University of Alberta

Differential Effects of Early-life Stress on Airway-inflammation in Mice

by

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Abstract

Neonatal stress can induce permanent psychological, neurological, and physiological changes that influence the immune system. For example, early-life stress increases the severity and susceptibility to asthma in children. Accordingly, we investigated if maternal separation, an early-life stress, can worsen airway inflammation and airway hyperresponsiveness (AHR) in a Balb/c mouse model of asthma. Separated (3hrs daily; 10 days) and unseparated mice were sensitized and challenged with chicken-egg ovalbumin (OVA) starting at 31-days post-birth to induce an asthma phenotype. Challenging with OVA increased airway inflammation and AHR compared to sham (saline) challenge. However, challenging maternally separated mice with OVA resulted in significantly less total inflammatory cells, eosinophils, interferon-gamma, and interleukin-4 in BAL compared to unseparated controls. AHR was unaffected by the stress. These findings indicate that early-life stress may provide some beneficial effects in certain situations and demonstrates the importance of early-life environmental factors in development of airway inflammatory diseases like asthma.

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List of Abbreviations

ACTH	Adrenocorticotropic hormone
AHR	Airways hyperresponsiveness
BAL	Bronchoalveolar lavage
CON	Unseparated Control (group)
CRH	Corticotropin releasing hormone
CRF	Corticotropin releasing factor
CCS	Corticosteroid
ELISA	Enzyme-linked immunosorbant assay
HPA	Hypothalmic-Pituitary-Adrenal (axis)
IBD	Inflammatory Bowel Disease
IFN	Interferon
IgE	Immunoglobulin E
IL	Interleukin
IP	Intraperitoneal injection
MLI	Mean Linear Intercept
MS	Maternally Separated (group)
MS+	Enhanced Maternally Separated (group)
OVA	Chicken Egg Ovalbumin
OVA/OVA	Sensitized and Challenged with Chicken Egg Ovalbumin
OVA/SAL	Sensitized with OVA and challenged with Saline
PBS	Phosphate Buffered Saline
Penh	Enhanced Pause

RIA	Radio immunoassay
SAL	Saline
SE	Standard Error
TNF	Tumor Necrosis Factor
VAF	Virus Antibody-Free (facility)
WBP	Whole Body Plethysmography

Chapter 1: General Introduction

I. Asthma: A Stressful, Inflammatory Disorder

Asthma is a chronic inflammatory disease of the airways defined by persistent symptoms of wheezing, chest tightness, and coughing derived from variable reversible airway obstruction and airway hyper-responsiveness (AHR). Asthma can be characterized as a syndrome because of the wide variety of pathogenesis, mechanisms, and manifestations. The resulting symptoms are largely due to epithelial damage, infiltration of the bronchial mucosa with lymphocytes, eosinophils and other inflammatory cells, increased mucus production, and edema that when present chronically can lead to airway smooth muscle hyperplasia, constriction, and remodeling (1, 2). Both inflammatory and mesenchymal cells of the airways release chemical and cytokine mediators that can directly lead to recruitment of more inflammatory cells and induce smooth muscle hypertrophy and hyperplasia. These changes are responsible for the development of bronchoconstriction and airway hyperresponsiveness where the airways constrict more forcefully than normal individuals in response to a bronchoconstrictor.

II. T_H1 and T_H2 Responses in Asthma

The wide variety of physiological and immunological changes that occur in asthmatics is believed to be a result of an allergic T_H2 response (3) that is identified by the activation and differentiation of $CD4^+$ T-cells to the T_H2 cell subtype. This is in contrast with a T_H1 response that is seen in cell mediated immunity (3).

