
UNIVERSITY OF NORTH TEXAS
HEALTH SCIENCE CENTER AT FORT WORTH
1970

ABSTRACT

The mechanisms by which stress can exacerbate asthma are still unknown. The purpose of this study was to examine the immunological links between stress controllability and asthma pathogenesis. Our studies reveal specificity of stress control and immune activation resulting in hyper-inflammatory reactions in response to allergic airway challenge. We anticipate that these studies can serve as a translational piece to facilitate clinical studies of stress and asthma prevalence.

The purpose of this project was to establish a murine model of stress controllability and demonstrate the impact of stress on the development of immune allergic airway hypersensitivity as a major feature of asthma. I hypothesized that given the ability to control the degree of stress would translate into less severe allergic airway hypersensitivity. We also hypothesized that distinct changes in immune responses generated in response to uncontrolled stress would reflect the extent of airway hypersensitivity.

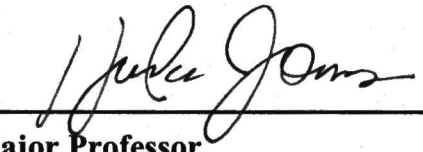
Mice were exposed to daily regimen of uncontrollable stress, controllable stress or no stress concurrently with allergen exposure. Behavioral disposition to stress was monitored in conjunction with evaluation of severity of asthma and immune status. Our results demonstrate that exerting control over stress conditions leads to distinct changes in immunological status corresponding with positive behavioral responses and less

disease severity. We anticipate that our studies will facilitate application of stress management in control of immune status as a biomarker for asthma progression.

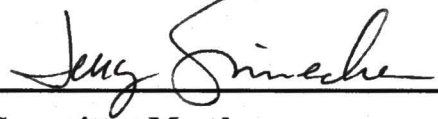
INVESTIGATING THE ROLE OF STRESS IN A MURINE MODEL OF ASTHMA

Aniket Deshmukh, B.S.

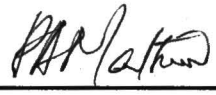
APPROVED:



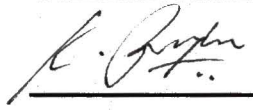
Major Professor



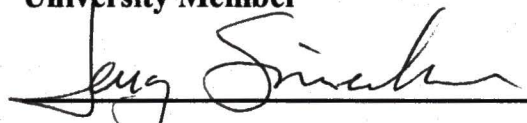
Committee Member



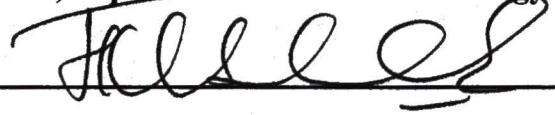
Committee Member



University Member



Chair, Department of Molecular Biology and Immunology



Dean, Graduate School of Biomedical Sciences

INVESTIGATING THE ROLE OF STRESS IN MURINE MODEL OF ASTHMA

THESIS

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas

Health Science Center at Fort Worth

in Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

Aniket Deshmukh, B.S.

Fort Worth, Texas

July 2008

ACKNOWLEDGEMENT

This thesis grew out of dialogues with a combination of an environment including the immunologist and psychologist as one mentor Dr. Harlan Jones.

Through his Socratic questioning Harlan brought me closer to the reality I had initially perceived eventually enabling me to grasp its rich complexity. His comments on chapter drafts are themselves a course in critical thought upon which I will always draw. His capacity to combine critique with an immediate empathy and commitment towards workers and others engaged in struggle will always inspire me.

This work would not have been possible without the support and encouragement of my colleague and friend Xavier Gonzales, Byung-Jin-Kim.

I would also like to thank the chairman of Department Prof. Jerry Simecka and Prof. P. Mathew who served as the member on my committee.

Last but not the least, I would like to thank my family and friends for their support throughout my educational career. Moreover my grandfather, late Wasudeorao Deshkar, who moulded my life and inspired me to recognize my passions and fulfill my dreams. Their unflinching courage and conviction will always motivate me. Most importantly I would like to thank God for his mercy, grace, strength and love.

TABLE OF CONTENTS

CHAPTER I

INTRODUCTION TO STUDY

Asthma is a chronic lung disorder of enormous public health importance that affects 10 to 12% of the population and disproportionately affects children, minorities, and persons of lower socioeconomic status (Statcher et al., 2008). It is the most frequent cause of pediatric emergency room use and hospital admission and is the leading cause of school absences. In particular, approximately 11% of adult population in Texas has had a diagnosis of asthma in their lifetime (Griswold et al., 2005). Epidemiologically, asthma morbidity and mortality has increased since the mid-1970s (CDC, surveillance summary). Thus, despite advances in medical treatments and prevention, asthma remains a major healthcare concern.

Asthma is a chronic condition involving the respiratory system in which the airways occasionally constrict, become inflamed, and are lined with excessive amounts of mucus, often in response to one or more triggers. These episodes may be triggered by such things as exposure to an environmental stimulant such as an allergen, environmental tobacco smoke, cold or warm air, perfume, pet dander, moist air, exercise or exertion, or emotional stress. The outward clinical features include wheezing, shortness of breath, chest tightness, and coughing. Asthma attacks are episodic, but airway inflammation is chronically present and as a result can lead to diminished lung function.

During an asthma episode inflamed airways react to environmental triggers such as smoke, dust, or pollen. The airways narrow and produce excess mucus, making it difficult to breathe. Asthma is commonly a result of an immune response in the bronchial airways. The airways of asthmatics are "hypersensitive" to certain triggers (allergens). In response to exposure to these triggers, the bronchi (large airways) contract into spasm during acute stages. At later stages, inflammation follows, leading to a further narrowing of the airways and excessive mucus production, which leads to coughing and other breathing difficulties. Extrinsic asthma (atopic asthma) is the major type of asthma that develops in people highly susceptible to allergens such as pollens, food, and dust particles. In both asthmatics and non-asthmatics, a type of cell known as antigen presenting cells, or APCs ingests inhaled allergens that find their way to the inner airways. APCs then "present" allergen to other immune system cells. In most people, these other immune cells (TH0 cells) usually ignore the allergen molecules. In asthmatics, however, these cells transform into a different type of cell (TH2), for reasons that are not well understood. The resultant TH2 cells activate an important arm of the immune system, known as the humoral immune system. The humoral immune system produces antibodies against the inhaled allergen. Later, when an asthmatic inhales the same allergen, these antibodies "recognize" it and activate a humoral response. The ensuing immune response stimulates downstream activation of other immune cells such as mast cells, releasing histamine and other mediators, causing physiological changes in the airway vessels and musculature. Thus, provoking a brisk inflammatory response that requires medical attention.

The etiology of asthma remains very complex, suggesting that a multitude of known and unknown co-existing risk factors participate in asthma pathogenesis. Within the past 20 years stress as a major risk factor of disease has emerged within the scientific community (Vitetta et al., 2005). Psychological stress can be defined as a perceived stress that influences an individual's ability to cope with events on a daily basis. There is emerging evidence to support stress responses as a major risk factor for asthma pathogenesis. Although the pathophysiology of asthma has been well characterized in humans and experimental models, little is known about its influence on stress (e.g. psychological) and the mechanism of interaction of stress and immune components. Symptoms of asthma triggered in allergic individuals (Mackenzie et al., 1886) suggest an important role for immune-neuroendocrine interactions in asthma. Depressed emotional status and increased levels of anxiety are commonly associated with asthma crises (Lawrence et al., 2002; Lehrer et al., 1993); on the other hand, the distress associated with a recurrent disease can be responsible for generating pathological anxiety in patients suffering from long-term asthma (Dahlen and Janson et al., 2002; Rietveld et al., 2000). In a clinical study the IL-4 cytokine level was detected higher in asthmatic children with higher study load, lower socioeconomic level showing a clear link between stress, asthma and Th2 response (Chen et al., 2006). As a result, investigators are suggesting that the relationships between the stress response and immune systems may hold the key in defining the risk of asthma.

As shown in fig. 1, it is clear that psychological stress impacts immune function and health. Clinical and experimental evidence indicates that the duration and course of

stress are pivotal factors that determine the nature of stress-induced immune changes and health related outcomes (Glaser and Kiecolt-Glaser et al., 2005; Segerstrom and Miller et al., 2004). Pyschoneuroimmunology is a growing discipline focused on understanding how stressors influence immune function and regulate disease. A stressor is any stimuli that cause a psychological or psychosomatic response in an individual, otherwise known as stress (Elliott and Eisdorfer, 1982). In response to adverse conditions of various origins (physical, psychological, environmental), the central nervous system (CNS) conveys signals in the form of neurotransmitters and neuropeptides to peripheral tissues as a means to neutralize or protect against potential harm. However, under certain circumstances, prolonged or repeated activation of the stress response system can facilitate mal-adaptation to the adverse event, resulting in inappropriate pathological responses. Exposure to stressful situations has been associated with health status (Schindler et al., 1985; Yang et al., 2006). In both experimental models of disease and in clinical settings, stress has been linked to various disease states including: coronary heart disease, colitis, asthma, rheumatoid arthritis, tumor malignancies and psoriasis (Gil et al., 2006; Fava et al., 2007; Davis et al., 2008; Andersen et al., 1994; Ader et al.,1993). Stressors are broadly known to induce the activation of the two major compartments of the CNS. In response to stressful conditions, the "stress response" is initiated with the immediate activation of the sympathetic nervous system, resulting in the release of the catecholamines, epinephrine and norepinephrine (Sternberg et al., 2006). Secondly, the hypothalamic-pituitary-adrenal axis (HPA) is activated resulting in the release of corticotropin releasing hormone (CRH) and downstream induction of the adrenal

glucocorticosteroids (McEwen et al., 2006). Together, with the activation of other neuroendcrine mediators (e.g. parasympathetic), they coordinate the early and later stages of the stress response in an attempt to regain physiological homeostasis.

Many acute and chronic disease states as mentioned above develop from a dysregulation in immune function mediated by an imbalance in inflammatory and anti-inflammatory responses. As researchers' knowledge of the bidirectional relationships between the immune system and CNS expanded, the realization of stress as a valid risk factor became an accepted idea. Belief that the central nervous system could facilitate immune responses originated from previous *in vitro* and *in vivo* studies demonstrating that neuroendocrine response factors of the central and peripheral nervous systems influences immune function including lymph node architecture, cellular proliferation, differentiation, cytolytic function and cytokine secretion (Dhabhar et al., 1996; Dhabhar et al., 1996; Calcagni et al., 2006; Bryndina et al., 2002). Direct influences of stress responses on immune cells have been shown to occur through the expression of specific receptors. For example, immune cells express adrenergic receptors (Callahan et al., 2002; del Rey et al., 2002; Kavelaars et al., 2002), and experimental evidence of altered disease susceptibility is produced by a disruption of sympathetic activity (Elenkov et al., 1995). An immune response generated in response to sympathetic activation is mediated through two adrenergic receptor types, alpha (alpha-1 and alpha-2 and beta-1, beta-2, beta-3). The adrenergic receptors are a class of G protein-coupled receptors that are targets of epinephrine and norepinephrine. Many cells possess these receptors, and the binding of an agonist will generally cause a sympathetic response (e.g. the fight-or-flight response).

In terms of immunomodulatory effects, stressful events are broadly characterized as being either immunosuppressive or immunoenhancing depending on the type, intensity regional compartment, genetic disposition and the specificity of stress response factors (Butcher et al., 2004; Kiank et al., 2006; Gross et al., 1981). There are two main categories of stress: acute (short term) and chronic (long term). Acute stressors may include unpleasant films, understimulation/work underload, overstimulation/work overload, unexpected or uncontrollable noise, prestige or status loss, electric shock, uncontrollable situations, physical illness, surgery, threats to self-esteem, and traumatic experiences. Chronic stressors may include sleep deprivation, daily "hassles", work overload or underload, role strains, or social isolation. There are, of course, many more things that can cause stress, but these are the stressors most commonly used in experimental research and most commonly seen in the general population (Elliott and Eisdorfer, 1982). While there is no way to predict conclusively how an individual will respond to different stressors, stress does cause some common changes. Emotionally, stress can lead to feelings of depression, anxiety, and anger (McEwen & Stellar, 1993). But even these seemingly simple reactions have high degrees of expressivity, and different individuals are affected in different ways.

The respiratory system is innervated by autonomic nervous system (i.e. Sympathetic and Para-Sympathetic nerves) which is part of central nervous system (brain). Bronchial constriction is a function of the sympathetic system and dilation is the function of parasympathetic system. Normal functioning of the pulmonary system requires a balance between sympathetic and parasympathetic systems. When a foreign

allergen attacks the respiratory system, the innervating nerves collectively signal and carry it to the brain for the information to be processed and the reaction to be propagated to peripheral tissues. Thus, if a dysregulation in this network occurs, it is conceivable that unwanted physiological changes may participate in the initiation and progression of an asthmatic event by direct influences on the respiratory airway or through mechanisms that modify the immune response to a given trigger.

My project "Investigating the Role of Stress in the Murine Model of Asthma" specifically focused on allergic atopic asthma, the most common type of asthma occurrence. The purpose of this project was to establish a murine model of stress controllability and demonstrate the impact of stress on the development of immune allergic airway hypersensitivity as a major feature of asthma. I hypothesized that given the ability to control the degree of stress would translate into less severe allergic airway hypersensitivity. We also hypothesized that distinct changes in immune responses generated in response to uncontrolled stress would reflect the extent of airway hypersensitivity, eosinophilic inflammation characterizing this condition.

Its underlying mechanisms are complex and may vary, but appear to be all together caused by an intrapulmonary allergen-driven T helper type 2 (Th2) response characterized by increased secretion of cytokines (IL-4, IL-5, IL-13, and others) by $CD4^+$ T lymphocytes (Sousa Mucida et al., 2003). In persuade of my hypothesis, I constructed two specific aims: 1) To determine the correlation between behavioral response and further downstream severity of pulmonary inflammation in a murine model of ovalbumin induced allergic inflammation. 2) To determine the link between changes in cellular

distribution and corresponding activation of immune cell responses causing allergic inflammation

In asthmatic patients, the allergic reaction progresses in two distinct phases representing segments of the phenomenon: an early response mediated by IgE and mast cells, and a late phase, during which Th2 lymphocytes, cytokines, and other cells build an inflammatory milieu that play a major role in the disease (Maddox and Schwartz et al.,2002). To date, the exact role that stress plays in altering the immune responses associated with asthma remain largely unknown. Physicians, scientists, and laymen have long believed that stress contributes in the exacerbation of asthma. However, it has only been in the past two decades that convincing scientific evidence has accumulated to substantiate this hypothesis followed by real life experiences.

CHAPTER II

EXPERIMENTAL DESIGN AND METHODS

Mice:

Adult (6-8 weeks of age) female Balbc/J mice (Jackson Laboratories, PA) were used in all studies. Mice were maintained under specific pathogen-free conditions under 12 hr light-dark cycle. Mice were housed 4 per cage under optimal temperature and humidity controlled conditions and provided proper care as directed by the institutional animal care and usage committee. All animals were allowed to acclimate to the surrounding environment prior to experiment.

Stress paradigm:

The stress paradigm involved the use of learned helplessness behavior apparatus as shown in fig. 3 (med-associates, Kentucky). Each unit consists on three compartments; each compartment consists of a box made up of plexiglass having a wheel connected to the computer which records the total number of wheel turns in each session for each mouse where one mouse. These recorded wheel turns are reflecting the performance of each mice exposed to shock using the instrument. The probes were attached to the hanging tail of the mouse through which the regulated current flows to elicit a shock. The total number of animals was divided in three groups. One is sham (no stress); controlled (ability to turn off shock); uncontrolled (inability to turn off shock). The sham (no shock) animals remained untouched during the stress paradigm. Stress was induced in mice by placing them in a sterile plexiglass box described above on alternate days starting from day 7 (5 days into the paradigm). Each animal was stressed (shocked) for

exactly 15 minutes on the day of the stress performed between the hours of 2.00 PM to 3.00 PM. As the type of stressor was the tail shock, a 0.2 mA current was provided over a random interval of time of 15 minutes. This flow of current was regulated by the software which also records the wheel turns as interpretation of behavioral activity during the shock treatment.

Allergen sensitization and challenge:

Female Balb/cJ mice received an intraperitoneal injection of 25 μg ovalbumin (Sigma-Aldrich, St. Louis, Missouri) along with aluminum hydroxide gel (13 mg/ml) (Sigma-Aldrich) diluted in the PBS (Mediatech Inc., Herndon, VA) on day 0 and 7. These mice received 10 μg ovalbumin in PBS on day 12, 13, 14 intranasally. For intranasal administration mice were anaesthetized using the in situ anesthesia chamber using isoflurane and oxygen cylinder (Accu Labs, Chicago, IL). On day 15, mice were sacrificed after anaesthetizing with xylazine & ketamine.

Blood Collection:

The animals were anesthetized using the ketamine & xylazine. Whole blood samples were collected through retro-orbital bleeding using sterile heparinized blood collecting tubes. Blood samples were allowed to coagulate for 10-20 min. prior to centrifugation followed by collection of serum. The serum was further aliquoted and stored in -80^0 C for further analysis.

Cell Isolation:

The animals were anesthetized using ketamine & xylazine. Specifically, lungs were perfused twice via tracheal instillation of total 2 ml of wash media using blunted syringe

needle. BALF containing cells was aspirated and the volume of the fluid retrieved was recorded and stored for further analysis.

