PULMONARY INNATE IMMUNE MODULATION IN THE PATHOGENESIS OF ALLERGIC ASTHMA

by

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To

Mom and Dad

who opened every door
but let me choose my own path

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List of Abbreviations

AHR	Airways Hyperresponsiveness
APC	Antigen Presenting Cell
BAL	Bronchoalveolar Lavage
COPD	Chronic Obstructive Pulmonary Disease
CRA	Cockroach Allergen
DC	Dendritic Cell
ECP	Eosinophil Cationic Protein
ELISA	Enzyme-linked Immunosorbant Assay
EPO	Eosinophil Peroxidase
HDE	House Dust Extract
IRAK	IL-1 Receptor Associated Kinase
LBP	LPS Binding Protein
LPS	Lipopolysaccharide
LTA	Lipoteichoic Acid
MBP	Major Basic Protein
MPO	Myeloperoxidase Assay
OD	Optical Density
OVA	Ovalbumin
PBS	Phosphate Buffered Saline
PAR	Protease Activated Receptor
TLR4	Toll-like Recepror-4
TNFα	Tumor Necrosis Factor-α
RSV	Respiratory Syncitial Virus

Abstract

Recent studies have shown that the innate immune response plays a significant role in the adaptive immune response in asthma. However, controversy exists regarding whether innate immune activating substances such as particulate matter, and bacterial components such as lipopolysaccharide (LPS) protect against or potentiate allergic diseases. Our studies first sought to determine whether LPS at the challenge phase of asthma affects the adaptive immune response. Because LPS is ubiquitously present in ambient air, we determined whether repeated LPS inhalation alone could modulate the pulmonary immune environment, thereby affecting the onset and progression of asthma.

Because increasing emphasis is being placed in removal of bacterial components from the home as a means of attenuating asthma severity, we determined the affect of LPS during the challenge phase of asthma in a clinically relevant model. Asthma was induced by sensitization to cockroach allergens, and challenge with a house dust extract (HDE) collected from the home of an asthmatic child. Depletion of LPS from the HDE resulted in attenuation of IgE, and augmented Th1 and Th2 cytokine production in the lung. The LPS-depleted HDE also exacerbated airways hyper-reactivity (AHR), suggesting that LPS removal from the home environment does not protect against the asthmatic phenotype.

We sought to determine whether repeated inhalation of LPS would cause a transient state of refractoriness to further LPS challenge, termed LPS tolerance. Mice receiving two LPS exposures were protected from excessive inflammation through

attenuated TNFα and IL-6, but maintained neutrophil recruitment. We then developed a more clinically relevant model of chronic LPS inhalation and found selective suppression of TNFα, but augmented IL-6. LPS tolerance also protected from AHR through the mechanism of reduced muscarinic receptor expression.

We then determined whether LPS tolerance can affect allergic asthma. Induction of LPS tolerance immediately prior to allergen sensitization resulted in protection from airway eosinophilia, IgE production and AHR in response to allergen challenge.

These findings suggest that LPS inhalation causes local immune modulation that can affect both the sensitization and challenge phases of asthma. Further, we demonstrated that transient innate immune modulation, can have lasting effects on adaptive immunity.

Chapter 1

Introduction

Asthma is a chronic allergic disease characterized by airway eosinophilia, IgE production and bronchial hyperresponsiveness. Its incidence has seen a marked increase in the Western world over the past 20 years (1). While genetic factors have been shown to be involved in predisposition to asthma, this increase is too drastic to be explained merely by the incidence of genetic changes (2). Asthma is generally characterized as an inappropriate immune response to environmental allergens, however a large body of data demonstrates the role of the innate immune response in the development of asthma. Activators of the innate immune response, such as lipopolysaccharide, are ubiquitous in the environment and are significant components of ambient air. Thus, understanding the role these molecules play in dictating the adaptive immune response is of critical importance.

The inflammatory response to LPS

LPS signaling

LPS is a component of the cell wall of Gram negative bacteria. Signaling occurs through binding of LPS to circulating LPS-binding protein (LBP) (4). This complex delivers LPS monomers to the surface molecule CD14, which is recognized by Toll-like receptor-4 (TLR4) on the surface of immune cells such as monocytes, macrophages and

dendritic cells (3). Activation of TLR4 results in signal transduction through MyD88 adapter protein dependent and independent pathways. The MyD88 dependent pathway activates the interleukin-1 receptor associated kinase family of proteins and initiates early translocation of NF-kB into the nucleus (4, 5). This results in production of proinflammatory mediators, such as tumor necrosis factor-α (TNFα) and interleukin-6 (IL-6). The MyD88-independent pathway activates the adapter protein TIR-domain-containing adapter-inducing interferon-β (TRIF), leading to IFNβ production through the nuclear translocation of interferon regulatory factor-3 (IRF-3) (6).

Inflammatory response to LPS

Activation of the innate immune response by LPS results in significant production of acute phase cytokines TNFα and IL-6 (7). In the lung, TNFα is produced mainly by macrophages, and has been shown to be a major regulatory factor in host defense against respiratory pathogens (8, 9). Exogenous pulmonary administration of recombinant TNFα has been shown to induce bronchial hyperresponsiveness, thus implicating TNFα production as the mechanism underlying AHR in response to LPS inhalation (10). IL-6 is also predominantly secreted by airway macrophages and has been shown to have both pro-and anti-inflammatory functions in mouse airways, depending on the type of pathogen (11).

LPS challenge has also been shown to result in a robust neutrophilic response (12). The CXC chemokines KC (CXCL1) and MIP-2 (CXCL2) are potent chemotactic cytokines responsible for the recruitment of neutrophils (13). They are termed CXC chemokines because the first two cysteine residues are separated by one amino acid (14).

Further, the production of these neutrophil-specific chemokines has been shown to be dependent on TNFα in several models of lung inflammation (15, 16).

LPS tolerance

Repeated LPS exposure can induce a state of hyporesposiveness to further LPS challenge. This diminished ability to respond to LPS is termed LPS tolerance (17). Initially, LPS tolerance was observed in *ex vivo* macrophage culture in the form of decreased production on TNFα and IL-6 in response to repeated LPS challenge (18, 19). However, some *in vitro* models have demonstrated differential regulation of these acute phase cytokines as well as CXC chemokines (20, 21). Upregulation of IL-10 has also been seen in response to tolerance induction, and implicates this mediator as having an anti-inflammatory role concomitant with TNFα attenuation (22, 23). Studies in animal models have demonstrated a role for LPS tolerance in models of sepsis and systemic bacterial infection (24, 25). Prior injection of LPS confers a survival advantage to polymicrobial sepsis through the mechanism of enhanced bacterial clearance (26, 27). Further, isolated circulating monocytes, and whole blood of septic patients stimulated with LPS produce attenuated concentrations of TNFα compared to healthy controls (28, 29).

Despite these findings, the molecular mechanisms governing LPS tolerance have not been fully elucidated. New evidence is emerging implicating the IRAK family of kinases in the regulation of inflammatory gene expression in response to repeated LPS challenge. Human THP-1 cells tolerized to LPS showed significantly attenuated levels of IRAK-1, which is involved in the MyD88-dependent pathway of TLR4 activation (30). This mechanism has also been shown to be involved in cross tolerance between TLR2

and TLR4 ligands (31). Downregulation of both TNFα and IL-1β was observed in cells pretreated with LPS and restimulated with lipoteichoic acid (LTA). More recent evidence also shows the upregulation of IRAK-M, produced only in monocytes (32). IRAK-M is a negative regulator of TLR signaling, which inhibits the dissociation of IRAK-1 and IRAK-4 from MyD88, thus preventing downstream gene transcription (33). IRAK-M is upregulated soon after LPS challenge in monocytes isolated from septic patients (34, 35). This finding has also been demonstrated by *ex vivo* stimulation of monocytes pretreated with LPS (36).

Although the body of literature regarding LPS tolerance in response to systemic LPS administration is expanding, little data exists investigating the role of repeated LPS inhalation. Epidemiologic data have demonstrated both positive and negative correlations between LPS inhalation and disease. High levels of environmental LPS, such as those present on farms and in agricultural and textile mills correlate with reduced incidence of atopy and asthma (37, 38). Further, individuals residing or working in these environments have reduced incidences of certain lung cancers (38). Conversely, LPS inhalation has been correlated with increased incidence and severity of asthma, as well as with causing persistent inflammation and decreased lung function (39-41).

Little precedent exists to show that LPS inhalation can induce a state of tolerance or immune suppression, however we hypothesized that because the lung is constantly exposed to environmental pathogens and microbial products, such as LPS, the lung must possess mechanisms that prevent excessive inflammation, without inducing global immune suppression.

Development of the asthmatic phenotype

Allergen Sensitization

The development of the allergic phenotype can be subdivided into the sensitization and challenge phases. Sensitization represents the initial encounter of allergen with the immune system, and requires allergen uptake by antigen presenting cells (APC) such as dendritic cells (DC) (42). Once exogenous pathogens are phagocytosed by dendritic cells, they then traffic to the draining lymph node, during which time they undergo maturation and upregulate T cell costimulatory molecules such as CD80 and CD86. Processed antigens are then presented to naïve T cells in the context of MHC class II molecules. This induces the activation and differentiation of these T cells into allergen-specific $CD4^+$ T helper cells (43, 44).

Sensitization also leads to the development of allergen-specific IgE. The production of this antibody is dependent on T cell expression of CD40L. CD40L ligation with CD40 on the surface of B cells, in conjunction with IL-4, IL-13, and LPS signaling results in NF-κB and STAT6 activation (45). This ultimately initiates class switch recombination and transcription of the immunoglobulin epsilon gene. Secreted IgE molecules are then loaded onto the surface of mast cells and basophils in the lung through the high affinity Fc epsilon receptor RI (46). Interestingly, class switch recombination and IgE production have also been shown to occur in the bronchial mucosa of atopic and non-atopic individuals (47, 48). This process occurs through local B cell expression of both epsilon germline transcripts as well as activation-induced cytidine deaminase (AID) mRNA, both of which are required for IgE antibody secretion (45). This ongoing synthesis of IgE has been implicated in potentiating asthma severity.

Challenge Phase

The challenge, or effector phase of asthma can also be subdivided into an early and late phase. The early phase response can occur within minutes of allergen encounter, and is characterized by allergen cross-linking of IgE on the surface of mast cells (49). This induces mast cell degranulation and the release of mediators such as histamine, tryptase and cysteinyl leukotrienes (50). Each of these mediators has a specific receptor which is expressed on the surface of airway smooth muscle cells. Histamine binds to H1 receptors, while tryptase signals through the activation of protease activated receptor-2 (PAR-2), and cysteinyl leukotrienes signal through receptors Cys-LT1 and Cys-LT2 (51). These receptors share the conserved structure of G-protein coupled receptors. Signaling through these receptors induces calcium influx into airway smooth muscle cells, causing contraction of alpha-smooth muscle actin (52). This leads to smooth muscle contraction and acute bronchial hyperresponsiveness.

The early phase is also characterized by the secretion of chemotactic factors responsible for the influx of inflammatory cells. The inflammatory cells characteristic of the asthmatic response include neutrophils, lymphocytes and eosinophils, which are considered the principle effector cells of asthma (53). The CXC chemokines CXCL1 and CXCL2 are secreted in order to recruit neutrophils into the lung (13). Chemotactic cytokines specific for lymphocytes and eosinophils are members of the CC chemokine family, in which the first two cysteine residues are adjacent (14). CCL2, CCL3 and CCL5 have all been shown to be significantly upregulated in the bronchoalveolar lavage fluid of asthmatics, and show strong correlation with subsequent lymphocyte infiltration (54). The eotaxin family of chemokines represents potent eosinophil chemoattractants. Three

members of this family, CCL11, CCL24 and CCL26 have been identified in humans, while only CCL11 and CCL24 are expressed in mice. All members of the eotaxin family of genes are significantly upregulated in asthmatics, both in human subjects and in animal models (55-57).

In order to further potentiate the influx of inflammatory cells into the allergic lung, studies have demonstrated the upregulation of various chemokine receptors on the surface of inflammatory cells. CCR4, the receptor for CCL17, and CCR22 is upregulated on $CD4^+$ T cells in asthmatics (57). Further, chemokine receptor blockade is currently being investigated as a therapeutic modality for the treatment of asthma. Blockade of CCR2, a receptor for CCL2, can prevent asthmatic inflammation in an animal model (57). Several low molecular weight antagonists of the eotaxin family receptor CCR3 are also being investigated as possible therapeutic agents for asthma treatment (58, 59).

The production of these chemokines results in the development of the late phase of the response to allergen challenge, which can develop several hours to days after allergen encounter (60). This is characterized by marked influx of neutrophils, lymphocytes and eosinophils. Once in the lung, lymphocytes can produce cytokines such as IL-5 which is a key mediator in eosinophil activation, and IL-13 which can induce goblet cell hyperplasia and mucus hypersecretion resulting in bronchoconstriction (61). Activation and degranulation of eosinophils results in the release of eosinophil major basic protein (MBP), eosinophil peroxidase (EPO) and eosinophil cationic protein (ECP), which can cause damage to the lung epithelium (62). Degranulation of neutrophils causes release of elastase and subsequent matrix metalloproteinase activation and degradation of type III collagen (63). Both of these processes can lead to lung damage if untreated. This

highlights that short term treatment of bronchoconstriction and AHR must be combined with anti-inflammatory treatments to prevent long-term inflammatory cell infiltration as a means to protect against airway remodeling and damage.

Respiratory infections and asthma onset

Epidemiological evidence suggests that early viral and bacterial infections can influence the development of atopy. Viral infections, such as influenza, RSV and rhinovirus correlate strongly with childhood wheezing, and children who develop repeated viral infections have an increased risk of developing chronic asthma (64). It has been suggested that virus-induced epithelial damage may increase the permeability of the airway mucosa, increasing the potential for allergen sensitization (65). Further, viral infection can induce signaling via TLR3 and TLR7 (66). It is possible that the innate immune response induced by different TLR ligands can mediate different outcomes in response to allergen encounter.

Clinical incidence of asthma has been shown to correlate inversely to the incidence of infections, such as tuberculosis and typhoid (67). The neonatal immune system is suggested to skew strongly toward Th2 responses (68). However, the Th1 immune response is required for clearance of these pathogens. It is therefore thought that the initiation of Th1 responses early in life may mediate protection from atopy by skewing the immune balance away from the Th2 phenotype (69). Atypical bacterial infection has also been shown to play a role in asthma severity. Early studies suggested that infections with *Mycoplasma pneumonia* and *Chlamydophila pneumoniae* were associated with later onset of asthma. More recent studies have also linked infection with increased asthma severity (70-72). These infections are common in children under the

age of 5 and have been shown to correlate with subsequent development of wheezing (73). These infections may potentiate and exacerbate asthma through skewing of the immune response to a Th2 phenotype (74). Here again, conflicting data make it difficult to elucidate the specific role of infections on the development of atopy. The key seems to lie in the age and the state of the immune system of the individual at the time of infection. If these infections occur early enough in life, they may lead to protection, however if the immune system has matured beyond a certain point, they may potentiate asthma development.

The role of innate immunity on the onset and progression of asthma

The hygiene hypothesis states that lack of exposure to infectious agents and increased use of antibiotics at a young age can potentiate the development of allergic diseases. On the other hand, frequent microbial infections and decreased environmental cleanliness are thought to skew toward protective immunity (75). While this theory has been the basis of much investigation, new evidence suggests that this hypothesis may oversimplify the role of infectious microbes and their byproducts in the development of allergy. Several studies have suggested that the type of infectious agent can potentiate or protect against allergy.

Early epidemiological studies showed negative correlations between TLR4 stimulation by LPS and sensitization (76). However, studies in mice lacking functional TLR4 suggested that TLR4 may potentiate airway eosinophilia (77). LPS administration, concomitant with OVA challenge has been shown to protect against IgE and airway eosinophilia in several mouse models, while others show that LPS is necessary for allergen sensitization (78-80). Further, LPS contamination of commercial OVA

preparations dramatically affects the allergic response. Commercial OVA depleted of LPS using Polymyxin B significantly increased AHR and airway inflammation compared to intact commercial preparations (81). Therefore, further evidence is needed to truly elucidate the role of microbial activation of the innate immune response on the development of asthma.

While the dose and timing of LPS exposure is of clear importance in determining the outcome of allergen encounter, the ability to draw further conclusions from these studies is limited. This is due to the difference in models employed in these studies, as well as the fact that studies with purified molecules make it difficult to relate findings in animal models to clinical data, which are the result of exposure to complex mixtures of immunogenic compounds. The majority of current models employ chicken egg ovalbumin (OVA) as the sensitizing agent (77). This model is widely used due to the availability of OVA-specific T cell receptor transgenic mice that greatly facilitates the study T cell activation and proliferation (82). However, this protein lacks intrinsic protease activities which are characteristic of other more clinically relevant allergens such as cockroach, or house dust mite allergens. In fact, a study comparing OVA and CRA sensitization in guinea pigs showed that CRA induced a more robust allergic response, and caused decreased lung function that persisted for longer than in OVA sensitized and challenged animals (83).

Finally, the use of adjuvants plays a significant role in the resulting asthmatic phenotype. The commonly used adjuvant aluminum hydroxide has been shown to skew the immune response toward a Th2 type phenotype (84, 85). Further, the route of sensitization must be considered. Evidence exists showing that intraperitoneal

sensitization with adjuvant/allergen mixtures can take place in the absence of TLR4 and MyD88 signaling, whereas intranasal sensitization requires innate signaling through these molecules (86, 87). A recent study using LPS adsorbed to alum adjuvant for sensitization dose-dependently attenuated airway eosinophilia, AHR, IgE secretion, and concentrations of IL-4 and IL-5 (88). Therefore, the development of more clinically relevant models that mimic the route of allergen exposure, and contain microbial components, such as LPS, which may themselves have adjuvant effects is essential to determining the mechanisms underlying asthma onset and progression. This is also key to relating findings from mouse models to human data.

CRA model of asthma induction

Initial investigations into the ability of CRA to induce asthmatic inflammation began over 35 years ago, showing children with asthma to be sensitized to CRA (89). Since then, cockroach allergens have been shown to induce airway eosinophilia and hyperreactivity in mice (90). More recently, CRA has been shown to be a major factor in allergen sensitization in asthmatic children in the inner city (91). Sensitivity to CRA in these children resulted in increased missed days of school, hospital visits, and nights with lost sleep compared to children sensitized and exposed to other allergens such as dust mite or cat dander. Infestation with the German cockroach *Blattella germanica* is most common in the United States (92). They produce the potent allergens Bla g1, Bla g2, Bla g4 and Bla g5. Of these, Bla g2 is an aspartic protease and has been shown to induce robust IgE production in allergic patients (93).

We have developed a clinically relevant model of asthma-like pulmonary inflammation that is induced by sensitization with cockroach allergens and challenge

with a house dust extract containing high levels of both CRA and LPS (55, 94). This model has been shown to induce the hallmark features of asthma, including airway eosinophilia, IgE production and airways hyperresponsiveness to aerosolized methacholine (94). We first used this model to assess the role LPS plays during the effector phase of asthma. Then, given the ubiquitous presence of LPS in the environment, we sought to determine whether acute LPS inhalation can induce a refractory state to further LPS stimulation. We further elucidated the nature of the selective immune suppression in a clinically relevant model of repeated LPS inhalation. Finally, we determined the effect of prior LPS inhalation on the development and progression of CRA-induced asthma.

Chapter 2

LPS Depletion Exacerbates Airways Hyperreactivity and Cytokine Production while Attenuating IgE in a Murine Model of House Dust-Induced Asthma

Introduction

Asthma is a chronic allergic disorder characterized by IgE production, airway eosinophilia and bronchial hyperresponsiveness (95). Numerous studies have shown that allergen alone is not responsible for the onset and severity of the asthmatic response (reviewed in (75)). Microbial components, such as lipopolysaccharide (LPS) are ubiquitously present in the environment, as well as in the ambient air. In addition to being potent activators of innate immunity, these compounds have a key role in allergen sensitization (96). The presence of these microbial components is associated with environmental and household cleanliness, and increasing emphasis is being placed on their removal as a means to protect against exacerbation of allergic diseases. However, the hygiene hypothesis postulates that the lack of exposure to these pathogens at a young age increases one's susceptibility to allergen sensitization, accounting for the dramatic increases in allergic diseases such as asthma in the developed world (97, 98).

While LPS plays a key role in sensitization, little is known about the synergistic effect of LPS and allergen during the effector phase of asthma. We sought to determine whether or not the removal of LPS from house dust obtained from the home environment can protect against asthma severity after sensitization. We employed a clinically relevant

model of asthma-like pulmonary inflammation based on immunization with a crude CRA extract containing LPS contamination, followed by sensitization with a house dust extract (HDE) collected from the home of an asthmatic child. This HDE has been shown to contain high levels of both CRA and LPS, and can induce the cardinal features of asthma, such as airway eosinophilia, airways hyperresponsiveness and IgE production (76, 94). In order to investigate the role of LPS at the time of allergen challenge, LPS was depleted from the HDE using a commercially available column. The current study shows that removal of LPS from the HDE exacerbates AHR and cytokine production, reduces IgE, but does not alter airway eosinophilia or neutrophil recruitment.

Experimental Design

Detailed experimental methods are provided in Appendix A.

Depletion of LPS from House Dust Extract

LPS was depleted from the house dust extract (HDE) mixture using the EndoTrap Blue column (Profos AG, Regensburg, Germany), according to the manufacturer's protocol, with several modifications. HDE was diluted 1:1 in sterile PBS. The depletion column was equilibrated with the provided equilibration buffer modified to contain 400mM NaCl. The HDE mixture was applied to the column and eluted. Concentrations of Bla g1 and Bla g2 were measured by ELISA and LPS was measured by *Limulus* amoebocyte lysate assay before and after application to the column. The eluted mixture was immediately aliquoted and stored at -70°C until use.

Asthma induction protocol

House dust collection was performed as previously described (94). For immunization, 61.9 ng of German cockroach-allergen (99) (Greer Laboratories, LeNoir, NC, Item #

B46) was diluted to a final volume of 50 μL in sterile phosphate buffered saline (PBS) immediately prior to use. This mixture was emulsified in 50 μL TiterMax Gold adjuvant (CytRx, Norcross, GA). Due to limitations in the availability of the HDE, and given the fact that our lab has demonstrated that asthma induced by the HDE is CRA specific, the commercial CRA preparation was used for immunization (55). Each mouse was injected i.p. with 100 μL of the adjuvant/allergen mixture on day 0. The house dust extract and LPS-depleted house dust extract were heat inactivated at 56ºC for 30 minutes immediately prior to use. Mice were challenged on day 14 and day 21 with either the intact HDE preparation, or LPS-depleted HDE, each containing 61.9 ng CRA in a total volume of 50 μL. Challenge was carried out by direct intratracheal administration of the house dust mixture (100). Briefly, mice were lightly anesthetized and suspended by their front incisors on a vertical board. Their tails were taped down to support the body weight. The tongue was gently extended and the liquid was placed at the base of the oropharynx so that it was inhaled. Airway hyperresponsiveness was measured prior to sacrifice, and animals were euthanized by cervical dislocation following ketamine/xylazine anesthesia. Plasma, bronchoalveolar lavage fluid and lung homogenates were prepared at various timepoints after final challenge. The 0 hour timepoint refers to animals sacrificed on day 21 without receiving the second pulmonary challenge.

Sample Collection and Analysis

Airways hyperresponsiveness to methacholine challenge was measured by unrestrained whole body plethysmography. Mice were exanguinated and euthanized by cervical dislocation and BAL was performed. The left lung was removed into protease inhibitor cocktail for later myeloperoxidase and eosinophil peroxidase assays, as well as for

cytokine analysis. Cytospin slides were prepared from cells recovered in the BAL fluid to determine absolute numbers of inflammatory cells. Cytokines, chemokines and plasma antibodies were measured by standard ELISA. All data are represented as mean ± SEM. Statistical significance was determined by Student's *t*-test using GraphPad Prism version 4.0.3. (GraphPad Software, San Diego, CA). Statistical significance was achieved when $p \leq 0.05$ at the 95% confidence interval.

Results

Depletion of LPS from the house dust extract

Dust was collected from the homes of asthmatic children as described previously (94). This dust was assayed extensively and shown to contain significantly higher concentrations of cockroach allergens Bla g1 and Bla g2 in comparison to the other measured indoor and outdoor allergens. This extract was used in previous studies at a 1:10 dilution, which successfully induced the hallmark features of asthma, including airways hyperresponsiveness, eosinophilia and IgE production (101-103). In the present study, we specifically sought to determine whether removal of the potent innate immune activating agent LPS from the home environment can attenuate asthma severity in sensitized mice. To address this question, LPS was depleted from the HDE using a commercially available column as described in materials and methods. Our previous attempts at LPS removal using Polymyxin B columns also removed 99.9% of Bla g1 from the HDE, and were therefore not appropriate for the present studies. Although use of this column resulted in minor reduction in CRA concentration, the current method allowed us to retain significant concentrations of both Bla g1 and Bla g2 in the HDE post

LPS removal. Therefore, with appropriate dilution, we were able to maintain the CRA concentration of 61.9ng for all treatments.

Asthma was induced by i.p. immunization with 61.9 ng CRA, containing 9 ng of LPS contamination (Table 1). This dose was chosen because 61.9 ng of CRA is present in a 1:10 dilution of the HDE. Mice were challenged on days 14 and 21 with either the intact HDE or LP-depleted HDE, each containing of 61.9 ng CRA. Table 1 shows that the intact HDE contains roughly 182ng LPS, while the LPS-depleted HDE contains only 3ng LPS. Our efforts did not result in complete removal of LPS from the HDE, highlighting the difficulty involved in removing this pathogen from the home environment.

		Total CRA (Bla g1 + Bla g2) (ng/dose)	**LPS (ng/dose)**
Immunization	**CRA**	61.9	11.6
Challenge	**LPS-depleted HDE**	61.9	3.0
Challenge	**HDE**	61.9	182.1

Table 1: CRA and LPS levels present in extracts used to induce asthma-like pulmonary inflammation.

TNFα production in BAL fluid post final challenge

Sensitization to cockroach allergens plays a key role in the development of asthma, however the level of LPS present in the home has also been shown to have a major effect on the severity of respiratory diseases (76, 104, 105). Consistent with the low levels of LPS present in the LPS-depleted HDE, TNFα production was significantly decreased in this group at 2 hours post final challenge (Figure 2.1). Previous studies have shown that this early timepoint reflects the point at which TNFα production peaks in the BAL fluid (102).