Accordingly, a switching between T_H1 and T_H2 responses occurs early in life which allows for activation of different cells and mediators. Antigen presenting cells display antigen to progenitor T-cells (T_0) in the context of certain co-stimulatory molecules depending on the antigen. The presentation of antigens in the context of the B7.2 costimulatory molecule by dendritic cells is one example that leads to the conversion of the progenitor T-cells to T_H2 cells (3). T_H2 cells, along with several others including eosinophils and mast cells, when stimulated, produce cytokines such as interleukin (IL)-4, 5, 6, 9, 13, which are commonly considered to be associated with T_H2 responses (2, 3). These cytokines promote the recruitment and activation of T_H2 cells, eosinophils, mast cells, and shift B-cells towards IgE production (Figure 1-1). The presence of IgE can prime mast cells, so when they bind antigen they release histamine and additional cytokine (3). Additionally, the T_H2 response leads to depression of the T_H1 response, whereby common anti-bacterial or anti-cell mediated immune activities are inhibited (4). These cytokines include IL-2, 10, and Interferon (IFN)-γ (2, 3). However, some studies suggest that some of these cytokines, like IFN-γ, play an important role in certain models of asthma (5).

III. Factors Associated with Asthma

Several genome-wide screens and association studies have found strong correlations between genetic markers that impact the expression of T_H2 cytokines like IL-3, 4, 5, 9, 13 and atopic asthma (6). Additionally, genetic factors are strong predictors of atopy and asthma, illustrated by high asthma rates within families (6).

