M D, Wenzel S E, Amer Thoracic S and European Resp Soc Task Force A. 2009. An Official American Thoracic Society/European

Respiratory Society Statement: Asthma Control and Exacerbations Standardising Endpoints for Clinical Asthma Trials and Clinical Practice. American Journal of Respiratory and Critical Care Medicine **180**(1): 59-99.

Rodriguez D, Keller A C, Faquim-Mauro E L, de Macedo M S, Cunha F Q, Lefort J, Vargaftig B B and Russo M. 2003. Bacterial Lipopolysaccharide Signaling through Toll-Like Receptor 4 Suppresses Asthma-Like Responses Via Nitric Oxide Synthase 2 Activity. Journal of Immunology **171**(2): 1001-1008.

SadeghiHashjin G, Folkerts G, Henricks P A J, Verheyen A, vanderLinde H J, vanArk I, Coene A and Nijkamp F P. 1996. Peroxynitrite Induces Airway Hyperresponsiveness in Guinea Pigs in Vitro and in Vivo. American Journal of Respiratory and Critical Care Medicine **153**(5): 1697-1701.

Saleh D, Ernst P, Lim S, Barnes P J and Giaid A. 1998. Increased Formation of the Potent Oxidant Peroxynitrite in the Airways of Asthmatic Patients Is Associated with Induction of Nitric Oxide Synthase: Effect of Inhaled Glucocorticoid. Faseb Journal **12**(11): 929-937.

Sheller J R, Polosukhin V V, Mitchell D, Cheng D S, Peebles R S and Blackwell T S. 2009. Nuclear Factor Kappa B Induction in Airway Epithelium Increases Lung Inflammation in Allergen-Challenged Mice. Experimental Lung Research **35**(10): 883-895.

Sonoki T, Nagasaki A, Gotoh T, Takiguchi M, Takeya M, Matsuzaki H and Mori M. 1997. Coinduction of Nitric-Oxide Synthase and Arginase I in Cultured Rat Peritoneal Macrophages and Rat Tissues in Vivo by Lipopolysaccharide. Journal of Biological Chemistry **272**(6): 3689-3693.

Stacey M A, Sun G, Vassalli G, Marini M, Bellini A and Mattoli S. 1997. The Allergen Der P1 Induces Nf-Kappa B Activation through Interference with I Kappa B Alpha Function in Asthmatic Bronchial Epithelial Cells. Biochemical and Biophysical Research Communications **236**(2): 522-526.

Tournoy K G, Kips J C, Schou C and Pauwels R A. 2000. Airway Eosinophilia Is Not a Requirement for Allergen-Induced Airway Hyperresponsiveness. Clin Exp Allergy **30**(1): 79-85.