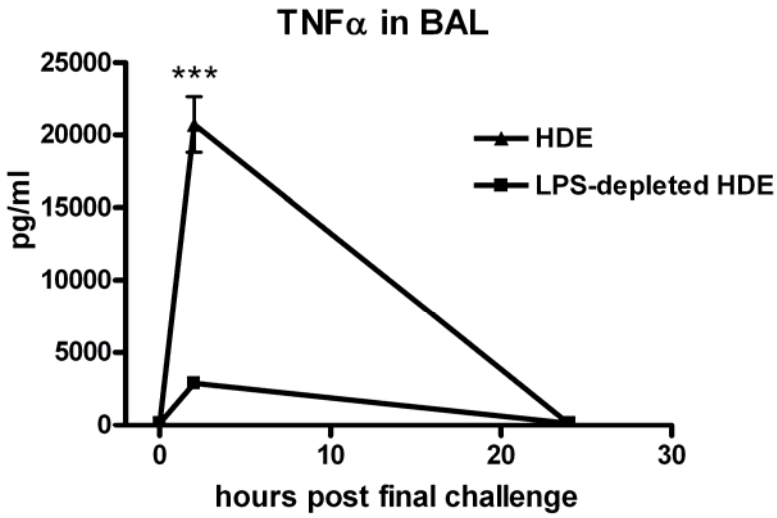


Figure 2-1: TNFα production in bronchoalveolar lavage fluid. TNFα was measured by standard ELISA at the timpoints indicated. The 0h sample was collected prior to the second pulmonary challenge. Data are represented as the mean ± SEM. n= 6-10 mice per g group. ***$p \leq 0.0001$ comparing the LPS-depleted HDE group to HDE the group

Role of LPS in antibody production in response to allergen challenge

The presence or absence of TNFα has been shown to affect the antibody response to allergen. In the absence of TNFα, class switch recombination resulting in IgE, as well as IgG1 production is significantly attenuated (80). We sought to determine whether attenuated levels of TNFα produced in response to challenge with the LPS-depleted HDE has an effect on antibody production. Interestingly, mice challenged with the intact HDE were able to produce significantly more IgE compared to mice challenged with LPS-depleted HDE (Figure 2.2A). However, compared to naïve BALB/c mice, challenge with the LPS-depleted HDE still induces class switch recombination leading to IgE production, although to a significantly lesser degree than HDE challenge. Plasma levels of IgG1 were also significantly decreased in mice challenged with the LPS-depleted HDE as seen in figure 2.2B. Similar to the case of IgE, IgG1 production seems to depend on the amount of LPS present at the time of allergen challenge. In contrast, challenge with the LPS-depleted HDE resulted in significantly increased production of plasma IgG2a (Figure 2.2C). Challenge with the intact HDE seems to inhibit IgG2a production, as these mice do not produce IgG2a above the levels found in naïve mice. Therefore, the level of TLR4 activation achieved with the HDE extract may inhibit class switch to IgG2a production. Taken together, these data suggest a threshold of TLR4 activation must be reached in order to skew the antibody response to allergen towards a Th2 phenotype.

Inflammatory cell recruitment in the BAL fluid post allergen challenge

The composition of inflammatory cells in the BAL fluid was assessed 24 hours after the final intratracheal challenge. Cytospin preparations demonstrate the similar numbers of eosinophils and neutrophils present between mice challenged with the intact

HDE and LPS-depleted HDE, in contrast to that of naïve mice containing predominantly macrophages (Figure 2.3). We observed a slight but statistically insignificant reduction in eosinophil numbers in the BAL fluid of the LPS-depleted HDE group (Figure 2.4A). The eosinophil-specific chemoattractant eotaxin-1 (CCL11) was significantly decreased at 24 hours post final challenge, however no difference in eotaxin-2 (CCL24) was observed in the BAL fluid (Figure 2.4 B&C).

We also assayed eosinophil-specific peroxidase activity (EPO) in the lungs of mice that had been subjected to BAL, as a measure of eosinophil presence in the lung tissue. No significant difference in EPO was measured between mice challenged with the LPS-depleted HDE and the intact HDE mixture (Figure 2.4D). However, a statistically significant decrease in eotaxin-1 (CCL11) was measured in the lung homogenate of mice challenged with the LPS-depleted HDE (Figure 2.4E). No difference was found in eotaxin-2 (CCL24) levels between groups (Figure 2.4F). While statistically significant differences in eotaxin-1 were observed in both the BAL and lung homogenate, it should be noted that eotaxin-2 is produced at higher concentrations in both the alveolar compartment and the lung tissue. This suggests that while eotaxin-1 production may be affected by the presence or absence of LPS, eotaxin-2 is not, rendering an equivalent biological outcome (eosinophil recruitment) in both groups.

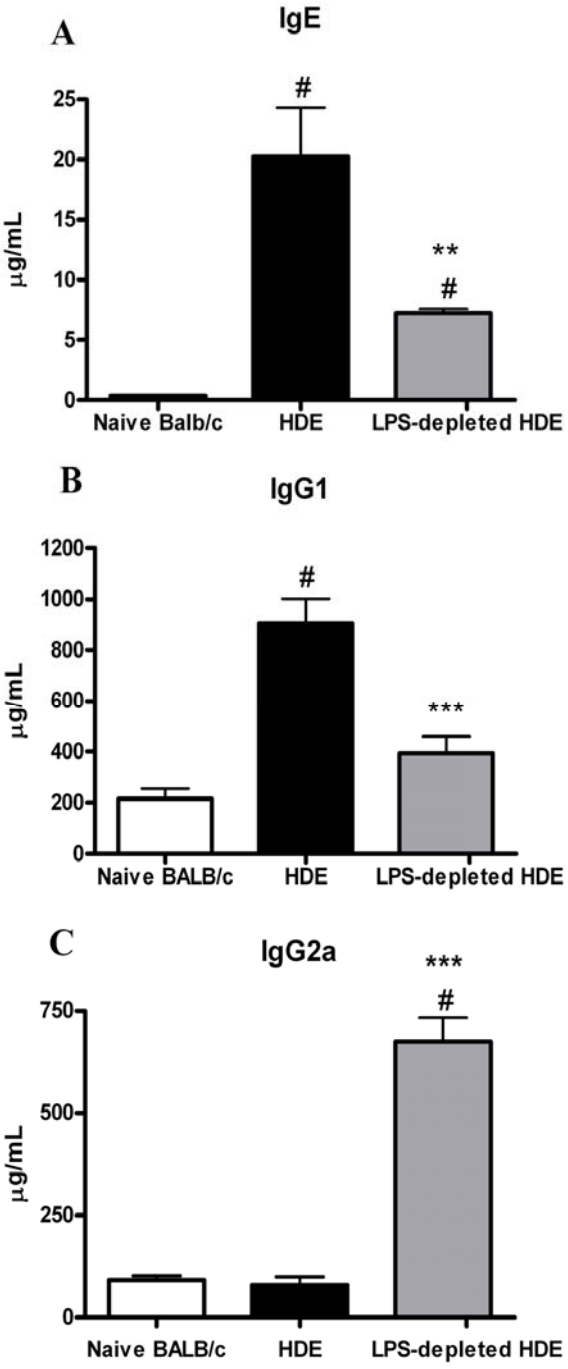


Figure 2-2: Plasma antibody production in immunized and challenged mice. Plasma levels of A) IgE, B) IgG1, and C) IgG2a were measured by standard ELISA from EDTA-plasma collected 24 hours post final challenge. Data are represented as the mean ± SEM. n= 6

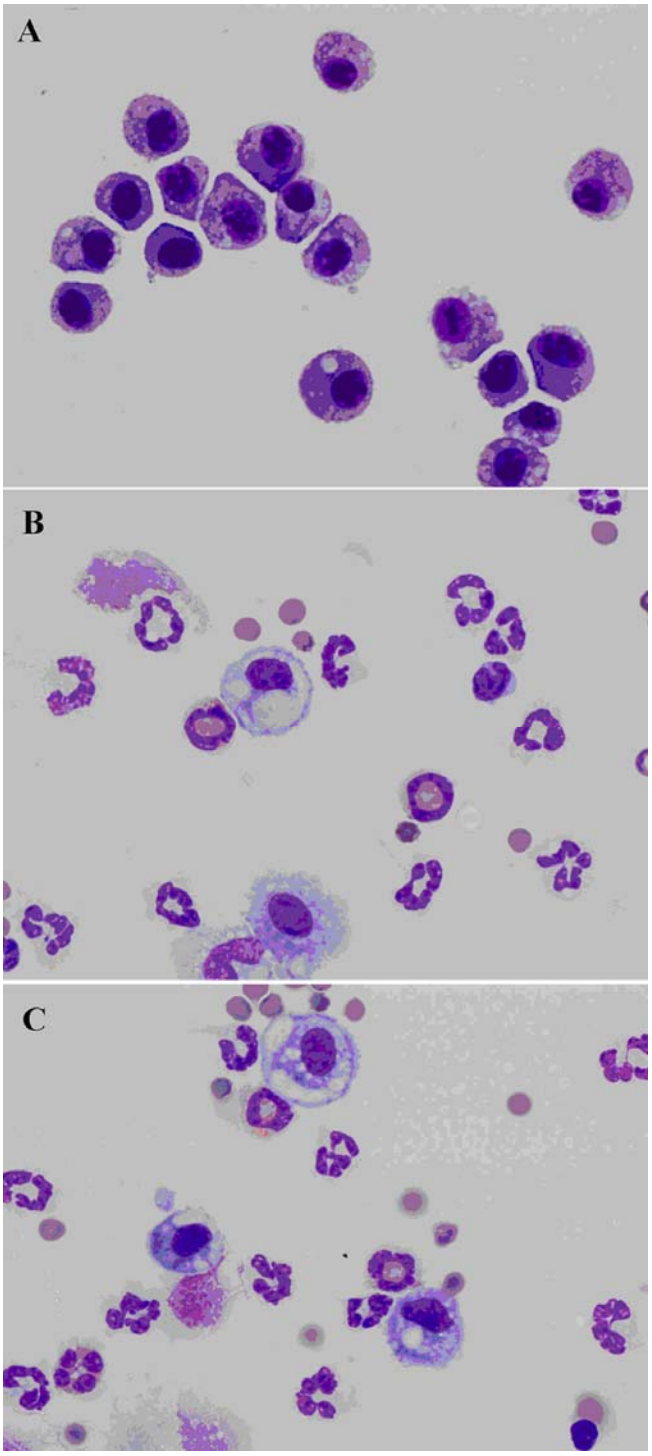


Figure 2-3: Cytospin preparations of cells recovered from A) Naïve mice, B) HDE challenged mice at 24 hours post final challenge and C) LPS-depleted HDE challenged mice at 24 hours post final challenge. Each is represented at 1000x.

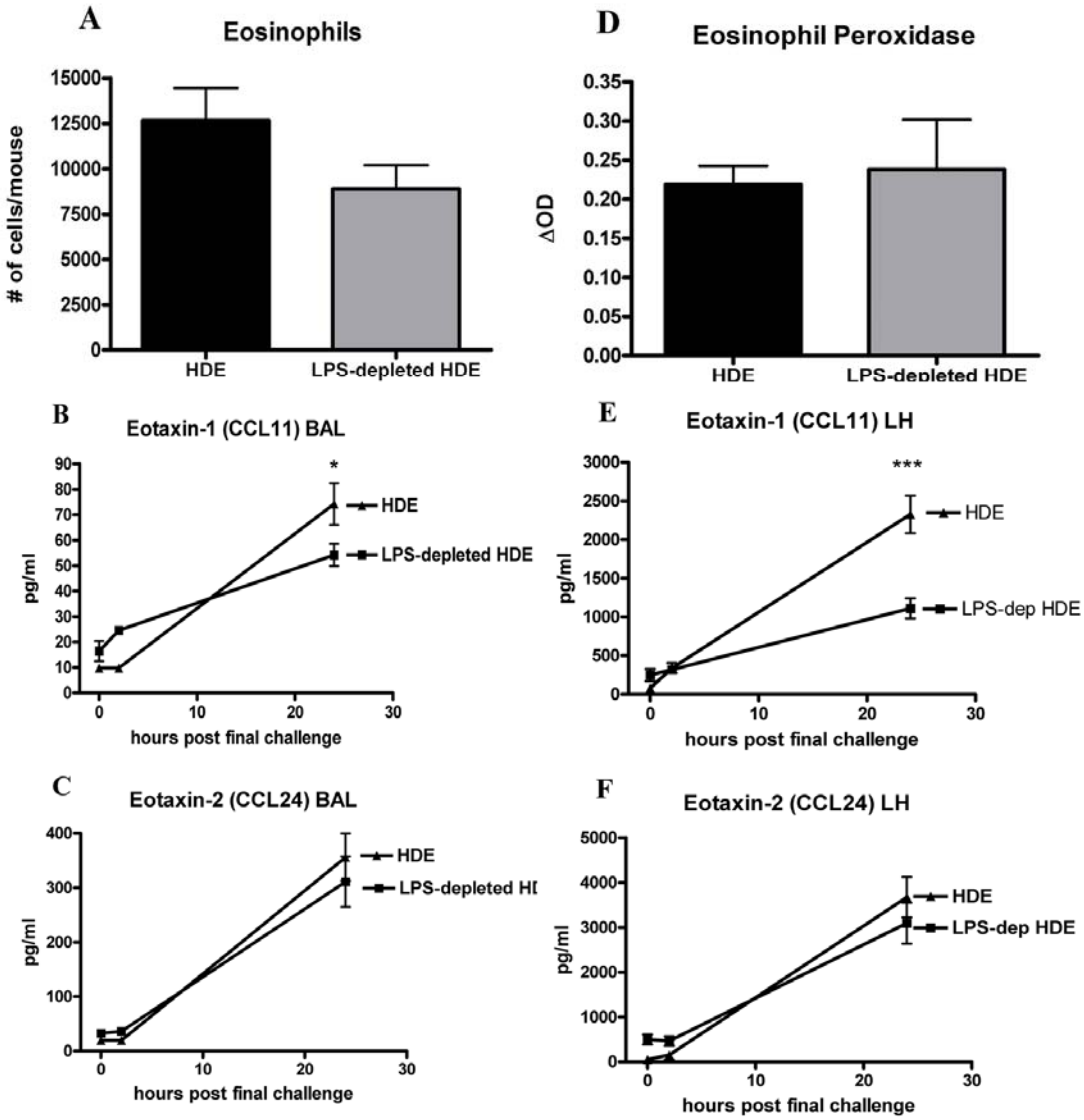


Figure 2-4: Eosinophil recruitment and production of eosinophil-specific chemokines in response to allergen challenge. Cytospin preparations from cells collected in the BAL fluid at 24h post final challenge were made and 300 cell differential counts performed to determine the absolute numbers of eosinophils present (A). Concentrations of the chemokines B) eotaxin-1 and C) eotaxin-2 in the BAL fluid were measured by standard ELISA. D) Eosinophil peroxidase activity was measured in the lung tissue after BAL at 24 hours post final challenge. Concentrations of E) eotaxin-1 and F) eotaxin-2 in the lung homogenate were assayed by standard ELISA. Data are represented as the mean ± SEM. n= 6-10 mice per group. * $p \leq 0.05$ and *** $p \leq 0.0001$.

We also assayed the number of neutrophils recruited in response to the pulmonary challenge. Interestingly, comparable numbers of neutrophils were present in both groups despite drastically reduced levels of LPS in the LPS-depleted HDE (Figure 2.5A). This suggests that other components of the HDE are able to activate the innate immune response in order to recruit neutrophils. The concentrations of neutrophil chemoattractants CXCL1 and CXCL2 were assayed as the mechanism of neutrophil recruitment into the alveolar space and lung tissue. BAL levels are CXCL1 are significantly decreased at 2 hours post final challenge in mice challenged with the LPS-depleted HDE, however no difference is seen in production of CXCL2 (Figure 2.5 B&C). Despite this slight decrease in chemokine production, we suspect that the concentrations at which they are being produced is adequate to induce the levels of neutrophil recruitment seen in figure 2.5A.

Myeloperoxidase activity (MPO) in the lung tissue was also assayed as a measure of neutrophil presence in the lung tissue. A slight increase in MPO activity was measured in the lung tissue of mice challenged with the LPS-depleted HDE (Figure 2.4D). This increase is reflected by significant increases in both CXCL1 and CXCL2 in the lung homogenate at all timepoints assayed (Figure 2.5E & F).

Th1 and Th2 cytokine production in the BAL post intratracheal allergen challenge

It has been suggested previously that the presence of TNFα can induce increased levels of Th2 cytokines in the BAL fluid of allergen challenged mice (102). Further, asthma is largely characterized as a Th2 mediated disease, accompanied by high IL-4 and IL-5 production and low IFNγ (106). To determine whether the reduction in TNFα seen in LPS-depleted HDE challenged mice had an effect on cytokine production, we assayed

both Th1 and Th2 cytokines 24 hours post final challenge. No differences in IL-4 concentrations in the BAL fluid was measured between groups, however IL-5 and IL-13 were significantly increased in LPS-depleted HDE challenged mice (Figure 2.6B, C &D). In conflict with the Th2 paradigm, IFNγ gamma was also significantly increased in the LPS-depleted HDE mice (Figure 2.6A), showing that in our clinically relevant model, the concept that Th2 cytokines dominate the allergic response does not hold true.

Induction of airways hyperresponsiveness after allergen challenge

Several studies have shown that both LPS inhalation and pulmonary TNFα administration induce AHR, however little data exist regarding the synergistic role of allergen and endotoxin on AHR (107-109). Therefore, we sought to determine whether the levels of LPS affect AHR severity. Challenge with 50 mg/mL aerosolized methacholine resulted in roughly 300% higher AHR at 4 hours post final challenge in mice exposed to the LPS-depleted HDE above that seen in HDE challenged mice (Figure 2.7). This suggests that the presence of LPS in the challenge mixture may protect against AHR, and that AHR in our model is not mediated by TNFα production.

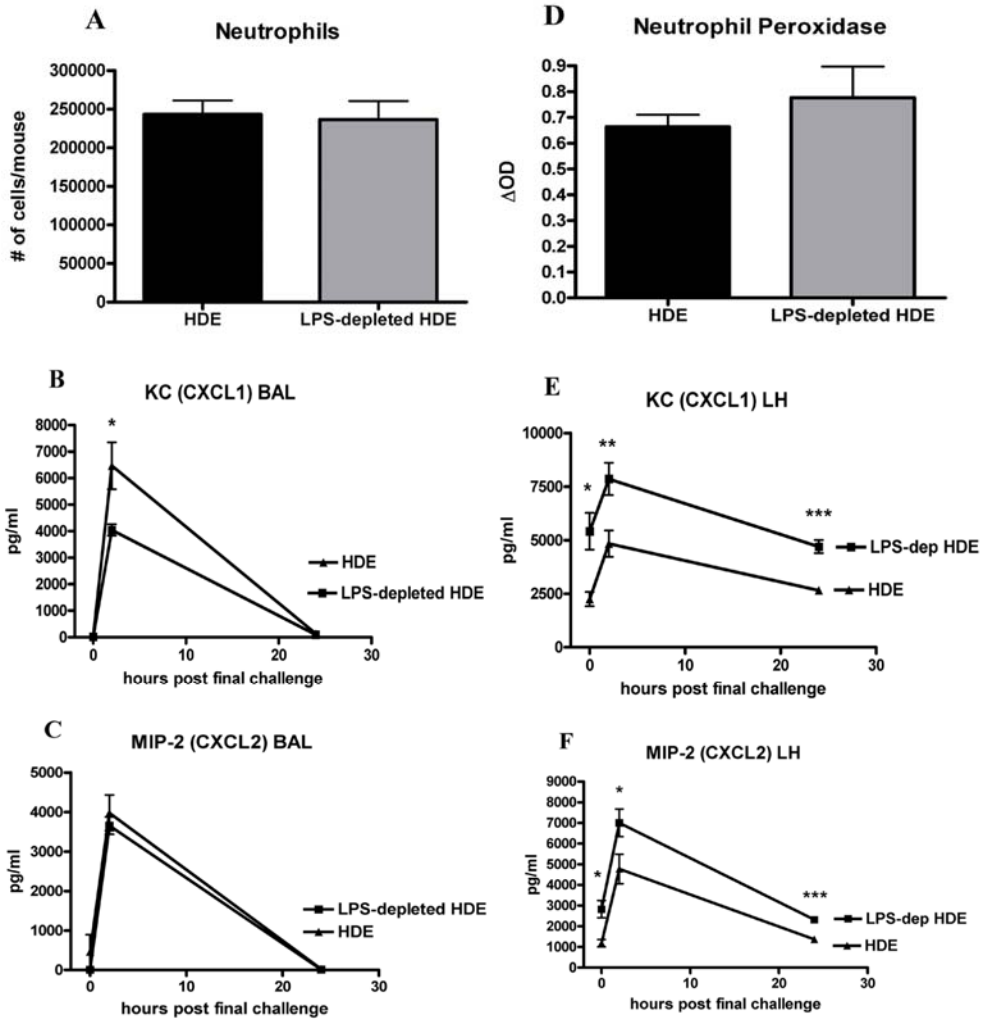


Figure 2-5: Neutrophil recruitment and production of neutrophil-specific chemokines in response to allergen challenge. Cytospin preparations from cells collected in the BAL fluid at 24h post final challenge were made and 300 cell differential counts performed to determine the absolute numbers of A) neutrophils present. Concentrations of chemokines B) CXCL1 and C) CXCL2 in the BAL fluid were measured by standard ELISA. D) Myeloperoxidase activity was measured in the lung tissue after BAL at 24 hours post final challenge. Concentrations of E) CXCL1 and F) CXCL2 in the lung homogenate were assayed by standard ELISA. Data are represented as the mean ± SEM. n= 6-10 mice per group. * $p \leq 0.05$, ** $p \leq 0.001$ and *** $p \leq 0.0001$ comparing the LPS-depleted HDE group to HDE the group.

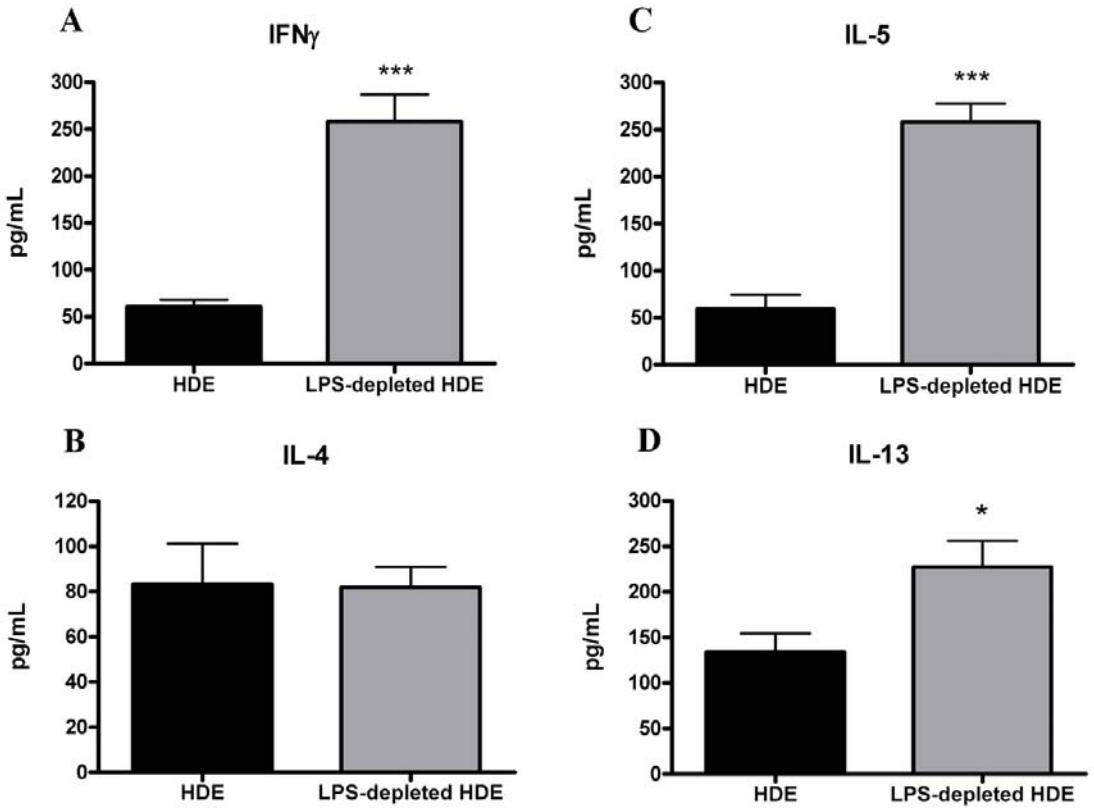


Figure 2-6: Th1 and Th2 cytokine production in the BAL fluid at 24 hours post final challenge. Cytokines were assayed by standard ELISA. Data are represented as the mean ± SEM. n= 6-10 mice per group. * $p \leq 0.05$ and *** $p \leq 0.0001$ comparing the LPS-depleted HDE group to HDE the group.

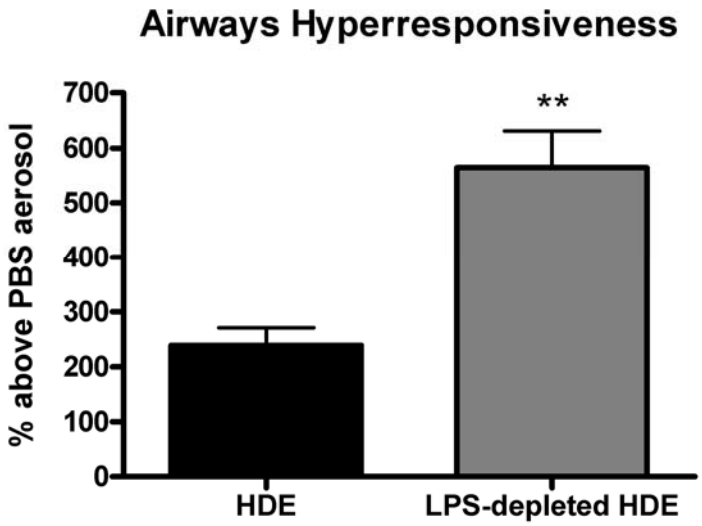


Figure 2-7: Airways hyperresponsiveness to aerosolized methacholine at 4 hours post final challenge. Mice were challenged with 50mg/mL aerosolized methacholine and PenH values are represented as percent above challenge with aerosolized PBS. Data are represented as the mean ± SEM. n= 6-10 mice per group. ** $p \leq 0.001$ comparing the LPS-depleted HDE group to HDE the group.

Discussion

The effect of exposure to household dust on the development of atopy and asthma has recently become a focus of investigation; however this avenue of study has resulted in more controversy than consensus. Epidemiological evidence shows that the presence of cockroach allergens in inner city homes is a strong risk factor for the development of atopy and asthma (110, 111). In fact, cockroach allergen sensitization was found to be responsible for exacerbations in a large proportion of asthmatic children in inner city Washington, D.C. Further, asthma exacerbations were not the result of sensitization to other allergens such as cat dander and dust mite allergens which were also present in these homes (91, 99). While allergen exposure plays a key role in sensitization and development of asthma, exposure to innate immune activators such as TLR ligands, as well as environmental pollutants such as diesel particulates can drive the adaptive immune response toward immune tolerance or sensitization (76, 112).

This area of study is becoming progressively more important in light of the increasing public emphasis being placed on the cleanliness of the home environment as a means to protect children against atopy and allergy.

This study sought to examine whether the presence of LPS in the household environment modifies the allergic response in the case of established hypersensitivity to cockroach allergens. Our model specifically evaluated the challenge phase of asthma, rather than the role of endotoxin in the sensitization phase. Recent studies have employed house dust extracts in order to determine their roles in asthma severity, and have suggested that house dust extracts can induce allergic phenotypes or can skew towards tolerance, depending upon the dose and timing of the exposure (112, 113). However,

these studies focused on the immunomodulatory effects of the house dust extracts on mice presensitized to OVA, and thus study house dust as an exacerbating factor, rather than a causative agent of asthma. In contrast, the current study directly examines the synergistic effects of allergen and LPS contained in the HDE in mice presensitized to CRA, which is the major allergen component of the HDE. LPS contamination in allergen preparations has been raised as an issue in many models of allergy, however we believe that approaching these studies using household components with naturally occurring LPS contamination more closely and accurately reflects the environment in which one develops asthma. A major strength of our current work is highlighted by the fact that the effect of LPS in the development of asthma can be investigated using a relevant allergen mixture, without the use of exogenous aeroallergens.

Optimization of our LPS depletion protocol resulted in removal of 98.4% of the LPS from the extract. Removal of 100% of the LPS was not possible, however this also reflects reality since it is currently impossible to remove 100% of LPS present in the home. Therefore, the aim of our study was to determine whether or not stringent efforts at maintaining a clean home environment in the context of LPS can attenuate asthmatic symptoms.