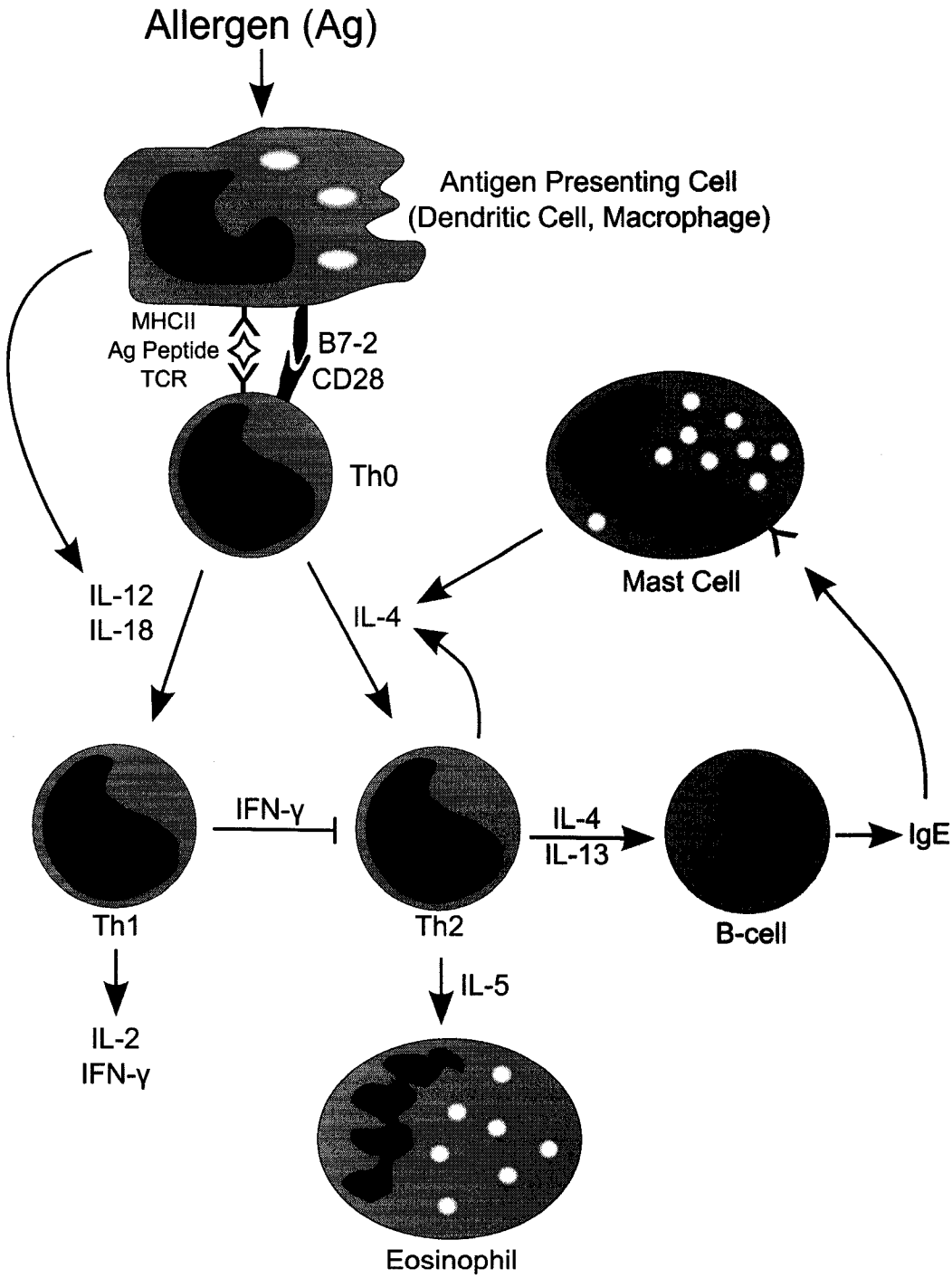


Figure 1-1: Relationships between Cytokines and Cells during an Allergic Challenge.

This diagram represents some of the interactions present in an antigen presentation in context with costimulatory molecules that stimulate a T_H2 response.

However, although such relationships between genetic factors and asthma is strong, there is no doubt that the predisposition towards asthma and its severity are strongly influenced by environmental factors (6). For example, although the prevalence of asthma globally is on the rise, it is more prevalent in more developed countries, likely due to improved hygienic conditions (7). The idea that environmental factors can affect asthma is enshrined in the *hygiene hypothesis* which states that exposure to bacterial products and unhygienic conditions early in life has the potential to modify the immune response away from a T_H2 phenotype (8). Since the development of this idea, the literature has begun to provide insight that additional environmental factors, such as psychosocial factors, can also strongly influence asthma (9).

One of these key factors is stress. A number of observational studies indicate that stress is closely related to asthma severity in both children and adults and may also be involved in the development of the disease. Between 20 and 35% of asthmatics experience exacerbations during periods of stress (10). The mental health of asthmatic children is an important predictor of asthma morbidity (11), and has been linked to asthma mortality (12, 13). Psychological distress in children is associated with asthma that is more difficult to manage (14), and with more frequent and lengthier hospital admissions (15) and functional disability (16).

Although there is evidence that stress adversely affects asthma, what is missing from these studies is a clear indication of the mechanism of the interactions between them. A workshop by the National Heart Lung and Blood

Institute (17) suggested that the role of psychological stress in asthma should be an area of intense study. Since then it has been increasingly recognized that psychosocial factors, as environmental stressors, may have important roles in the severity of asthma symptoms and potentially in the induction of allergic diseases in general.

IV. Stress

Stress, or the general adaptation syndrome, was first described in a physiological context by Hans Selye (18, 19) who noted that it is "*a specific syndrome which consists of all the non-specifically induced changes within a biological system.*" Stress is considered the common denominator of all the adaptive reactions in the body and as such stress cannot be directly detrimental for health. The concept that stress in certain cases may precipitate physiological changes leading to disease was first explored by Hans Selye (19), who called that kind of stress "distress". This stress response may be defined as the psychophysiologic reaction to noxious physical or psychological stimuli.