Single cell suspensions from lung tissue were prepared as previously described (Jones et al., 2002). Briefly lungs were infused with sterile PBS to eliminate contaminating RBCs. Lung tissue was separated into single lobe and pooled together for each group finely minced and placed into digestion medium containing 300 units/ml collagenase type II (Worthington, Lakewood, NJ.) and 0.01% DNAse (Sigma-Aldrich, St. Louis, MO). Lung tissue was digested for 1 hr per pooled group. After digestion, lung supernatants were passed through a nylon mesh filter (LabPak, Depew, NY.) into sterile 15 ml conical tubes and washed 2X with RPMI (Hyclone, Logan, UT.) wash medium supplemented with 1% antibiotic cocktail. Lymphocytes were prepared by ficoll-hypaque (Lympholyte M, Cedarlane Laboratories ltd., Ontario, CA.) centrifugation. Contaminating RBCs were removed using ammonium chloride potassium (ACK) lysis buffer as described (Kruisbeek, 1999). Further the total viable cell count was performed using a haemocytometer (Hausser Scientific, Horsham, PA).

Radioimmunoassay:

The kit for corticosterone quantification was purchased from MP Biomedicals, LLC, Orangeburg, NY. All the tubes for the assay including sample as well as calibrators were set up. The reagents were brought to room temperature prior to use. The collected serum was diluted with steroid diluent. 0.1 ml of this diluted serum collected as mentioned above and corticosterone calibrators were added to the respective tubes. Then 0.2 ml corticosterone – ^{125}I (tracer). Then 0.2 ml anti-corticosterone was added to all tubes

except blank. Then all assay tubes were vortex mixed and incubated at room temperature for two hours. After incubation 0.5 ml precipitant solution was added to all tubes and vortexed thoroughly. All assay tubes were centrifuged for 15 minutes. The supernatant was decanted. The precipitate was counted on the gamma counter. The blank readings were subtracted from all sample readings and graph was plotted.

Histopathology and Immunohistochemistry:

Mice were sacrificed and the dissected lungs were perfused with instillation of 2 ml of 2% paraformaldehyde (Sigma Chemical, Co., St. Louis, MO) intratracheally and then the whole lung was collected and fixed in 2% paraformaldehyde at 4^0C for 4 hours following overnight incubation in 30% solution of sucrose at 4^0C. The next day each individual lobe of the lung were frozen using the OCT (Optimum Cutting Temperature) embedding medium (Sakkura Finetechnical, Torance, CA). 10 μm thin tissue sections were prepared on SuperFrost/plus microscope slides (Fisher Scientific) using Ultrapro 5000 cryostat (Vibratome, St. Louis, MO). The tissue sections were stained with Hematoxylin and Eosin (H&E) (FisherBrand). The tissues were stained Periodic Acid Schiff's Base Staining (PAS) for further confirmation. Immunohistochemical Staining (IHC) analysis was performed using Image Pro X software (Olympus, Centervalley, PA). The Images were captured on Olympus AX70 fluorescent microscope (Olympus, Centervalley, PA).

ELISA:

Enzyme-Linked Immunosorbent Assays (ELISAs) were performed using OptEIA sets for mouse interferon-γ (IFN- γ), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-10 (IL-10) from BD Biosciences (Franklin Lakes, NJ) and interleukin-13 (IL-13) from eBioscience (San Diego, CA).

Briefly flat bottom 96 micro-well plates were coated with 100 µl (per well) capture antibody (OptEIA anti-mouse monoclonal antibodies for different cytokines IL-4, IL-5, IL-10, IL-13, IFN-γ diluted 1:250 in coating buffer (0.1M carbonate or 0.1M phosphate). The plate was sealed with a plastic wrap and incubated overnight at 4^0C. The plate was then washed 5 times with ≥ 200 µl/well wash buffer (PBS with 0.05% Tween-20). After the last wash the plate was inverted and blotted on a paper towel to remove residual buffer. This wash procedure was used throughout the rest of the protocol unless otherwise noted. After washing, the plate was blocked with ≥ 200 µl/well assay diluent (PBS with 10% FBS) and incubated overnight at 4^0C. The plate was washed, and then samples (with suitable dilutions) and standards (OptEIA serially diluted recombinant mouse IL-4, IL-5, IL-10, IL-13, IFN-γ) were pipetted into the appropriate wells of the plate (100 µl/well of sample and standard). The plate was sealed and incubated overnight at 4^0C. After wash procedure, 100 µl/well of detection antibody (OptEIA biotinylated anti-mouse IL-4, IL-5, IL-10, IL-13, IFN-γ monoclonal antibody) diluted 1:250 in assay diluent were added, and the plate was sealed and incubated for an hour at RT (room temperature). After washing, 100 µl/well enzyme reagent (OptEIA avidin-horseradish peroxide conjugate) diluted 1:250 in assay diluent was added, and the plate was sealed and incubated for 30 minutes

at room temperature. For the final plate wash, plates were rinsed with wash buffer 7 times, letting the buffer sit in the wells for ≥ 30 seconds per rinse. 100 µl/well TMB (Tetramethylbenzidine, BD Pharmingen) were added and the plate was incubated for approximately 20 minutes (normally) unsealed at RT in dark. 50 µl/well stop solution (0.25 M HCl) was added to halt the reaction and the absorbance (at 450 nm) of the plate was read on an ELISA plate reader using the computer program Gen5 (Bio-Tek Instruments).

Flow Cytometry:

Three color flow cytometric staining was performed using the following flourochromes. The antibodies that were used included FITC (Fluroscein isothiocyanate), R-PE (R-phycoerythrin) and cy-chrome. 1 X 10^6 lymphocytes from lung single cell suspensions were washed with washing buffer (2% FBS in 1X PBS with 2mM EDTA) and incubated with an optimal concentration of anti-FcBlock Antibody (clone 2.4G2) (BD Pharmingen, SanDiego, CA) to block non-specific binding to FcRs for 30 min. multicoloured staining was performed by incubating cells at 4^0C with compatible combinations of pre-tittered concentrations of following antibodies: anti-GR1$^+$FITC, anti-CD8$^+$FITC, anti-CD3$^+$PE, anti-CD4$^+$PE-Cy7, anti-B220$^+$FITC, anti-MHC II$^+$PE, anti-CD19$^+$PE-Cy5, anti-CD11c$^+$Alexa Flour 488, anti-F/480$^+$PEcy7. Unstained lymphocytes served as negative controls. Antibodies were bought from BD Pharmingen. Gating of the lymphocytes was identified by forward-scatter/ side-scatter profile. Data acquisition was done on FC500 flow cytometer (Beckman Coulter, Miami, FL). Further analysis was done on CXP software (Beckman Coulter).

Statistical Analysis:

Statistical analysis was performed using GraphPad prism Version 4.0 for Windows (GraphPad Software, San Diego, USA). For multi-experimental group analysis, data were subjected to analysis of variance (univariant ANOVA) followed Post Hoc tests (Tukey) for group differences. For analysis of two-group differences, student's t test was employed. All data are expressed as the mean ± SEMs. The two tailed level of significance was set up at $p \leq 0.05$.

CHAPTER III

RESULTS

A. Behavioral and physiological response to stress using the learned helplessness paradigm:

I. Behavioral adaptations according to stress controllability:

The response to adverse stimulus is a valid measurement of behavioral disposition. Wheel turn frequency was measured as an indication of altered behavioral response to electric shock stimulus. A similar wheel turn response to electrical shock was initially observed among mice subjected to either controllable or uncontrollable stress during day one of stress exposure. However, significantly ($p \leq 0.05$) higher wheel turns were observed between PBS (non-asthmatic) and OVA (asthmatic) challenged mice having the capacity to extinguish the stress stimulus as compared to mice lacking control throughout the execution of the stress protocol. An additional marked increase ($p \leq 0.05$) in wheel turn frequency was observed among PBS challenged mice subjected to the controllable stress event as compared to the OVA challenged mice (Figure 5).

II. Stress exposure elevates blood corticosterone:

It is well documented that alterations in corticosterone (CORT) levels in mice is associated with stress exposure. CORT concentrations within the serum of experimental groups were measured upon the completion of the stress paradigm. A significant ($p \leq 0.05$) elevation in serum CORT was elevated with stress exposure in PBS and OVA

challenged mice as compared to non-stressed mice. Mice exposed to uncontrollable stress demonstrated the highest level of CORT in serum (Figure 6).

III. Blunted weight gain as a marker for mal-adaptive physiological and behavioral response to stress:

The effect of stress exposure on weight changes was monitored between PBS (non-asthmatic) and OVA (asthmatic) challenged mice exposed to controllable and uncontrollable stress as compared with non-stressed (sham) counterparts. For each subject body weight measurements were taken on each day of stress exposure. OVA (asthmatic) challenged mice exposed to uncontrollable stress experienced a significant ($p \leq 0.05$) decline in weight gain as compared to mice capable of extinguishing stress. OVA (asthmatic) alone also resulted in a marked ($p \leq 0.05$) decline in weight as compared to their PBS (non-asthmatic) counterpart (Figure 7).

B. Qualitative and quantitative changes in the lungs of OVA-challenged mice according to stress severity:

I. Hematoxylin and eosin (H&E) and Periodic acid-schiff base (PAS) staining:

To validate the stress paradigm to have an effect on pulmonary changes to OVA challenge, Histopathological changes in the lungs were compared between non-stressed (sham), controllable and uncontrollable stressed mice given OVA airway challenge using H&E and PAS staining techniques. Maximal severity of cellular infiltrates around bronchioles and within alveolar spaces was observed in OVA challenged (asthmatic) mice exposed to uncontrollable stress as compared to controllable and non-stressed

(sham) counterparts. No distinguishable differences were observed between controllable and non-stressed (sham) OVA-challenged (asthmatic) mice (Figure 8 & 9). An additional characteristic of allergic airway disease is airway obstruction produced by goblet cell hyperplasia and mucus secretion within and surrounding the respiratory airways. PAS staining of mucus containing goblet cells, indicated by intense pink coloration was observed with greatest intensity within the bronchioles of OVA challenged (asthmatic) mice exposed to uncontrollable stress as compared to controllable and non-stressed (sham) counterparts. As controls, PBS sensitized mice challenged with OVA did not demonstrate histological changes along the airways (data not shown).

II. Quantitative increase in total lung mononuclear cells in the lungs of OVA-challenged mice exposed to stress:

PBS-challenged mice exposed to uncontrollable stress demonstrated a significant ($p \leq 0.05$) increase in total lung leukocytes. Whereas exposure of PBS-challenged mice subjected to controllable and uncontrollable stress led to a concomitant decrease ($p \leq 0.05$) in total LRN leukocytes. OVA alone led to significant ($p \leq 0.05$) increases in the total number of lung & LRNs leukocytes. Uncontrollable stress led to a significant ($p \leq 0.05$) increase in total lung leukocytes among OVA-challenged mice as compared to non-stressed counterparts. In contrast, uncontrollable stress led to a significant decrease in total LRNs of OVA-challenged mice as compared to OVA-challenged sham and control counterparts. These results indicate regional differences in cellular responsiveness to stress and OVA challenge (asthma) (Figure 10). Mice challenged with PBS demonstrate

no difference in lung histology as compared to PBS sensitized mice challenged with OVA (data not shown).

C. Stress causes a preference in the number and type of respiratory immune cells among OVA-challenged mice:

The respiratory tract is comprised of a heterogeneous population of immune cells. We assessed whether differences in the distribution of immune cells along the respiratory tissue would be observed in response to stress conditions. Total immune cell numbers and phenotypic examination of leukocytes isolated from lung interstitial tissues and lower respiratory draining lymph nodes (LRNs) was determined between non-stressed, controllable and uncontrollable OVA-challenged (asthmatic) mice as compared to PBS-challenged (non-asthmatic) counterparts. Cell-specific phenotyping was performed using antibodies characteristic of specific cell surface molecules that were labeled with fluorochromes to be analyzed by flow cytometery techniques. The results stated here reflect the major population found in the lung and draining lymph node tissues.

I. Determination of neutrophils and eosinophils cell numbers in lung:

OVA challenge led to a significant ($p \leq 0.05$) increase in both neutrophils and eosinophils. Stress exposure alone did not have an effect on the number of eosinophils population. In contrast, uncontrollable stress led to a significant ($p \leq 0.05$) increase in population of neutrophils. A further significant increase ($p \leq 0.05$) in eosinophils was only found given exposure to uncontrollable stress and OVA challenge as compared to the controllable stressed and non-stressed (sham) counterparts. In contrast, a sequential

significant ($p \leq 0.05$) increase in neutrophils was found given controllable and uncontrollable stress as compared to non-stressed (sham) counterparts. Thus, stress exposure had the greatest effect on the neutrophil subpopulations (Figure 11 & 12).

II. Determination of antigen presenting cell numbers (APCs) in lung and LRNs:

I determined the distribution of dendritic cells (DC) and macrophages in the lungs and LRNs of mice exposed to OVA alone or under conditions of controllable and uncontrollable stress as compared to their PBS counterparts. Distinct populations were characterized as $CD11c^{+}F4/80^{-}$ (DCs), $CD11c^{-}F4/80^{+}$ (Macrophages) and a third population expressing both $CD11c^{+}$ and $F4/80^{+}$ surface markers. Macrophages (cell number) were the major population found in the lungs of naïve mice followed by the double stained population (cell number) and DCs (cell number).

In lungs, OVA challenge alone resulted in a significant ($p \leq 0.05$) increase in the total number of DCs (Fig. 15). An increase in double positive APCs was apparent. In contrast, no significant changes in cell numbers were found among the macrophage population.

The distribution of DCs, double positive APCs and macrophages was also demonstrated in the LRNs in response to stress and OVA challenge. Resident APCs comprised mainly of DC and the double positive cell population. OVA challenge alone led to significant ($p \leq 0.05$) increase in total APC subtypes with the greatest increase found among the double positive population followed by DC and macrophages respectively. In contrast to the lung, controllable and uncontrollable stress led to a significant ($p \leq 0.05$) concomitant decrease in the number of double positive and DC

populations with exception to macrophages which showed an equal decrease in cell number given both controllable and uncontrollable stress (Figure 13A, 14A, 15A, 16A, 17A, 18A).

III. Determination of $CD4^+$ T helper, $CD8^+$ cytotoxic and B lymphocyte numbers in lung and LRNs:

In lungs and LRNs $CD4^+$ T lymphocytes were the major population followed by B lymphocytes and $CD8^+$ T lymphocytes. In lungs and LRNs OVA exposure resulted in a significant ($p \leq 0.001$) increase in $CD4^+$ T lymphocytes and B lymphocytes. In lungs a sequential increase in $CD4^+$ T cells was observed among OVA-challenged mice exposed to controllable and uncontrollable stress in contrast to LRNs. However, only uncontrollable stress led to a significant ($p \leq 0.05$) increase in B lymphocytes in lungs. In contrast, B lymphocytes were significantly ($p \leq 0.05$) decreased in LRNs in response to uncontrollable stress (Figure 19, 20 & 21).

C. Characterization of MHC II^+ expression on antigen presenting cells by Mean Fluorescence Intensity (MFI):

We demonstrated the effect of stress on the maturation of APCs by determining the number and intensity of MHC II^+ cell surface expression by macrophages, DC and double positive cell populations within lung and LRNs. In the lungs, mice subjected to OVA alone led to a significant ($p \leq 0.05$) increase in the MFI of MHC II^+ macrophages and DCs. An equivalent MFI of MHC II^+ double positive cells were present between PBS and OVA-challenged mice. A similar pattern was observed among mice subjected to controllable stress and uncontrollable stress. In total $CD11c^+$

MHC II^+ cells were preferentially increased in the lungs that were exacerbated given exposure to uncontrollable stress. A direct relationship between the increase or decrease in MHC II^+ cell numbers and MHC II mean fluorescent intensity (MFI) was observed with DCs having the highest MFI (Figure 13B, 15B, 17B).

APC MHC II^+ MFI was also examined in LRNs. MHC II^+ expressing cells were the major phenotype of macrophages, DCs and double positive cells among naïve mice. OVA challenge alone lead to significant expansion of MHC II^+ macrophages, DCs and double positive cells with the greatest increase among DCs. Introduction of controllable and uncontrollable stress resulted in a concomitant significant ($p \leq 0.05$) decrease in MHC II^+ cells. There was no significant difference among MFI of the MHC II^+ cells in LRNs (Figure 14B, 16B, 18B).

D. Uncontrollable stress produces preferences in pulmonary cytokine responses and cellular distribution a factor contributing for disease severity:

Effect of stress and OVA (asthmatic condition) on cytokine content in the bronchiolar lavage environment:

Preferences in Th2-associated cytokine secretion are known to correspond with increased allergic airway hypersensitivity. I determined the changes in Th2 associated cytokines in bronchiolar lavage fluid (BALF) given stress exposure and OVA challenge. IL-4, IL-5, IL-10, IL-13 and IFN-γ cytokines were measured within BALF. All cytokines tested were below detection level in non-allergic (non-asthmatic) non-stressed groups. OVA-challenge (asthma) alone led to significant ($p \leq 0.05$) increases in IL-4, IL-5, IL-10 and IL-13. Stress alone led to significant ($p \leq 0.05$) increases in IL-10 and IL-13. A

sequential significant ($p \leq 0.05$) increase in IL-4, IL-5, IL-10 and IL-13 was observed between OVA-challenged (asthmatic) mice exposed to controllable and uncontrollable stress as compared to non-stressed groups (Figure 22). IFN-γ was not detected in all experimental groups.