In the lung, TNFα is primarily produced by alveolar macrophages and is secreted within 1 hour of LPS challenge (114, 115). We initially wanted to determine if depletion of LPS could significantly attenuate this acute phase cytokine. In our model of asthma-like pulmonary inflammation, TNFα production in the BAL fluid peaks at 2 hours post final challenge (102). Our results show that mice challenged with the LPS-depleted HDE produce significantly less TNFα compared to those challenged with the HDE, verifying

that removal of LPS from the HDE has physiological ramifications. Several studies have implicated TNFα and TNFα signaling in the development of adaptive immunity, particularly in the context of class switch to IgE production (80, 116). Therefore, we wanted to determine if LPS could modulate the amount of IgE produced in response to allergen challenge. We found that mice challenged with the LPS-depleted HDE had significantly attenuated levels of plasma IgE compared to those challenged with the HDE. It should be noted that LPS-depleted HDE challenged mice had significantly higher levels of IgE than naïve mice, indicating that class switch recombination was taking place in these mice, but to a lesser extent than HDE challenged mice (Figure 2.2A). These data highlight the requirement of LPS signaling, and particularly TNFα, for production of IgE. Further, these data demonstrate that the amount of TNFα produced can modulate the level of IgE production.

We observed that LPS depletion significantly decreased IgE and IgG1 production, while augmenting IgG2a. In fact, the dose of LPS in the intact HDE seems to abrogate IgG2a production as these mice do not produce this antibody above the levels circulating in naïve mice. These data suggest that a threshold of TNFα exists that is responsible for dictating the development of a Th1 or Th2 type antibody response. We believe that this may the mechanism by which high-dose LPS exposure can skew towards a protective response with limited IgE production (117, 118). In the absence of TNFα, class switch to IgE production does not occur, however once TNFα production surpasses the threshold, a Th1 antibody response in the form of reduced IgE and increased IgG2a develops. The absence of TNFα, or in cases of excessive TNFα production, IL-4 production may be inhibited, thereby inhibiting class switch recombination to IgE production (116, 119).

While the presence of moderate doses of LPS at the time of allergen sensitization has been shown to induce a protective Th1 type cytokine response, our study shows that a very low level of LPS exacerbates both Th1 and Th2 cytokine responses in the lung (Figure 2.6). It has been suggested that allergen sensitization with adjuvants such as aluminum hydroxide can artificially skew towards a Th2 type allergic response (84, 85). However, our data clearly indicate that LPS can dictate the classical Th1/Th2 distinctions, irrespective of the use of adjuvants. Our observation that removal of LPS exacerbates both Th1 and Th2 cytokines suggests that the classical Th1/Th2 paradigm does not strictly apply in this model of allergic inflammation. However, more studies are needed to precisely determine the source of these cytokines.

Limited epidemiological data exist investigating whether removal of house dust can limit future asthma exacerbations. One such study carried out in the UK found no correlation between removal of house dust and amelioration of asthma symptoms in presensitized individuals. However, intervention in homes of children who were highly susceptible to developing atopy was effective in reducing asthma diagnosis (120, 121).

Taken together our data show that in a clinically relevant model of house dust induced asthma, the removal of LPS from the environment does not protect against the development of asthma-like pulmonary inflammation. In fact, in the context of AHR, as well as in the case of Th1 and Th2 cytokine production, removal of LPS can exacerbate the immune response. This finding calls into question the efficacy of clearance of these environmental components from the home as a means of attenuating asthma severity. Further studies are needed to determine whether or not the hygiene hypothesis holds true

in the case if LPS contamination in the home and allergic sensitization, and what levels of LPS are acceptable in the home environment.

Chapter 3

Acute Pulmonary LPS Tolerance Decreases TNFα without Reducing Neutrophil Recruitment

Introduction

Pulmonary LPS exposure plays a key role in the exacerbation of lung diseases such as chronic obstructive pulmonary disease (COPD) and asthma. However, little is known about the effects of repeated LPS exposure in the lung microenvironment. While quick recognition of pathogens is indispensable to the host, excessive inflammation in response to prolonged exposure can prove detrimental (122, 123). As such, compensatory mechanisms to avoid excessive inflammation and organ damage have developed. LPS tolerance represents one such method (124). To date, LPS tolerance has been characterized mainly as it pertains to septic shock, focusing on individual cell populations, mainly monocytes and macrophages. Elucidating the manner in which LPS tolerance modulates the local immune environment is crucial to determine how the host limits organ injury to LPS exposure without inducing immunosuppression. We have developed a novel murine model of pulmonary LPS tolerance induced by intratracheal (i.t.) administration of LPS. Here, we show that the lung is protected against excessive inflammation, without compromising the ability to mount a neutrophilic response.

Experimental Design

Detailed experimental methods are provided in Appendix A.

Induction of Acute LPS Tolerance

Mice were given PBS or 100ng E. coli LPS O111:B5 (Sigma-Aldrich, cat# 62325) in a total volume of 50μL by direct intratracheal challenge under isofluorane anesthesia (100). Briefly, mice were lightly anesthetized and suspended by their front incisors on a vertical board. Their tails were taped down to support the body weight. The tongue was gently extended and the liquid was placed at the base of the oropharynx so that it was inhaled. Mice received the second challenge of either PBS or 100ng LPS 48h later. Animals were sacrificed at various timepoints thereafter as indicated in the figure legends. In order to determine if systemic tolerance was induced, mice received an intratracheal challenge of PBS or 100ng LPS at time 0. They were then injected intraperitoneally (i.p.) with 100ng LPS in 100uL PBS at 48h. Blood was collected from the facial vein at the times indicated in the figure legend. EDTA plasma was assayed by ELISA for TNFα and IL-6.

Sample Collection and Analysis

Mice were euthanized by cervical dislocation and BAL fluid was collected. Cytospin slides were prepared from BAL fluid cells to determine the absolute numbers of neutrophils present in the alveolar space. The left lung was removed into protease inhibitor cocktail for myeloperoxidase assay and cytokine analysis. LPS levels in the BAL were measured by *Limulus* amoebocyte lysate assay. Cytokines and chemokines were measured by standard ELISA. mRNA was extracted from BAL cells and lung tissue and semi-quantitative real-time PCR was performed for the genes indicated in the figures. All data are presented as mean ± standard error. Statistical analysis was performed using

One-Way ANOVA. Turkey's post test for pairwise comparison was performed when the F value was significant. Statistical significance was achieved when $p<0.05$ at 95% confidence interval.

Results

Compartmentalization of pulmonary LPS tolerance

In order to determine whether i.t. administration of LPS induced systemic LPS tolerance, mice received an in intratracheal challenge of PBS or 100ng LPS at time 0, and received an intraperitoneal injection of LPS at 48h. Blood was collected by facial vein bleed at the indicated timepoints, plasma was recovered and assayed for TNFα and IL-6. Blood was sampled from each mouse repeatedly rather than sacrificing the mice at each time point, in order to reduce the number of animals used in the experiment. Plasma TNFα levels peaked in both groups at 1 hour post final challenge and begin to drop thereafter, while IL-6 levels peak 2 hours post final challenge (Fig. 3.1). Animals receiving i.t. LPS challenge and those receiving PBS produced equivalent concentrations of both TNFα and IL-6, indicating that i.t. LPS administration does not result in systemic tolerance to LPS. In fact, the total cytokine production over the 6 hour time course as calculated by the area under the curve, was similar for TNFα (Tolerant vs. non-tolerant; (mean±SEM) 2037 ± 298 vs. 1974 ± 302) and IL-6 (23982 ± 7122 vs. 20687 ± 4397). Due to limited sample volumes, additional cytokines and chemokines could not be assayed.

Acute phase cytokine expression in the lung after LPS tolerance induction

Attenuation of acute phase cytokines, especially TNFα protein and mRNA production are hallmark features of LPS tolerance (18, 125). As such, we sought to first

verify that LPS tolerance can be induced by isolated intratracheal LPS exposure. For these experiments, tolerant mice received two intratracheal exposures of LPS, one at time 0 and again at 48h.

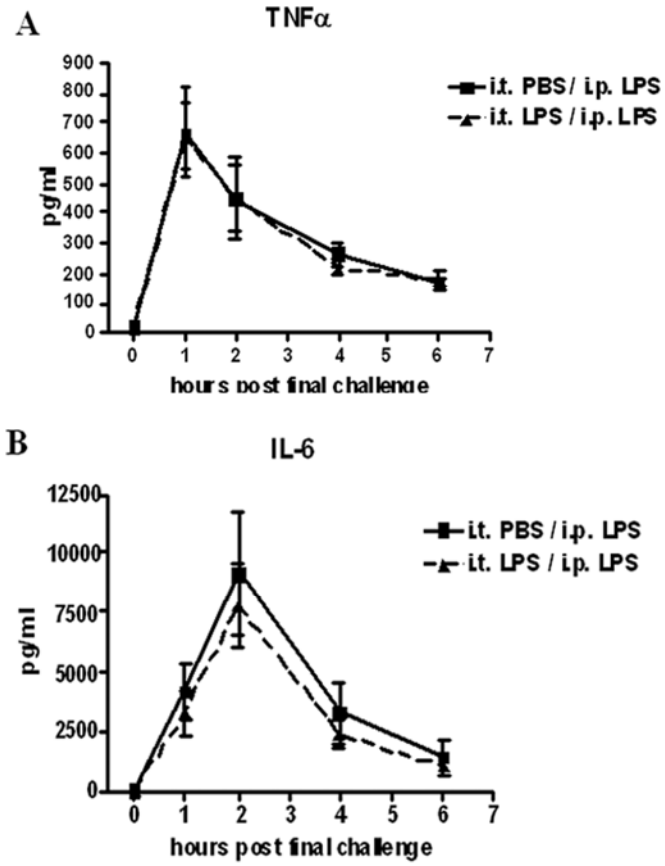


Figure 3-1: Compartmentalization of pulmonary LPS tolerance. Mice were given either PBS or 100ng LPS on Day 0 by direct intratracheal installation. After 48h, mice were injected intraperitoneally with 100ng LPS. Blood was collected by facial vein bleed at the indicated timepoints post final challenge and plasma A) TNFα and B) IL-6 levels were measured. Values are expressed as mean ± SEM. n=6 mice per group.

Non-tolerant mice received PBS for the time 0 challenge and LPS at 48h. Two additional control groups, one receiving PBS for both challenges (PBS/PBS) as well as one receiving LPS for the first and PBS for the second challenge (LPS/PBS) were also included. The LPS/PBS group was included to determine whether the first LPS exposure had any residual effects lasting until the 48h challenge.

TNFα protein was reduced approximately 78% in the BAL fluid of LPS tolerant mice compared to non tolerant animals at 4 hours post final challenge (Fig. 3.2A). In addition, TNFα mRNA was significantly reduced in cells isolated from the BAL fluid at 30 minutes post final challenge (Fig 3.2B). At this early time point (30 minutes), the cells recovered in the BAL fluid of non-tolerant mice contained roughly $2.2x10^4$ neutrophils, while BAL fluid from the tolerant group showed a significant contribution of neutrophils ($\sim 9.8x10^4$). This also represents the time point of maximal TNFα mRNA production (114). TNFα protein in the lung tissue of tolerant mice at 4 hours was reduced, but not significantly (Fig 3.2C), while mRNA was significantly reduced at 1 hour (Fig 3.2D).

Production of IL-6 protein at 4 hours in the alveolar compartment was significantly reduced in the LPS tolerant animals (~25%), although the reduction was not nearly as dramatic as TNFα. IL-6 mRNA measured in the tissue, however was reduced by 400 fold compared to non-tolerant mice (Fig. 3.3). We suspect that although this reduction is significant, the 100 fold increase in IL-6 mRNA seen in the tolerant mice approaches the maximal capacity of the cells to translate the protein.

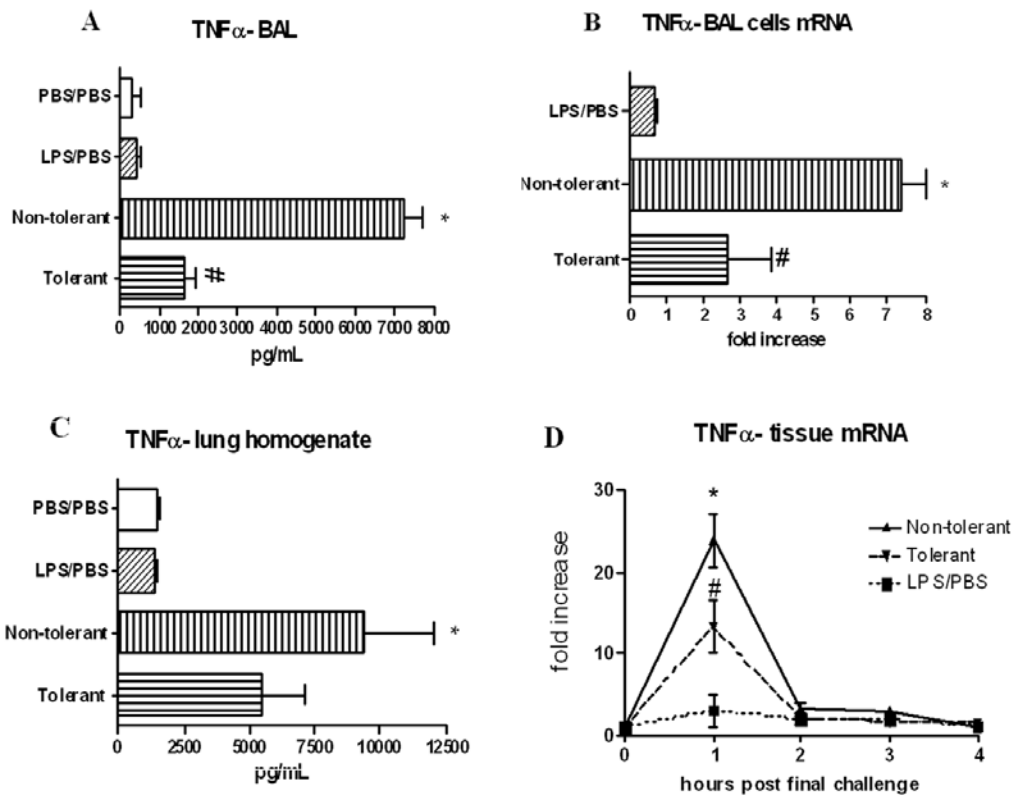


Figure 3-2: TNFα expression after induction of LPS tolerance. A) BAL TNFα protein levels 4h post final challenge and B) mRNA expression from cells recovered from the BAL 30min post final challenge. C) Lung homogenate protein levels 4h post final challenge and D) kinetics of mRNA expression in lung tissue. mRNA results are expressed as fold increase above PBS/PBS group and all data are expressed as mean ±SEM. n=8-10 mice per group. *$p<0.05$ compared with PBS/PBS group. #$p<0.05$ compared to non-tolerant group.

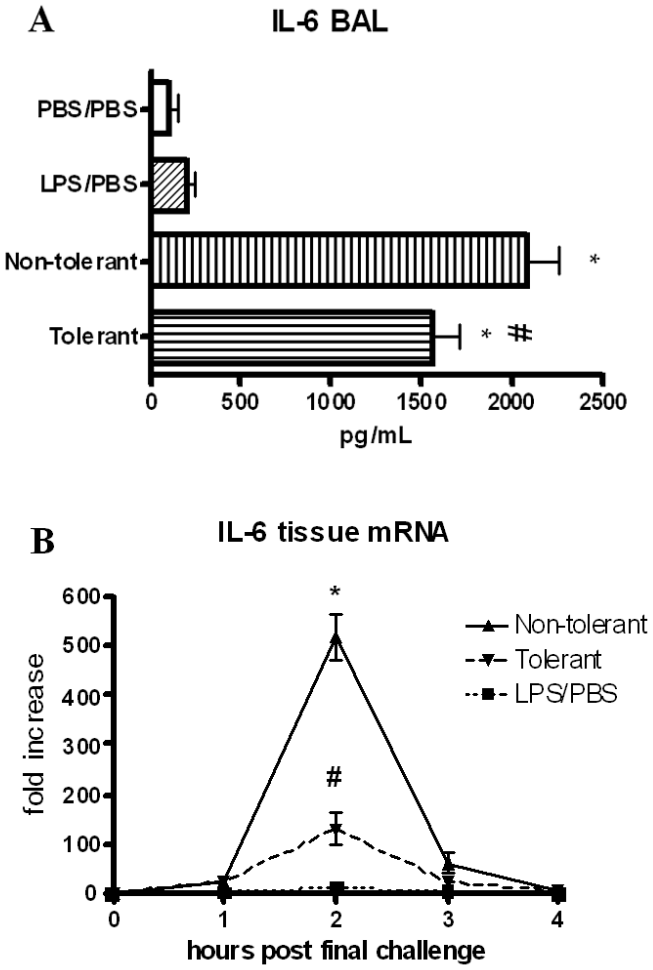


Figure 3-3: IL-6 expression upon induction of LPS tolerance. A) IL-6 protein levels in BAL and lung homogenate 4h post final challenge. B) Lung tissue IL-6 mRNA at various timepoints post final challenge. mRNA was expressed as fold increase above PBS/PBS group. Data are expressed as mean ±SEM. n=8-10 mice per group. For protein, *$p<0.05$ compared with PBS/PBS group. For mRNA *$p<0.05$ compared with LPS/PBS group. #$p<0.05$ compared to non-tolerant group for both protein and mRNA.

Further mRNA induction would not induce much more protein expression, accounting for the fact that a similar dramatic reduction in IL-6 protein was not observed (126). Additionally, the anti-inflammatory mediator IL-10 has been shown to play a role in various models of LPS tolerance (127, 128). However, we found no detectable IL-10 in the BAL fluid of non-tolerant or tolerant mice, further highlighting the specificity of pulmonary LPS tolerance (data not shown).

TNF soluble receptor production

We also assessed production of the TNF soluble receptors TNF-SRI and TNF-SRII for their contribution to the altered immune environment that develops in response to LPS tolerance. Both TNF-SRI and TNF-SRII were significantly increased in LPS tolerant mice in the BAL fluid 4 hours post final challenge, (Fig. 3.4) indicating that a compensatory anti-inflammatory response is induced upon repeated LPS stimulation that is independent of the reduction in TNFα levels. Additionally, TNF-SRII likely plays a greater physiological role as it is expressed at 10 fold higher concentrations than TNF-SRI.

TNFα ELISA validation

Reduced TNFα protein, combined with increased soluble receptor expression, called into question the ability of our ELISA to measure TNFα complexed with soluble receptors. In order to address this issue, high (3000pg/mL) and low (300 pg/mL) concentrations of TNFα were mixed with various concentrations of either TNF-SRI or TNF-SRII, allowed to incubate at room temperature and then assayed by sandwich ELISA for TNFα. These concentrations of soluble receptors spanned the range observed in our experimental results. As shown in figure 3.5, our assay is able to measure both free

and complexed TNFα. Comparison by one way ANOVA showed no significant difference in TNFα for all soluble receptor concentrations, showing that soluble receptors do not inhibit measurement of TNFα. This indicates that the observed reduction in TNFα is a relevant physiologic observation and not false observation due to assay limitations.

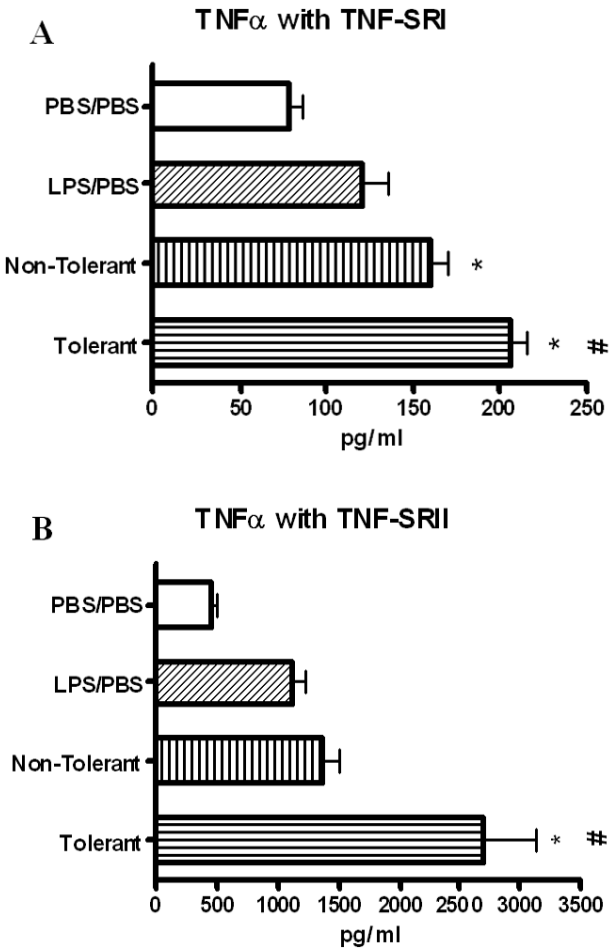


Figure 3-4: Expression of A) TNF-SRI and B) TNF-SRII in BAL fluid at 4 hours post final challenge. Data expressed as mean ±SEM. n=8-10 mice per group. *$p<0.05$ compared with PBS/PBS group. #$p<0.05$ compared to non-tolerant group.

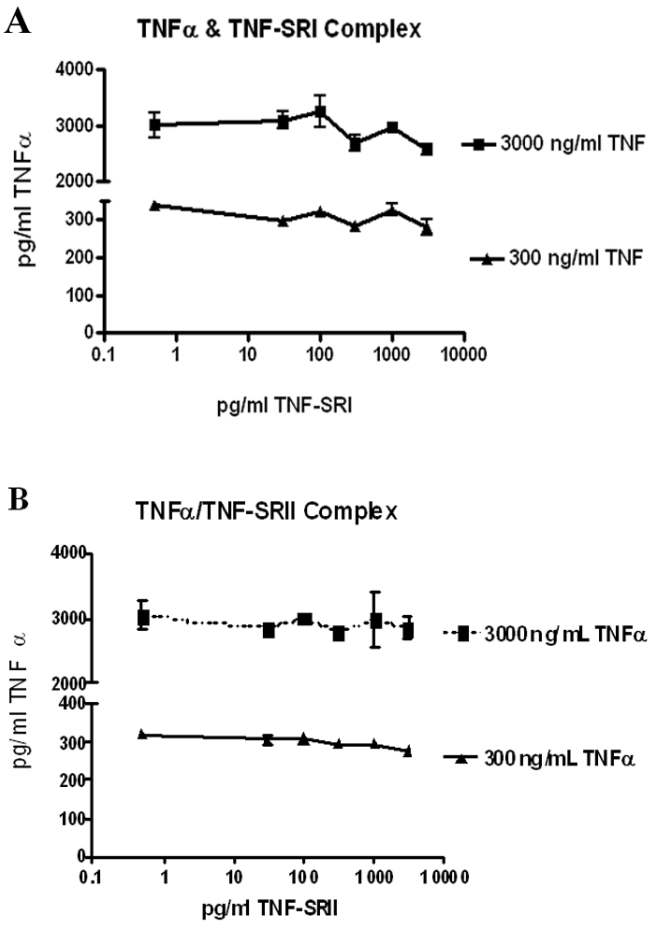


Figure 3-5: Validation of TNFα ELISA: Measurement of TNFα in complex with A) TNF-SRI and B) TNF-SRII. High (3000pg/mL) and low (300pg/mL) concentrations of recombinant TNFα were mixed with various concentrations of TNF-SRI and TNF-SRII and assayed by standard ELISA. Data shown are a combination of two experiments. Soluble receptor concentrations are represented on a log scale.

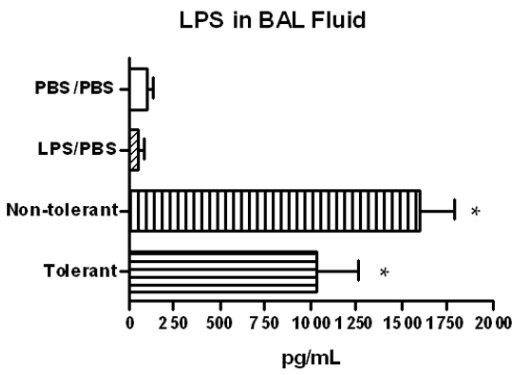


Figure 3-6: LPS clearance from the lung microenvironment. LPS levels were measured in the BAL fluid 4 hours post final challenge by endpoint LAL assay. *$p<0.05$ compared with PBS/PBS group.

LPS clearance from the lung microenvironment

We determined LPS levels in the lung in order to assess whether or not LPS was still present and able to signal several hours post final challenge, and to determine whether or not LPS tolerance modulates the ability of mice to clear LPS. LPS tolerant animals have a slight, but not significant reduction in LPS concentrations in the lung as determined by limulus assay (Fig 6), indicating that the reduction in TNFα levels was not the result of increased LPS clearance.

CXC chemokine expression and neutrophil influx

We next assayed whether LPS tolerance affected the expression of CXC chemokines and the ability of LPS tolerant animals to recruit neutrophils to the lung. Figures 3.7A and 7B show no attenuation in the production of CXCL1 or CXCL2 in LPS tolerant animals in the alveolar compartment. However, slight reductions in both CXCL1 and CXCL2 were observed in the organ homogenates (Fig 3.7C and D). CXCL1 mRNA peaked at 2 hours post final challenge, while CXCL 2 mRNA peaked at 1 hour, and LPS tolerance induction had no effect on the mRNA production of either of these chemokines (Fig 3.7E and F).

Cytospin preparations and 300 cell differential counts were performed in order to assess the ability of LPS tolerant animals to recruit neutrophils to the lung in response to LPS challenge. LPS tolerant animals are able to recruit comparable numbers of neutrophils at 4 hours compared to non-tolerant animals (Fig 3.8A). No statistically significant difference was observed for tissue myeloperoxidase activity in LPS tolerant mice, indicating that similar numbers of neutrophils were present in the lung tissue (Fig. 3.8B).