When the body perceives a stress, either physically or psychologically, short term activation of the neuroendocrine and autonomic nervous systems promotes adaptation and survival. This has been termed *allostasis* meaning literally "reestablishing stability through change" (20). During allostasis physiological systems operate at a higher or lower level than during "normal" homeostasis; for example, emotional distress may lead to elevated heart rate and blood pressure, as well as elevated glucocorticoid levels that in turn depress production of inflammatory cytokines. Providing allostatic responses are shut off when they

are no longer needed, the body is able to adapt to and survive the immediate challenge without suffering long term consequences. However if the same response systems are activated over a longer period of time or remain active when no longer needed, these adaptive changes lead to other consequences including receptor desensitization and tissue damage that may precipitate or exacerbate disease processes. This has been termed "allostatic load," and it refers to the price the tissue or organism pays for an overactive or inefficiently managed allostatic response.

There are two main methods by which stress exerts its effects on the body. The first, the sympathetic nervous system, is part of the autonomic nervous system. It is responsible for many immediate effects as a result of perceived stress or injury. When stimulated, efferent nerves induce a wide scope of effects upon the body, such as an accelerated heart rate, bronchodilation, reduced motility and blood flow to the gastrointestinal tract, perspiration, and pupil dilation (10). Accordingly, the sympathetic nervous system targets specific organs and tissues that provide immediate benefit in stressful situations.

Alternatively, the Hypothalamic-Pituitary-Adrenal (HPA) axis, through the endocrine release of hormones, affects most tissues and cells throughout the body. Release of the hypothalamic corticotrophin releasing hormone (CRH or CRF) stimulates release of adrenocorticotropic hormone (ACTH) from the pituitary which in turn promotes the release of corticosteroids from the adrenal cortex (21). These stress hormones have a wide variety of effects on growth, development, and the immune system which will be described below.

In humans, stress can manifest itself in various ways based on its duration. Acute stress, dubbed the *flight or fight response* or positive stress, occurs when a perceived threat or challenge requires a small period of time to adapt, like an immediate threat of an exam (22). This stress leads to the immediate activation of the HPA axis and sympathetic nervous system. As explained above, both of these mechanisms allow stress, detected by the brain, to alter physiological function throughout the body. This leads to beneficial effects on the individual (23). The sympathetic nervous system acts to prepare the body physically to react to the stressor but the HPA axis acts on the immune/inflammatory response by depressing the responses as soon as they begin to prevent them from proceeding unchecked.

These effects are opposite to those of chronic stressors where the presence of long term stress maintains the output of stress molecules via the HPA axis and sympathetic stimulation. Over a long period of time this may lead to a state of exhaustion (22). Many receptors for the molecules, especially those involved in negative feedback mechanisms of corticosteroid release become desensitized, thus leading to high levels of stress molecules in the body. The reference to stress in clinical studies usually refers to chronic psychological stress. These types of stress include reduced social status, death of a friend or family member, loss of employment, long term anxiety, and depression.

V. Effects of Stress on the Immune System

Stress has a variety of immunomodulatory effects, both positive and negative. The literature demonstrates that psychological stress may influence inflammatory and immune cell trafficking, cell proliferation, and cell function including cytokine and inflammatory mediator production (24). Stress can modulate these responses through the nervous system via connections between autonomic nerves and the immune system by the release of hormones and neuropeptides that can interact with immune cells (25).

Acute stress induces an adrenal hormone-mediated redistribution of immune cells from the blood to other compartments of the immune system (24). Immune cells in these cases redistribute to the skin where they may enhance skin immune function (26, 27), partly mediated by IFN-γ (28). Similarly, short exposures to stressful experimental tasks have been shown to induce suppression of T-cell mitogenesis and increase the number of circulating CD8+ and natural killer cells (29, 30). These effects appear to be mediated by the autonomic nervous system (31) and are associated with alterations in the production of IL-1β, IL-2 and IFN-γ (32, 33).

Although acute stress augments many aspects of the immune response, it almost counter-intuitively has been shown to inhibit other aspects of the immune response. In humans for example, stress induced by examinations can down regulate T-cell function and alter cytokine levels (17). This stress leads to a shift in the immune response away from an anti-bacterial T_H1 response towards an allergic T_H2 response (34).