CHAPTER IV

DISCUSSION

Asthma remains a major health problem in the United States. For many years, stress has been believed to play a role in the onset and progression of asthma, but the underlying mechanisms remain unclear. As an inflammatory disease, stress-induced alterations in immune function may provide a link between stress and asthma pathogenesis. The purpose of this project was to establish a murine model of stress controllability and demonstrate the impact of stress on the development of immune allergic airway hypersensitivity as a major feature of asthma. I hypothesized that given the ability to control the degree of stress would translate into less severe allergic airway hypersensitivity. We also hypothesized that distinct changes in immune responses generated in response to uncontrolled stress would reflect the extent of airway hypersensitivity.

Animal models of stress are commonly used to investigate physiological function related to disease (Joachim et al., 2008). The fear conditioning model of learned helplessness provides the ability to study the effects of controllable verses uncontrollable stress conditions on physiological function. To date, no study exists which implements this model of stress to study the effects of immune regulation of asthma pathogenesis. Thus, studies were performed to establish a model of stress controllability and allergic airway hypersensitivity in mice.

Stress induced alterations in central nervous system functioning is known to affect peripheral physiological homeostasis (Gavillet et al., 2008) and facilitate disease susceptibility. Most notable are the known effects of altered CNS function on behavioral dispositions such as temperament, physical activity and appetite (Levin et al., 2007). In establishing a murine model of stress and allergic lung hypersensitivity (ALH), we proposed that implementing the ability of stress controllability would reveal distinct differences in behavioral and physiological measures and hence correlate with the extent of ALH.

Weight fluctuation is a well-known characteristic of health status that can be provoked by stress as well as acute and chronic illness, commonly referred to as chachexia (Veenema et al., 2008). Neuronal signal pathways of the CNS control this physiological response (Agnati et al., 2006). For example, stress response factors released by distinct centers in the CNS such as corticotropin releasing hormone (CRH) by the hyperpituitary axis (HPA) convey signals to the peripheral nervous system, eventually translating into specific physiological function. Among such pathways, CRH-induced corticosterone production has been reported to play a major role in regulation of behavioral and biological functions (Romeo et al., 2007; Pechnick et al., 2006). Disruption of CNS control due to psychological aberrations can have pronounced effects on the maintenance of weight and other physiological functions. Weight gain was suppressed among mice exposed to airway allergen challenge, typifying a chachexic phenotype. Importantly, my results demonstrated that by elevating the degree of stress through implementing a lack of control, weight gain was profoundly diminished among

mice exposed to allergen challenge. Moreover, these observations corresponded with elevations in serum corticosterone levels with significantly higher concentrations found in the serum of mice exposed to stress. Thus, the involvement of allergic pulmonary challenge and stress acted in synergy via the coordination between CNS and peripheral physiological function.

The coordinate elevation in corticosterone and stress-induced chachexia, not only translates into physiological abnormalities such as weight homeostasis, but also causes mal-adaptive behaviors (Pechnick et al., 2006). Previous studies have documented corticosterone as a tool to distinguish neurological responses (Pechnick et al., 2006). To assess behavioral disposition, many studies (Wright et al., 2006) employ specific behavioral tests such as water maze test for studying cognitive functions as well as the investigation of active avoidance learning through the use of the Shuttle-Box apparatus (Buselmaier et al., 1981). In my studies, I determined behavioral temperament by recording the number of wheels turns by mice subjected to the stress alone or in combination with OVA airway challenge. In this respect, I documented a direct association between behavioral disposition at the time of stress and alterations in weight gain among mice exposed to stress as well as mice exposed to allergen challenge accompanying stress. Specifically, by demonstrating that diminished weight gain corresponded with decreased wheel turn frequency among allergen-challenged mice given either controllable or uncontrollable stress, substantiates the link between stress, behavioral and physical status.

Based upon the above observations, I hypothesized that controllability of stress would translate into distinct characteristics of inflammatory responses generated by allergen airway challenge. From previous studies (Zhang-Hoover et al., 2005), it is clear that allergies are associated with the infiltration of inflammatory cells along the respiratory airways. Thus, given my hypothesis, I examined whether histopathological changes would ensue and manifest in response to uncontrollable stress and would be blunted give the ability to escape from the stressor. Indeed, my results demonstrated that cellular infiltration along the airways was increased with uncontrollable stress as exemplified by Hematoxylin/Eosin and Periodic acid Schiff-base (PAS) staining of lung tissue as compared to controllable stress. These results are in parallel with previous clinical findings suggesting that quality of stress can predict asthma severity and therefore serves as a formidable tool for more in-depth study.

The relationship among psychological stress, neuroendocrine activity, and immune function has been well documented in numerous clinical studies and a variety of experimental models with implications for human health (Ader et al. 2001; Glaser and Kiecolt-Glaser et al., 2005). As an inflammatory disease immune responses play a major role in disease pathogenesis. Specifically, allergic airway hypersensitivity produced by a predominant Th2 cellular response is believed to have immunopathological consequences. Yet, there is increasing evidence that Th2 mediation is not the sole regulator of asthma pathogenesis. In fact, a more rational idea is that although Th2 responses favor disease progression, it is likely that an imbalance in all immune responses cumulatively determine disease severity. Importantly, studies have

demonstrated that stress-induced neuroendocrine responses can influence immune function among asthmatics (Joachim et al., 2008), primarily demonstrating the effects of stress on Th2 cellular function. However, more studies are needed to define the underlying mechanisms mediating Th2-predominant responses. Moreover, further correlation of stress and other cellular functions in response to asthma is needed to fully understand the role of stress and disease pathogenesis.

A definition of the cellular responses occurring over the airways that respond to allergenic challenge given stress is likely to provide important insight in understanding the mechanisms favoring the preferential Th2 response. Thus, studies were performed to evaluate the distribution of various cellular phenotypes along respiratory tissues (lung and draining lower respiratory lymph nodes).

Antigen presenting cells (APCs) are a vital part of the innate immune system, involved in capturing antigen and presenting it to T cells. DCs are the major antigen presenting cells. Antigen presentation of potential allergens is essential for the activation of Th2 responses. Thus, studies were conducted to evaluate the responsiveness by pulmonary APCs. Previous studies have demonstrated the diversity of APC subsets having distinct functional roles in protection and pathogenesis of pulmonary disease (Zhang-Hoover et al., 2005). A particular interest in my studies was to define the relationship between preferences in APC distribution patterns and the observed Th2 dominant response facilitated by exposure to stress. Thus, I examined the phenotype characteristics of pulmonary DC and macrophages in the lung and LRNs that responded to allergen challenge in response to controllable and uncontrollable stress conditions.

DCs and macrophage cell-types are traditionally considered the major APC populations involved in regulation of T cell effector function DCs and macrophages have been traditionally defined as having the $CD11c^+$ (DCs) and $F4/80^+$ (macrophages) surface molecules respectively. The total cell number of DC and macrophages examined in this study were defined based expression of $CD11c^+$, $F4/80^+$ and MHC II^+.

In the resident lung, immature macrophages were the predominant cell population as compared to DC cells and the $CD11c^+$ $F4/80^+$ population. Exposure of mice to uncontrollable stress alone led to a corresponding increase of all immature cell phenotypes (lacking MHC II+ expression). As expected allergen exposure alone led to a preferential increase in the absolute number of MHC II^+ phenotypes typical of antigen-driven APC maturation. Interestingly, allergen challenge and only uncontrollable stress caused a further significant increase in mature DCs and $CD11c^+$ $F4/80^+$ cells, but not macrophages. Whether or not the absolute number of MHC II^+ cells within each respective population would correlate with the propensity of MHC II surface intensity was also evaluated. Accordingly, DCs and $CD11c^+$ $F4/80^+$ demonstrated the greatest MHC II surface intensity, reflective of a higher activation status respectively in response to uncontrollable stress as compared to macrophages. Thus, based on these findings, I propose that a preference in MHC II^+ expression on $CD11c^+$ cells in response to stress is likely to increase the efficiency of APC-T cell interaction and hence be responsible in orchestrating the observed Th2 cellular response associated with ALH under stress conditions. Importantly, we observed that among mice capable of controlling their stress

exposure, dampening of ALH was directly associated with a blunting of DC MHC II^+ exposure in lung.

The major lymphocytes of the adaptive immune compartment are traditionally $CD4^+$, $CD8^+$ and B lymphocytes. As Th2 cells are a subset of $CD4^+$ T cells, I expected to observe a preferential increase in this population along the respiratory tissues. Indeed, $CD4^+$ T cells were the predominant lymphocytes among $CD8^+$ and B lymphocytes within the lung parenchyma among subjects challenged with and without allergen challenge. In addition, mice subjected to allergen challenge under uncontrolled stress conditions demonstrated a significant increase in $CD4^+$ T cells as compared to mice which could control their stress experience followed by a further decrease in the number of $CD4^+$ T cells among non-stressed mice exposed to allergen. It is also worth noting that the effect of stress under the non allergic conditions demonstrated an increase in $CD4^+$ T cells. These findings suggest that stress independent of allergen exposure causes a preferential expansion of $CD4^+$ T cells, presumably having a Th2 phenotype. B cells only showed a significant increase in their population in response to allergen that was increased in response to stress. IgE antibody levels in blood secreted by differentiated plasma cells play a major role in acute allergic responses through IL-4 and IL-13-mediated B cell maturation and immunoglobulin class switching. In particular, in previous studies under sensitized conditions in allergic asthma, IgE levels are found to be elevated. Crosslinking IgE help in the capture of antigen by binding with the Fc receptor attached to mast cells and eosinophils. It causes the degranulation and release of histamine and cationic proteins

leading to constriction of bronchial muscles induced by stress exposure. Therefore, B cells are a proof of augmented immune ALH responses.

In addition to examining APC and lymphocyte distribution patterns, we also observed changes in neutrophils and eosinophils. These populations are also involved in inflammatory processes through the production and release of oxidative species that can initiate acute and chronic tissue damage. Our data demonstrates that uncontrollable stress leads to a dramatic increase in both cell populations and point to additional targets for future studies investigation the direct and indirect neuroendocrine-mediated pathways affecting their function.

Similarly, we also considered the effects of stress on the distribution of major lymphocytes within the draining lymph nodes (LRN) of the respiratory airways. Structurally, the LRN is an important site for the induction B and T cell responses. Distinct differences in the distribution and response between DC, macrophages and $CD11c^+$ $F4/80^+$ cells were found in response to stress within LRN as compared to lung. Mainly, $CD11c^+$ $F4/80^+$ MHC II^+ cells predominated in number within the LRN in response to stress. Whereas macrophages were not significantly influenced by stress within the lungs, their response within the LRN was significantly altered given uncontrollable stress. Based upon these findings, preferences in the response of APCs to stress depend on location and cell phenotype. The link between these two important compartments is that the migration of cells is taking place from LRNs towards lungs at the faster rate with increase in level of stress in allergic as well as non-allergic groups. A possible mechanism for the observed increase in migration rate may be due to increased

secretion of CCL21 a chemokine that is responsible for migration of APCs and T cells (Förster et al., 2008). Another reason of decrease in cells in LRNs can be failure of recruitment of immature cells or suitable precursor cells affected by stress. Based upon these findings, it is clear that stress exposure elicits a complex milieu of immune responses involving distinct cell types residing and mobilizing between various lymphoid and peripheral tissues.

Interestingly, my results demonstrated that $CD4^+$ T and B cells decreased with increasing stress intensity. In contrast, $CD8^+$ T cells were not influenced by stress. One potential explanation for such findings could be that stress may alter cellular recruitment favoring peripheral cellular retention or local proliferation, leading to deleterious pulmonary inflammatory responses. In this regard, previous studies have demonstrated a dysregulation in chemokine activity a local generation of lymph tissue in response to stress exposure (Sugama et al., 2006). Thus, understanding the dynamic influences of neuroendocrine activation and cellular trafficking would provide valuable information developing potential strategies in controlling cellular inflammatory processes. Both neutrophils and eosinophils were not found in LRNs.

To confirm that preferences in CD4+ T cells responses in the lungs of mice exposed to uncontrollable stress would be associated with pronounced Th2 responses, we demonstrated the type of cytokines detected in the BALF of similarly treated mice. The results from my murine model of ALH demonstrated that Th2 polarized changes are augmented with the subject's inability to escape stress as compared to subjects which could extinguish the stress stimulus as well as those not subjected to the stressor. This

was evident by significant increases in IL-4, IL-5, IL-10, IL-13 and the lack of IFN-γ cytokine detected within the bronchiole lavage fluids among allergen-challenged mice subjected to uncontrolled stress as compared to controllable and non-stressed mice. These results are supported by, numerous studies demonstrating that stress and altered glucocorticoid, catecholamines, corticosterones hormones induce a shift from Th1 to Th2 immune response (Daynes et al., 1989; Stanulis et al., 1997; Elenkov et al., 1999). Briefly introduced above, these hormones are secreted as a result of activation of both sympathetic nervous system (SNS) and HPA axis. My findings are in agreement with these documented facts. The corticosterone levels in allergic and non-allergic animals are equivalent but vary between controllable, uncontrollable and non stressed mice. This ultimately implicated differences in corticosterone levels as an indication of the degree of stress and a potential modulator of the observed Th2-dominant responses to allergen-induced ALH. Based upon these findings, I concluded that the increase in corticosterone peripherally favored a Th2 phenotype. However, one must consider the potential influence of other T cell dependent and independent effector functions not examined in this study to be involved in stress-induced alterations in ALH. Furthermore, it should also be realized that other neuroendocrine factors are likely to participate in the regulation of asthma pathogenesis in the midst of stress. Thus, further investigation is needed to fully understand the relationships regulating immune effector function associated with asthma pathogenesis.

To summarize, this study established a mouse model for stress controllability providing an investigation of the immunological consequences of stress controllability

and exacerbation of ALH. In addressing my hypothesis, we demonstrated the following: a) distinct physiological and behavioral responses were produced in response to controllable and uncontrollable stress corresponding with ALH, b) the type of stress led to differences in lung lesion severity c) APC activation was supportive of the preferential increase in $CD4^+$ T cells responses and d) the preference of stress-induced Th2 cellular cytokine responses produced along respiratory airways corresponded with the severity of stress. We believe these findings pave the way to define the specific pathways that direct neuroendocrine activation by immune cells of a certain phenotype. We anticipate that in defining such interactions, will provide experimental evidence that stress is a valid risk factor and lead to the development of novel approaches using neuro-immune-based therapies to fight asthma.

CHAPTER V

FIGURES

Fig. 1

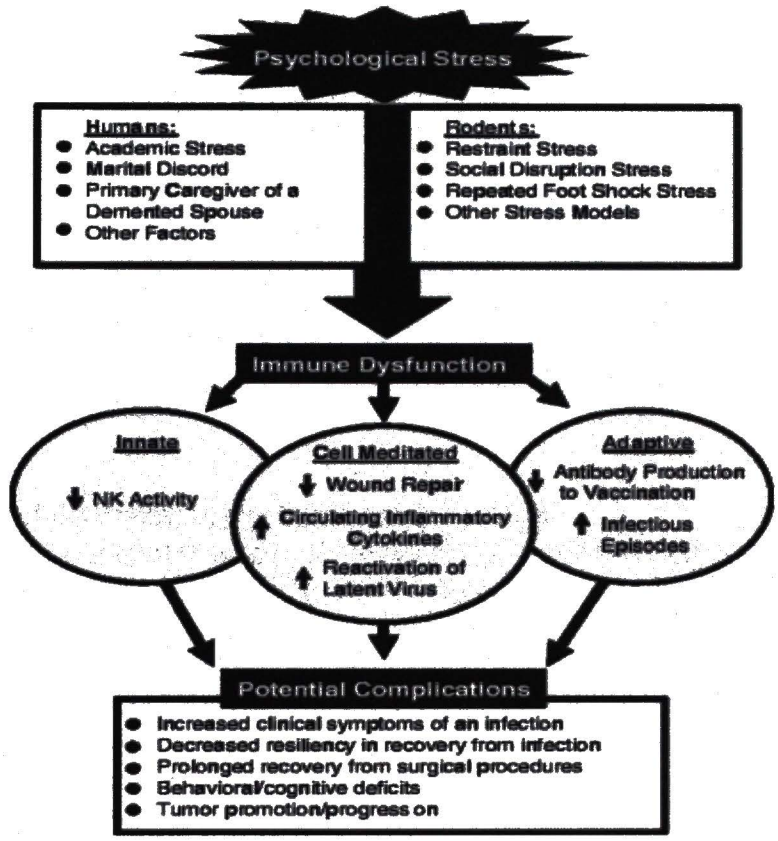


Fig. 1: Stress in day to day life
The above model depicts the major impact on immune system due to stress.
Reference: Sagerstrom et al., 2006