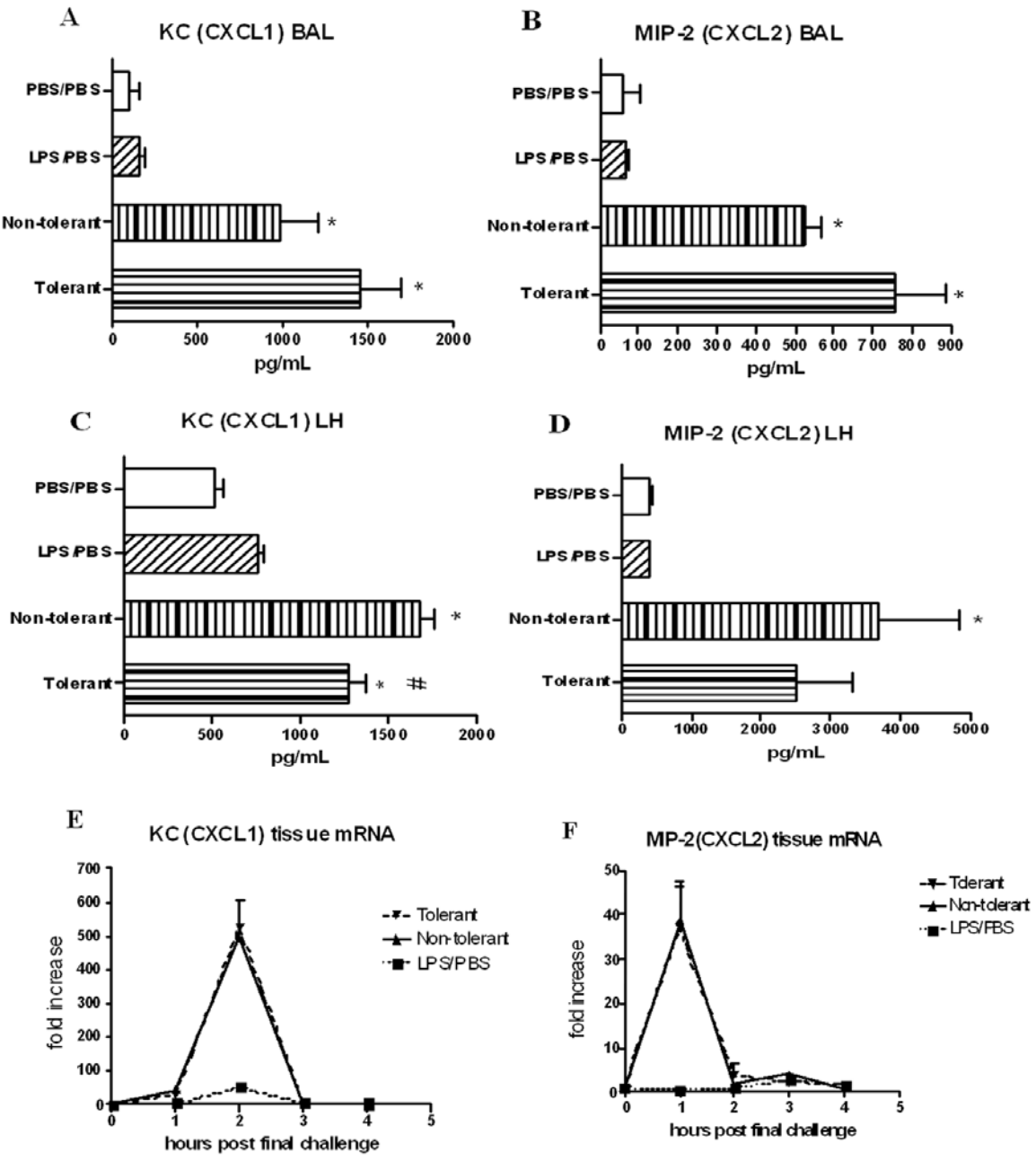


Figure 3-7: Neutrophil chemoattractant protein expression after induction of LPS tolerance. A) KC (CXCL1) and B) MIP-2 (CXCL2) expression in BAL fluid at 4 hours post final challenge. C) KC and D) MIP-2 expression in lung homogenate at 4 hours. E) KC and F) MIP-2 lung tissue mRNA expression at various timepoints post final challenge. mRNA was expressed as fold increase above PBS/PBS group. Data are expressed as mean ±SEM. n=8-10 mice per group. For protein *$p<0.05$ compared with PBS/PBS group. For mRNA *$p<0.05$ compared with LPS/PBS group. #$p<0.05$ compared to non-tolerant group for both protein and mRNA.

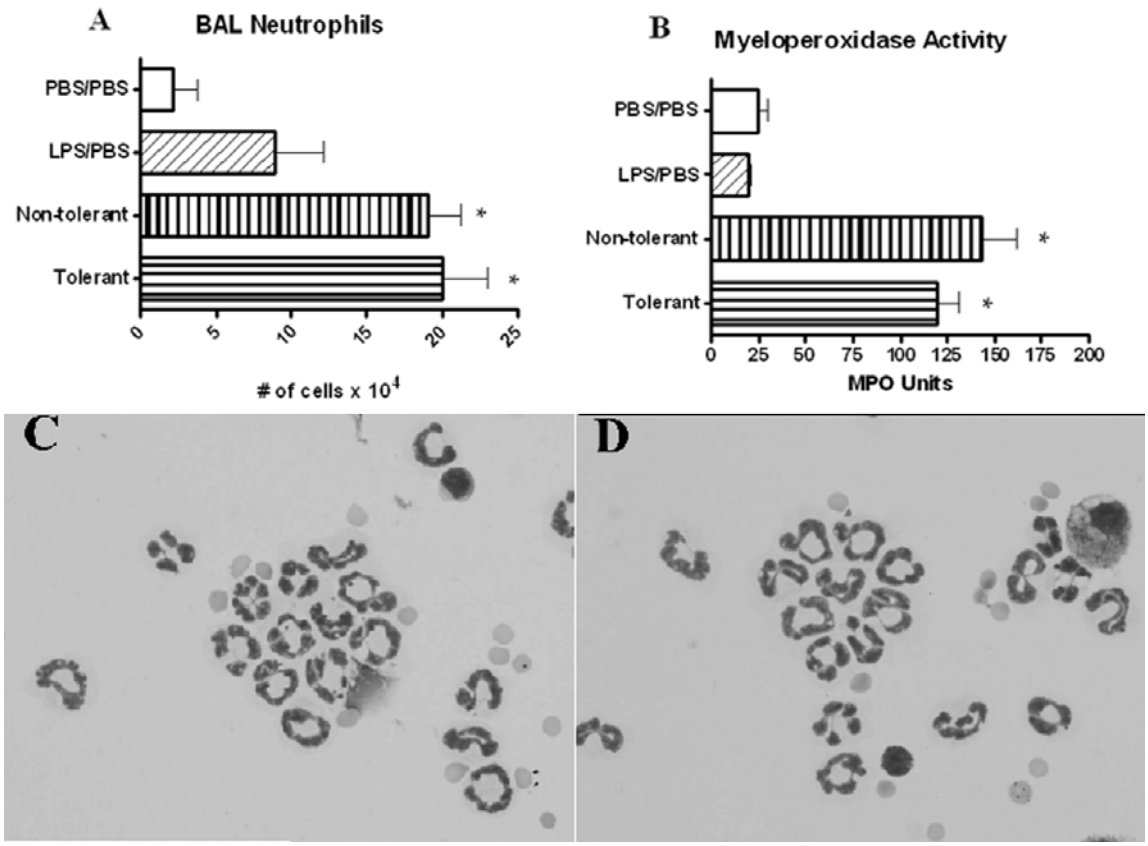


Figure 3-8: Neutrophil recruitment and myeloperoxidase activity 4 hours post final challenge. A) BAL fluid was collected and cytospins prepared from collected cells. Data expressed as absolute cell number per mouse. B) After BAL, the right lung was collected and homogenized for myeloperoxidase assay. Data are expressed as mean ±SEM. n=8-10 mice per group. *$p<0.05$ compared to PBS/PBS group. Cytospin preparations of cells recovered for BAL fluid at 4 hours from C) Non-tolerant mice, and D) Tolerant mice and represented at 1000x.

Discussion

LPS tolerance has long been defined as refractoriness to LPS following an initial LPS challenge. Previous exposure to LPS offers protection against subsequent lethal LPS challenge in mice and this tolerance has been shown to be principally mediated by macrophages (129, 130). LPS tolerance induction has been well studied in models of systemic bacterial infection and has been shown to enhance bacterial clearance in mice (131). Additionally, previous efforts have characterized LPS tolerance in various organs, including findings that TLR4 expression is decreased in the livers of LPS tolerant mice (132).

Most of these studies involve intraperitoneal or intravenous administration of LPS, or *ex vivo* stimulation of isolated primary cells. Little data documents *in vivo* LPS tolerance and even less evaluates LPS tolerance specific to the lung microenvironment. Inhalational LPS exposure has been shown to cause various respiratory conditions, including wheezing, and inflammation, and has been shown to modulate asthma severity. Given that the lung is a unique route of pathogen exposure we have developed a novel model of *in vivo* pulmonary LPS tolerance (133, 134).

Previous in vivo studies induce LPS tolerance by pre-exposure to sublethal doses of LPS, followed by re-challenge with a lethal dose. We chose to develop a dosing regimen that more closely resembled inhalational exposure in the context of lung disease. In particular, 100ng was chosen to recapitulate low-dose LPS exposure in several models of murine asthma (80, 118). We also chose the 48 hour interval between pre-exposure and re-challenge based on studies demonstrating that the potential to induce LPS

tolerance is exhausted by 72 hours post pre-exposure (135). Therefore, the 48 hour exposure interval was one that was likely to induce tolerance.

Intratracheal LPS exposure followed by intraperitoneal exposure 48 hours later does not result in systemic tolerance induction, highlighting the fact that this model is specific for pulmonary LPS tolerance (Fig. 3.1). Other studies have indirectly studied pulmonary LPS tolerance by systemic LPS administration followed by *ex vivo* stimulation of alveolar cells (36, 135). This experimental approach requires relatively high LPS doses (up to 100μg/mouse), in order to cause vascular permeability and LPS leakage into the airways, where it can be taken up by alveolar macrophages. These models are clinically relevant in the case of systemic bacterial infection and sepsis, but do not model day-to-day pulmonary LPS inhalation, and are therefore appropriate for the study of LPS-mediated lung diseases. Our model of direct pulmonary exposure closely mimics environmental LPS exposure by inhalation (94, 136).

We have also shown that LPS tolerant mice are not impaired in their ability to clear LPS, as there is no significant difference in the concentration of LPS remaining in the lung 4h post final challenge (Fig. 3.6). The prolonged presence of LPS may be responsible for CXC chemokine production, as well as the unimpaired neutrophil recruitment. As shown in figure 3.8A, the BAL compartment of LPS tolerant mice is composed of roughly $8.9x10^4$ neutrophils at the time of the second LPS challenge. The increased number of neutrophils in comparison to the non-tolerant group ($2.2x10^4$) is a key immune modulation that results from pre-exposure to LPS, and likely has a role in further chemokine production seen in the tolerant group. Lung histopathology of H&E stained lungs, which were not subjected to BAL, showed no significant presence of

neutrophils in the tissue and alveolar space of both tolerant and non-tolerant mice 4 hours after the second LPS challenge (data not shown).

The production of compensatory anti-inflammatory mediators TNF-SRI and TNF-SRII were significantly increased in tolerant mice (Fig. 3.4). We have validated that our ELISA assay for measuring TNFα detects free, as well as soluble receptor bound TNFα, showing that the observed decrease in TNFα protein is not merely the result of an assay limitation (Fig 5). Still, this does not rule out the possibility that increased soluble receptor production may be responsible for increased binding and clearance of secreted TNFα (137).

Several studies have implicated the interleukin receptor-associated kinase (IRAK-M) as a principle mediator of LPS tolerance in macrophages (35, 138). However, since IRAK signaling takes place upstream of NF-κB activation, which is responsible for regulating TNFα, IL-6, and the CXC chemokines, it was not expected that IRAK-M plays a major role in the differential regulation of cytokines and chemokines seen in this model. Further, whole lung nuclear extracts in this model showed no difference in NF-κB activation between non-tolerant and tolerant mice (data not shown).

Inhibition of TNFα is an emerging therapeutic target for inflammatory diseases such as asthma and Chronic Obstructive Pulmonary Disease (COPD). Although controversial, serious side effects such as increased incidence of infection and pneumonia have been reported to result from use of these therapies (139, 140). The specific immune modulation induced by our model of LPS tolerance suggests a mechanism by which anti-TNFα treatments for asthma have resulted in increased incidences of pneumonia and have been shown to inhibit treatment of pneumonia (141, 142). Pharmacological TNFα

inhibition has been shown to induce a local state of immunosuppression which includes reduced numbers of neutrophils (102, 143). This may render the lung more susceptible to infection by inhibiting the influx of neutrophils in response to bacterial challenge. Our data show that with LPS tolerance, TNFα is suppressed, but neutrophils are still present, which may offer protection against bacterial infection. Further, we have shown that the mechanism of continued neutrophil recruitment is the sustained production of CXC chemokines in LPS tolerant mice.

LPS tolerance may also offer protection in the case of allergic asthma. In an ovalbumin model, TLR4 deficient mice show significant attenuation of the hallmark features of asthma such as IgE production and eosinophil recruitment. These were restored by intratracheal administration of recombinant TNFα 1 hour after ovalbumin sensitization (80). In the LPS tolerant state, TNFα levels are low, which may offer protection against allergic sensitization. Indeed, much epidemiological data exist correlating repeated LPS exposure with reduced incidence of asthma, such as that seen in children raised on farms (144, 145). Further studies need to be conducted to determine whether or not those who do not develop asthma are tolerized to LPS and more importantly, whether they respond poorly to LPS challenge in terms of TNFα production.

The selective immune modulation following induction of LPS tolerance suggests a novel mechanism by which immune regulation is achieved in the lung. The selective suppression of TNFα may be a mechanism by which the lung prevents excess inflammation and injury caused by TNF α-induced cytokines, while the sustained ability to recruit neutrophils may offer protection against bacterial challenge. However further studies are required to understand LPS-induced lung injury in the context of LPS

tolerance. Additionally, further studies aimed at understanding the local immune modulation induced by pulmonary LPS tolerance are crucial to understanding how this can affect the onset and progression of various diseases such as asthma, pneumonia and COPD.

Chapter 4

Chronic Pulmonary LPS Tolerance Induces Selective Immunosuppression and Protects Against AHR through Muscarinic Receptor Downregulation

Introduction

Inhalation of endotoxin (LPS) containing particulates is a serious concern for many individuals and can exacerbate chronic lung conditions such as asthma and chronic obstructive pulmonary disease (COPD). While LPS inhalation is widely studied in the context of these chronic lung diseases, little information exists about the effects of repeated LPS inhalation in the absence of underlying disease. Inhalational LPS exposure can occur in the household from cigarette smoke, as well as in the workplace of agricultural and textile workers (146). Environmental exposure to high levels of LPS has been associated with decreased lung function and inflammation, but has also been associated with reduced risk of atopy and reduced rates of certain lung cancers (37-39). Given these conflicting data, and the evidence that the lung is constantly exposed to potentially pathogenic compounds, we hypothesized that the lung must utilize compensatory mechanisms to avoid excessive inflammation and potential organ damage.

We have recently developed a novel model of acute pulmonary LPS tolerance (147). This study provided the first global characterization of the effect of repeated LPS inhalation in the mouse. In the current study, we aimed to expand this model into a

clinically relevant model of chronic, high-dose LPS exposure that more accurately recapitulates repeated inhalation in the environmental setting.

Experimental Design

Detailed experimental methods are described in Appendix A.

Induction of Chronic Pulmonary LPS Tolerance

In order to examine the effects of repeated LPS exposure, we developed a protocol which will be referred to as chronic pulmonary LPS tolerance. Mice were exposed to either PBS or 1μg E. coli LPS O111:B5 (Sigma-Aldrich, cat# 62325) in a total volume of 50μL, for four consecutive days by direct intratracheal installation while under isofluorane anesthesia (100). Briefly, mice were lightly anesthetized and suspended on a vertical board by their front incisors. The tails were taped to the board to support the body weight. The tongue was gently extended and the liquid placed at the base of the oropharynx so that it was inhaled. Tolerant mice were challenged with LPS for four consecutive days, while non-tolerant mice were challenged with PBS. Twenty-four hours later, both groups were challenged with 10μg LPS. Airways hyperresponsiveness was measured prior to sacrifice. Animals were euthanized and samples collected at various timepoints indicated in the figure legends.

Sample Collection and Analysis

Airways hyperresponsiveness to methacholine challenge was measured by unrestrained whole body plethysmography at 4 hours post final challenge. Mice were exanguinated and euthanized by cervical dislocation. Complete blood count was performed on blood collected 24 hours post final challenge. Immediately after sacrifice, BAL was performed and cytospin slides were prepared from BAL fluid cells to determine the absolute

numbers of neutrophils present in the alveolar space. The left lung was removed into protease inhibitor cocktail for myeloperoxidase assay and cytokine analysis. LPS levels in the BAL were measured by *Limulus* assay. Cytokines and chemokines were measured by standard ELISA. mRNA was extracted from BAL cells and lung tissue and semi-quantitative real-time PCR was performed for the genes indicated in the figures. All data are represented as mean ± SEM. Statistical significance was determined by Student's *t*-test using GraphPad Prism version 4.0.3. (GraphPad Software, San Diego, CA). Statistical significance was achieved when $p \leq 0.05$ at the 95% confidence interval.

Results

Body weight and hematologic parameters after induction of chronic LPS tolerance

In order to induce chronic LPS tolerance, mice received direct intratracheal installation of 1μg LPS for four consecutive days and then received 10μg LPS 24 hours later. Non-tolerant mice received PBS for the first four challenges and 10μg LPS 24 hours later. This model employs relatively high doses of LPS for the induction of tolerance. As such, we determined whether repeated inhalation of high dose LPS caused systemic physiological changes. Although the body weight did decrease slightly over time there was no statistical difference between non-tolerant and tolerant animals (Figure 4.1). Day 0 represents the day before the first intratracheal challenge. Since either stress or LPS may induce lymphocyte apoptosis, we measured total circulating white blood cells and lymphocyte numbers 24 hours post final challenge and found no significant alteration in these parameters as a result of repeated LPS inhalation (Figure 4.1 B&C). Finally, hemoglobin concentrations were also found to be unchanged after induction of LPS tolerance (Figure 4.1D). These data indicate that the model used in these studies

does not cause major systemic illness, and that the subsequent effects examined are a result of local pulmonary immune modulation.

Acute phase cytokine expression after induction of chronic LPS tolerance

We have shown that induction of acute pulmonary LPS tolerance results in significant reductions in both TNFα protein, as well as IL-6 (147). Induction of chronic LPS tolerance also resulted in significant reduction in TNFα protein (~56%) at both 2 and 24 hours post final challenge, along with a significant reduction in TNFα mRNA expression in the lung tissue 1 hour post final challenge (Figure 4.2 A&B). We have previously validated that the ELISA used to measure TNFα detects this cytokine both free and in complex with both TNF-SRI and TNF-SRII, verifying these results are physiologically relevant (147). In contrast to TNF, chronic LPS tolerance did not result in reduced IL-6 expression. This is in marked contrast to our previous observation in the acute LPS tolerance model showing that IL-6 was significantly reduced in the BAL fluid (147). In this model we saw that BAL fluid IL-6 protein at 2 hours post final challenge was significantly increased (~53%) in LPS tolerant mice compared to the non-tolerant group (Figure 4.2C). This was accompanied by a statistically insignificant increase in IL-6 mRNA expression in lung tissue (Figure 2D).

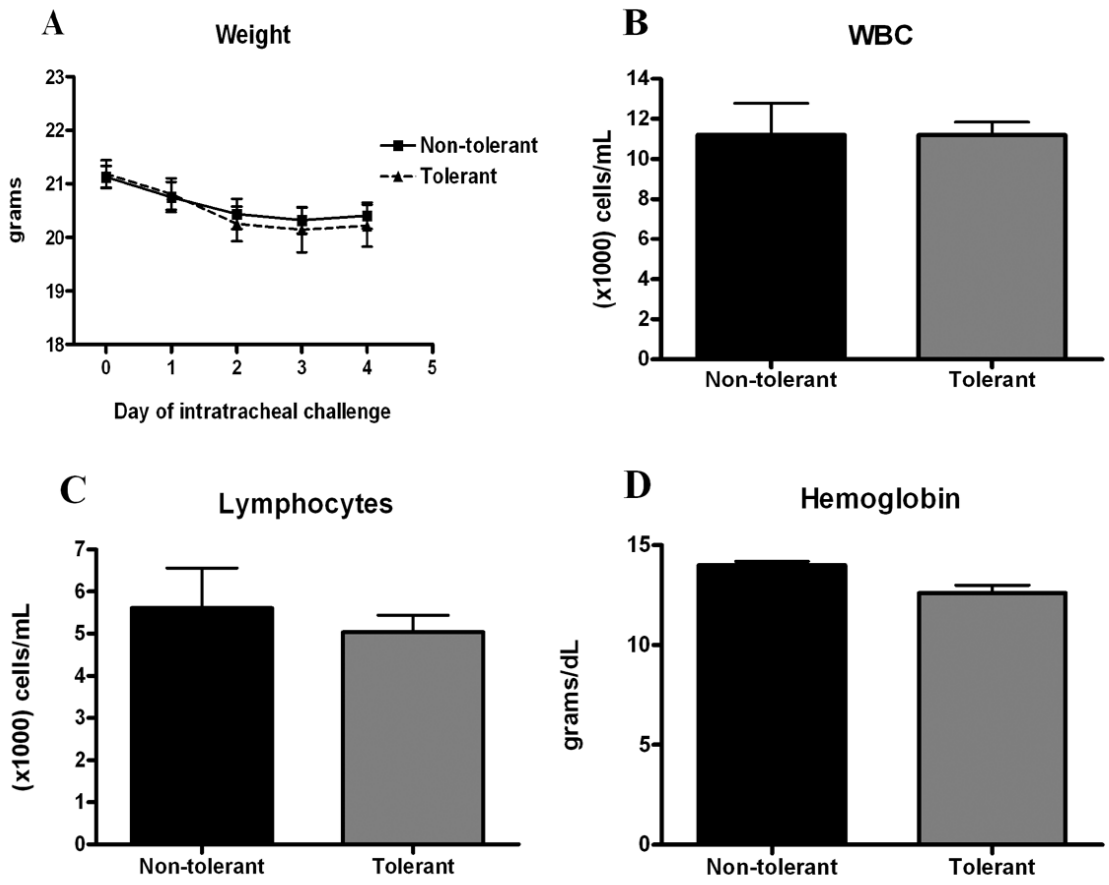


Figure 4-1: Physiologic parameters in response to LPS tolerance. A) Mouse weights were measured the day prior to, and each day mice received intratracheal challenges. Complete differential was performed on blood collected from the tail vein at 24 hours post final challenge and B) total white blood cell counts, C) lymphocyte counts and D) hemoglobin concentrations are represented. Data are expressed as mean ± SEM. n= 6-12 mice per group. There were no differences between the tolerant and non-tolerant animals in any of the measured parameters.

Production of TNF soluble receptors after induction of chronic LPS tolerance

We assessed whether chronic LPS inhalation can upregulate compensatory anti-inflammatory proteins such as TNF soluble receptors. As seen in figure 4.3, repeated LPS inhalation induces significant expression of both TNF-SRI and TNF-SRII. While no TNFα is present at time 0 (Figure 4.2A), both TNF-soluble receptors are substantially upregulated. We suspect that TNF-SRII plays a more potent physiological role in LPS

tolerance given that it is produced at roughly 20 times higher concentrations. The therapeutic TNFα inhibitor Etancercept, a TNF-SRII-IgG fusion protein is widely used to treat excessive inflammation in several chronic inflammatory conditions (148). Our data demonstrate that inducing inflammation causes the physiological upregulation of a mediator that has been a successful target for the treatment of chronic inflammation.

Neutrophil recruitment and expression of CXC chemokines

Cytospin preparations and 300 cell differential counts were made from cells recovered in the BAL fluid at 24 hours post final challenge. Cytospin preparations demonstrate comparable numbers of neutrophils recruited into the alveolar space in non-tolerant and tolerant mice, in contrast to predominantly macrophages which are present in naïve mice (Figure 4.4).

We found that the number of neutrophils recruited to the alveolar space was not affected by repeated LPS inhalation. At early timepoints (0 and 2 hours) LPS tolerant mice have significantly more neutrophils in the BAL fluid, which were recruited in response to the first four LPS exposures. By 24 hours however, both groups recruited comparable numbers of neutrophils into the BAL fluid (Figure 4.5A). Linear regression analysis of both groups between 2 and 24 hours shows no significant difference between the slopes of the regression lines indicating that the capacity of LPS tolerant animals to respond to a high dose LPS challenge was not compromised. Myeloperoxidase assay at 24 hours post final challenge showed no significant increase in activity between groups suggesting that comparable numbers of neutrophils are present in the lung tissue of non-tolerant and tolerant mice (Figure 4.5B).

We further assayed CXC chemokine production as potential mechanism responsible for recruitment of neutrophils. No biologically significant difference in KC (CXCL1) concentrations were measured in the BAL fluid between non-tolerant and tolerant mice (Figure 4.6A). However, a statistically significant reduction in CXCL1 mRNA in the tolerant mice was present in the lung tissue at 1 hour post final challenge (Figure 4.6B). Interestingly, a specific reduction in BAL MIP-2 (CXCL2) expression was measured at 2 hours post final challenge in the LPS tolerant mice. This was accompanied by a significant reduction on CXCL2 mRNA in the lung tissue (Figure 4.6C&D). Although CXCL2 was reduced, based on the data in figure 4.5 the levels of both chemokines combined must have been sufficient to still recruit neutrophils to the lung.

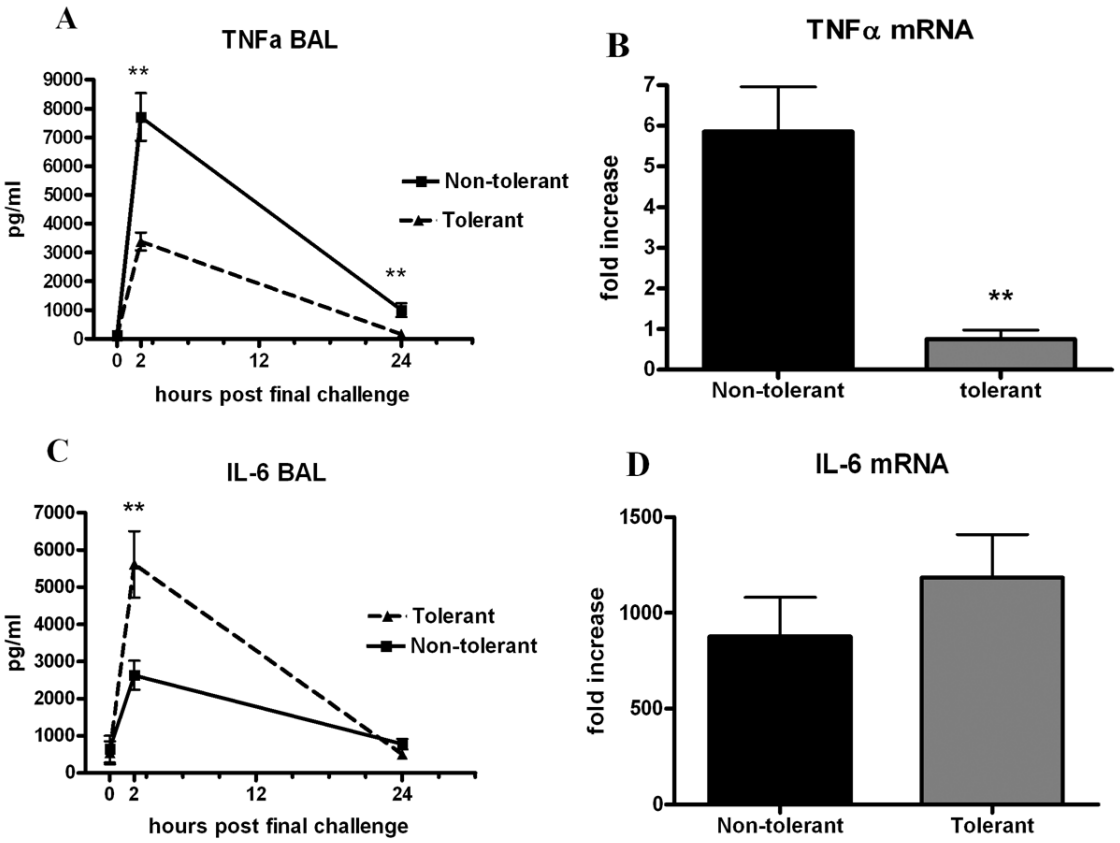


Figure 4-2: Acute phase cytokine expression after induction LPS tolerance. A) BAL TNFα concentrations at the indicated timepoints post final challenge and B) lung tissue TNFα mRNA expression 1 hour post final challenge. C) BAL IL-6 concentrations at the indicated timepoints post final challenge and D) lung tissue IL-6 mRNA expression 1 hour post final challenge. 0h samples were harvested immediately before the final LPS challenge. mRNA data were calculated as fold increase over 0 hour non-tolerant group and are expressed as mean ± SEM. n= 6-10 mice per group. **p<0.01 compared to non-tolerant group.