In contrast to the effect of acute stress, which generally augments immune responses, chronic stress seems to depress the migration of immune cells from the blood (35) an effect that correlates with the attenuation of responsiveness to corticosteroids. Although the HPA axis is active in both acute and chronic stress, the temporal nature of the stress response induces opposite effects in chronic stress as compared to acute stress. These differences may be due to the differing mechanisms that take place with regard to acute and chronic stress states, as well as exhaustion of body resources that occurs as the body attempts to remain in a constant state of "fight or flight" as it attempts to cope with repeated or chronic stressors. Also, the chronic excitation of the HPA axis inhibits the expression of corticosteroid receptors in the brain that result in increased corticosteroid levels (21).

In humans, chronic stress plays an important role in disease, usually resulting in exacerbation of symptoms, primarily in inflammatory diseases. Mental stress, anxiety, and anger are positively correlated with cardiac ischemia (36), periodontal disease (37), Inflammatory Bowel Disease (IBD) (38-40), and it is hypothesized to play major roles in autoimmune diseases such as Graves' disease, multiple sclerosis, rheumatoid arthritis, and others (41-44). Also, patients with conditions believed to be affected by stress, such as atopic dermatitis, are more sensitive to stress-induced immune system dysfunction than normal individuals (45). Chronic stress also promotes lymphocyte apoptosis via a mechanism independent of the HPA axis (46).

Working knowledge into the functions of the HPA axis, the primary mechanism whereby the effects of stress are carried throughout the body, has greatly increased in the last few decades. In addition with increasing knowledge of the sympathetic nervous system, stress has begun to be recognized as a fundamental physiological process that regularly influences well being and disease states.

VI. Human Studies Relating Stress and Asthma

There are innate problems with studying the effects of stress on human asthma in experimental settings. It is difficult to impart chronic clinical stresses on human subjects, and so good evidence relating the effects of stress and asthma are relatively sparse. However, there are observational and epidemiological studies that have been conducted that have looked at the relationship between stress and asthma.

a) Effects of Suggestion and Hypnosis on Asthma

Many early studies examined the link between the nervous system and asthma using suggestion (hypnotic or conscious) and described its effects on asthma symptoms. A review by Isenberg *et al.* (10) asserts that 35% to 40% of asthmatics develop bronchoconstriction following relevant suggestion or following other stressful stimuli. This effect is greater and more consistent in asthmatics, although even non-asthmatic individuals develop bronchoconstriction as a result of suggestion. Pastorello *et al.* used suggestion to indicate that a saline solution was a bronchodilator or bronchoconstrictor but

found no significant changes in respiratory measures (47). In contrast, a study in elderly male patients showed that the recollection of an asthma attack either consciously or through hypnotism was sufficient to cause a significant rise in minute ventilation (48). In some of these studies the effect of suggestion in normal controls was not studied. Despite this important criticism, a number of studies show an increase in airway resistance and reduction in forced expiratory volume following hypnotism and suggestion of anger, fear, and recollection of asthma in adults (49, 50) and children (51).

b) Observational Studies Relating Stress and Asthma

Long term observational studies in asthmatic patients tend to show stress induced effects similar to the suggestion studies above. In one set of studies (52, 53), Sandberg *et al.* followed a large cohort of asthmatic children over 18 months using a diary system to record positive and negative events, and showed a significant correlation between stressors and the development of asthma exacerbations. The first study concluded that severe acute stresses in the absence of chronic stress increased the risk of an asthma attack significantly between 2-6 weeks after the event. However in children with chronic stress, an acute severe stress could precipitate an increased risk of asthma attack within the first 2 weeks. Another study conducted by Smyth *et al.* also found an association between stress and changed moods with lower peak expiratory volumes in asthmatic patients (9). Other studies have associated psychological distress in children with asthma that is more difficult to manage (14), with more frequent and lengthier admissions to the hospital (15), and functional disability (16). One

of these studies was also able to correlate the length and number of hospitalizations with anxiety, stigma of being asthmatic, neuroticism, and hostility (15). Similar findings linking psychological morbidity to asthma mortality in children have been conducted in Australia (13), the US (12), and in adults in England (54). All of these studies show that stress plays a role in the development of symptoms of chronic asthma and on the probability, number, and severity of asthma exacerbations. In addition, asthma induces stress and negative emotional responses, which some studies have correlated with further deterioration of asthma (55). To summarize, most long term observational studies show strong harmful effects of clinical stress on asthma.