Fig. 2

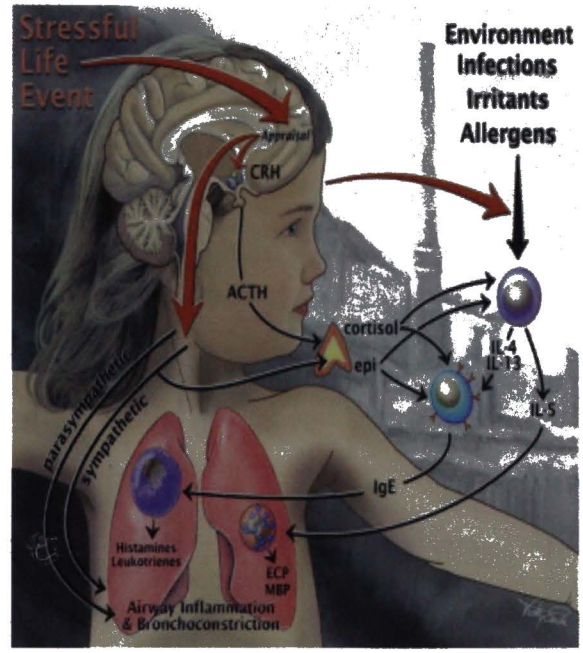
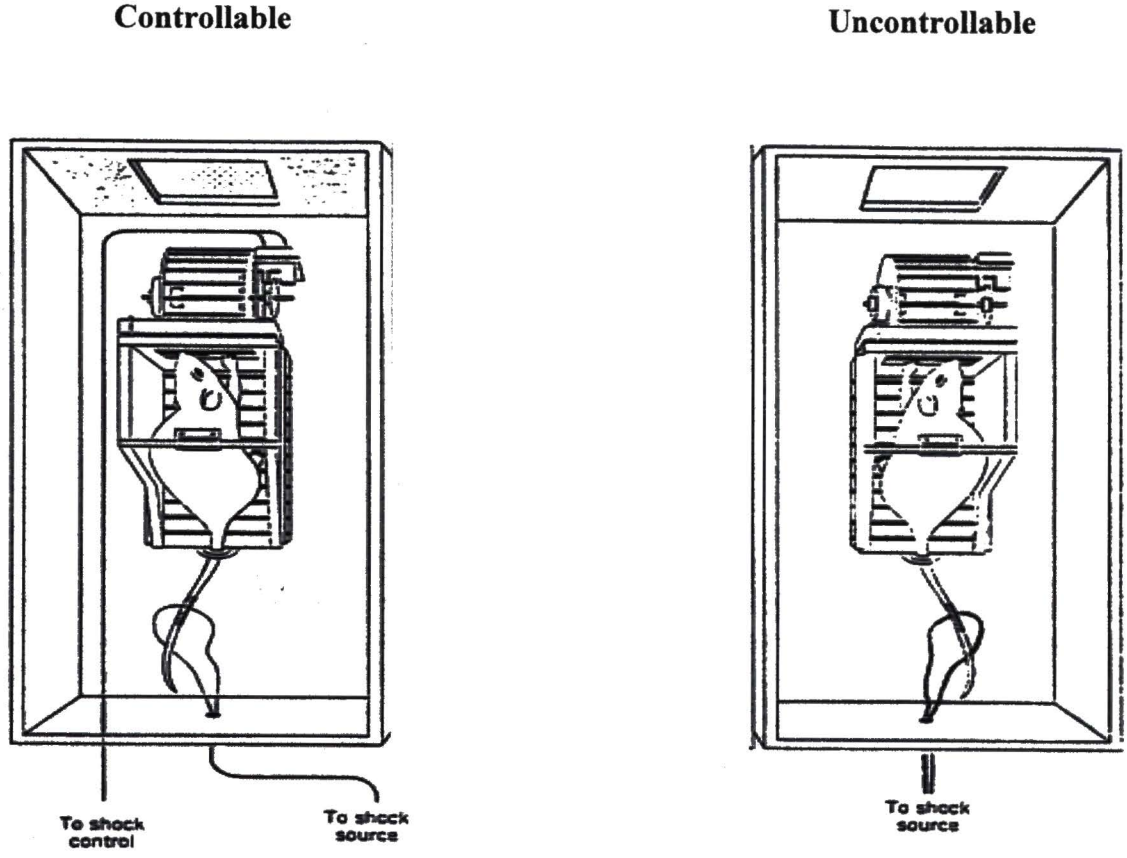


Figure 2: Model depicting brain-immune interaction
Model depicting the interaction of psychological stress with environmental triggers in influencing asthma exacerbations. The basic premise of the model is that stress operates by altering the magnitude of the airway inflammatory response that irritants, allergens, and infections bring about in persons with asthma. The figure provides an overview of the relevant biological pathways to airway inflammation and bronchoconstriction, including the hypothalamic-pituitary-adrenal (HPA) axis, the sympathetic-adrenal-medullary (SAM) axis, and the sympathetic (SNS) and parasympathetic (PNS) arms of the autonomic nervous system.
Reference: Chen et al., 2007

Fig. 3



Reference: Weiss JM et.al 2000

Figure 3: Stress Apparatus
Above shown is one unit of the Tridiac Shock Instrument which consists of the two compartments as shown. One is controllable which allows mice to control the tail shock, and the other compartment is uncontrollable which doesn't allow mice to control the tail shock.
Reference: Weiss JM et al., 2000

Fig. 4

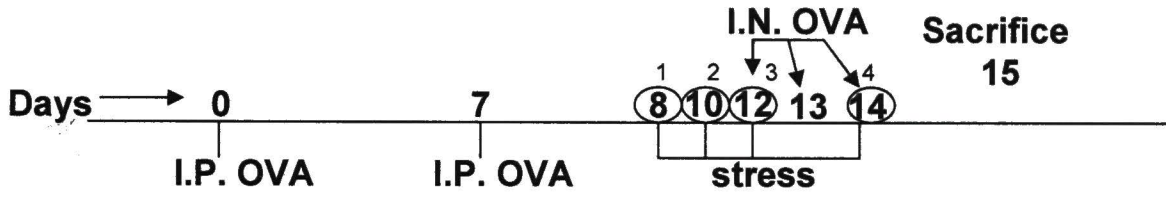


Figure 4: Stress paradigm and allergen sensitization & challenge
In all the experiments the above shown protocol is used. On day 0 all the animals were sensitized with ovalbumin (OVA) and adjuvant aluminum hydroxide. On day 7 all these animals were once again sensitized. On day 8, 10, 12 and 14, the animals were stressed in the above shown shock instrument. On day 12, 13 & 14 randomly selected animal groups were challenged with OVA and sham treatment group was challenged with PBS intranasal from each stress group. On day 15 mice were sacrificed and further tissues were collected and processed.

Fig. 5

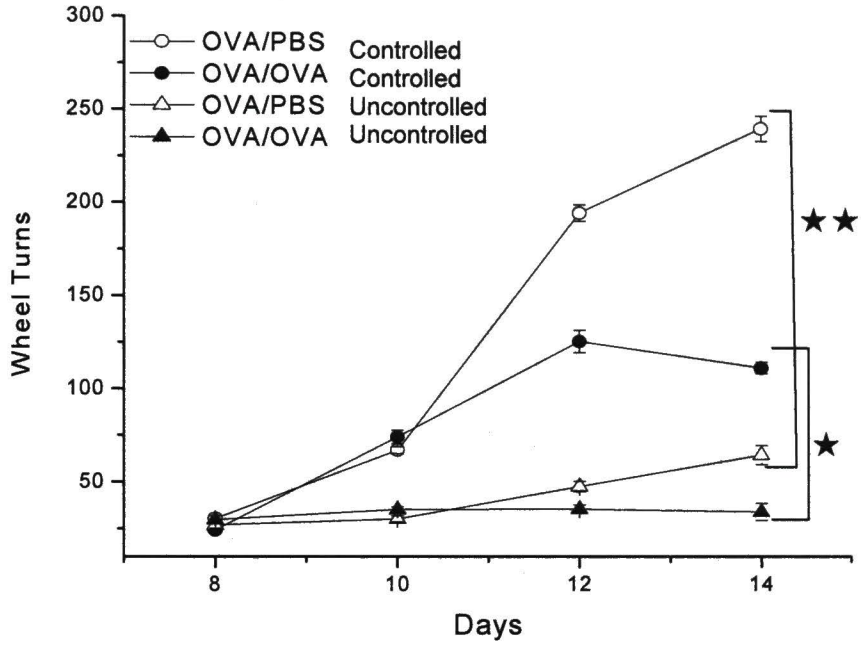


Figure 5: Kinetics of Wheel Turns

The total number of wheel turns during each session of the shock in the given paradigm was recorded for each mouse per day and then plotted as a function of stress days during stress paradigm. In this figure each data point indicates N=10 obtained from three independent experiments.

(★$P \leq 0.05$, ★★$P \leq 0.01$, ★★★$P \leq 0.001$)

Fig. 6

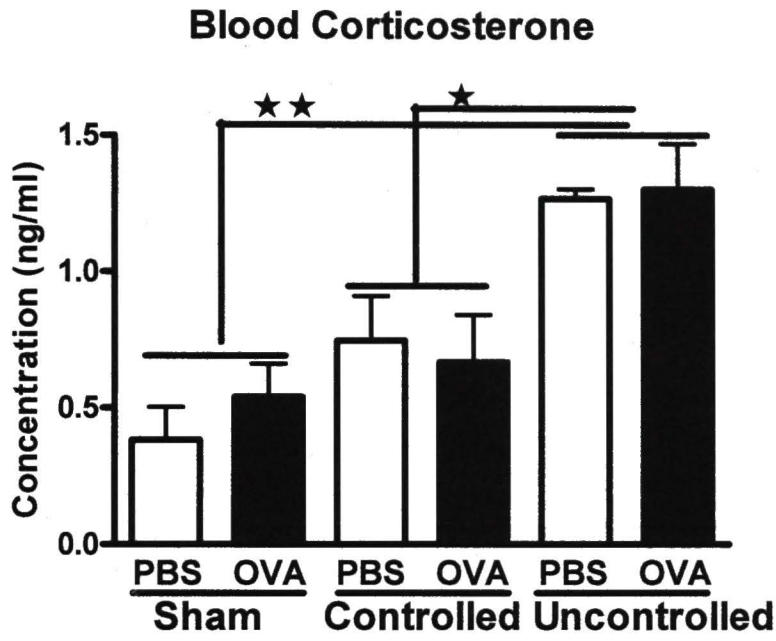


Fig. 6: Blood Corticosterone Level

The serum collected from N=7 mice was tested for the concentration of corticosterone for each mice using the RIA corticosterone kit and plotted as bar graph, concentration of corticosterone as a function of different experimental groups. No significant difference was observed between allergic and non-allergic group. There was significant increase in level of stress with the increase in level of stress from sham (no stress) stress to uncontrollable stress. (★ $P \leq 0.05$, ★★ $P \leq 0.01$, ★★★ $P \leq 0.001$)

Fig. 7 A) Non-allergic Group

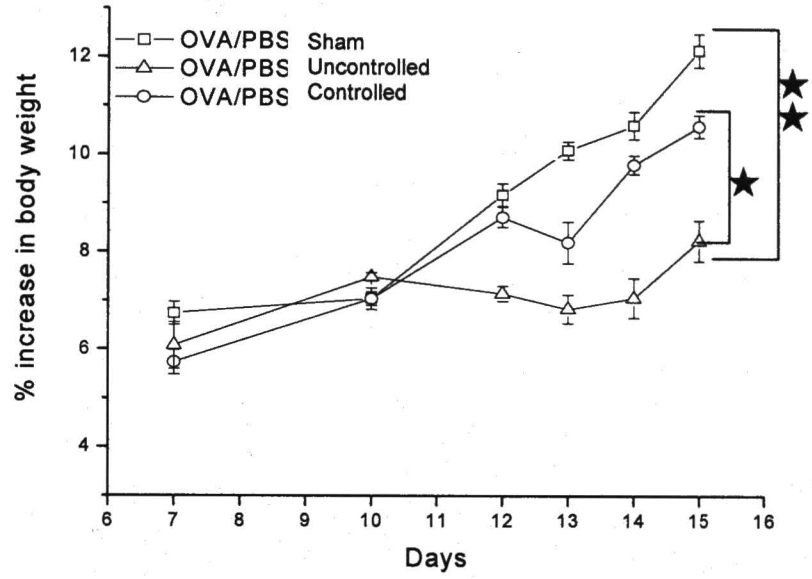


Fig. 7 B) Allergic Group

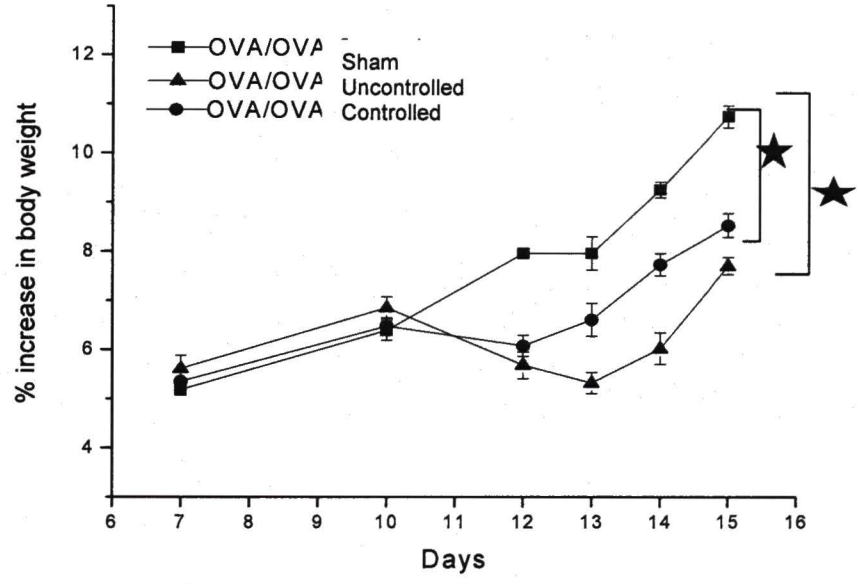


Fig. 7: Kinetics of body weight changes over the stress paradigm
The body weight on day 0 and on subsequent days of the paradigm was noted. The percentage change in the body weight over the initial body weight was calculated and plotted as function of respective days in the paradigm in asthmatic as well as non asthmatic group separately. A) It (with hollow points) shows comparative changes due to different stress conditions in no allergic group animals. B) It (with solid points) shows comparative changes due to different stress conditions in allergic group animals.
(★$P \leq 0.05$, ★★$P \leq 0.01$, ★★★$P \leq 0.001$)

Fig. 8

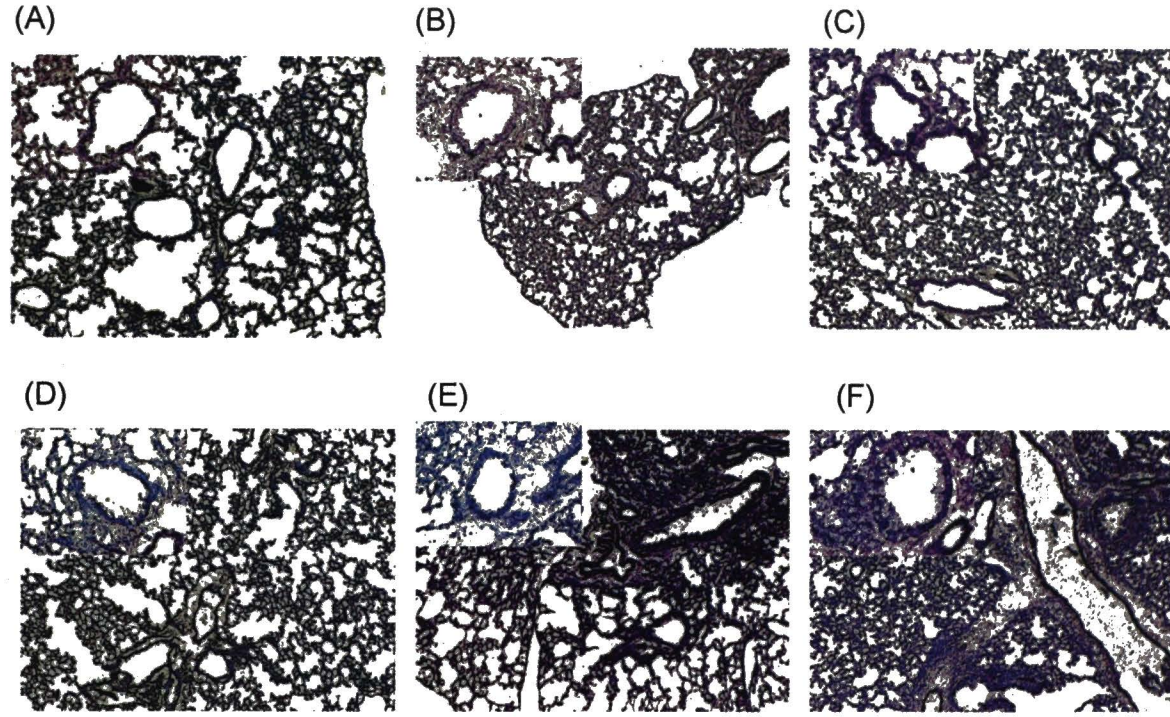


Fig. 8: Hematoxylin and Eosin staining (magnification 4X)
The lung tissue sections of 10 um thickness on the glass slide were stained with hematoxylin to stain the granular nucleus and with eosin to stain the cytoplasm to overall study the disease severity of the lung tissue. Above shown is the representative of one experiment where N=3. The qualitative changes were prominently seen in allergic group. OVA/PBS indicates the non-allergic group and OVA/OVA indicate the allergic group.