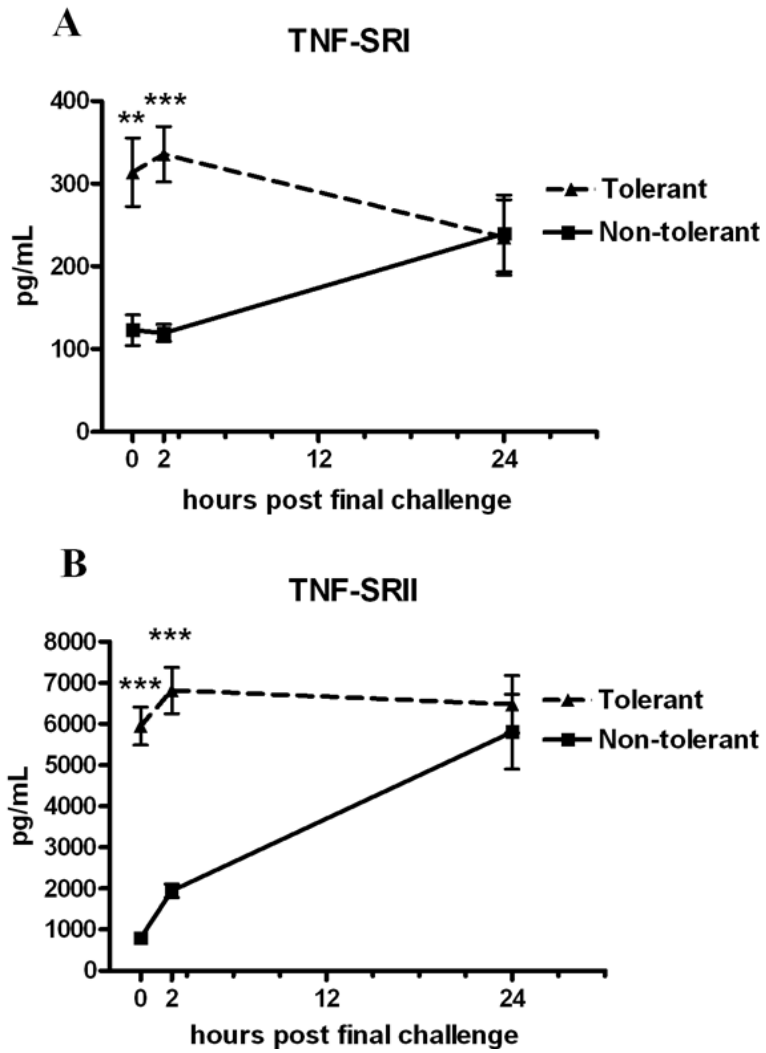


Figure 4-3: TNF soluble receptor expression in response to inducti of LPS tolerance. A) TNF-SRI and B) TNF-SRII concentrations in BAL fluid at the indicated timepoints post final challenge. 0h samples were harvested immediately before the final LPS challenge Data are expressed as mean ± SEM. n= 6-10 mice per group. **p<0.01 and ***p<0.0001 compared to non-tolerant group.

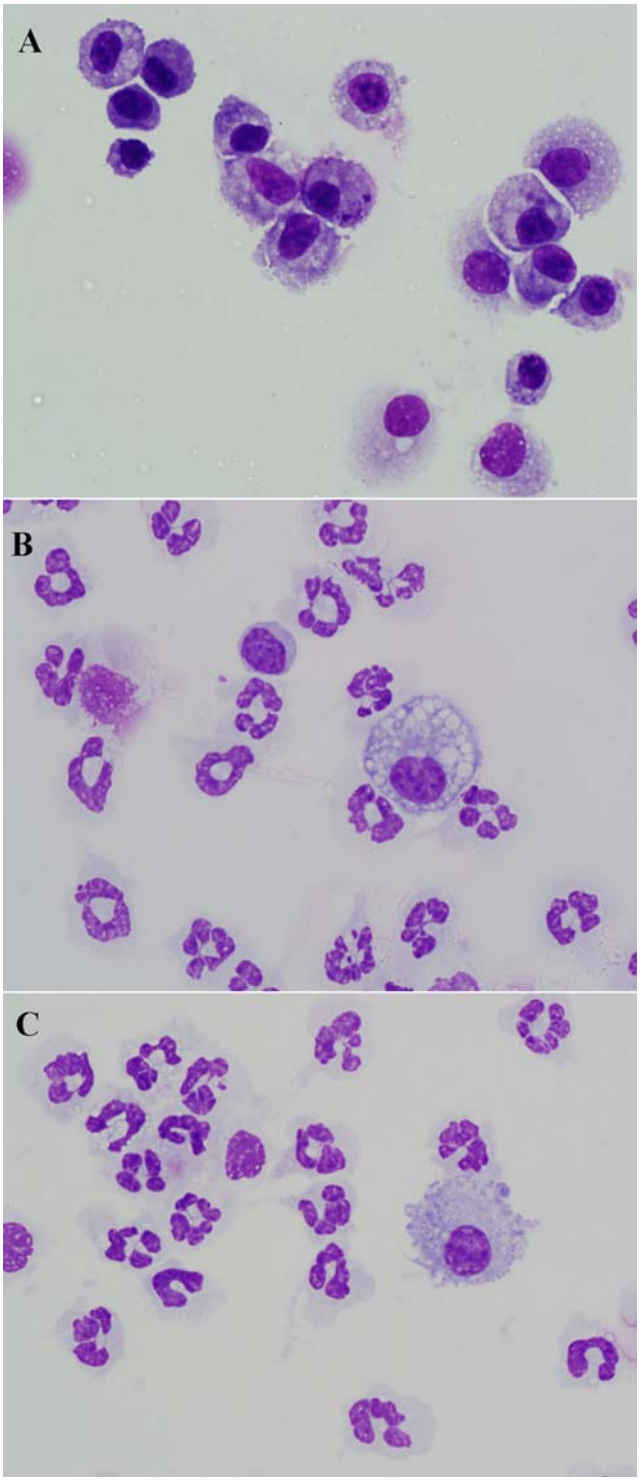


Figure 4-4: Cytospin preparations of cells recovered the BAL fluid of A) Naïve, B) Non-tolerant and C) LPS tolerant mice at 24 hours post final LPS challenge. Each is represented at 1000x magnification. The cells from naïve mice are virtually all macrophages while numerous neutrophils are observed in the other groups.

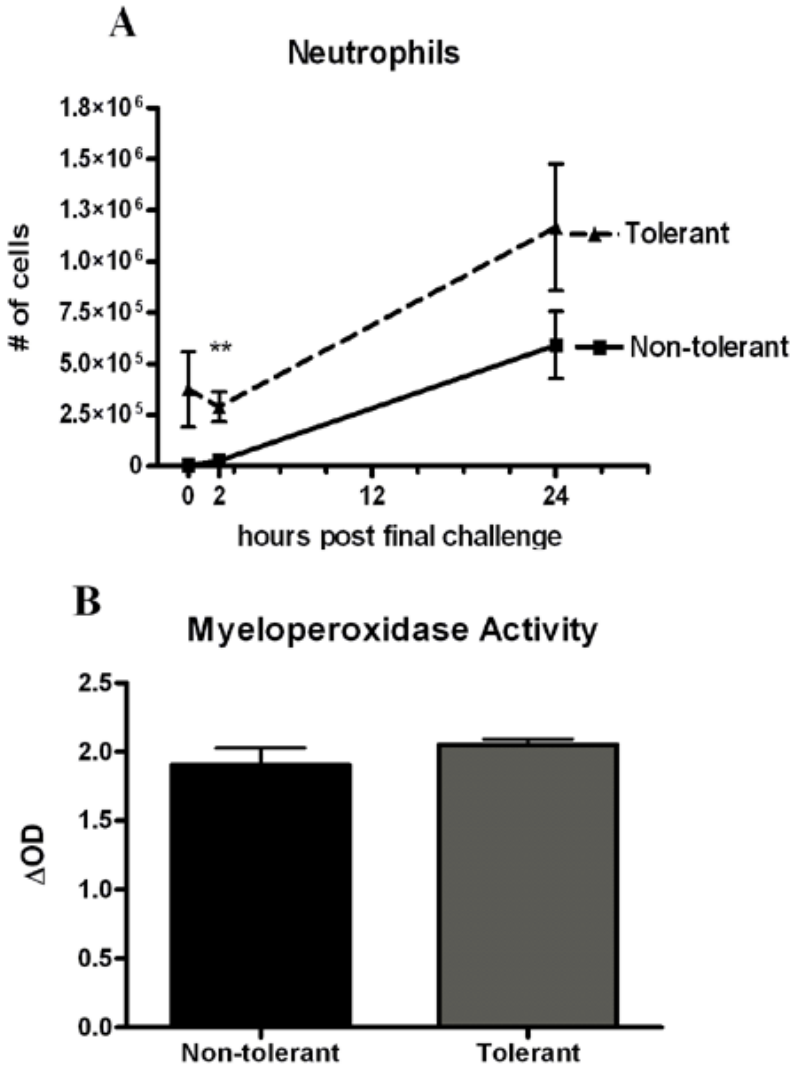


Figure 4-5: Neutrophil influx and myeloperoxidase activity in response to LPS tolerance. A) BAL neutrophil numbers at various timepoints post final challenge. Cytospins were prepared and quantified for collected cells and data are represented as absolute cell number per mouse. B) After BAL, the right lung was homogenized and sonicated for myeloperoxidase assay. Data are expressed as mean ± SEM. n= 6-10 mice per group. **$p<0.01$ compared to non-tolerant group.

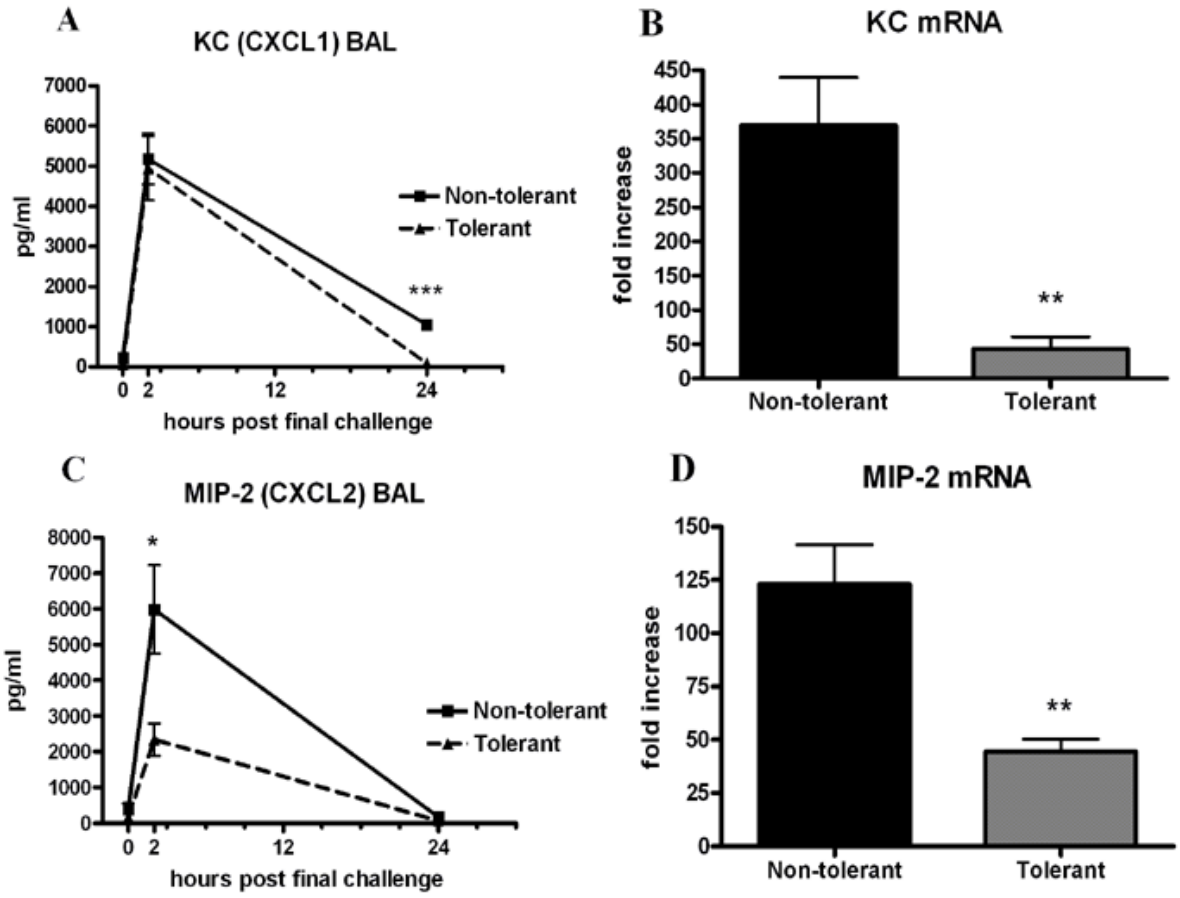


Figure 4-6: Neutrophil chemotactic protein expression after induction LPS tolerance. A) BAL KC (CXCL1) concentrations at the indicated timepoints post final challenge and B) lung tissue KC (CXCL1) mRNA expression 1 hour post final challenge. C) BAL MIP-2 (CXCL2) concentrations at the indicated timepoints post final challenge and D) lung tissue MIP-2 (CXCL2) mRNA expression 1 hour post final challenge. 0h samples were harvested immediately before the final LPS challenge. mRNA data are expressed as fold increase above naïve mice and are represented as mean ± SEM. n= 6-10 mice per group. *$p<0.05$ and **$p<0.01$ compared to non-tolerant group.

Airways hyperresponsiveness and mediators of AHR

Inhalation of LPS has been shown to induce airways hyperresponsiveness in mouse models (107, 149). We measured AHR at 4 hours post final challenge in both non-tolerant and tolerant mice and found that induction of LPS tolerance is protective against AHR severity (Figure 4.7A). We then assayed for the mediators of AHR, cysteinyl leukotrienes and tryptase, as possible mechanisms governing the decrease in AHR seen in LPS tolerant mice. Surprisingly, both cysteinyl leukotrienes and tryptase measured in the BAL fluid 2 hours post final challenge were significantly elevated in LPS tolerant mice (Figure 4.7 B&C). This indicates that while repeated LPS exposure may induce production of the mediators of AHR, a divergent mechanism must be present that governs the methacholine-induced AHR assayed in this experiment.

Muscarinic receptor expression in response to chronic LPS tolerance

The finding that methacholine-induced AHR was attenuated in LPS tolerant mice, even in the presence of significant concentrations of potent mediators of AHR, led us to investigate the expression of pulmonary methacholine receptors as a possible mechanism underlying the protection from AHR. Methacholine is a non-specific muscarinic receptor agonist which causes bronchial smooth muscle contraction (150). As such, we measured mRNA levels of the M2 and M3 muscarinic receptors. Our data show decreased expression of both M2 and M3 receptors in the lung tissue at 1 hour post final challenge in LPS tolerant mice compared to non-tolerant mice. Further, these data are expressed as fold change relative to the 0 hour non-tolerant group. These mice have been exposed only to the mechanical stress of aspirating 50μL of PBS. A fold increase of 1.0 would represent basal expression equivalent to that observed after this mechanical stress (151).

However, in the tolerant group, both M2 and M3 receptors were decreased below basal expression levels, suggesting that downregulation of these receptors occur upon induction of LPS tolerance.

While LPS has been shown to potentiate smooth muscle responses to cholinergic stimuli, we provide the first evidence that LPS challenge can affect the level of expression of either of these muscarinic cholinergic receptors (152). While we have demonstrated mRNA downregulation, but not receptor expression, our data demonstrate a clear dissociation between AHR (decreased) and the mediators causing AHR (increased). The likely bridge between the methacholine challenge and the induction of AHR lies in the ability of methacholine to activate the appropriate receptors. Therefore, our finding that both M2 and M3 receptors are downregulated is likely the mechanism by which AHR is reduced in tolerant mice.

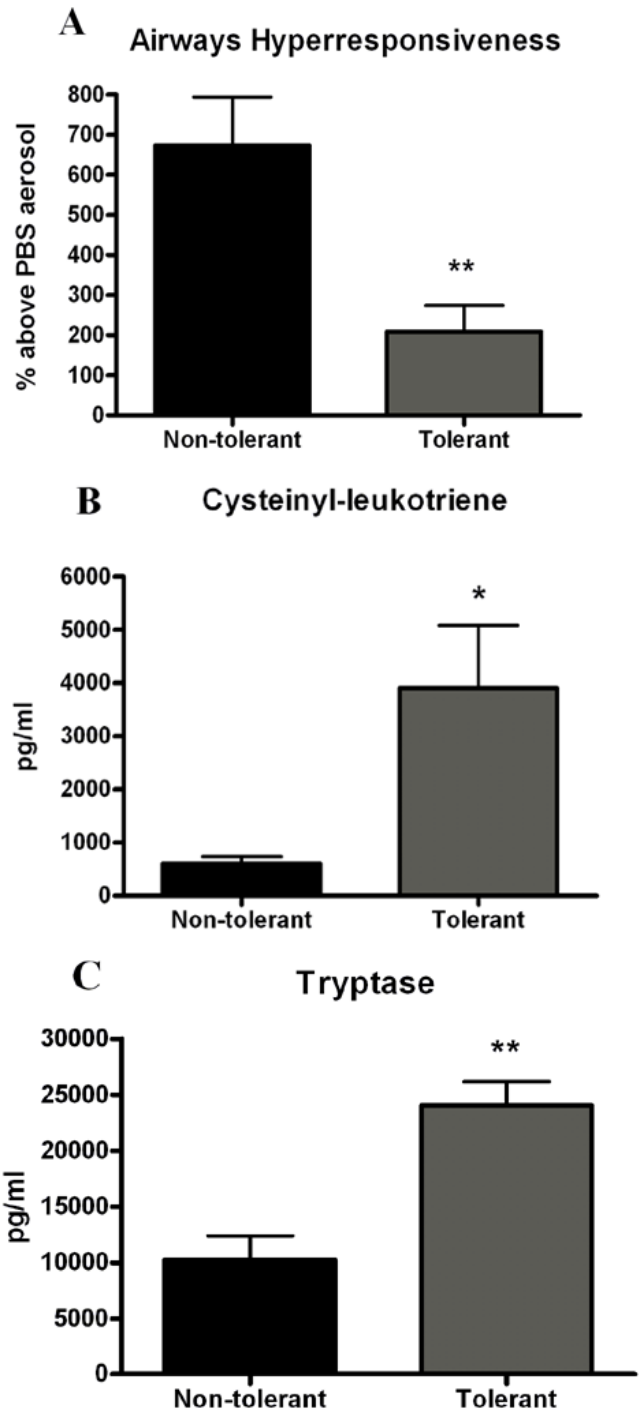


Figure 4-7: AHR and mediators of AHR in response to LPS tolerance. A) Airway hyperresponsiveness in response to 50mg/mL aerosolized methacholine at 4 hours post final challenge. B) BAL cysteinyl leukotriene concentrations at 2 hours post final challenge. C) BAL tryptase concentrations at 2 hours post final challenge. Data are expressed as mean ± SEM. n= 6-10 mice per group. *p<0.05 and **p<0.01 compared non-tolerant group.

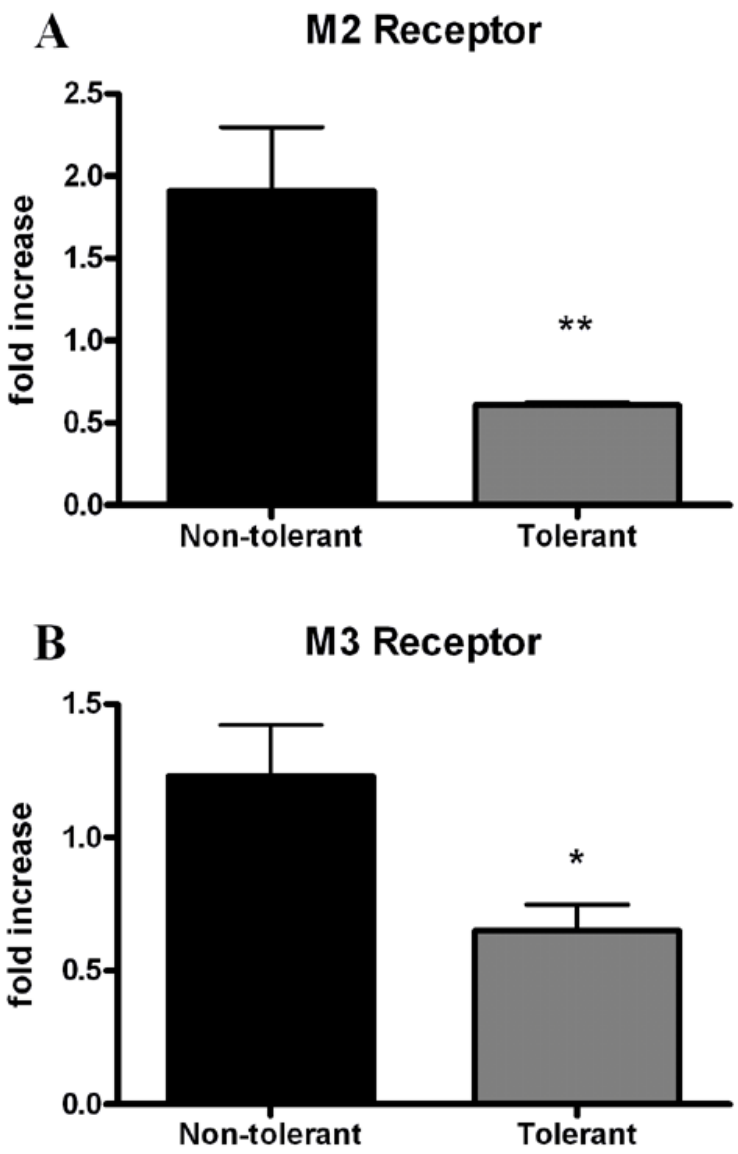


Figure 4-8: mRNA expression of M2 and M3 muscarinic acetylcholine receptors in non-tolerant and tolerant mice at 1 hour post final challenge. Data are calculated as fold increase over 0 hour non-tolerant group and are expressed as mean ± SEM. n= 6 mice per group. *p<0.05 and **p<0.01 compared to non-tolerant group.

Discussion

While the role of LPS in the onset and exacerbation of chronic lung diseases such as asthma and COPD have been widely studied, the effect of repeated LPS exposure in the lung microenvironment has been a largely neglected field of study. Because the lung is constantly exposed to environmental pathogens, the local immune environment requires tight regulation (153, 154). The manner by which LPS modulates the local immune environment is of particular importance in determining the mechanisms by which the lung protects itself from excessive inflammation and lung damage, while retaining the capacity to mount an immune response to exogenous pathogens.

We have previously demonstrated that mice exposed to a single intratracheal installation of low dose LPS become refractory to further LPS stimulation in the context of TNFα and IL-6 production, but are unaffected in their ability to recruit neutrophils in response to subsequent LPS challenge (147). Having demonstrated that LPS tolerance can be induced in the lung, we sought to determine whether this refractoriness would persist in a more appropriate model, specifically chronic LPS inhalation. Epidemiological data shows that the levels of LPS present in the homes of cigarette smokers, as well as in the workplace of agricultural and textile workers are significantly higher than those measured in the air of non-smoking households, and other indoor occupational settings (146). As such, we have developed a more clinically relevant model of pulmonary LPS exposure involving four exposures to high dose LPS. In order to test the induction of tolerance, mice were then exposed to ten times the dose of LPS for the final challenge.

Because the doses of LPS we used in this model are relatively high, we first sought to demonstrate that tolerance induction would not induce a systemic immune

response or global physiological changes. Our data show no differences between groups in terms of body weight, as well as other parameters, such as total peripheral white cell count and hemoglobin concentrations. This confirmed that chronic LPS tolerance induced in this model is an organ specific phenomenon.

Our findings demonstrate that after chronic exposure to high levels of LPS, downregulation of both TNFα protein and mRNA still occurs. This, coupled with the finding that IL-6 levels are increased in the BAL fluid of LPS tolerant mice indicates that pulmonary tolerance is not the result of global immunosuppression (Figure 4.2). Further, we show that LPS tolerant mice recruit equivalent numbers of neutrophils into the alveolar space in response to the final LPS challenge. More importantly, linear regression analysis of the timeframe between 2 and 24 hours post final challenge showed no significant difference in the slopes of the regression lines. This indicates that although tolerant animals have a significant number of neutrophils already present in the BAL fluid at zero hours, their capacity to recruit neutrophils in response to the final LPS challenge was not impaired.

We further sought to elucidate the mechanism responsible for the recruitment of neutrophils. We found no statistical difference in KC (CXCL1) production between non tolerant and tolerant mice, however a significant decrease in mRNA was measured at 1 hour post final challenge. We hypothesize that induction of LPS tolerance may affect mRNA stability, possibly by increased deadenylation of the mRNA body, promoting subsequent degradation (155, 156). Additionally, we have previously shown that CXCL1 mRNA production peaks at 2 hours post final challenge in the lung tissue. LPS tolerance may also affect the rate at which CXCL1 mRNA is transcribed (147). We also measured

a significant reduction in MIP-2 (CXCL2) expression in the BAL fluid, which was accompanied by a significant decrease in mRNA. This finding further highlights the specificity of immune regulation in the lung microenvironment. To our knowledge, differential regulation of CXCL1 and CXCL2 in the lung in response to LPS challenge has not previously been reported.

The most striking finding of this study was that chronic LPS tolerance protects against methacholine-induced airways hyperresponsiveness, even in the presence of significantly higher concentrations of both cysteinyl leukotrienes and tryptase. Since both of these molecules were produced in response to LPS challenge, and are primary mediators of AHR, we sought to determine if the mechanism responsible for the protection from AHR was due to an alteration in the response to methacholine itself (51, 157). Methacholine, a synthetic choline ester, is a non-specific agonist for muscarinic cholinergic receptors (158). Decreased expression of these receptors, or a defect in their function, would reduce smooth muscle contraction and therefore attenuate AHR (159). Both M2 and M3 muscarinic receptors are expressed in the lung and bronchi (151). Upon methacholine challenge, M3 muscarinic receptors cause smooth muscle contraction, while M2 receptors inhibit smooth muscle relaxation (160). Decreased expression of both receptors would manifest as an attenuated contractile response to inhaled methacholine.

Our data provide the first evidence that LPS challenge can alter muscarinic receptor expression, and that the mechanism of reduced AHR seen in LPS tolerant animals may be a reduction in expression of both M2 and M3 muscarinic receptors. We measured a significant decrease in mRNA expression of both M2 and M3 muscarinic receptors in response to LPS tolerance. More importantly, a single LPS challenge induced

transcription of the genes for both these receptors, as seen in the non-tolerant group, and LPS tolerance induced downregulation of this message to below basal expression levels.

This study introduces a new model of chronic pulmonary LPS tolerance, and demonstrates a novel mechanism of immune regulation in the lung. Chronic LPS inhalation protects against excessive inflammation by attenuation of TNFα production, and selectively protects against persistent AHR through the mechanism of decreased muscarinic receptor expression. LPS tolerance does not impair the ability to mount a neutrophilic response to exogenous pathogens, however one cannot rule out the possibility that the persistence of neutrophils in the lung can lead to organ injury and long-term loss of function. As such, more studies must be carried out to determine the role of chronic LPS inhalation on lung injury.

These data may also suggest the mechanism underlying the decreased incidence of asthma and atopy seen in farm communities and in the developing world. Studies in murine models have shown that in the absence of TNFα or TNFα signaling, hallmark features of asthma, such as eosinophilia and IgE production are attenuated (116). In fact, in TLR4 deficient mice, OVA-induced asthma like inflammation was restored by exogenous administration of TNFα post sensitization (80). Further studies are required to determine whether individuals exposed to high levels of inhalational LPS are refractory in the context of TNFα production, and to determine whether this is the mechanism by which these individuals are protected against atopy and asthma.