c) Experimental Stress on Human Asthma

So, is asthma induced by stress, or does stress worsen asthma symptoms? A third form of studies used to address this question involved inducing short term stress in an individual. Asthmatics that were stressed with a method of forced oscillation had significantly higher airway impedance as compared to controls (56). Other methods of inducing stress, via an examination or computer puzzle test, have also been used. Adolescents with asthma undertaking a stressful computer puzzle test have increased breathlessness (57). Asthmatic students respond to allergen challenge with increased airway inflammation during stressful examination periods compared to low stress periods (58).

Other studies are less conclusive. One study found no significant relationship between daily life stress and serum IgE levels or bronchial hyperresponsiveness (59). Studies have shown that stress-induced changes in

physiological parameters of breathing may be similar in asthmatic and non-asthmatic children, but these changes may have higher clinical significance in asthmatics due to their higher baseline airway resistance (60). Such changes may be the result of differences in coping behavior, as passive stressful tasks like watching videos of bloody surgeries or other stress inducing events cause increased airway resistance in asthmatics, whereas active stressful tasks like arithmetic do not have this effect (49). Similarly, relieving stress by writing about stressful experiences, a task that helps to improve coping, improves a number of physiological parameters of asthma for long periods (61).

d) Other Explanations for the Interaction between Stress and Asthma

Explanations for the purported enhancement of asthmatic symptoms by stress, which do not involve direct alteration of airway physiology or inflammation, have also been suggested. Stress can change an asthmatic's perception of breathlessness and lead them to believe that their condition is deteriorating despite an absence of changes in physiologic parameters of breathing (57). Additionally, stress might affect self-management strategies and adherence to treatment plans and, therefore can lead to deterioration of asthma control during periods of stress. It is also commonly believed that asthma can induce stress in patients, although one study demonstrated that in most patients, asthma exacerbations do not provoke a physiological stress response (62). These are possible explanations for the observation of Rimington *et al.* (63) that increases in "Hospital Anxiety and Depression" scores lead to scores of symptom measurement above what is expected by lung function and other

objective measurements. Finally, psychological stress correlates with an increased risk of acute respiratory infections (64, 65), an important trigger of asthma exacerbations. Although the above explanations are possible, abundant epidemiological evidence suggests that the regulatory mechanisms affected by stress are more complicated than previously supposed, and directly affect the pathophysiology of the asthmatic response.

Overall, the consensus has emerged that stressful situations and events affect the severity of asthma. Observational, hypnotic, and experimental stress studies all show that the induction of stress in a clinical setting leads to changes in physiological parameters in asthmatics. What is not clear is if this increased severity of asthma is the direct result of increased inflammation during high stress states or results from other effects of the nervous or endocrine systems directly on pulmonary physiology. Liu *et al.* (58) showed that stressed individuals can develop higher degrees of inflammation but did not show any baseline differences between high and low stress states.

Several other important questions also remain. Many of the studies seem to identify only a subpopulation that is more greatly affected by stressors than others. Is this a result of the cumulative effect of stress, or do genetic or environmental aspects make certain individuals more prone to the effects of stress? Does stress only exacerbate existing disease, or is it also responsible for the initial development of atopy in the individual? Our ability to answer these questions and determine the mechanisms of the effects of stress on asthma in humans is limited. To understand the effect of stress on asthma and to discern

the mechanisms involved, it is vital to use animal models.