A) OVA/PBS Sham B) OVA/PBS Controlled C) OVA/PBS Uncontrolled
D) OVA/OVA Sham E) OVA/OVA Controlled F) OVA/OVA Uncontrolled

Fig. 9

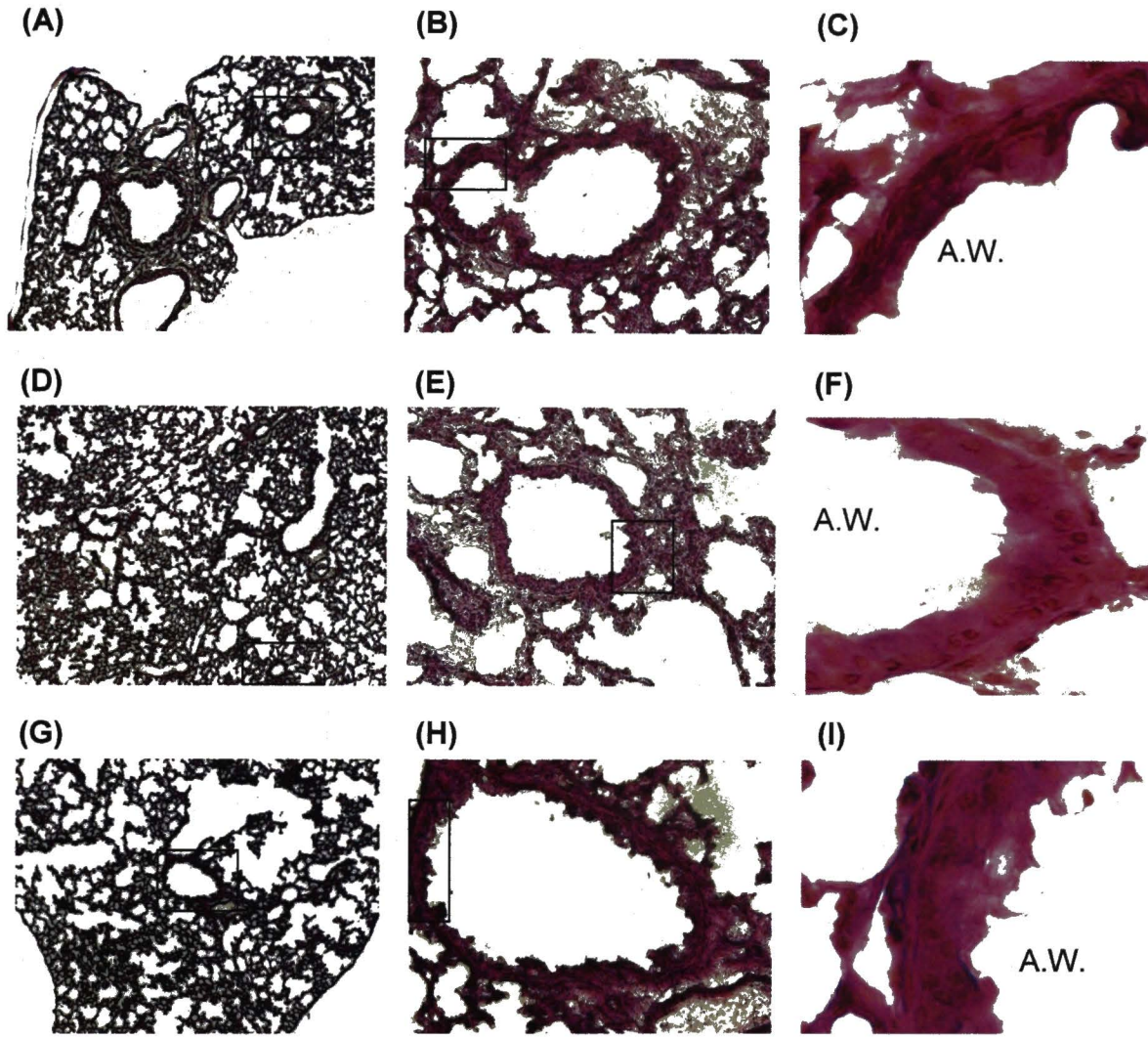


Fig. 9.1: Periodic acid & Schiff's base staining (magnification 4X, 20X, 100X)
The lung tissue sections on the glass slide from non allergic group were stained with PAS staining to study the increased mucus secretion that stained pink because of presence of glycosides in mucus. Above shown is the representative of one experiment where N=3.
4X: A) No Allergy Sham D) No Allergy Controlled G) No Allergy Uncontrolled
20X: B) No Allergy Sham E) No Allergy Controlled H) No Allergy Uncontrolled
100X: C) No Allergy Sham F) No Allergy Controlled I) No Allergy Uncontrolled
Note: A.W. indicates the Air Way

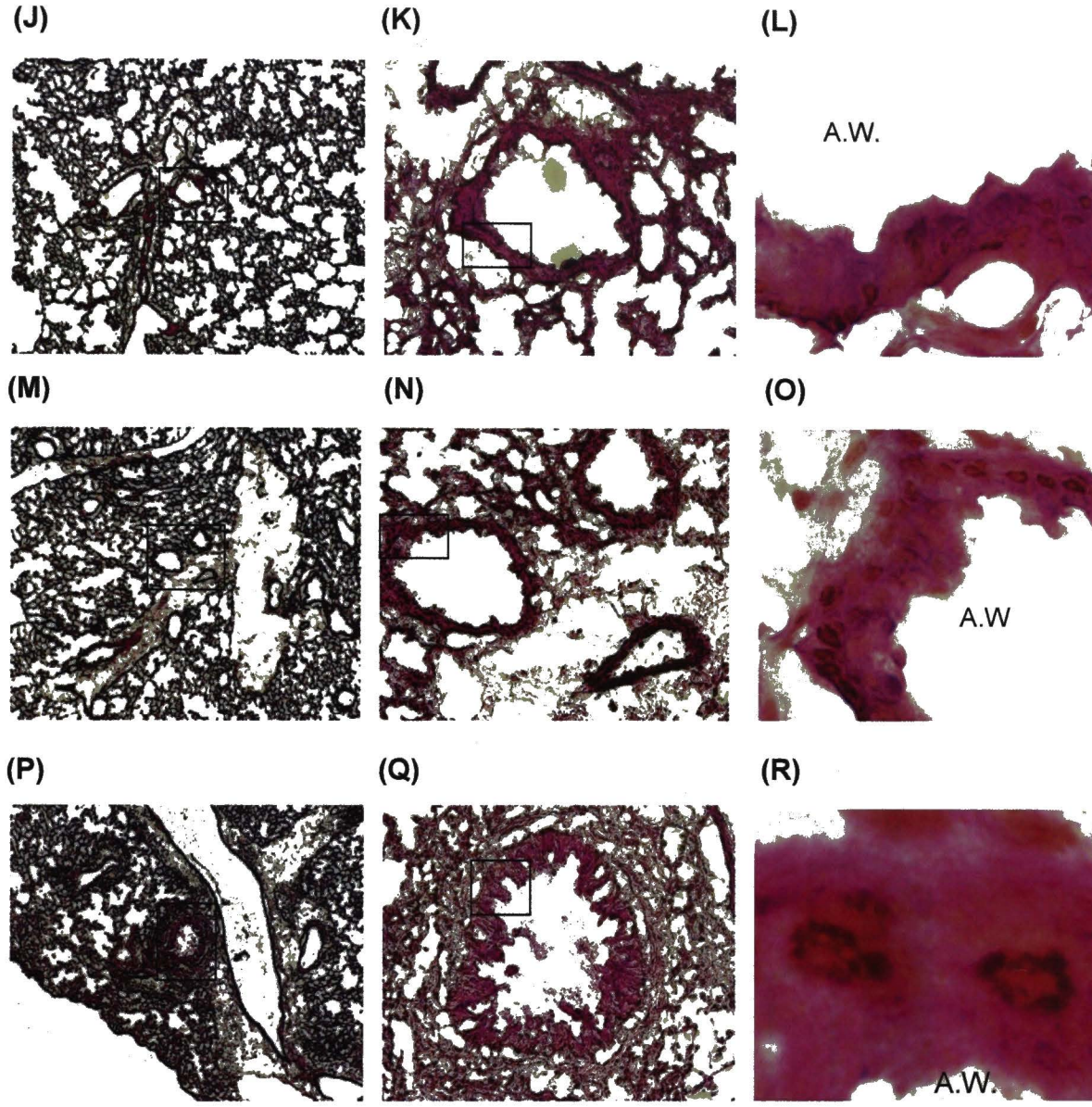


Fig. 9.2: Periodic acid & Schiff's base staining (magnification 4X, 20X, 100X)
The lung tissue sections on the glass slide from allergic group were stained with PAS staining to study the increased mucus secretion that stained pink because of presence of glycosides in mucus. Above shown is the representative of one experiment where N=3.
4X: J) Allergy Sham M) Allergy Controlled P) Allergy Uncontrolled
20X: K) Allergy Sham N) Allergy Controlled Q) Allergy Uncontrolled
100X: L) Allergy Sham O) Allergy Controlled R) Allergy Uncontrolled
Note: A.W. indicates the Air Way

Fig. 10 A)

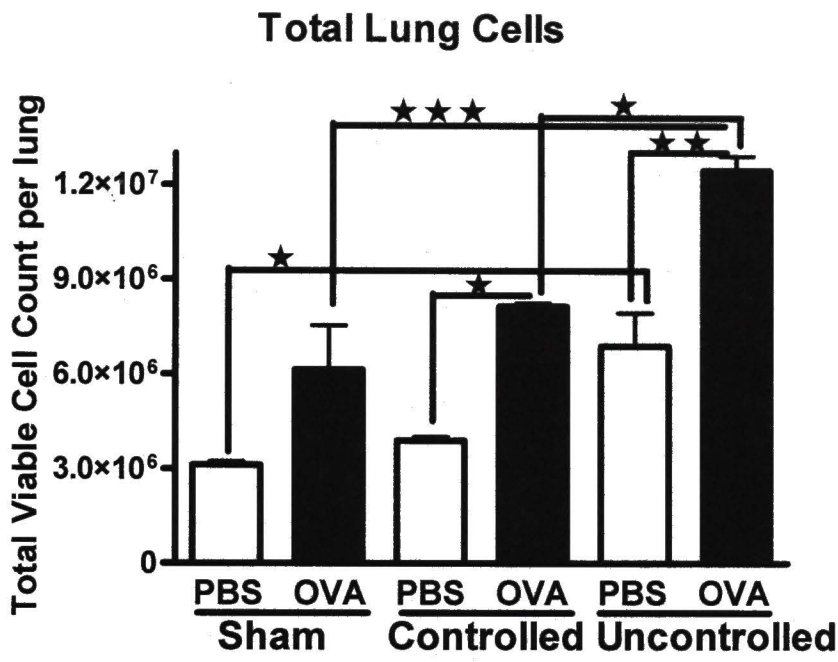


Fig. 10 B)

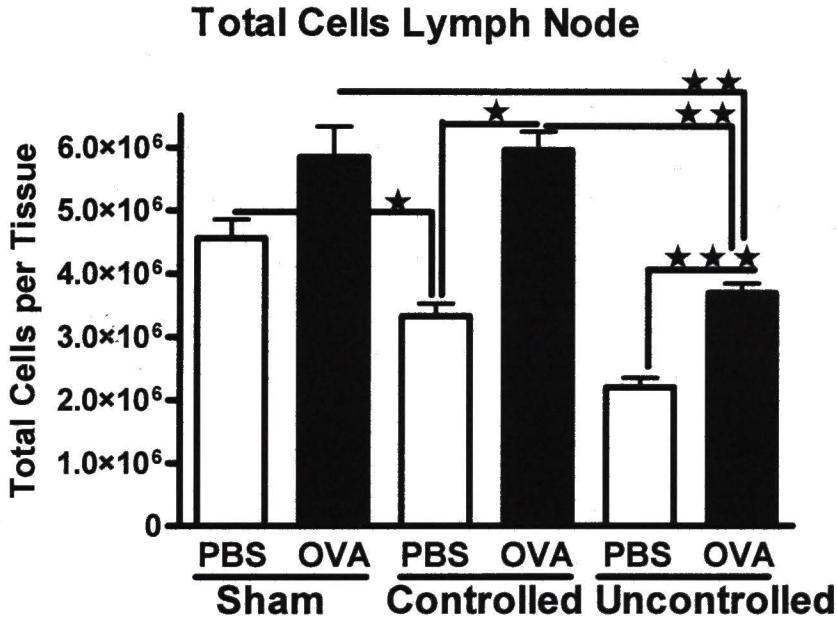


Figure 10: Total Cell Count

A) Results are represented in total cell numbers from the lung tissue. The data summarizes 3 independent experiments showing mean ± errors.

B) Results are represented in total cell numbers from the lower respiratory lymph node tissue. The data summarizes 3 independent experiments (N=12) showing mean ± errors.

(★$P \leq 0.05$, ★★$P \leq 0.01$, ★★★$P \leq 0.001$)

Fig. 11

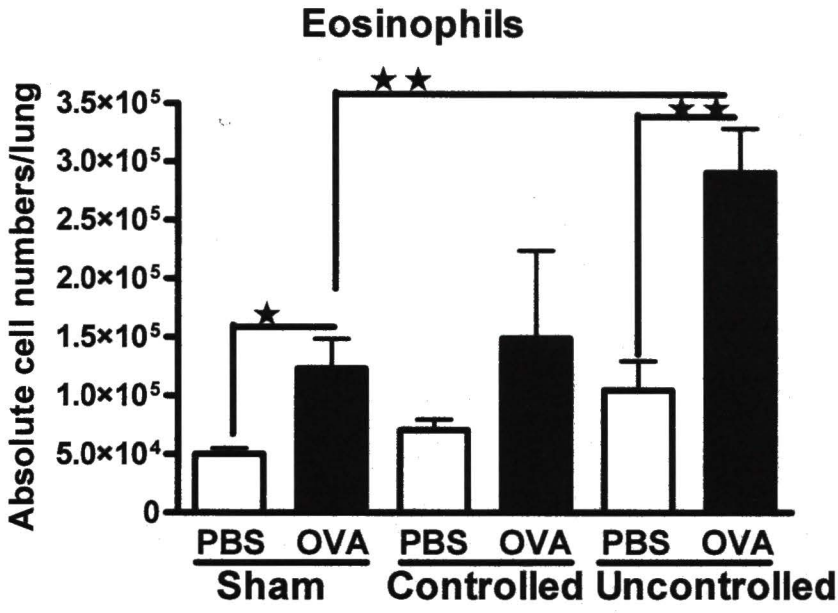


Figure 11: Distribution of Eosinophils in lungs

Results are represented in total cell numbers for cell population from the lung tissue. The data summarizes 3 independent experiments (N=12) showing mean ± errors. The above graph represents eosinophils population. The cell numbers significantly increases with the increase in stress and OVA treatment as compared to the increase given PBS treatment.

(★$P \leq 0.05$, ★★$P \leq 0.01$, ★★★$P \leq 0.001$)

Fig. 12

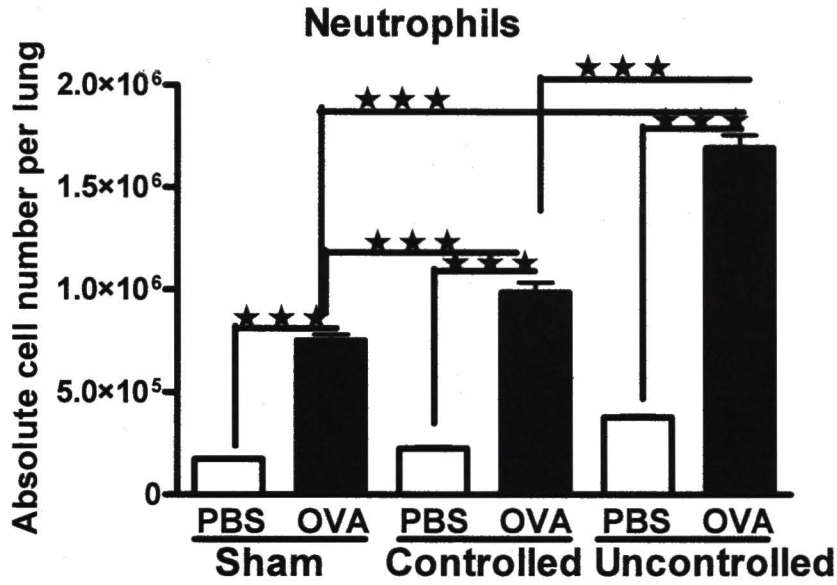


Figure 12: Distribution of Neutrophils in lungs
Results are represented in total cell numbers for particular cell population from the lung tissue. The data summarizes 3 independent experiments (N=12) showing mean ± errors. The above graph represents neutrophils population. The cell numbers significantly increases with the increase in stress and OVA treatment as compared to the increase given PBS treatment.
(★$P \leq 0.05$, ★★$P \leq 0.01$, ★★★$P \leq 0.001$)