Chapter 5

LPS Tolerance Protects Against Airways Hyperreactivity, IgE Production and Eosinophilia in a Mouse Model of Asthma-Like Inflammation

Introduction

Compounds which activate the innate immune system, such as LPS, are a significant component of ambient air, and are extremely difficult to remove from the environment. Inhalation of LPS is key factor in asthma exacerbations and has been shown to play a role in allergen sensitization in various mouse models. Epidemiological studies have shown that the severity of asthma in inner city children is greater than that seen in children raised in farming environments (111). Further, the levels of LPS present in farm environments are significantly higher than those measured in inner city homes (146). Therefore, we sought to investigate whether tolerance to LPS, induced by repeated inhalation was the mechanism responsible for attenuated asthma severity in response to cockroach allergen sensitization and challenge. LPS tolerance was induced by four consecutive days of LPS exposure by direct intratracheal installation. Both allergen sensitization and challenge were also carried out by intratracheal exposure without the use of adjuvants. Because adjuvants are thought to artificially skew the immune system toward a Th2 response, their use is controversial (161). By using a clinically relevant allergen and the natural route of exposure, the model used in the current studies more accurately recapitulates the clinical setting.

Experimental Design

Detailed experimental methods are described in Appendix A.

Induction of LPS tolerance and asthma-like pulmonary inflammation

LPS tolerance was induced by exposing mice to 1μg LPS E. coli LPS O111:B5 (Sigma-Aldrich, cat# 62325) in a total volume of 50 μL sterile PBS for four consecutive days by direct intratracheal installation (100). Non-tolerant mice were challenged with 50 μL sterile PBS. Briefly, mice were lightly anesthetized with isofluorane and suspended on a vertical board by their front incisors. The tail was taped down to support the body weight. The tongue was gently extended with forceps and the liquid placed at the base of the oropharynx so that it was inhaled. Twenty-four hours after the final PBS or LPS challenge, asthma was induced by the same protocol in all animals. On day 0, mice were sensitized to cockroach allergen by intratracheal administration of 2 μg CRA (Greer Laboratories, LeNoir, NC, Item # B46) in PBS. No adjuvants were used in this model of asthma induction. On day 14, mice were challenged intratracheally with 1 μg CRA, and received the same challenge on day 21. This CRA preparation contained 200 ng LPS contamination for sensitization and 100 ng LPS at challenge as determined by *Limulus* assay. Airways hyperresponsiveness was measured and animals were sacrificed at various timepoints as indicated in the figure legends. The 0 h timepoint represents sacrifice immediately prior to receiving the day 21 CRA challenge.

Airways hyperresponsiveness to methacholine challenge was measured by unrestrained whole body plethysmography at 4 hours post final allergen challenge. Mice were exanguinated and BAL was performed at various timepoints post final challenge, as indicated in the figure legends. The left lung was removed into protease inhibitor cocktail

for later cytokine and chemokine analysis, as well as myeloperoxidase and eosinophil peroxidase assays. The right lung was removed into 70% ethanol for processing for histopathology. PAS staining was performed for mucus hypersecretion, and Congo Red staining was used to identify tissue eosinophils. All data are represented as mean ± SEM. Statistical significance was determined by Student's *t*-test using GraphPad Prism version 4.0.3. (GraphPad Software, San Diego, CA). Statistical significance was achieved when $p \leq 0.05$ at the 95% confidence interval.

Results

Activation of innate immunity after allergen sensitization and challenge

Previous studies of LPS tolerance have defined it as a transient phenomenon. It is generally accepted that if more than 72-96 hours elapses between initial LPS exposure and rechallenge, the acute inflammatory response is not blunted (135, 162, 163). After LPS tolerance induction, mice were sensitized and challenged with CRA. Figure 5.1 shows that prior induction of LPS tolerance has no persisting effect on the innate immune response to allergen challenge. No difference in neutrophil recruitment to the alveolar space, or in pulmonary production of the CXC chemokines KC and MIP-2 was observed (figure 5.1A-C). More importantly, we saw no lasting defect in the ability of LPS tolerant mice to produce TNFα 21 days after LPS tolerance induction. In fact, these mice produced slightly more of this acute phase mediator than non-tolerant mice at 2 hours post final allergen challenge.

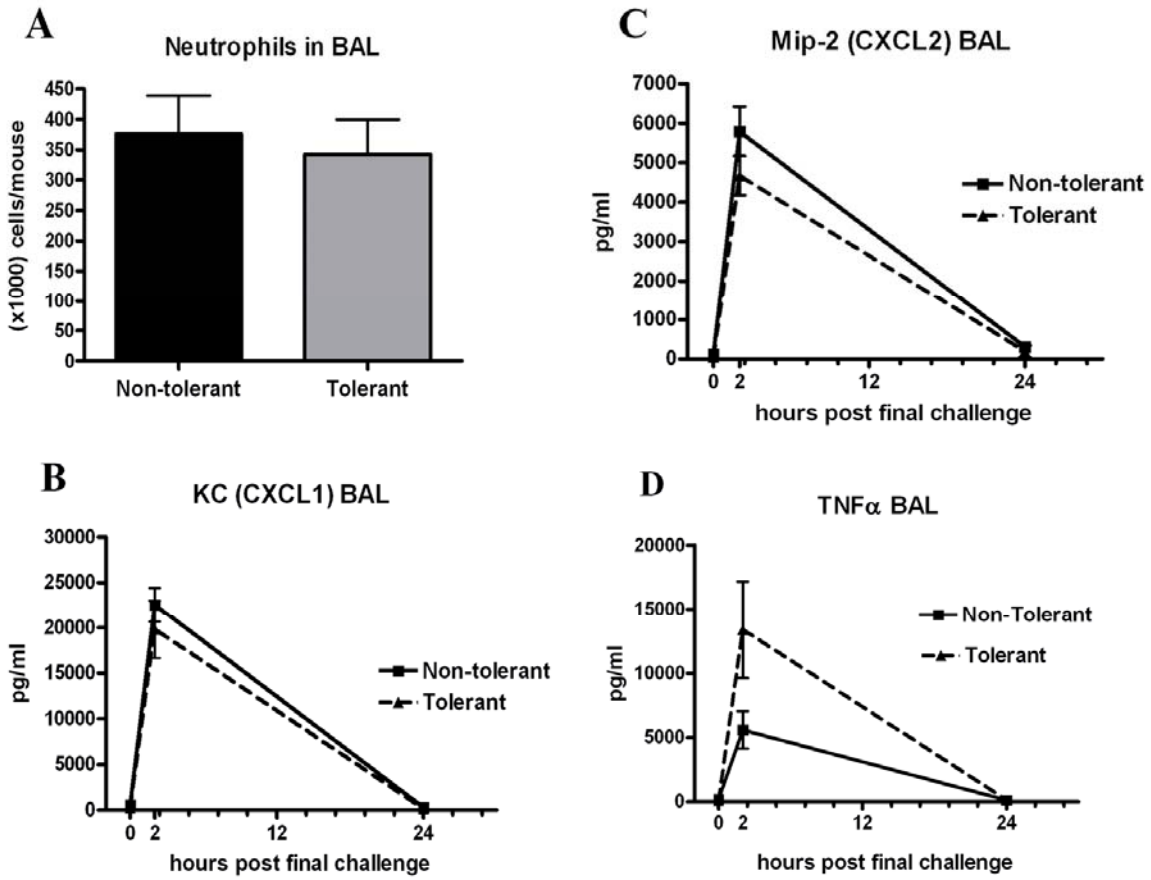


Figure 5-1: Neutrophil recruitment and mediators of innate immunity after final allergen challenge. Cytospin preparations from cells collected in the BAL fluid were made and 300 cell differential counts performed to determine the absolute numbers of A) neutrophils present. Concentrations of chemokines B) CXCL1, C) CXCL2 and D) TNFα in the BALfluid were measured by standard ELISA. Data are presented as mean ± SEM. n=10-14 mice per group.

Airways hyperresponsiveness and mediators of AHR

We have previously demonstrated that mice made tolerant to LPS are protected from methacholine-induced AHR in the absence of allergen challenge. Here, we examined whether LPS tolerance can modulate AHR in response to allergen sensitization and challenge. Mice were challenged with increasing doses of aerosolized methacholine at 4 hours post final allergen challenge. LPS tolerant mice had significantly attenuated AHR compared to non-tolerant mice (Figure 5.2A). We then measured cysteinyl leukotrienes in the BAL fluid at 2 hours post final challenge as a possible mechanism responsible for the protection from AHR seen in LPS tolerant mice. Interestingly, cysteinyl leukotriene production was significantly increased in LPS tolerant mice, suggesting that decreased leukotriene production was not responsible for the methacholine-induced hypo-responsiveness measured in this model (Figure 5.2B).

PAS staining was performed in lung sections harvested at 24 hours post final challenge in order to determine if LPS tolerance affected goblet cell hyperplasia and subsequent mucus production. We observed decreased mucus staining in the airway epithelial cells of LPS tolerant mice compared to the non-tolerant group (Figure 5.3A&B). Quantification of this staining using the NIH ImageJ software confirmed these observations, showing significantly less positive PAS staining in the tolerant lungs (Figure 5.3C).

Eosinophil influx and eosinophil-specific chemokine production

Eosinophil infiltration is a cardinal feature of the late phase of the asthmatic response (164). Absolute numbers of eosinophils in the BAL fluid of LPS tolerant mice were significantly attenuated (Figure 5.4A). We also measured eotaxin-1 (CCL11) and

eotaxin-2 (CCL24) as the possible mechanism responsible for the defect in eosinophil recruitment seen in tolerant mice. We measured significant reductions in both CCL11 and CCL24 in the BAL fluid of LPS tolerant mice (Figure 5.4 B&C).

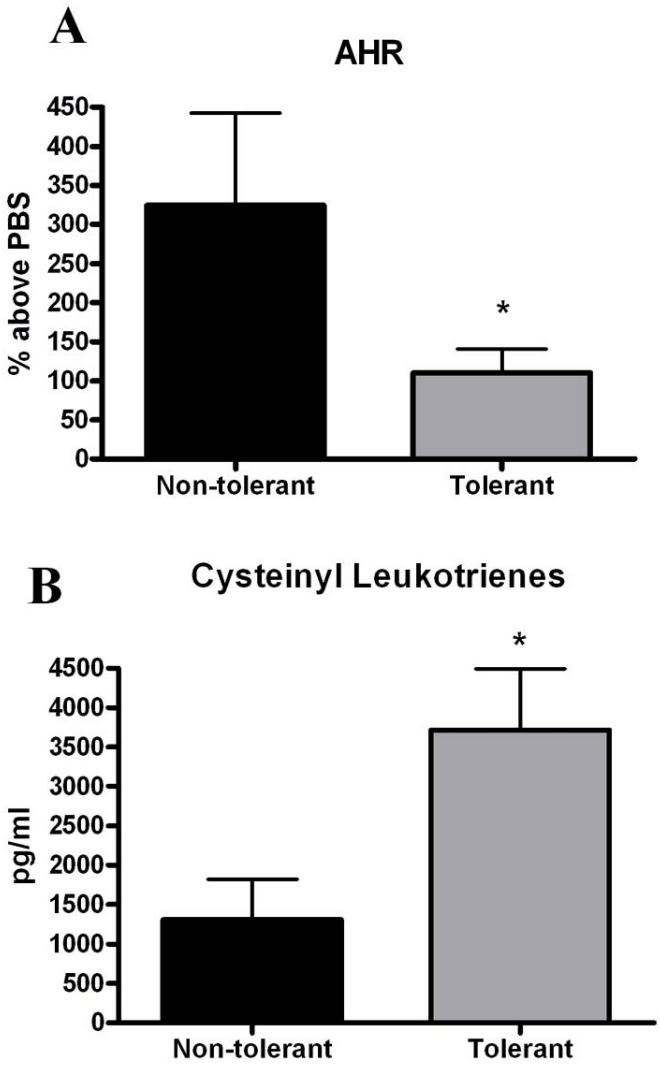


Figure 5-2: Airways hyperresponsiveness and cysteinyl leukotriene production after final allergen challenge. Mice were challenged with 50mg/mL aerosolized methacholine at 4 hours post final allergen challenge. A) PenH values are represented as percent above challenge with aerosolized PBS. B) Cysteinyl leukotriene concentrations were measured in the BAL fluid 2 hours post final challenge by enzyme-linked immunoassay. Data are presented as mean ± SEM. n=8-10 mice per group. *$p<0.05$ compared to non-tolerant group.

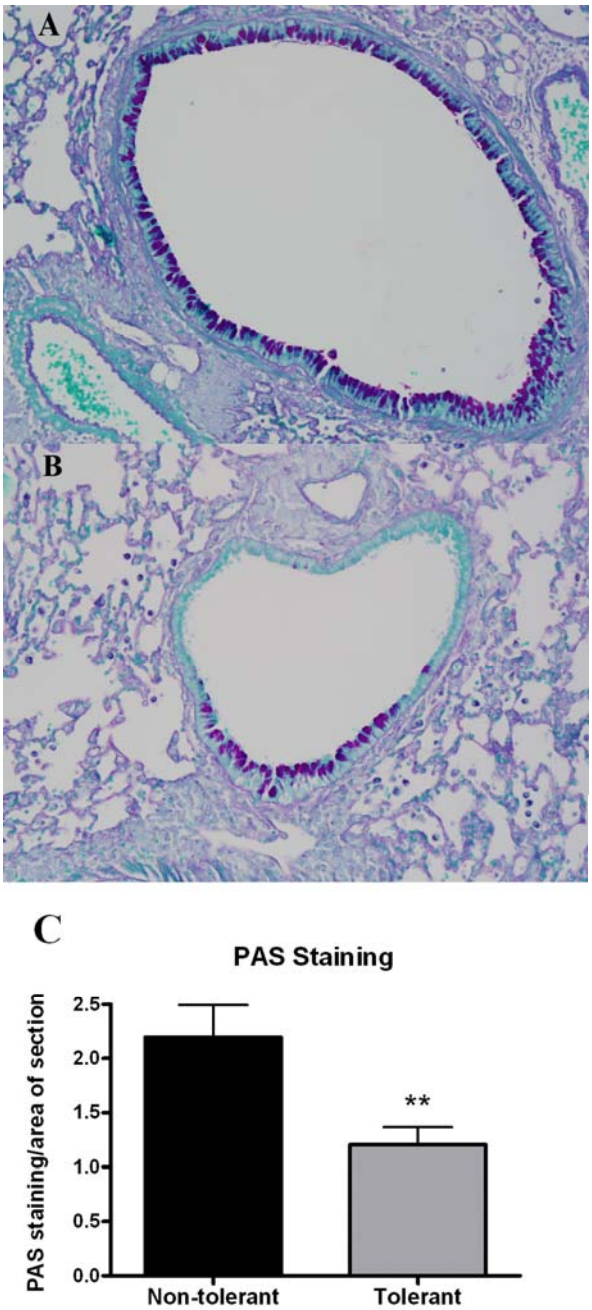


Figure 5-3: PAS staining and quantification. Representative images of PAS stained A) non-tolerant and B) tolerant mice 24 hours post final allergen challenge. C) The amount of positive PAS staining was quantified using NIH ImageJ software and expressed as area of PAS staining per area of the entire lung section. Data are presented as mean ± SEM. n=12 mice per group. $^{**}p<0.01$ compared to non-tolerant group.

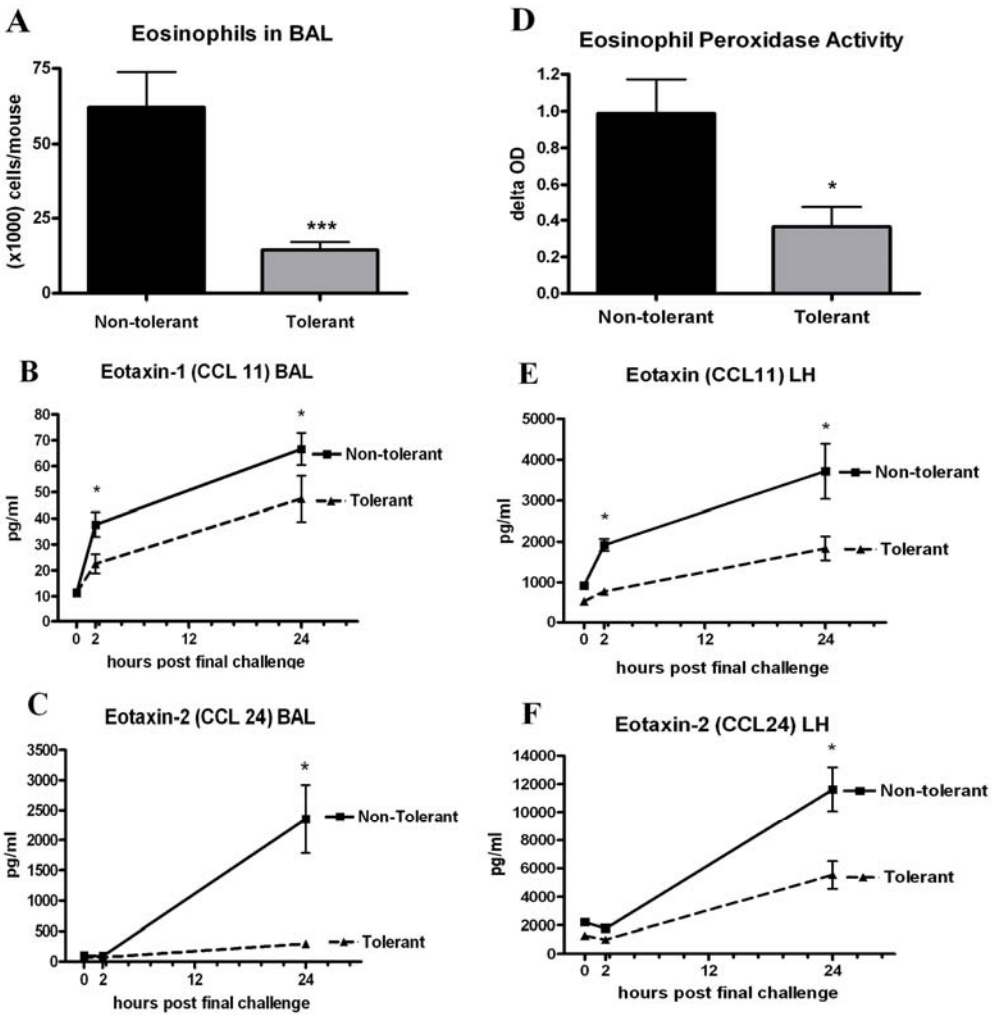


Figure 5-4: Eosinophil recruitment and production of eosinophil-specific chemokines in response to allergen challenge. Cytospin preparations from cells isolated from the BAL fluid were made and 300 cell differential counts performed to determine the absolute numbers of eosinophils (A). Concentrations of the chemokines B) eotaxin-1 and C) eotaxin-2 in the BAL fluid were measured by standard ELISA. D) Eosinophil peroxidase activity was measured in the lung tissue after BAL at 24 hours post final challenge. Concentrations of E) eotaxin-1 and F) eotaxin-2 in the lung homogenate were assayed by standard ELISA. Data are represented as the mean ± SEM. n= 10-14 mice per group. * $p \leq 0.05$ and *** $p \leq 0.0001$.

In order to rule out the possibility that reduced chemokine expression was causing eosinophils to sequester in the lung parenchyma, we measured eosinophil peroxidase

activity as an indicator of eosinophils in the lung tissue. Eosinophil peroxidase activity 24 hours post final challenge, as well as lung homogenate CCL11 and CCL24 concentrations were significantly decreased in LPS tolerant mice (Figure 5.4D-F). In contrast, RANTES production was equivalent between groups at all timepoints assessed (data not shown).

Tissue eosinophil numbers were further confirmed by Congo Red staining of paraffin-embedded lung sections. Eosinophils were identified by cellular and nuclear morphology, as well as pink cytoplasmic staining. Representative digital images of non-tolerant and tolerant lungs show an apparent reduction in eosinophil numbers in the tolerant lungs (Figure 5.5A&B). Quantification of these sections clearly documented decreased numbers of eosinophils in the lungs of LPS tolerant mice (Figure 5.5C).

Th1 and Th2 cytokine production in BAL fluid

Asthma is largely characterized as a Th2 mediated disease accompanied by high concentrations of IL-4 and IL-5, and low levels of IFNγ (106). Given the apparent protection from airway eosinophilia, we investigated whether LPS tolerance alters the production of Th1 and Th2 cytokines, possibly skewing the inflammatory response to one mediated primarily by IFNγ. We found a slight but statistically insignificant increase in both IFNγ (Th1), as well as the Th2 cytokines IL-4, IL-5 and IL-13 in the BAL fluid of LPS tolerant mice at 2 and 24 hours post final challenge (Figure 5.6). Also, no statistical difference was seen in IL-12 levels in the BAL fluid (data not shown). This demonstrates that in this clinically relevant model of asthma, induced in the absence of exogenous adjuvants, the Th1/Th2 paradigm does not clearly apply. Further, while LPS tolerance

protects against eosinophilia, our data show that it also exacerbates cytokine production in response to allergen.

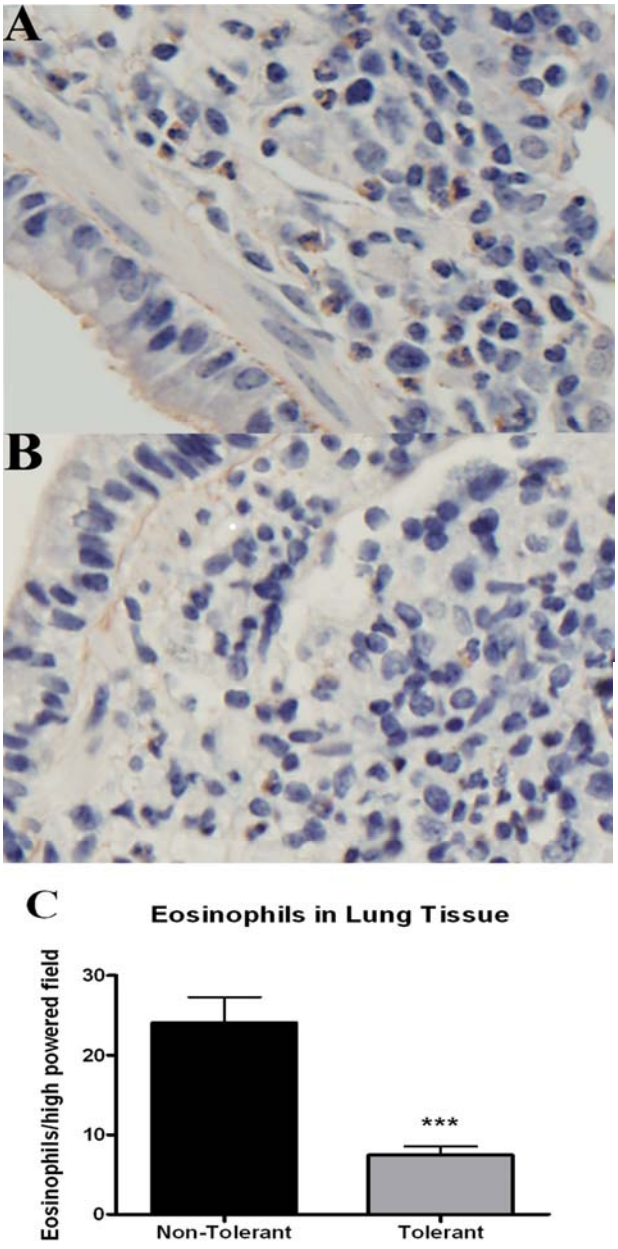


Figure 5-5: Tissue eosinophil staining and quantification. Representative images of paraffin sections from A) non-tolerant and B) tolerant mice at 24 hours post final allergen challenge were stained with Congo Red. Eosinophils were identified by morphology and red cytoplasmic staining. C) Number of eosinophils per high powered field was quantified. Data are presented as mean ± SEM. n=12 mice per group. ***$p<0.001$ compared to non-tolerant group.

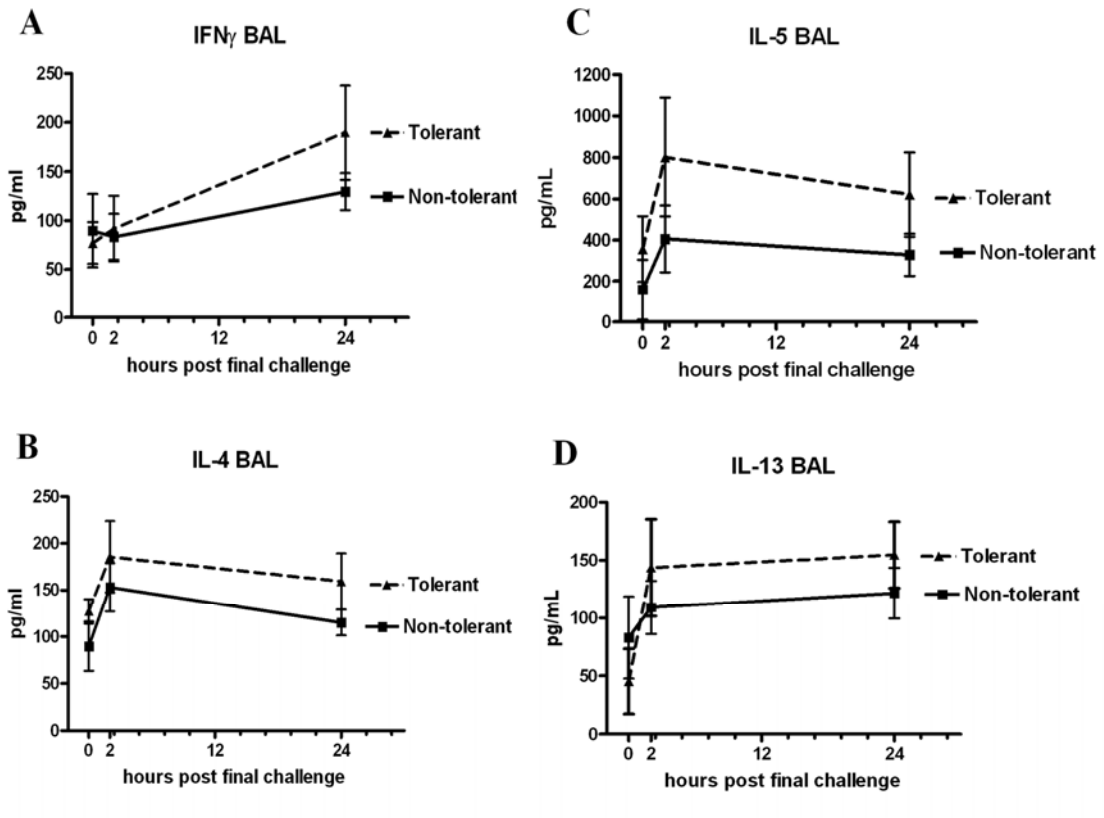


Figure 5-6: Th1 and Th2 cytokine production in BAL fluid at 24 hours post final allergen challenge. Th1 cytokine A) IFNγ and Th2 cytokines B) IL-4, C) IL-5 and D) IL-13 were assayed by standard ELISA. Data are represented as the mean ± SEM. n= 10-14 mice per group.

Antibody production in response to allergen sensitization and challenge

Antibody production is a key feature of the asthmatic response. Cross-linking of allergen-specific IgE antibodies on the surface of mast cells causes degranulation, subsequent AHR and can eventually lead to epithelial damage (51). Plasma levels of IgE were significantly decreased in LPS tolerant mice. In fact, LPS tolerant mice were not able to produce IgE above the levels circulating in naïve mice (Figure 5.7A). This suggests that LPS tolerance inhibits class switch recombination for IgE production. We further sought to determine whether LPS tolerance suppressed the production of IgG2a, traditionally associated with a non-allergic condition, as well as IgG1, associated with an allergic condition (165). We found that both non-tolerant and LPS tolerant mice were able to produce both IgG1 and IgG2a to levels substantially above those measured in naïve mice, and that no statistically significant difference was measured between groups for either antibody (Figure 5.7B&C). This indicates that LPS tolerance selectively suppressed production of IgE in response to allergen sensitization and challenge.

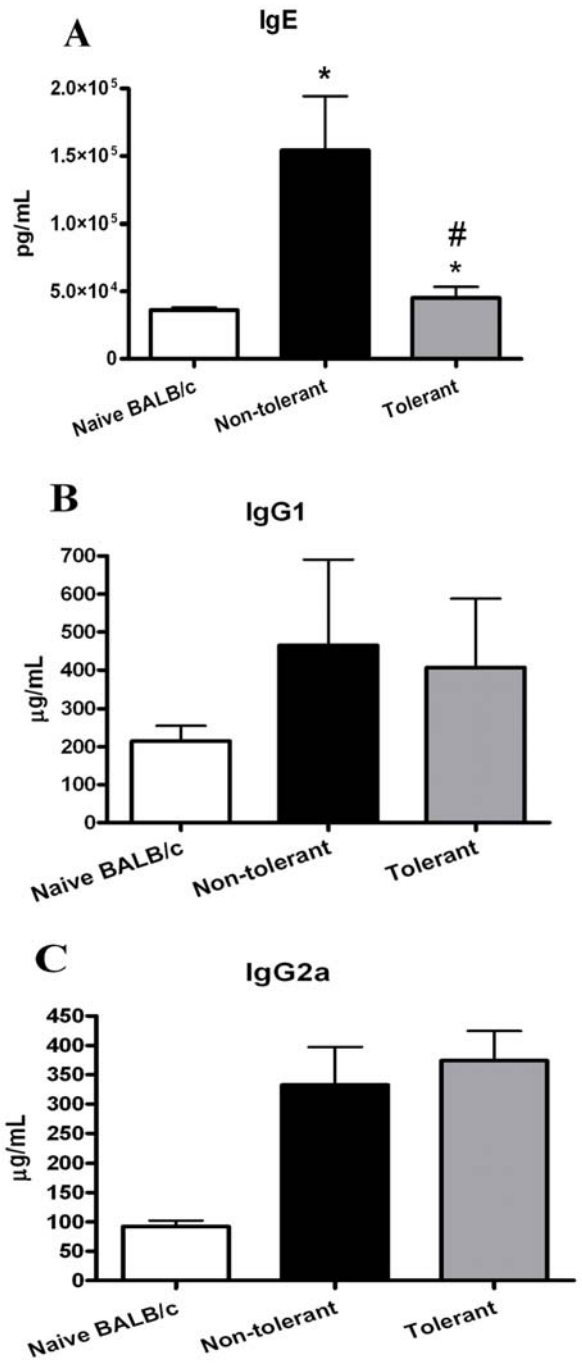


Figure 5-7: Plasma antibody production in response to allergen sensitization and challenge. A) IgE, B) IgG1 and C) IgG2a were measured in plasma collected at 24 hours post final allergen challenge by standard ELISA. Data are represented as the mean ± SEM. n= 12 mice per group. * $p \leq 0.05$ compared to naïve mice and #<0.05 compared to non-tolerant group.

Discussion

The effect of activators of the innate immune system on the development of the adaptive response to allergen has become an important focus of investigation. Epidemiological studies have produced conflicting results with regard to the effect of repeated LPS inhalation. Some studies have suggested a protective effect on the development of asthma, as well as certain forms of lung cancer (39, 166). However, others have demonstrated that high LPS levels correlate with increased asthma incidence and severity (167). Animal models have also correlated high dose LPS exposure at the time of sensitization with decreased allergen sensitization, whereas low levels of LPS seem to potentiate allergen sensitization (104, 168). We have shown that repeated LPS inhalation can result in a transient refractoriness to further LPS stimulation, referred to as LPS tolerance (147). Given that levels of LPS are significantly higher in the workplace of agricultural and textile workers than those measured in inner city homes, we sought to determine whether LPS tolerance is the mechanism that confers protection from asthma and atopy in individuals exposed to high levels of LPS (146).

In this model, LPS tolerance was induced by four consecutive days of high-dose intratracheal LPS administration, followed by induction of asthma by intratracheal exposure to CRA. Few models of adjuvant-free OVA-induced asthma exist, however these models utilize an average of 6 exposures and a total of 2 mg of allergen, while our model requires 3 exposures and a total of only 4 μg of allergen (169-171). Therefore, in the absence of adjuvant, significantly higher concentrations of OVA are required to induce an allergic response. The ability to induce asthma-like pulmonary inflammation with relatively low concentrations of allergen may lie not only in the use of an

environmentally relevant allergen, but also in the substantial LPS contamination naturally present in the allergen preparation. This further recapitulates the environmental setting in which LPS contamination is ubiquitous.

Asthma has long been considered a Th2 cell mediated disease, with the cytokines IL-4 and IL-5 driving the allergic phenotype. Further, IFNγ production is thought to be protective against the onset and progression of asthma (172). However, adjuvants were readily used in the development of animal models which led to the wide acceptance of this paradigm (173-175). This is of critical importance because the use of adjuvants can artificially skew the immune response toward a Th2 phenotype (84, 85). Our clinically relevant model showed no clear distinctions between Th1 and Th2 phenotypes. This is seen in the production of significant BAL fluid concentrations of both Th1 and Th2 cytokines.

Further, IgE and IgG1 antibodies are considered indicative of a Th2 allergic phenotype and IgG2a indicative of a protective Th1-type immunity. However, we show the selective abrogation of IgE, with no effect on IgG1 or IgG2a production. Therefore, this model implies the involvement of both Th1 or Th2 immune cells. Our model shows that the presence of LPS has adjuvant effects, but that these effects are different from those observed with the use of exogenous adjuvants.

We observed that induction of LPS tolerance prior to allergen sensitization results in the attenuation of airway eosinophilia measured after allergen challenge. We determined that the mechanism underlying this decrease is the significant reduction in CCL11 and CCL24 in both the BAL fluid and organ homogenate. We hypothesized that if the lack of eosinophils in the alveolar space and lung tissue was purely the result of a

defective chemotactic gradient, as seen in decreased chemokine production, increased numbers of circulating eosinophils would be measured. Analysis of the peripheral blood showed no differences in circulating eosinophils in non-tolerant mice compared to the LPS tolerant group (data not shown). Further, IL-5 is a key mediator in eosinophil activation and egress from the bone marrow into sites of inflammation (176). We found that plasma IL-5 levels were below the limit of detection of our ELISA (data not shown), however this does not rule out the possibility that eosinophils of LPS tolerant mice are deficient in their ability to egress from the bone marrow, through subtle differences in IL-5 concentrations.

LPS tolerant mice were also protected from AHR. We observed a clear dissociation between methacholine-induced airways hyperresponsiveness and mediators of AHR, such as cysteinyl leukotrienes. Our previous studies investigating pulmonary LPS tolerance resulted in a similar protection from AHR and concomitant increases in the mediators responsible of AHR induction. We showed in that previous model that protection from AHR was mediated through a reduction in M2 and M3 muscarinic receptor expression. It is of importance to determine whether muscarinic receptor downregulation lasts up to 21 days after LPS tolerance induction. The abundant production of cysteinyl leukotrienes indicates that mast cell function is not impaired in the tolerant mice, so it is unlikely that the protection from AHR is mediated by the abrogation of IgE production, and its resulting absence from the surface of mast cells. The increased concentration of cysteinyl leukotrienes may be the result of increased production of these mediators by mast cells. It has been shown that human mast cells stimulated with LPS produce higher concentrations of tryptase (177). Therefore, the prior

induction of LPS tolerance may cause mast cells to release increased concentrations of mediators such as cysteinyl leukotrienes in response to allergen challenge.

Our findings also demonstrate that the modulation of innate immunity induced by LPS tolerance is transient. Figure 1 demonstrates that CXC chemokine production and neutrophil recruitment induced 21 days after LPS tolerance are not affected. Further, TNFα, a hallmark feature of the LPS tolerant phenotype is no longer attenuated. This demonstrates the importance of the innate immune response at the time of sensitization. These data also reinforce our previous findings that a threshold of innate immune activation, in the form of LPS signaling dictates allergen sensitization or tolerance. Excessive innate immune activation, induced by LPS tolerance is protective against allergen sensitization, while one exposure to low-dose LPS (non-tolerant group) clearly potentiates the development of asthma.

Taken together, our data show that prior exposure to high doses of LPS protects against airway eosinophilia, AHR and IgE production. Further, these studies show that optimal allergen sensitization requires innate immune activation, and that this transient modulation of the innate immune response can have lasting effects on adaptive immunity. Our findings further suggest that the LPS tolerant state may be a mechanism by which individuals exposed to high doses of LPS are protected from the development of atopy and asthma. Further epidemiological studies are needed to determine whether lungs of individuals exposed to these LPS levels are refractory to further LPS stimulation, thereby conferring protection against allergen sensitization.

Chapter 6

Discussion

LPS tolerance in the lung microenvironment

These studies show for the first time that repeated LPS inhalation can induce a transient and specific refractoriness to further LPS challenge. While tolerant mice are deficient in their ability to produce TNFα, they produce significantly higher concentrations of IL-6. We have also demonstrated that LPS tolerance induction causes differential regulation of CXCL1 and CXCL2 expression, with CXCL2 being significantly reduced in tolerant mice. The reason for this differential regulation is unknown at this time and has, to our knowledge, not been reported elsewhere. However, given that these two chemokines share the CXCR2 receptor, the affinity of each chemokine for this receptor may have a role in determining the level of production in order to achieve the desired biological outcome (178). Human CXCL1 has been shown to bind to CXCR2 with the highest affinity of the CXC ligands specific for this receptor (179).

The IRAK family of kinases has been shown in both *in vivo* and *ex vivo* macrophage cultures to dictate the attenuated response to LPS seen in those models. IRAK signaling converges on the activation of NF-κB, which can induce activation of both acute phase cytokines and CXC chemokines. In acute LPS tolerant mice, we demonstrated that NF- κB activation was equivalent in LPS tolerant and non-tolerant

mice. Since alveolar macrophages are the primary producers of TNFα, we cannot rule out the possibility that IRAK-M is downregulated in these cells, but that the involvement of other cell types dictates the global inflammatory environment of the lung. It is possible that LPS tolerance is a cell type specific phenomenon and that the differential ability of macrophages and epithelial cells to become tolerized is the mechanism underlying the differential cytokine expression seen in both the acute and chronic models. Further studies are required to determine if various cell types play differing roles in LPS tolerance.

Mechanism of airways hyperresponsiveness

We demonstrated that airways hyperresponsiveness was attenuated both with induction of chronic LPS tolerance, as well as in CRA sensitized mice in which tolerance was previously induced. While we showed that M2 and M3 muscarinic receptors were downregulated in chronic LPS tolerance mice, it is not clear whether this same mechanism governs the reduced expression in the asthmatic mice. Both models show significant increases in the mediators of AHR, tryptase and cysteinyl-leukotrienes. It is of interest to determine whether the effects of muscarinic receptor expression induced by LPS tolerance last throughout the allergen sensitization and challenge protocol, resulting in attenuated AHR in response to allergen.

New evidence has emerged regarding the role of the surface molecule CD38 in airway smooth muscle contraction. CD38 is an ectoenzyme that is responsible for the conversion of β-NAD into cyclic ADP-ribose (180). Cyclic ADP-ribose is an intracellular calcium mobilizing agent that can induce calcium influx into the cytoplasm of airway smooth muscle cells from extracellular sources, or intracellular stores. The influx of

calcium then causes contraction of α-smooth muscle actin and subsequent AHR. The expression of CD38 has been shown to be upregulated in response to TNFα stimulation in isolated human airway smooth muscle cells (181, 182). Little to no *in vivo* data exist in determining the role of CD38 in the response to LPS or allergen, however future studies involving this enzyme may be of significant importance.

The role of TLR activators in the development of asthma

While this work has focused on the role of the TLR4 activating compound LPS, we cannot exclude the possibility that other microbial components, such as LTA, peptidoglycan, or bacterial DNA are signaling through Toll-like receptors and influencing the onset of asthma. This is particularly true because the exact composition of both the cockroach allergen and house dust extract (HDE) used in these studies is unknown at this time. TLR2 ligands such as LTA have been shown to participate in cross-tolerance, causing downregulation of cytokines in response to a primary challenge with TLR4 ligands (183). TLR2 ligands are also capable of potentiating airway inflammation and IgE production in response to OVA-induced asthma (184). In contrast, CpG DNA, a TLR9 ligand, can inhibit allergic inflammation of the airways. TLR9 signaling activates robust production of IFN-α and IFN-β in dendritic cells, which may skew the pulmonary immune environment toward protection (185, 186). Therefore, determining if allergen sensitization after LPS tolerance induction results in Type-1 interferon production would be of interest in determining the mechanism by which mice are protected from asthma.

Protease activity of allergens

The model of asthma induction used in the studies determining the effect of LPS tolerance on the asthmatic phenotype used direct intratracheal exposure for sensitization. This may be a key factor in our ability to sensitize animals with very small amounts of allergen, indeed concentrations that are 3 logs less than those used in ovalbumin studies. Bla g2, the principle cockroach allergen present in both the CRA mixture and HDE has aspartic protease activity (93). This protease activity may aid in sensitization by increasing airway mucosal permeability. This may make allergens more readily accessible to airway dendritic cells, facilitating antigen uptake (187). This permeability may take place through the cleavage and activation of protease activated receptors, whose expression is increased in the airway epithelium of asthmatic subjects (188). LPS has also been shown to upregulate the PAR-2 receptor on the surface of human epithelial cells (189). PAR receptors are also present on the surface of airway smooth muscle cells, which may implicate protease activity of allergens on the onset of bronchial hyperresponsiveness (190). Activation of this receptor can alter airway smooth muscle tone by inducing calcium influx into the cell and smooth muscle contraction.

Mast cells and basophils

Mast cells have been shown to reside in the naïve lung, while basophils are present in the circulation and are recruited to the lung in the allergic setting. Both mast cells and basophils are FcεRI+ and therefore can express IgE on their surface (191). Mast cells are localized in the BAL space, near airway vasculature, as well as in airway smooth muscle bundles, and this microlocalization can have an effect on the severity of methacholine responsiveness (192, 193). This suggests the possibility that mast cell

localization in the lung may be altered in our model of LPS tolerance. Repeated LPS inhalation may cause mast cells to localize in the BAL space and may inhibit localization in ASM bundles. This may be the mechanism by which increased concentrations of tryptase and cysteinyl leukotrienes were measured in the BAL fluid of LPS tolerant mice, while methacholine responsiveness was attenuated.

Further, mast cells express TLRs on their surface, and have been shown to present antigen in the context of both MHC class I and class II. They have therefore been implicated in having a role in allergen sensitization (194-196). Mast cell deficient mice do not develop airway eosinophilia in response to intra-nasal OVA exposure, while their wild-type counterparts do. However, if mast cell deficient mice are immunized by i.p. injection, their response is similar to that seen in wild type controls. While mast cells are able to take up and present antigen, it has also been shown that mast cells can traffic to the lymph node to present antigen to naïve T cells (197). Additionally, mast cells may present antigen locally to B cells in the airway mucosa in order to induce class switch recombination.

The vast majority of basophils express the chemokine receptor CCR3 and can be recruited to the asthmatic lung via the expression of CCL5, CCL11 and CCL24 (198). Paradoxically, our data show decreased expression of CCL11 and CCL24, but increased levels of cysteinyl-leukotrienes, which are released by both mast cells and basophils. This suggests that other factors, such as MCP-1 and MCP-3 may be responsible for basophil chemotaxis in our model (199). Further, in the allergic setting, infiltrating basophils express IL-4 mRNA and may therefore be a source of Th2 cytokines in the lung microenvironment, further exacerbating the allergic phenotype (200). The abundant

secretion of both tryptase and cysteinyl leukotrienes, as well as Th2 cytokines suggests that repeated LPS exposure may increase the number of resident mast cells in the lung and may potentiate the influx of basophils in response to allergen challenge. Further, LPS has been shown to induce degranulation of both mast cells and basophils. Therefore, it is possible that the potential of these cells for mediator release is increased upon repeated LPS exposure (201).

Our data show that IgE production in response to CRA sensitization is abrogated in LPS tolerant mice, however cysteinyl leukotriene concentrations are increased. This suggests that mast cell and basophil degranulation occurs via an IgE-independent mechanism. One such mechanism of IgE-independent mast cell activation is by direct stimulation by TLR ligands. Human basophils express mRNA and protein for both TLR2 and TLR4, but only reacted to stimulation with TLR2 ligands. TLR2 ligands were able to induce the release of IL-13, as well as histamine and leukotriene C4 in an IgE-independent manner (202). This apparent non-responsiveness to TLR4 stimulation suggests that basophils do not become tolerant in our model, but may become hyperresponsive to TLR2 ligands that are likely present in our CRA extract..

Further, complement derived peptides C3a and C5a can stimulate degranulation of both mast cells and basophils (203). These particular complement factors are produced mainly by alveolar macrophages and airway epithelial cells (204). We hypothesize that the differential mediator production observed in the lungs of LPS tolerant mice may be the result of the ability of various cell types to become tolerized. While it has been shown in several models that mediator production is reduced in LPS-tolerant macrophages,

airway epithelial cells may be the principle cell type responsible for the production complement-derived peptides (35, 163).

B Cells

The activation of B cells by ligation of surface CD40 to T cell CD40L is a primary means by which isotype switching to IgE production occurs (45). However, LPS stimulation is also a potent activator of B cell activation as well, leading to increased antigen presentation and B cell proliferation. This apparently non-specific signal can lead to increased production of antigen-specific IgE (205). The specificity for the antigen-specific antibody response is conferred by the binding of the polysaccharide moiety of LPS to B cell Ig receptors on antigen specific B cells (206). Evidence exists that suggests the dose of LPS used for B cell stimulation can dictate the outcome of the antigen-specific response. It is suggested that high-dose LPS can induce B cell paralysis, thereby inhibiting antigen-specific antibody production (207).

However, because LPS administration in our model is localized to the lung, it is unlikely that follicular B cells directly encounter LPS. Therefore, direct B cell tolerance likely effects the small number of lung resident B cells, but is likely not the only mechanism directing the abrogation of the IgE response in LPS tolerant mice. Additionally, a possible defect in antigen presentation by dendritic cells, leading to altered T cell maturation and subsequently defective CD40-CD40L interactions with B cells is likely (45). Abrogated B cell maturation would lead to decreased numbers of plasma cells in the bone marrow of LPS tolerant mice.

TNFα and allergen sensitization

Eisenbarth et.al. showed that mice defective in TLR4 did not develop OVA-induced pulmonary inflammation, however this phenotype was restored by the exogenous administration of TNFα (80). We initially hypothesized that the mechanism by which LPS tolerant mice were protected from asthma was through a reduction in TNFα produced in response to CRA sensitization. However, our studies demonstrated that TNFα levels 2 hours post sensitization were not attenuated in LPS tolerant mice (data not shown). However, IL-6 levels were significantly elevated, recapitulating the tolerance phenotype observed in our earlier studies with LPS exposure alone. We therefore hypothesized that LPS tolerance modulates allergen sensitization through a mechanism independent of TNFα production. Thus we observed that in this model, allergen sensitization can be abrogated in the presence of TLR4 signaling, however the mechanism driving this observation has yet to be elucidated.

The dose of LPS used for LPS tolerance induction must also be considered. Eisenbarth et.al. demonstrated that while high-dose LPS was protective against OVA-induced asthma, low-dose LPS was able to potentiate the allergic response. Our data suggest that the immune environment of the lung is differentially regulated by the dose of LPS used for tolerance induction. Therefore, low-dose LPS may potentiate CRA-induced asthma by increasing DC maturation and the subsequent production of allergen-specific T cells as well as IgE.

Dendritic cells in sensitization

Dendritic cells play an important role in bridging the innate and adaptive immune responses. LPS stimulation of dendritic cells induces DC maturation, resulting in

cytokine production and upregulation of costimulatory molecules (208, 209). Dabbagh et. al. showed that LPS signaling through TLR4 on DCs to be required for the development of the allergic response to OVA (210). Further, these studies demonstrated that DCs of TLR4 deficient mice expressed fewer costimulatory molecules such as CD80 and CD86 on their surface, and produced decreased levels of IL-12 in contrast to wild type controls. Further, these mice had significantly reduced airway eosinophilia, but were able to generate allergen-specific T cells. However these cells were deficient in their ability to produce IL-4 and IL-5.

Further, the importance of TLR4 signaling in DCs has been demonstrated by Eisenbarth et. al., who showed that low dose LPS was required to elicit asthmatic inflammation and IgE production, but that this was abrogated in animals receiving high dose LPS at sensitization. They further showed that in this model, DC maturation, and migration to the lung draining lymph node was impaired in TLR4-deficient mice. This led to the abrogation of production of allergen-specific T cells and was considered to be the mechanism by which TLR4 signaling modulates allergen sensitization. The difference between these findings and those of Dabbagh, et. al. with regard to the generation of mature T cells may lie in their use of intraperitoneal immunization, which has been shown to induce T cell priming independently of the innate immune response (86).

IL-12 production was previously suggested to be necessary for the induction of a tolerogenic response to allergen encounter (211, 212). However, more recent findings have demonstrated that overstimulation of DCs with LPS can induce a state of LPS exhaustion, resulting in refractoriness to further LPS stimulation and attenuated IL-12 production. However, these exhausted DCs could still skew the immune response toward

tolerance (213, 214). While IL-12 has been excluded as the factor responsible for inducing a protective immune response, these results still demonstrate that DCs can become refractory, or tolerant to excessive LPS stimulation. This suggests the possibility that DC tolerance to LPS may be the mechanism by which chronic LPS tolerant animals do not develop the asthmatic phenotype. Therefore, it is possible that the cytokine milieu secreted by LPS tolerant DCs alters T cell maturation, inducing protection from asthmatic symptoms.

The mechanism dictating DC exhaustion remains unclear. Recent evidence suggests that stimulation of DCs with TLR ligands induced rapid DC maturation, however this maturation is transient (215). These previously mature DCs "expire" and take on a $DC86^{low}$ de-matured phenotype. Unlike mature DCs, expired DCs are able to take up antigen with similar efficiency to immature DC. These DCs were also unable to prime naïve T cells. Cytokine production, such as IL-1β TNFα are rapidly upregulated upon LPs stimulation, but gradually decreases after prolonged challenge (214). Recent evidence shows that blocking of autocrine IL-10 signaling in mature DCs allows them to remain in the mature state, whereas exogenous addition of IL-10 induced DC expiration. Further, IL-10-induced expired DCs were unable to induce an OVA-specific CTL response in vivo (216). Therefore, exhaustion of pulmonary DCs by repeated LPS inhalation may be the mechanism by which LPS tolerant mice are protected against the hallmark features of allergen-induced asthma. This may provide the link between pulmonary innate immune modulation (LPS tolerance) and the adaptive immune response (allergic asthma).

Taken together, we demonstrate that repeated LPS inhalation can cause specific, yet transient modulation of the innate immune response. Because LPS is ubiquitously present in ambient air, its effects on the development of allergic responses cannot be ignored. We have shown that prior induction of LPS tolerance using high-dose LPS inhalation is protective against the development of CRA-induced asthma (Figure 6-1). Dendritic cells are the key cell populations in bridging innate and adaptive immunity. We propose that the mechanism by which LPS tolerant mice are protected from the development of asthma-like inflammation is through dendritic cell exhaustion, induced by increased IL-10 production (Figure 6-2). Further studies must be performed in order to determine whether repeated LPS inhalation induced DC expiration, and whether these expired DCs are able to induce adaptive immunity in CRA-induced asthma.

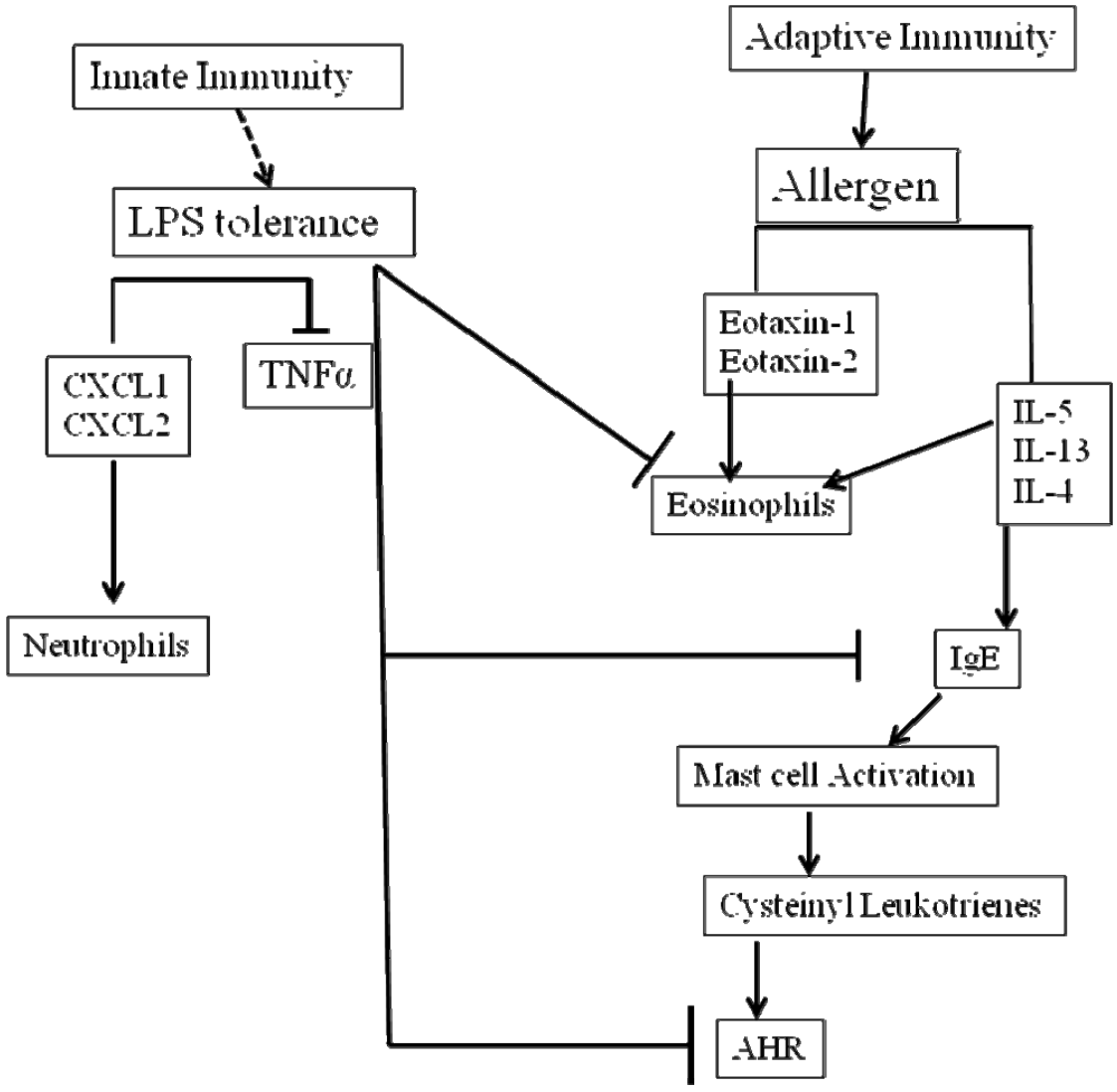


Figure 6-1: Schematic representation of LPS tolerance-induced modulation of the adaptive immune response. The transient modulation of innate immunity, induced by LPS tolerance has lasting effects on adaptive immunity, in the form of protection from airway eosinophilia, IgE and airways hyperresponsiveness.