Fig. 13 A) Cell Number

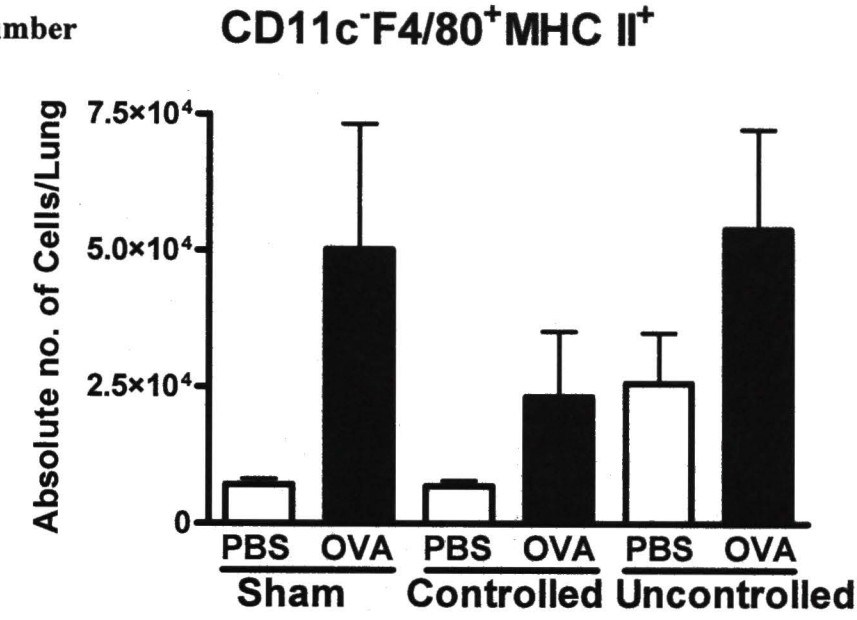


Fig. 13 B) Mean Fluorescence Intensity

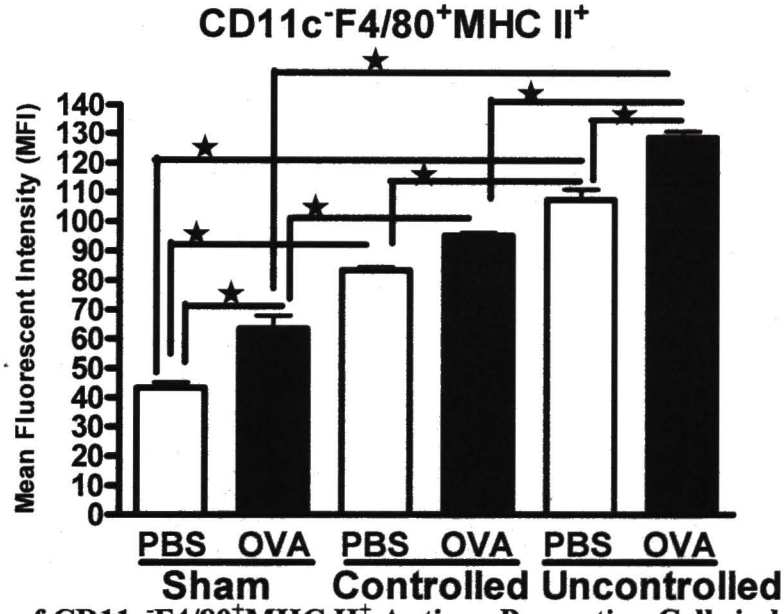


Figure 13: Distribution of CD11c⁻F4/80⁺MHC II⁺ Antigen Presenting Cells in lungs
A) The above graph represents CD11c⁻F4/80⁺MHC II⁺ cell population. Results are represented in total cell numbers for particular cell population from the lung tissue. The data summarizes 3 independent experiments (N=12) showing mean ± errors. No significant changes were seen among different treatment groups. B) The above graph represents CD11c⁻F4/80⁺MHC II⁺ cell population. Results are represented in Mean Fluorescence Intensity for particular cell population from the lung tissue. The data summarizes 3 independent experiments showing mean ± errors. Significant changes were seen among different treatment groups.
(★$P \leq 0.05$, ★★$P \leq 0.01$, ★★★ $P \leq 0.001$)

Fig. 14 A) Cell Number

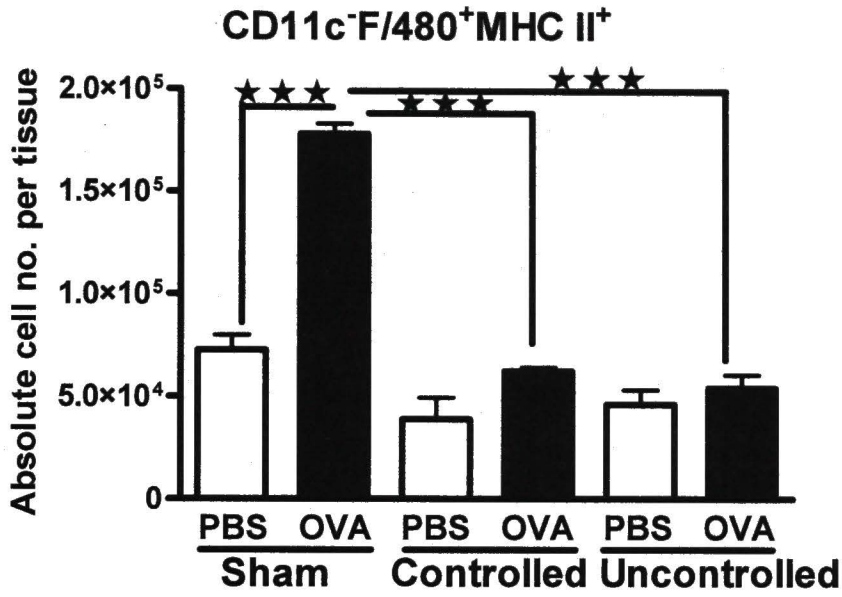


Fig. 14 B) Mean Fluorescence Intensity

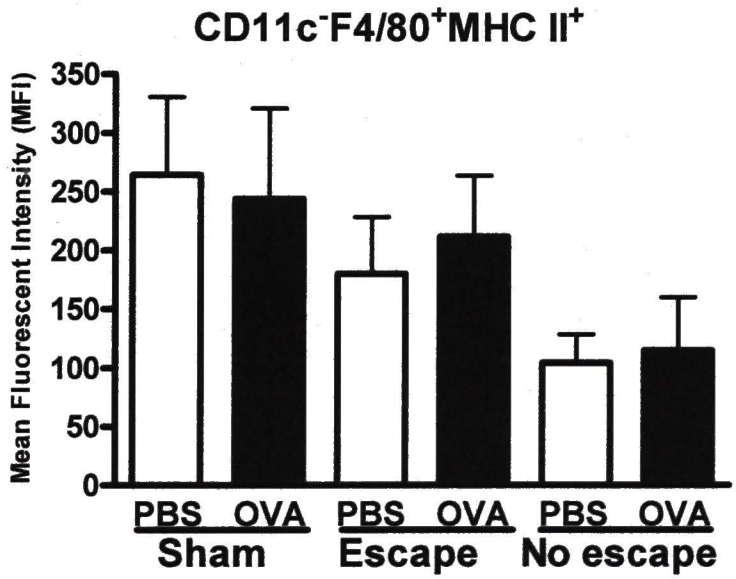


Figure 14: Distribution of CD11c$^-$F4/80$^+$MHC II$^+$ Antigen Presenting Cells in LRNs
A) The above graph represents CD11c$^-$F4/80$^+$MHC II$^+$ cell population. Results are represented in total cell numbers for particular cell population from the lower respiratory lymph node tissue. The data summarizes 3 independent experiments (N=12) showing mean ± errors. The cell numbers significantly decrease with the increase in stress and OVA treatment shows higher number of cells as compared to PBS treatment. B) The above graph represents CD11c$^-$F4/80$^+$MHC II$^+$ cell population. Results are represented in Mean Fluorescence Intensity for cell population from the lower respiratory lymph node tissue. The data summarizes 3 independent experiments showing mean ± errors.
(★$P \leq 0.05$, ★★ $P \leq 0.01$, ★★★ $P \leq 0.001$)

Fig 15 A)

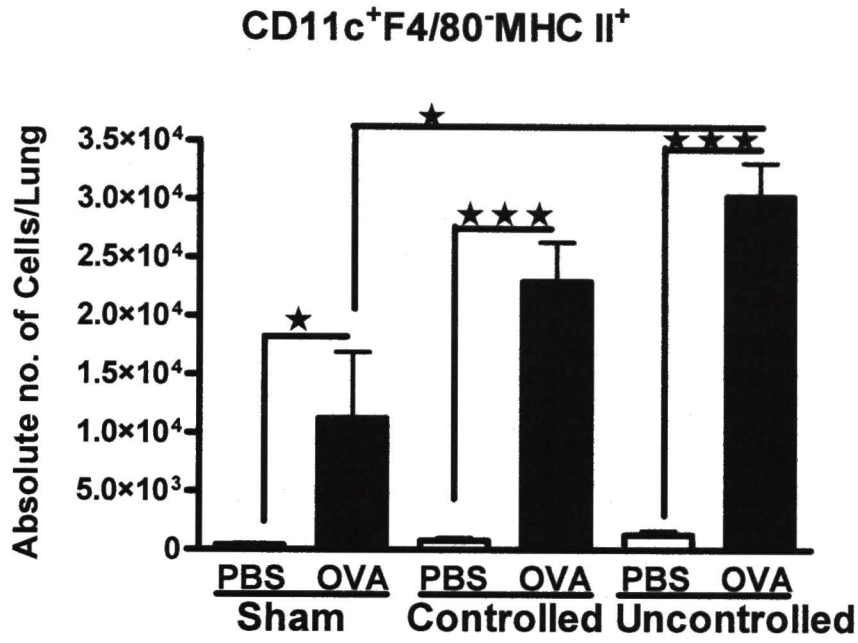


Fig. 15 B) Mean Fluorescence Intensity

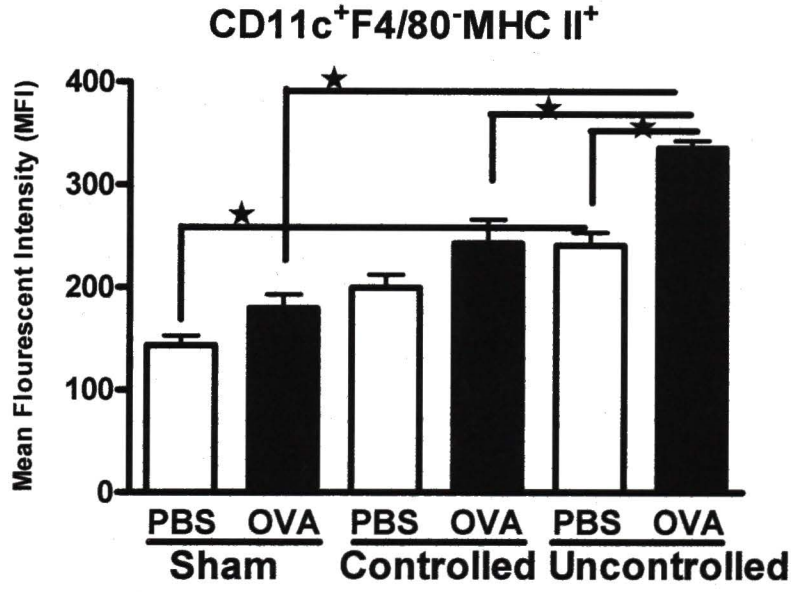


Figure 15: Distribution of CD11c$^+$F4/80$^-$MHC II$^+$ Antigen Presenting Cells in lungs The above graph represents CD11c$^-$F4/80$^+$MHC II$^+$ cell population. A) Results are represented in total cell numbers for particular cell population from the lung tissue. The data summarizes 3 independent experiments (N=12) showing mean ± errors. B) Results are represented in Mean Fluorescence Intensity for particular cell population from the lung tissue. The data summarizes 3 independent experiments showing mean ± errors. (★P ≤ 0.05, ★★ P ≤ 0.01, ★★★ P ≤ 0.001)

Fig. 16 A) Cell Number

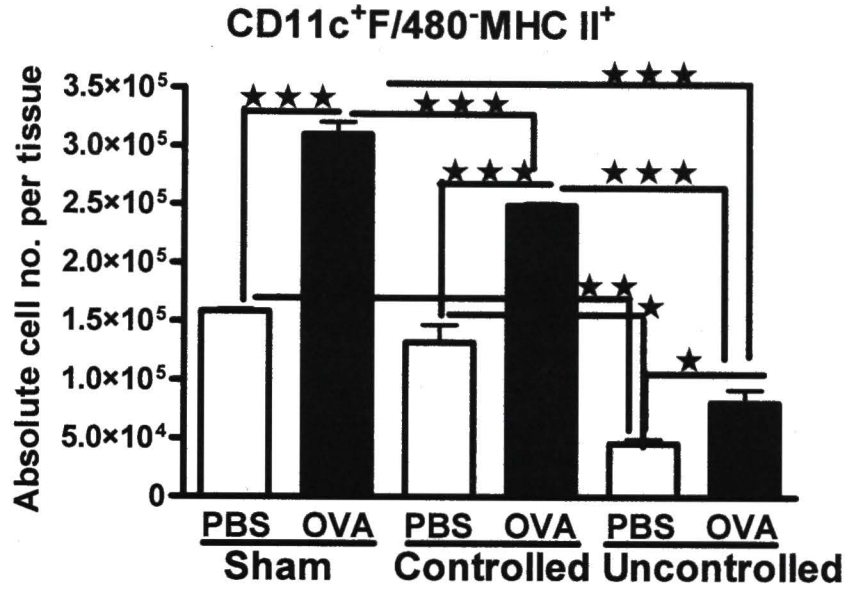


Fig. 16 B) Mean Fluorescence Intensity

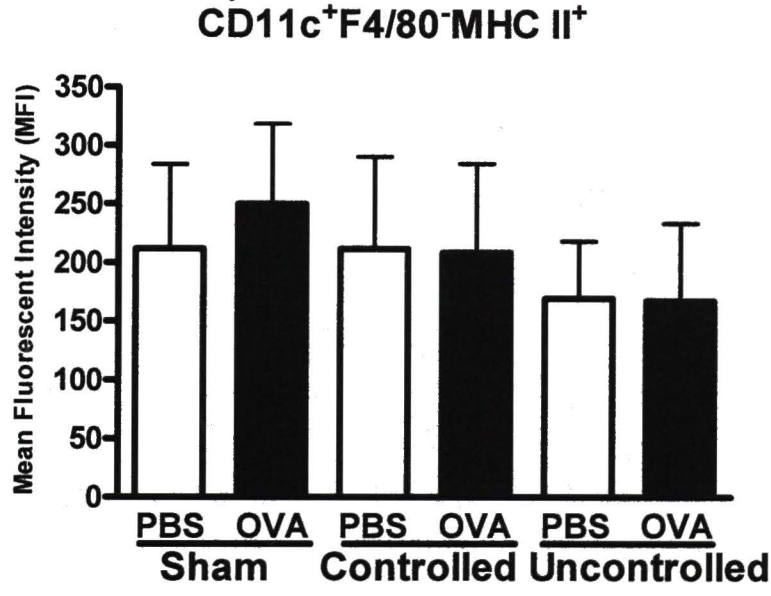


Figure 16: Distribution of CD11c$^+$F4/80$^-$MHC II$^+$ Antigen Presenting Cells in LRNs
The above graph represents CD11c$^+$F4/80$^-$MHC II$^+$ cell population. A) Results are represented in total cell numbers for cell population from the lower respiratory lymph node tissue. The data summarizes 3 independent experiments (N=12) showing mean ± errors. It represents MHC II$^+$ cells among given population. The cell numbers significantly decrease with the increase in stress and OVA treatment shows higher number of cells as compared to PBS treatment. B) Results are represented in Mean Fluorescence Intensity for cell population from the lower respiratory lymph node tissue. The data summarizes 3 independent experiments showing mean ± errors.
(★$P \leq 0.05$, ★★ $P \leq 0.01$, ★★★ $P \leq 0.001$)

Fig. 17 A)