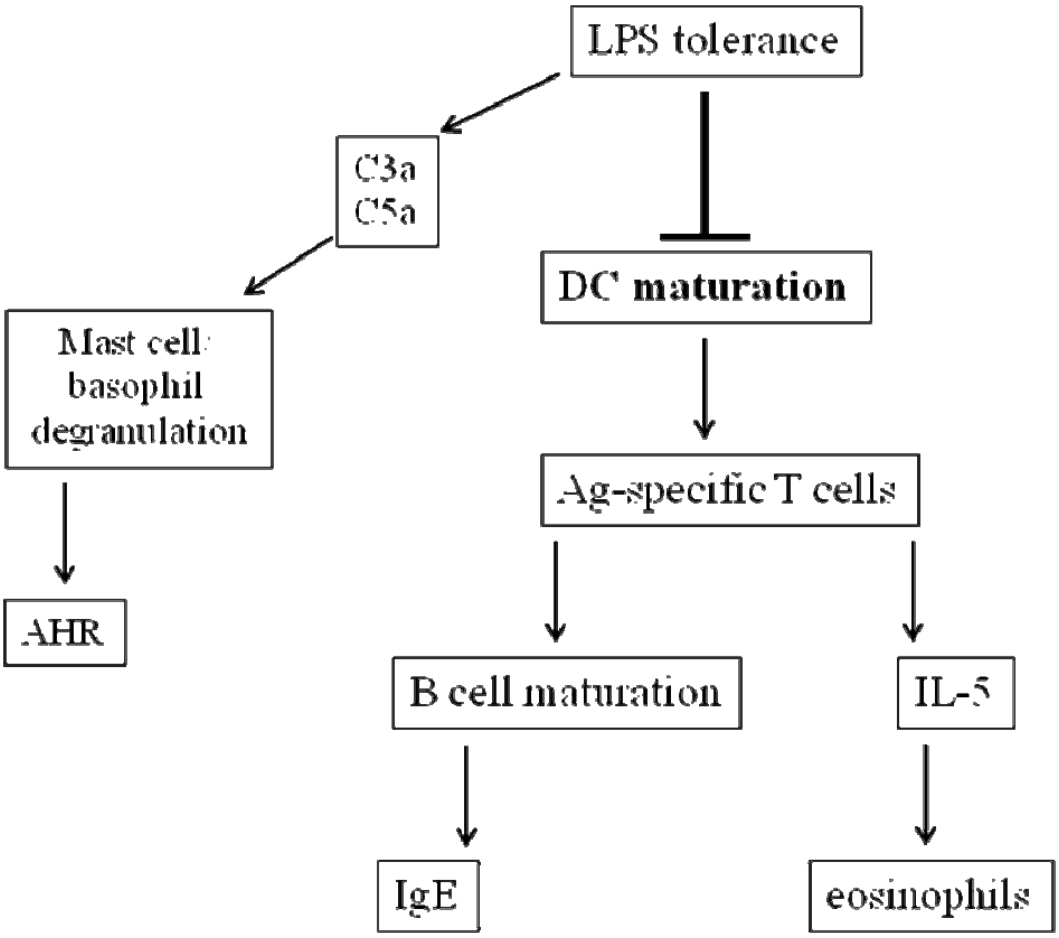


Figure 6-2: Proposed mechanism adaptive immune modulation by LPS tolerance. Repeated inhalation of LPS induces transient DC maturation followed by DC expiration. Expired DCs are impaired in their ability to induce differentiation of allergen specific T cells. Subsequently, circulating IL-5 is not present and eosinophi do not egress from the bone marrow into the circulation. B cell maturation is inhibit which abrogates differentiation into plasma cells, preventing IgE secretion into the circulation. LPS tolerance induces specific immune modulation in the form of increased complement derived peptides, such as C3a and C5a, which induce IgE independent degranulation of mast cells and basophils.

APPENDIX A

Experimental Methods

Animals

Female BALB/c mice 9-12 weeks old were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained under standard laboratory conditions. The mice were housed in a temperature and humidity controlled room with 12 hour light/dark cycles. Food and water were allowed *ad libitum*. All experiments were performed according to the National Institutes of Health guidelines and were approved by the Boston University Institutional Animal Care and Use Committee.

LPS Assay

LPS in the house dust extract and cockroach allergen preparations was assayed in pyrogen free water using the *Limulus* amoebocyte lysate assay (Lonza, Basel Switzerland, 50-647U). 96-well microplates and substrate solutions were warmed to 37°C. 50µL of sample and standard were added to the plate in duplicate followed by 50µL LAL regent. The plate was incubated at 37°C for 10 min. 100µL of substrate solution was then added to each well and the plate was incubated at 37°C for 6 minutes. The reaction was stopped with 50µL 25% glacial acetic acid. The absorbance was read at 405nm.

Bronchoalveolar lavage and lung homogenate preparation

Mice were exanguinated and BAL was performed by cannulating the trachea. The lung was lavaged with 2, 1mL aliquots of warm Hank's Buffered Salt Solution (HBSS, Gibco, Grand Island, NY). Both aliquots were centrifuged and the supernatant of the first wash was removed and frozen at -20°C for cytokine analysis. The supernatant from the second wash was discarded and the cell pellets from both aliquots were resuspended and combined. Total cell counts were obtained using a Beckman-Coulter particle counter model ZF (Coulter Electronics Inc., Hialeah, FL). Cytospin preparations were stained with Diff-Quick and 300 cell differential counts were performed to determine the absolute numbers of inflammatory cells. The right lung was removed, placed in ice cold protease inhibitor cocktail (Roche, Indianapolis, IN) containing 0.00005% Triton X in PBS, and homogenized with 3, 10 second passes in a Brinkmann Polytron PT3000 homogenizer. An aliquot was removed and sonicated in hexadecyltrimethylammonium bromide (HTAB) buffer for myeloperoxidase assay. A separate aliquot was removed and sonicated in 0.5% cetyltrimethylammoniumchloride (CTAC) (Sigma, St. Louis, MO) for eosinophil-specific peroxidase assay. The homogenized and sonicated mixtures were centrifuged at 15,000g for 15min. The homogenate supernatant was removed and stored at -20°C for cytokine analysis and the supernatant from the sonicated fractions were used immediately for peroxidase assays.

mRNA isolation and RT-PCR

mRNA was extracted using Trizol Reagent (Invitrogen, Grand Island, NY). PCR reactions were carried out using iScript One-Step PCR kit with Sybr Green (BioRad, Hercules, CA) using the following primer pairs: TNFα: Sense 5'-ACTCCCAGACCCGGTATCTT -3'; Antisense 5'- CTGCAATTGACCGTCTTCT -3',

IL-6: Sense 5'- ACGGCCTTCCCTACTTCACA -3'; Antisense 5'-TCCAGAAGACCAGAGGAAATTTT -3', KC: Sense 5'-TCAAGAACATCCAGAGCTTGAAG -3'; Antisense 5'-GGACACCTTTTAGCATCTTTTGG -3', MIP-2: Sense 5'-CACCAACCACCAGGCTACAG -3'; Antisense 5'-CAGTTAGCCTTGCCTTTGTTCA-3'; M2 Receptor: Sense 5'-TAAAGTCAACCGCCACCTTC-3', Antisense: 5'-ATAACGGAGGCATTGCTGAC-3'; M3 Receptor: Sense 5'-GCTCAGAGACCAGAGCCTATC-3', Antisense 5'-ACAGTTGTCACGGTCATCCA-3'; GAPDH: Sense 5'- AACGACCCCTTCATTGAC -3'; Antisense 5'- TCCACGACATACTCAGCAC -3'. Real-time PCR reactions were performed on a BioRad iQ5 light cycler. Data was normalized to housekeeping gene and expressed as fold increase above the 0 hour non-tolerant group using the $2^{-(\Delta\Delta Ct)}$ method (217).

Cysteinyl-Leukotriene Immunoassay

Cysteinyl leukotrienes levels in the BAL fluid were measured by an Enzyme-linked Immunoassay Kit (Cayman Chemicals, Ann Arbor, MI) according to the manufacturer instructions. All samples were run at two dilutions. Only $\%B/B_0$ values in the linear range of the standard curve were accepted. Sample values falling out of this range were further diluted and rerun.

Airways Hyperresponsiveness

Airways hyperresponsiveness was measured using unrestrained whole body plethysmography (Buxco Systems, Troy, NY). Mice were placed in the instrument chamber and allowed to acclimate for 5 minutes. Baseline measurements were recorded

for 5 minutes. Mice were challenged for 2 minutes with aerosolized PBS and increasing doses of methacholine (Sigma, St. Louis, MO). Each aerosol was followed by 5 minutes of monitoring and data collection. The partial pressure difference between the experimental and reference chambers is represented as the PenH parameter, which corresponds closely with invasive measurements (218). The data is represented as the percent increase in PenH above PBS aerosol.

ELISA

Cytokines and chemokines were measured by sandwich ELISA as previously described (219). Matched antibody pairs and recombinant standards were purchased from R&D Systems (Minneapolis, MN). Lung homogenate samples were assayed with the addition of 20% normal lung homogenate to the standards to adjust for the increased background caused by non-specific matrix effects. Antibody ELISAs were performed by the same standard protocols. Antibodies and standards were purchased from Bethyl Laboratories (Montgomery, TX). Cockroach allergen ELISAs were performed as previously described (55) with antibody pairs and recombinant standards from Indoor Biotechnologies (Charlottesville, VA).

Myeloperoxidase and Eosinophil Peroxidase Assays

Myeloperoxidase (MPO) and eosinophil peroxidase (EPO) assays were performed as described previously, with some modifications (220). EPO was performed by diluting the supernatant of the mixture sonicated in CTAC 1:3 in 10mM HEPES, pH 8, in quadruplicate in a 96-well plate. 150µL ice cold stop solution (4N H_2SO_4 + 2mM resorcinol) was added to 2 of the sample wells. 75µL substrate solution containing 6mM KBr, 1.5mM *o*-phenylenediamine (Sigma, St. Louis, MO, P9029), and 0.3% H_2O_2 in

50mM HEPES, pH8, was added to the two remaining sample wells and incubated in the dark for 30 seconds. 150µL ice cold stop solution was added and the absorbance was read at 490nm.

MPO was performed by diluting the supernatant of the mixture sonicated in HTAB 1:5 in 10mM citrate buffer, pH5. 150µL ice cold stop solution (4N H_2SO_4) was added to 2 of the sample wells. 75µL substrate solution containing 0.3mM 3,3′,5,5′-Tetramethylbenzidine, 120mM resorcinol, and 0.007% H_2O_2 in ddH_2O was added to the two remaining sample wells and incubated in the dark for 2 minutes. 150µL ice cold stop solution was added and the absorbance was read at 450nm. Data is expressed as ΔOD reflecting the difference in absorbance between the average of the sample and background wells.

Determination of TNFα and TNF-soluble receptor interactions

3000 pg/mL or 300 pg/mL of recombinant TNFα were mixed with increasing concentrations of either TNF-SRI or TNF-SRII, as indicated in the figure legend, in PBS + 2% FCS and incubated at room temperature for 1 hour. TNFα was then assayed by with the standard ELSIA.

Histopathology

Immediately after collection of the left lung, the right lung was harvested, fixed in 70% ethanol, paraffin embedded and processed for routine histology. Separate sections were also stained with periodic acid-Schiff (86) reagent and were examined under light microscope. Digital images if the entire section were taken and analyzed using NIH ImageJ Software. The outline of each figure was traced and the total area in pixels was calculated using the measure function. Positive PAS staining was selected using the

Color Deconvolution plug-in. The area of positive staining was measured using the measure feature. Positive staining is represented as area of PAS staining per 10^6 pixels.

Congo Red stain for tissue eosinophils

Paraffin sections were stained with Congo Red as previously described (221). Eosinophils were counted in four randomly selected high powered fields for each section and 8 sections per group were analyzed. Eosinophil numbers are represented as number of eosinophils per high powered field.

Measurement of physiological parameters

For determination of total peripheral white blood cell, lymphocyte numbers and hemoglobin concentrations, 20μL of blood was collected from the tail vein and an automated differential was performed using a Hemavet Mascot (Drew Scientific, Ramsey, MN). Body weight was determined daily.

APPENDIX B

The ELISA Standard Save: Calculation of sample concentrations in assays with a failed standard curve

Introduction

Given its ease of performance, high throughput capabilities and the increasing availability of recombinant standards and antibodies for various proteins, the ELISA has become the assay of choice for measuring proteins in various biological matrices (222, 223). The ELISA technique has recently been optimized to measure protein concentrations from as little as 10μL of sample, with proper dilution. This greatly benefits many in vivo applications in which only small volumes of sample can be collected in order to monitor disease progression over time (224, 225). Unfortunately, human error and other unidentifiable factors sometimes result in unsuccessful assays in which a reliable standard curve cannot be generated, leaving otherwise representative sample readings useless. This can prove detrimental when only small sample volumes can be collected, requiring both time and resources to repeat experiments.

Here, we outline a method by which the concentrations of samples may be calculated by deriving a separate standard curve. This method is not recommended as a primary means of data collection, but is a rescue protocol for instances in which there is a need for salvage. This method is applicable when it is determined that the defect is with the standard only. Standard curves are deemed failed if they meet the following criteria: five or more samples have ΔOD 0.5 higher than the most concentrated standard; a 4-

parameter regression cannot be calculated (ΔOD does not strictly decrease as standard concentration decreases); and r^2<0.97. In our experience, technical blunders involving the capture antibody result in little to no color development, while problems with detection antibody results in uniform faint color in all wells, including blanks. As such, experienced ELISA users can determine whether or not sample ΔOD values are usable as they will show a wide range of values. Specific examples of possible mistakes where only the standard has failed would be using mouse IL-6 standard in an ELISA for human IL-6, or selecting the IL-1 standard when the assay was meant to measure IL-10.

In this method, a new standard curve is generated using the ΔOD values of the samples themselves from the original assay. 8-10 samples with maximal range of ΔOD values are chosen and re-run with a verified working standard protein. The concentrations of these samples are then used to generate a new standard curve, which is applied to the original plate in order to determine sample concentrations. Linear regression analysis of these curves showed a 1:1 correlation between concentrations, with r^2 values ranging from 0.98-0.99 and slopes ranging from 0.97-1.10 for each cytokine assayed.

Methods

1. Initial Sample Run
    a. Samples are run according to previously optimized standard ELISA protocol (219, 226).
    b. Immediately prior to stopping the color reaction, the OD_{590} is measured.
    c. The reaction is stopped with sulfuric acid and ΔOD values are used for data analysis. At this point, if a working standard curve can be generated, the data

may be analyzed normally. If not, the following steps can be taken for preservation of sample data.

2. Troubleshoot Standard Curve
    a. Prepare fresh dilution buffer and rerun standard curve. In order to proceed with the assay, troubleshooting must be successful so that standard curves can be reliably generated.
3. New Standard Run
    a. Choose 8-10 samples to rerun to generate the new standard curve.
        i. Samples with the maximum and minimum ΔOD values are always chosen, along with others with ΔOD values that are evenly distributed within this range.
    b. These samples are rerun by standard ELISA protocol.
    c. Prior to addition of sulfuric acid solution, OD_{590} is monitored. The reaction should not be stopped until the OD_{590} values are equal to that read in the initial sample run. The plates can be left in a covered plate reader and reread until the desired OD_{590} is obtained. This is to ensure that differences in extent of color development do not affect sample concentrations calculated using the new standard.
    d. A non-linear 4-parameter regression is used to generate a standard curve. Note that any computer program capable of generating a 4-parameter regression can be used. The concentrations of the samples are then used to generate a new standard curve as follows:

i. Each well corresponding to a sample in the new standard run is re-labeled as a standard and its concentration is entered manually.
ii. Once all samples are renamed and concentrations entered, those wells are used to generate the new standard curve and 4-parameter equation.
iii. The new standard curve is then applied to samples from the initial sample run to interpolate concentrations of all the samples.

Results and Discussion

The OD_{590} is monitored in order to ensure consistent color development in each ELISA run. Plotting of OD_{590} versus ΔOD resulted in an r^2 value of 0.98, indicating that OD_{590} is an effective method to monitor color development (Figure B.1). Once concentrations are determined for the samples in the new standard run, those samples must be re-labeled as standards so they may be applied back to the initial run. In order to illustrate this, a sample 96-well plate layout for the new standard run is shown in Figure B.2. The chosen samples are rerun by the same method as the initial sample run. Once these concentrations are determined, these same wells are re-labeled as standards and the measured concentrations are entered manually. This new standard is applied back to the initial sample run to interpolate sample concentrations.

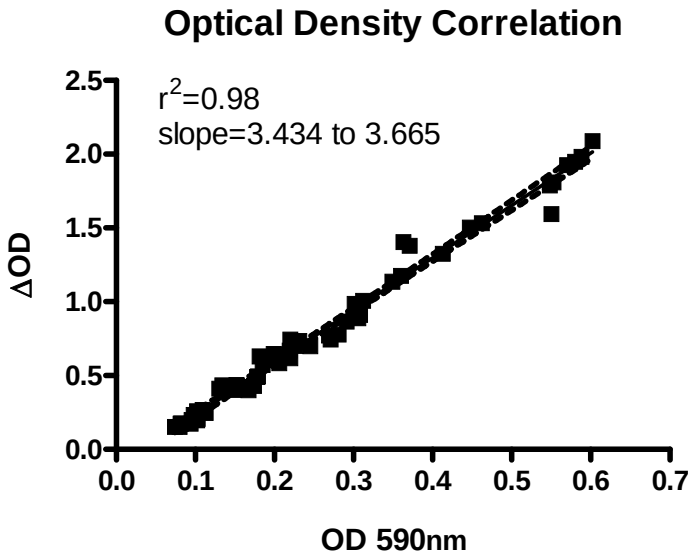


Figure B.1: Correlation of OD_{590} and ΔOD- Samples were read before and after addition of acid to stop color development. OD 590_{nm} was plotted against ΔOD to reveal a linear relationship.

In order to validate this method, 40 samples were prepared by spiking normal mouse plasma with a range of concentrations of the mouse recombinant cytokines TNFα, IL-4 and IL-6. Concentrations of the samples determined in the initial sample run plotted against those determined by applying the newly generated standard results in a linear correlation between the two methods with an $r^2 \geq 0.98$ for all cytokines (Figure B.3). Upon performing linear regression analysis, a slope of 1 indicates perfect agreement between the initial run and the application of the new standard. The slopes of the regression lines for all cytokines used were between 0.92 and 1.10, indicating that the new standard curve is a highly effective means by which to determine sample concentration.

A)	1	2	3	4	5	6
A	Standard 1 10,000 ng/ml	Standard 1 10,000 ng/ml	Sample 1 ΔOD: 1.38 2858.3 ng/ml	Sample 1 ΔOD: 1.4050 2961.9 ng/ml	Sample 9 ΔOD: 0.6310 916.8 ng/ml	Sample 9 ΔOD: 0.627 909.9 ng/ml
B	Standard 2 3330.0 ng/ml	Standard 2 3330.0 ng/ml	Sample 2 ΔOD: 1.5320 3562.5 ng/ml	Sample 2 ΔOD: 1.5940 3911.7 ng/ml		
C	Standard 3 1108.9 ng/ml	Standard 3 1108.9 ng/ml	Sample 3 ΔOD: 0.7450 1122.7 ng/ml	Sample 3 ΔOD: 0.7720 1174.5 ng/ml		
D	Standard 4 369.26 ng/ml	Standard 4 369.26 ng/ml	Sample 4 ΔOD: 0.1530 192.7 ng/ml	Sample 4 ΔOD: 0.1530 192.7 ng/ml		
E	Standard 5 122.96 ng/ml	Standard 5 122.96 ng/ml	Sample 5 ΔOD: 0.2340 307.79 ng/ml	Sample 5 ΔOD: 0.2460 324.80 ng/ml		
F	Standard 6 40.496 ng/ml	Standard 6 40.496 ng/ml	Sample 6 ΔOD: 0.6470 944.57 ng/ml	Sample 6 ΔOD: 0.6990 1037.3 ng/ml		
G	Standard 7 13.635 ng/ml	Standard 7 13.635 ng/ml	Sample 7 ΔOD: 0.2590 343.25 ng/ml	Sample 7 ΔOD: 0.2680 356.05 ng/ml		
H	Standard 8 0.001 ng/ml	Standard 8 0.001 ng/ml	Sample 8 ΔOD: 0.4110 564.24 ng/ml	Sample 8 ΔOD: 0.4350 600.45 ng/ml		

B)	1	2	3	4	5	6
A			Standard 2 2858.3 ng/ml	Standard 2 2961.9 ng/ml	Standard 5 916.79 ng/ml	Standard 5 909.89 ng/ml
B			Standard 1 3562.5 ng/ml	Standard 1 3911.7 ng/ml		
C			Standard 3 1122.7 ng/ml	Standard 3 1174.5 ng/ml		
D			Standard 9 192.7 ng/ml	Standard 9 192.7 ng/ml		
E			Standard 8 307.79 ng/ml	Standard 8 324.80 ng/ml		
F			Standard 4 944.57 ng/ml	Standard 4 1037.3 ng/ml		
G			Standard 7 343.25 ng/ml	Standard 7 356.05 ng/ml		
H			Standard 6 564.24 ng/ml	Standard 6 600.45 ng/ml		

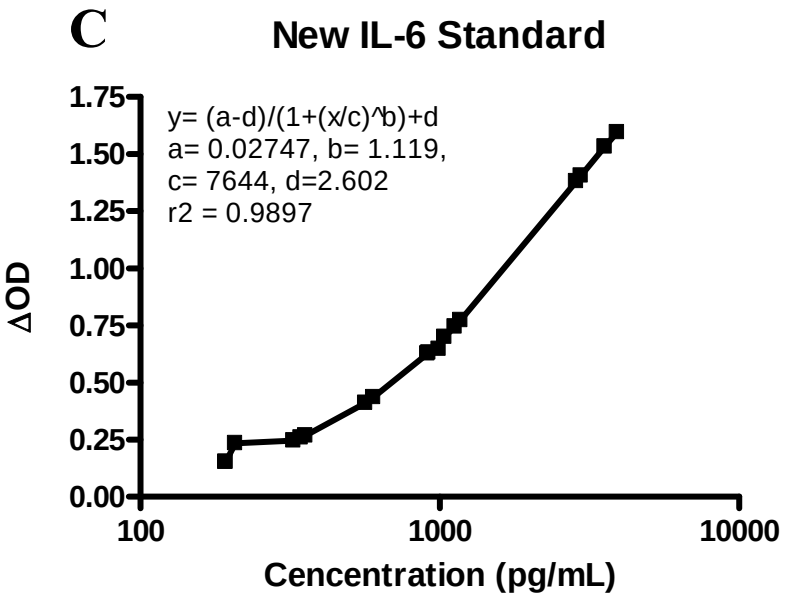
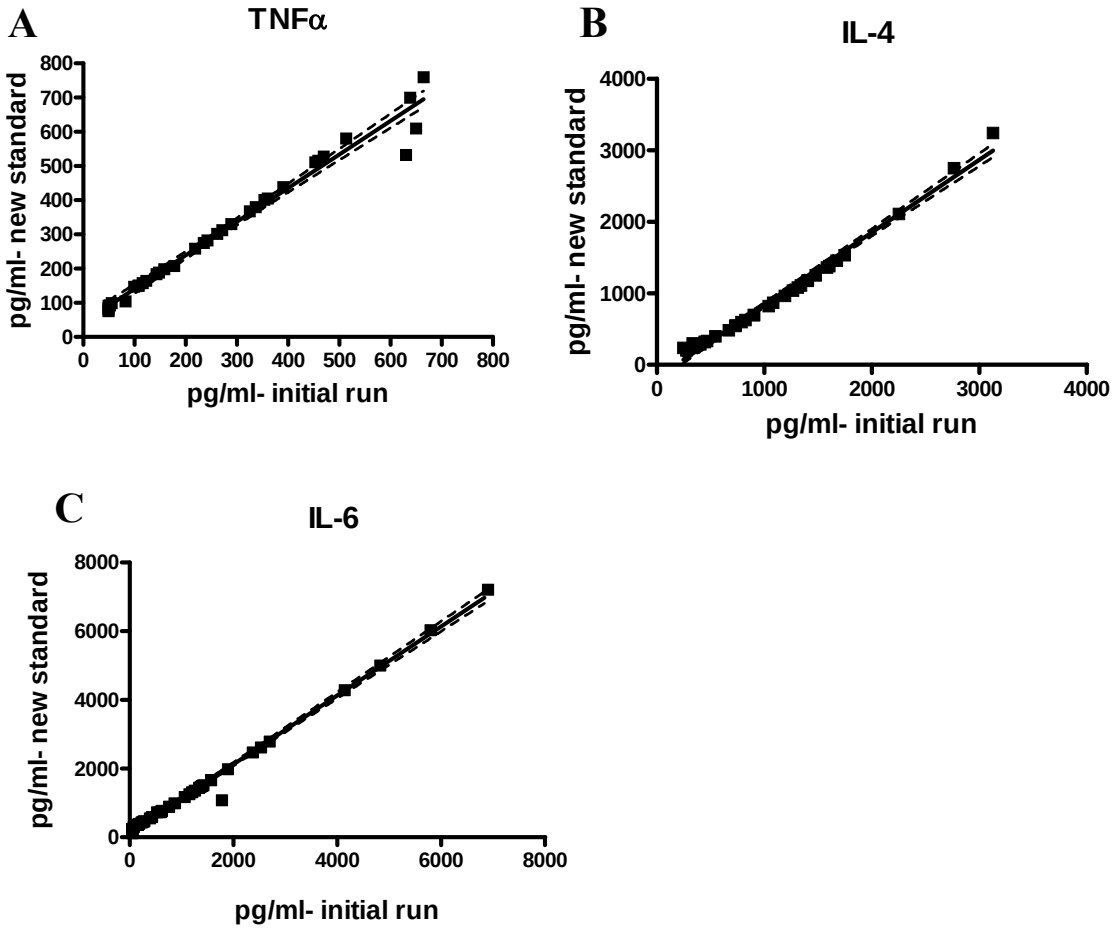


Figure B.2: 96-well plate layouts for generation of new standard curve. A) Plate layout with standard curve used to determine chosen sample concentrations. Standard concentrations are entered manually based on 1:3 dilutions of top standard. Sample concentrations are determined using standard curve. B) Re-labeling of sample wells as standards by manually inputting average concentrations of samples determined in the new standard run. C) Example of the new standard curve constructed for mouse IL-6.



D)	r^2 coefficient	slope
TNFα	0.98	0.92-1.0
IL-4	0.99	0.97-1.10
IL-6	0.99	0.97-1.0

Figure B.3: Validation of the accuracy of the new standard curve. A) Sample concentrations determined by the initial sample run were plotted against those determined using the newly generated standard. The graphs show a nearly 1:1 correlation for all cytokines used, indicating the accuracy of this method. B) r^2 values and slopes indicate the new standard correctly calculates the concentration.

This method is based upon the concept that an unsuccessful standard curve does not negate the usefulness of the entire ELISA assay. Additionally, this method is most

effective with samples that contain varying concentrations of the protein to be assayed. Samples with a wide range of concentrations will maximize the dynamic range of the new standard curve, making it more robust. We must emphasize the importance of measuring the OD_{590} for all ELISA assays having greater than 20 samples, as it provides the researcher the option to use this method if the standard curve is not satisfactory. Significant resources may be saved as this method requires only the time needed to rerun 10 or fewer samples, saving both the time and money otherwise required for repeating experiments to collect more sample.

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