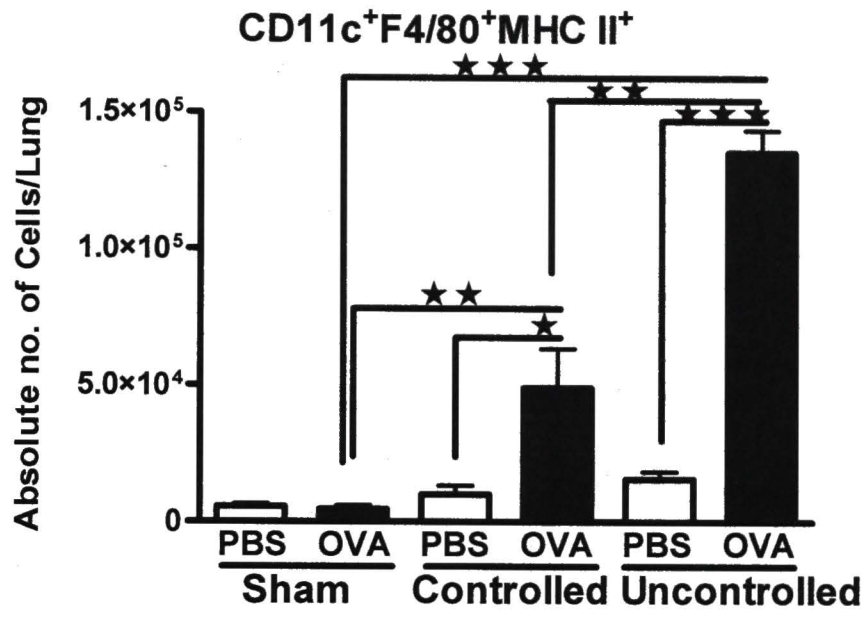


Fig. 17 B) Mean Fluorescence Intensity

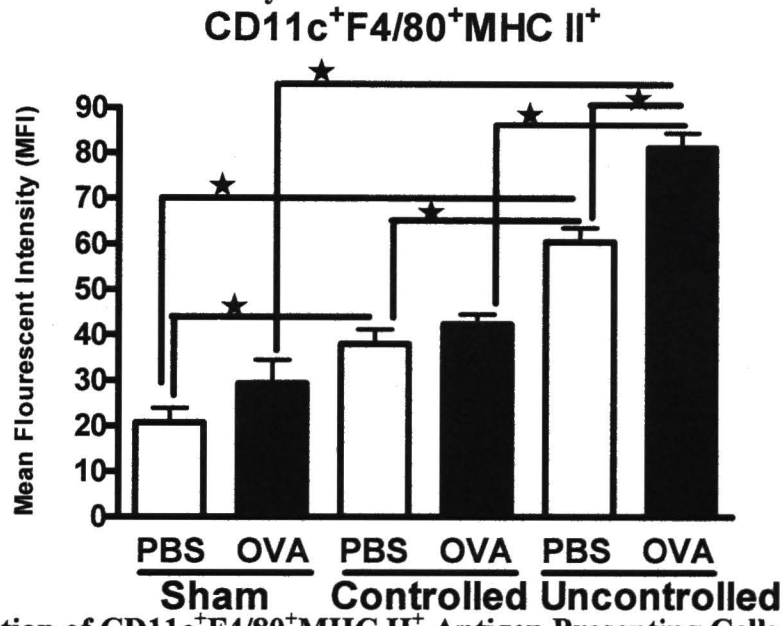


Figure 17: Distribution of CD11c$^+$F4/80$^+$MHC II$^+$ Antigen Presenting Cells in lungs
The above graph represents CD11c$^+$F4/80$^-$MHC II$^+$ cell population. A) Results are represented in total cell numbers for cell population from the lung tissue. The data summarizes 3 independent experiments showing mean ± errors. It represents MHC II$^+$ cells among given population. Significant increases were seen among different stress groups given OVA challenge. B) Results are represented in Mean Fluorescence Intensity for cell population from the lung tissue. The data summarizes 3 independent experiments (N=12) showing mean ± errors. It represents MHC II$^+$ cells among given population. Significant increases were seen among different stress groups given OVA challenge. (★$P \leq 0.05$, ★★ $P \leq 0.01$, ★★★ $P \leq 0.001$)

Fig. 18 A) Cell Number

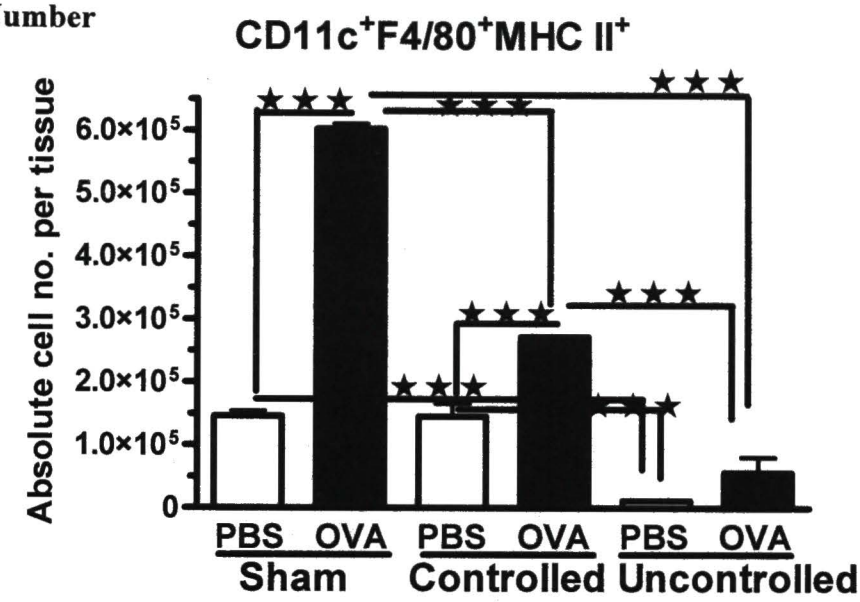


Fig. 18 B) Mean Fluorescence Intensity

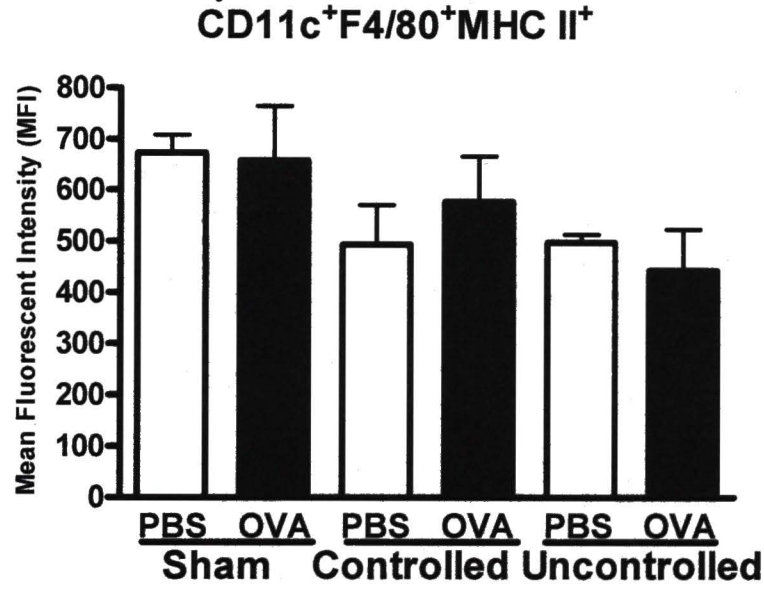


Figure 18: Distribution of CD11c$^+$F4/80$^+$MHC II$^+$ Antigen Presenting Cells in LRNs
The above graph represents CD11c$^+$F4/80$^+$MHC II$^+$ cell population. A) Results are represented in total cell numbers for cell population from the lower respiratory lymph node tissue. The data summarizes 3 independent experiments (N=12) showing mean ± errors. The cell numbers significantly decrease with the increase in stress and OVA treatment shows higher number of cells as compared to PBS treatment. B) Results are represented in Mean Fluorescence Intensity for cell population from the lower respiratory lymph node tissue. The data summarizes 3 independent experiments showing mean ± errors.
(★$P \leq 0.05$, ★★ $P \leq 0.01$, ★★★ $P \leq 0.001$)

Fig. 19 A)

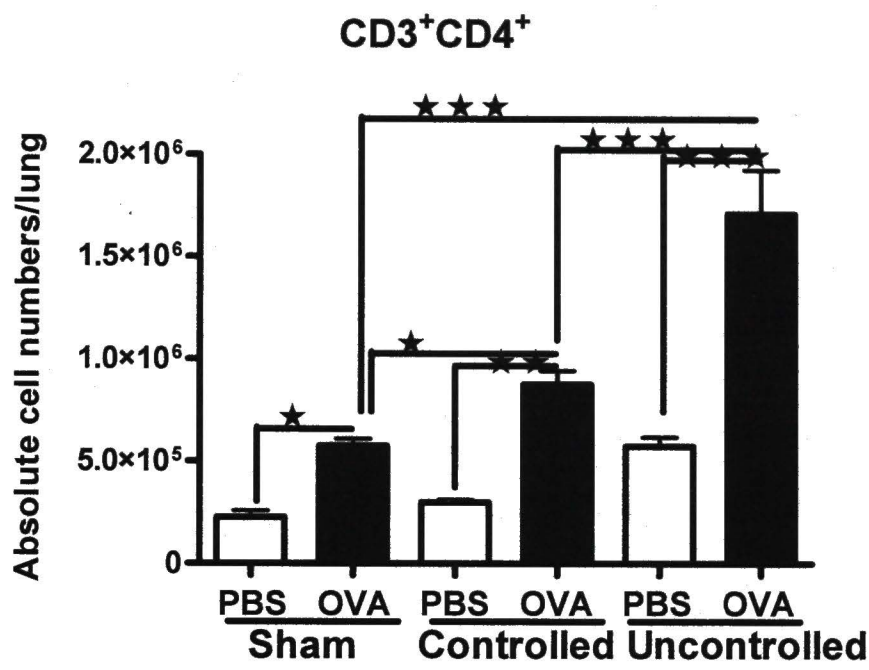


Fig. 19 B)

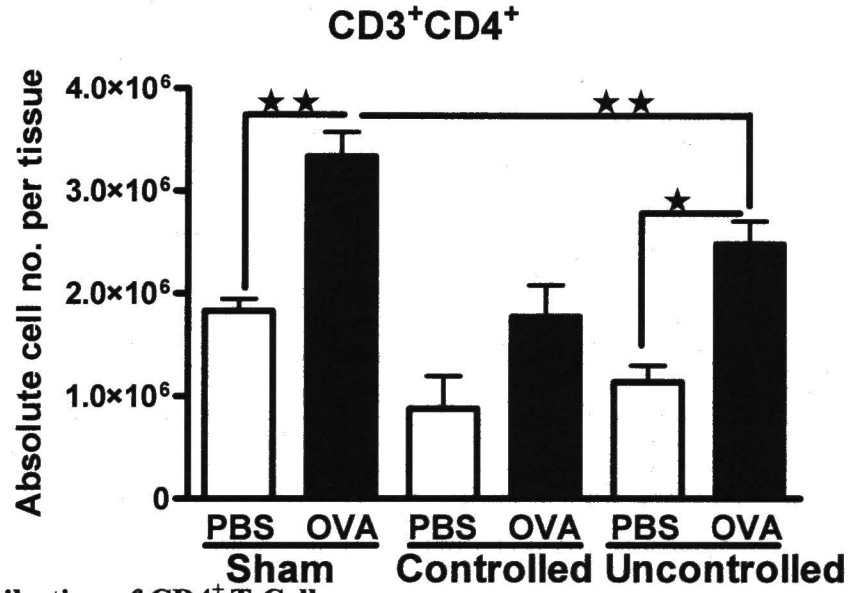


Figure 19: Distribution of $CD4^+$ T Cells

Results are represented in total cell numbers for particular cell population. The data summarizes 3 independent experiments (N=12) showing mean ± errors. A) The cell population represents $CD4^+$ T cells from lungs. The cell numbers significantly increases with the increase in stress and OVA treatment as compared to the increase given PBS treatment. B) The cell population represents the $CD4^+$ T cells from LRNs. The cell numbers show significant decrease with the increase in level of stress. The OVA treated group shows higher cell numbers as compared to PBS treated groups.

(★$P \leq 0.05$, ★★$P \leq 0.01$, ★★★$P \leq 0.001$)

Fig. 20 A)

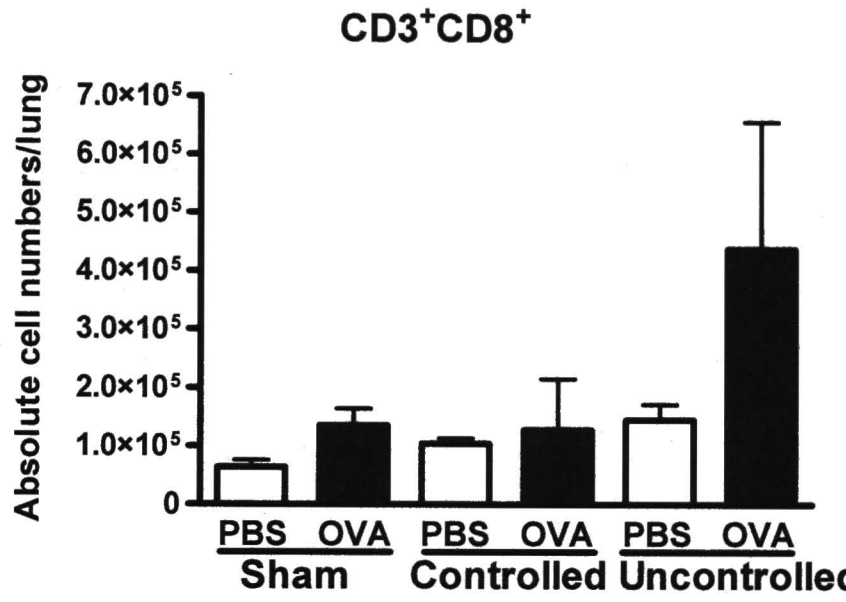


Fig. 20 B)

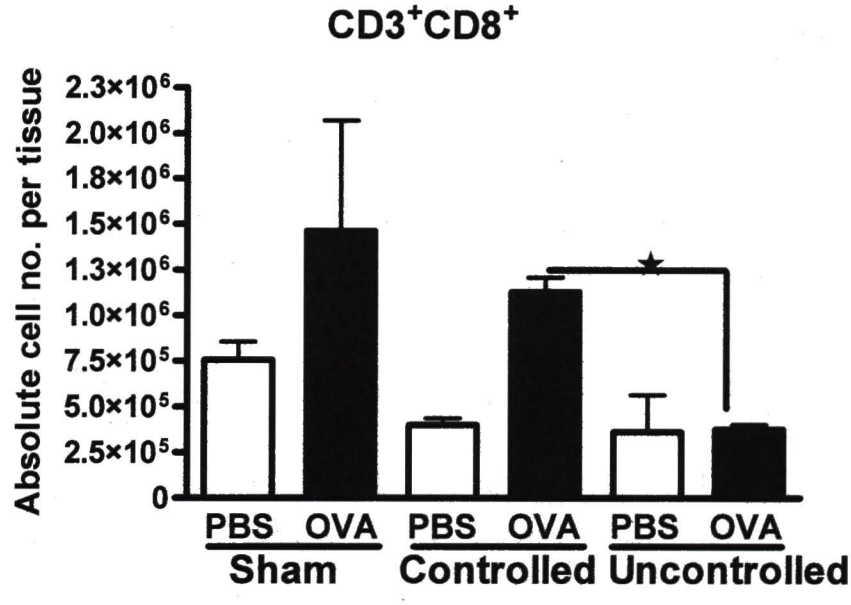


Figure 20: Distribution of $CD8^+$ T Cells

Results are represented in total cell numbers for particular cell population. The data summarizes 3 independent experiments (N=12) showing mean ± errors. The above graphs represent lymphocytes population. A) The cell population represents $CD8^+$ T cells from lungs. There is no significant change in cell numbers with different stress treatment and OVA, PBS challenge. B) The cell population represents the $CD8^+$ T cells from LRNs. The cell numbers did not show significant decrease with the increase in level of stress except from controlled to uncontrolled. The OVA treated group did not shows higher cell numbers as compared to PBS treated groups.

(★$P \leq 0.05$, ★★$P \leq 0.01$, ★★★$P \leq 0.001$)

Fig. 21 A)

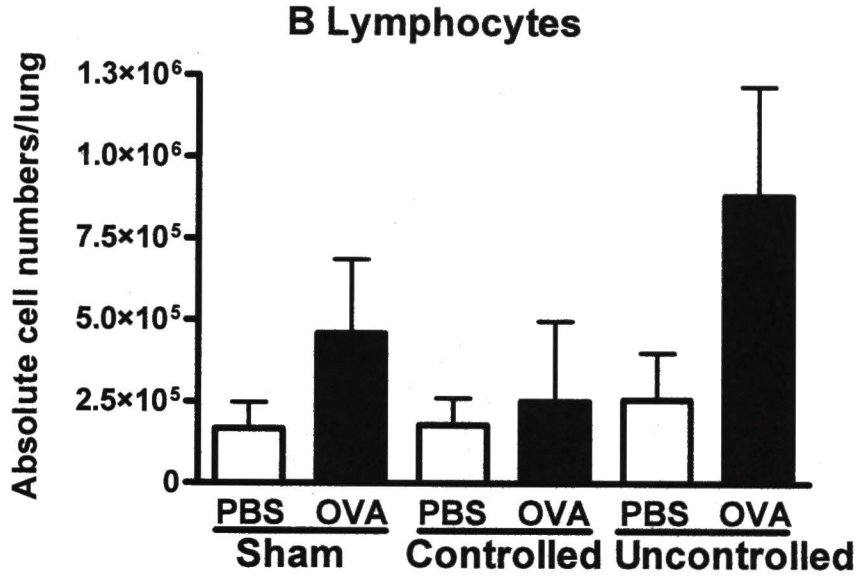


Fig. 21 B)

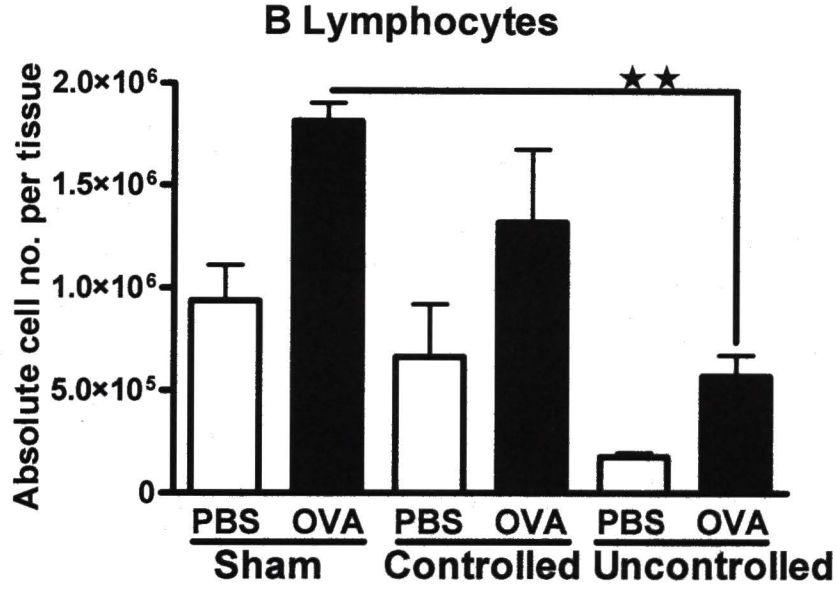


Figure 21: Distribution of B Lymphocytes

Results are represented in total cell numbers for particular cell population. The data summarizes 3 independent experiments (N=12) showing mean ± errors. The above graphs represent lymphocytes population. A) The cell population represents B cells from lungs. The cell numbers did not significant changes with the increase in stress and OVA treatment as compared to the increase given PBS treatment. B) The cell population represents the B cells from LRNs. The cell numbers show significant decrease with the increase in level of stress. The OVA treated group shows higher cell numbers as compared to PBS treated groups.

(★$P \leq 0.05$, ★★$P \leq 0.01$, ★★★$P \leq 0.001$)

Fig. 22 A)

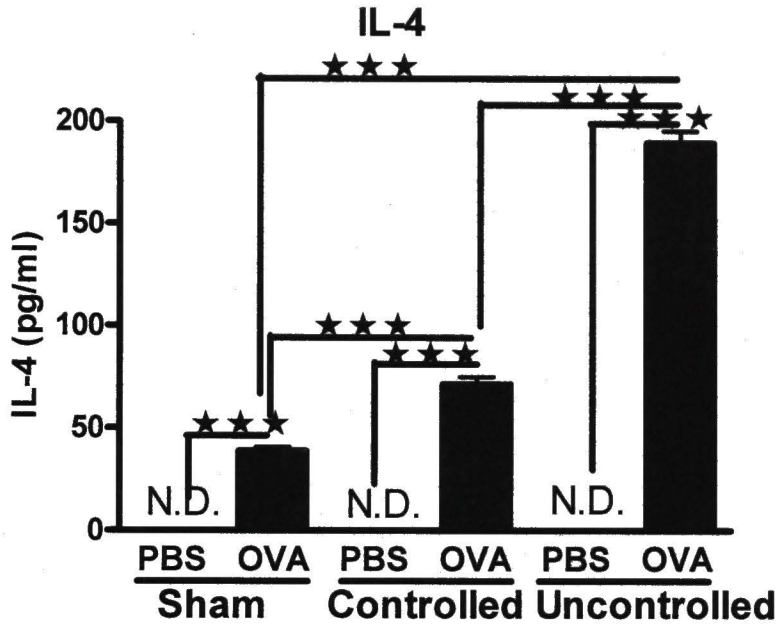


Fig. 22 B)

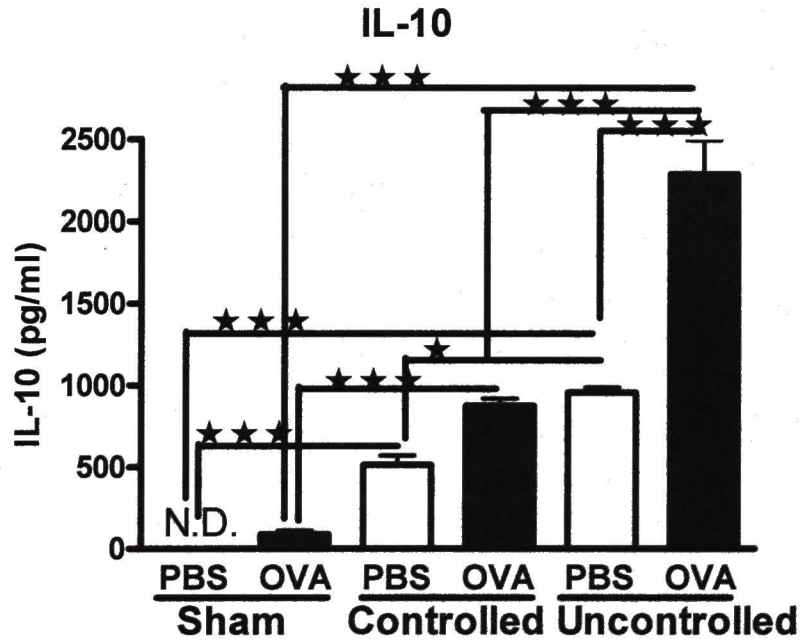


Fig. 22 C)

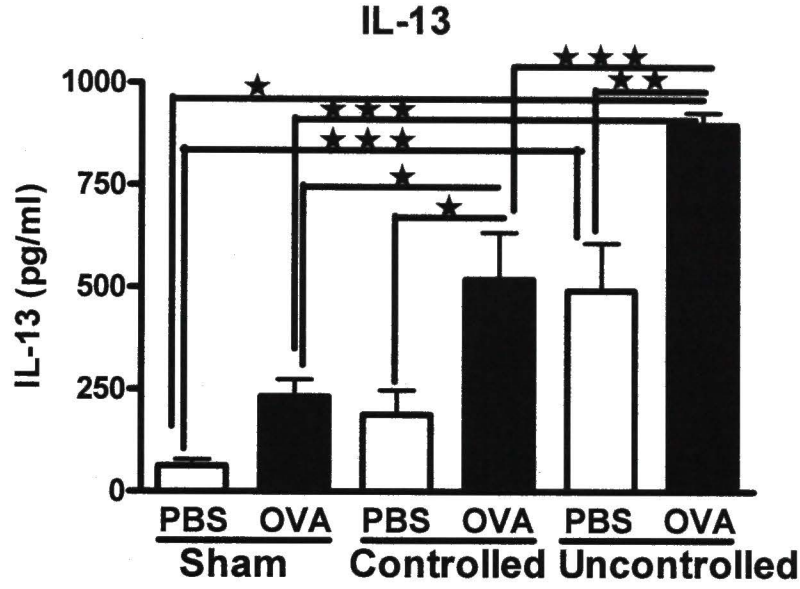


Fig. 22 D)

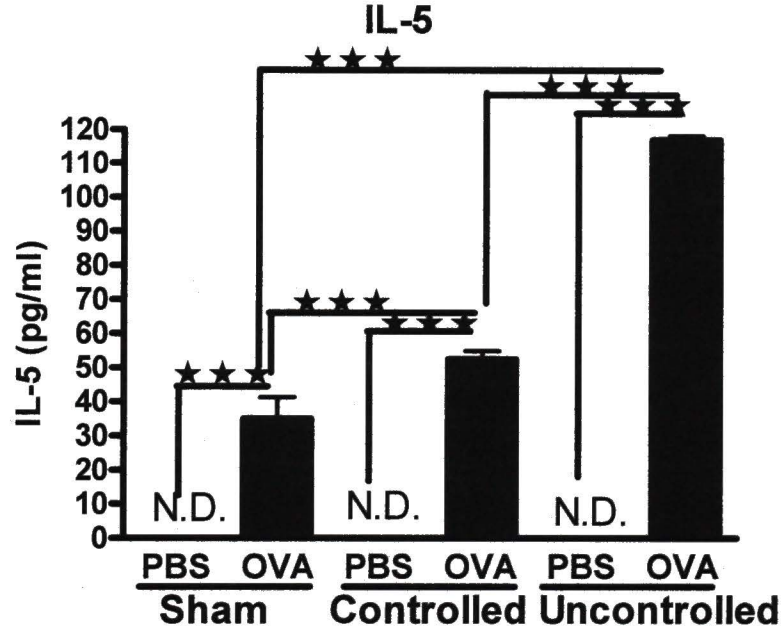


Fig 22: In-vivo cytokine environment at the site of inflammation
ELISA was carried out on Bronchioalveolar Lavage Fluid collected directly from the lung of BalbC/J mice. IL-5, IL-13, IL-4, IL-10 indicates the Th2 cytokines and IFN-γ was tested for Th1 cytokine environment. IFN-γ was undetected in these samples. Each sample was run as duplicates from N=5 showing mean ± errors. The white bar indicates OVA/PBS treated group and black bar indicates OVA/OVA group.
(★ $P \leq 0.05$, ★★ $P \leq 0.01$, ★★★ $P \leq 0.001$)

CHAPTER VI

REFERENCES

1) Ader, R. and Cohen, N. (1993) Psychoneuroimmunology: conditioning and stress. Annu Rev Psychol, *44:* 53-85.

2) Agnati LF, Zunarelli E, Genedani S, Fuxe K (2006) On the existence of a global molecular network enmeshing the whole central nervous system: physiological and pathological implications. Curr Protein Pept Sci. 7(1):3-15.

3) Altemus M, Rao B, Dhabhar FS, Ding W, Granstein RD (2001) Stress-induced changes in skin barrier function in healthy women. J Invest Dermatol 117(2):309–317.

4) Andersen, B. L., Kiecolt-Glaser, J. K., and Glaser, R. (1994) A biobehavioral model of cancer stress and disease course. Am Psychol, *49:* 389-404.

5) Buselmaier W, Vierling T, Balzereit W, Schwegler H (1981) Genetic analysis of avoidance learning by means of different psychological testing systems with inbred mice as model organisms. Psychological Research 43(3):317-333.

6) Butcher, S. K. and Lord, J. M. (2004) Stress responses and innate immunity: aging as a contributory factor. Aging Cell, *3:* 151-160.

7) Bryndina, I. G. and Danilov, G. E. (2002) [Substance P as a factor enhancing resistance of the surfactant lung system to chronic immobilization stress]. Ross Fiziol Zh Im I M Sechenova, *88:* 84-89.

8) Calcagni, E. and Elenkov, I. (2006) Stress system activity, innate and T helper cytokines, and susceptibility to immune-related diseases. Ann N Y Acad Sci, *1069:* 62-76.

9) Callahan, T. A. and Moynihan, J. A. (2002) The effects of chemical sympathectomy on T-cell cytokine responses are not mediated by altered peritoneal exudate cell function or an inflammatory response. Brain Behav Immun, *16:* 33-45.

10) Chen E and Miller GE. (2007) Stress and inflammation in exacerbations of asthma. Brain, Behavior, and Immunity, 21:993-999.

11) Cohen S, Hamrick N (2003) Stable individual differences in physiological response to stressors: implications for stress-elicited changes in immune related health, *Brain Behav Immun* 17:407–414.

12) Cohen S, Herbert TB (1996) Psychological factors and physical disease from the perspective of human psychoneuroimmunology, *Ann Rev Psychol* 47:113–142

13) Cohen S, Kessler R, Underwood GL (1995) Strategies for measuring stress in studies of psychiatric and physical disorders. In: Measuring stress: a guide for health and social scientists. New York: Oxford University Press. 3-26.

14) Dahlen I and Janson C (2002) Anxiety and depression are related to the outcome of emergency treatment in patients with obstructive pulmonary disease, Chest 122:1633–1637.

15) Dantzer R (2004) Innate immunity at the forefront of psychoneuroimmunology, *Brain Behav Immun* 18:1–6.

16) Davis, M. C., Zautra, A. J., Younger, J., Motivala, S. J., Attrep, J., and Irwin, M. R. (2008) Chronic stress and regulation of cellular markers of inflammation in rheumatoid arthritis: implications for fatigue. Brain Behav Immun, *22:* 24-32.

17) Daynes RA, Araneo BA (1989) Contrasting effects of glucocorticoids on the capacity of T cells to produce the growth factors interleukin 2 and interleukin 4. Eur. J. Immunol. 19:2319

18) Delfino RJ. (2002) Epidemiological evidence for asthma and exposure to air toxics: linkages between occupational, indoor, and community air pollution research. Environ Health Perspect.;110 (suppl 4):573–589.

19) del Rey, A., Kabiersch, A., Petzoldt, S., and Besedovsky, H. O. (2002) Involvement of noradrenergic nerves in the activation and clonal deletion of T cells stimulated by superantigen in vivo. J Neuroimmunol, *127:* 44-53.

20) Dhabhar FS (2003) Stress, leukocyte trafficking, and the augmentation of skin immune function. Ann NY Acad Sci 992:205–217.

21) Dhabhar, F. S. and McEwen, B. S. (1996) Stress-induced enhancement of antigen-specific cell-mediated immunity. J Immunol, *156:* 2608-2615.

22) Dhabhar, F. S., Miller, A. H., McEwen, B. S., and Spencer, R. L. (1996) Stress induced changes in blood leukocyte distribution. Role of adrenal steroid hormones. J Immunol, *157:* 1638-1644.

23) Elenkov, I. J. (2008) Neurohormonal-cytokine interactions: implications for inflammation, common human diseases and well-being. Neurochem Int, *52:* 40-51.

24) Elenkov IJ, Chrousos GP (1999) Stress hormones, Th1/Th2 patterns, pro/anti-inflammatory cytokines and susceptibility to disease. Trends Endocrinol. Metab. 10:359

25) Elliott, G.R., Eisdorfer, C. (1982). Stress and Human Health. New York: Springer Publishing Company.

26) Fava, G. A., Fabbri, S., Sirri, L., and Wise, T. N. (2007) Psychological factors affecting medical condition: a new proposal for DSM-V. Psychosomatics, *48:* 103-111.

27) Fong TA, Mosmann TR (1989) The role of IFN-gamma in delayed-type hypersensitivity mediated by Th1 clones. *J. Immunol.* 143:2887.

28) Förster R, Davalos-Misslitz AC, Rot A (2008) CCR7 and its ligands: balancing immunity and tolerance.Nat Rev Immunol.

29) Forsythe P, Ebeling C, Gordon JR, Befus AD, Vliagoftis H. (2004) Opposing effects of short- and long- term stress on airway inflammation. Am. J. Respir. Crit. Care Med.; 169: 220-226.

30) Frank E, Doyle WJ, Skoner DP, Rabin BS, Gwaltney Jr. JM (1998) Types of stressors that increase susceptibility to the common cold in healthy adults, *Health Psychol* 17:214–223.

31) Gavillet M, Allaman I, Magistretti PJ (2008) Modulation of astrocytic metabolic phenotype by proinflammatory cytokines. Glia.

32) Gil, K., Radziwillowicz, P., Zdrojewski, T., Piwonski, J., Landowski, J., and Wyrzykowski, B. (2006) [Depression as a risk factor of cardiovascular disease]. Kardiol Pol, *64:* 526-531.

33) GINA, 2002. Global Strategy for Asthma Management and Prevention. NHLBI/WHO workshop report, revised. National Institute of Health/National Heart, Lung and Blood Institute. NIH publication no. 02-5639.

34) Glaser R, Kiecolt-Glaser JK (2005) Stress-induced immune dysfunction: implications for health. Nat Rev Immunol 5(3):243–251

35) Godbout P, Glaser R (2006) Stress-Induced Immune Dysregulation: Implications for Wound Healing, Infectious Disease and Cancer. Journal of NeuroImmune Pharmacology; 4:421-427

36) Gonzales XF, Deshmukh A, Pulse M, Johnson K, Jones HP (2007) Stress-induced differences in primary and secondary resistance against bacterial sepsis corresponds with diverse corticotropin releasing hormone receptor expression by pulmonary CD11c(+) MHC II(+) and CD11c(-) MHC II(+) APCs. Brain Behav Immun.

37) Griswold SK, Nordstrom CR, Clark S, Gaeta TJ, Price ML, Camargo A (2005) Asthma Exacerbations in North American Adults. *Chest.*; 127:1579-1586

38) Gross, W. B. and Siegel, P. B. (1981) Long-term exposure of chickens to three levels of social stress. Avian Dis, *25:* 312-325.

39) Joachim RA, Noga O, Sagach V, Hanf G, Fliege H, Kocalevent RD, Peters EM, Klapp BF (2008) Correlation between immune and neuronal parameters and stress perception in allergic asthmatics. Clin Exp Allergy. 38(2):283-90.

41) Jones HP, Tabor L, Sun X, Woolard MD, and Simecka JW (2002) Depletion of $CD8^+$ T Cells Exacerbates $CD4^+$ Th Cell-Associated Inflammatory Lesions During Murine Mycoplasma Respiratory Disease.J. Immunol; 168:3493–3501.

41) Kavelaars, A. (2002) Regulated expression of alpha-1 adrenergic receptors in the immune system. Brain Behav Immun, *16:* 799-807.

42) Kelley KW (2004) From hormones to immunity: the physiology of immunology, *Brain Behav Immun* 18:95–113.

43) Kiank, C., Holtfreter, B., Starke, A., Mundt, A., Wilke, C., and Schutt, C. (2006) Stress susceptibility predicts the severity of immune depression and the failure to combat bacterial infections in chronically stressed mice. Brain Behav Immun, *20:* 359-368.

44) Lawrence DA (2002) Psychologic stress and asthma: neuropeptide involvement, Environ. Health Perspect. 110:A230–A231.

45) Lehrer PM, Isenberg I and Hochron SM (1993) Asthma and emotion: a review, J. Asthma 30:5–21.

46) Levin R, Nielsen TA (2007) Disturbed dreaming, posttraumatic stress disorder, and affect distress: a review and neurocognitive model. Psychol Bull. 133(3):482-528.

47) Mackenzie JN (1886) The production of the so-called "rose effect" by means of an artificial rose, with remarks and historical notes, Am. J. Med. Sci. 91:45–57.

48) Maddox L and Schwartz DA (2002) The pathophysiology of asthma, Annu. Rev. Med. 53:477–498.

49) McEwen, B. S. (2006) Protective and damaging effects of stress mediators: central role of the brain. Dialogues Clin Neurosci, *8:* 367-381.

50) Mucida DS, Keller AC, Fernvik EC and Russo M (2003) Unconventional strategies for the suppression of allergic asthma, Curr. Drug Targets Inflamm.