VII. Animal Models to Study the Relationship between Stress and Asthma

Human studies have established the association between stress and immune function. Since human studies are not often suitable to identify the mechanism of these interactions, much of the evidence regarding the pathways of the effects of stress on asthma comes from animal models. Using animal models, the level and type of stress can be controlled and quantified and other interventions can be used to define the pathways. As seen in some human studies, animal models of stress can utilize short term stressors and chronic or repeated stressors that may have different effects on allergic inflammation.

a) Short Term Stress

Research has provided insight into the effects of acute stress on allergic inflammation. Our group, using a Balb/c mouse model of allergic airway inflammation and restraint stress for 1 hour per day for 3 consecutive days, showed a significant reduction of inflammatory cells in bronchoalveolar lavage (BAL), but increased levels of IL-6, IL-9, IL-13, and no change in IL-10 and IFN-γ levels (66). The use of a specific corticosteroid type II receptor antagonist, RU486, reversed these BAL changes (66). Thus in this short term stress model, elevated levels of corticosteroids in stressed animals may result in a reduction of inflammatory cell infiltration in the airways and may alter cytokine levels in BAL. The importance of endogenous corticosteroids in the mouse model of asthma is also supported by the fact that CRH deficient mice develop increased

airway inflammation and elevated IL-4, 5, 13, RANTES, IFN-γ, and eotaxin levels in BAL after chicken egg ovalbumin (OVA) sensitization and challenge compared to normal controls (67). This effect is believed to be the result of low corticosteroid and catecholamine production from the adrenal gland because of the absence of the stimulus for their release.

Similar to our study, an elevation in corticosteroids was also seen in a rat model of water-avoidance stress. This was associated with increased tracheal epithelial short-circuit activity and an elevated response to CRH (68). Thus stress may induce inflammatory changes that affect asthma, but also affects physiological changes in the airways that may further contribute to asthma. Another physiological change can be seen in guinea pigs where injection of CRH, which not only is a stress hormone but also an indirect stimulator of corticosteroid release, reduces OVA-induced plasma extravation in airways via the inhibition of tachykinin release from primary sensory nerves (69).

These studies with different animal models show that short term stress results in the activation of CRH and corticosteroid release, which reduces inflammation and plasma extravation, and that this mechanism may involve the inhibition of tachykinin release and physiological changes in the tracheal epithelium. These observations helped initiate research into the important role that tachykinins play in airway inflammation, mast cell activation, cytokine secretion, bronchial hyperresponsiveness, and mucoid secretion (70-72). Thus the evidence obtained in animal models demonstrates beneficial effects of acute stress on inflammation and plasma extravation in asthma.

b) Long Term Stress

In contrast to short term stress, chronic or repeated stress induces the reverse phenotype with regard to inflammation in animal models of asthma. Extending the 1 hour daily restraint stress from 3 days to a week in the mouse model described above, our group showed a significant increase in BAL accumulation of all inflammatory cell types, although there was no significant change in the cytokines tested (66). A similar result was seen using repeated sound stress for 24 hours. There were increased BAL leukocytes (especially eosinophils) and increased hyperresponsiveness of tracheal smooth muscle (25). However in contrast to our studies, Joachim *et al.* (25) identified increased AHR in addition to airway inflammation in stressed versus non-stressed control mice. The two studies have a number of differences that may explain the discrepancies. Joachim *et al.* evaluated AHR using a different technique than in our study. More importantly however was that Joachim *et al.* used a different strain of mice (CBA/J instead of Balb/c). Perhaps genetic differences are important for the manifestation of stress-induced or exacerbated asthma and may explain why only a subgroup of patients with asthma experience increased severity during episodes of stress (10). Investigation into possible genetic markers that may responsible for these changes would be interesting. Also the stressor in this study (25) was of longer duration compared to stress in our study. This could indicate that different mediators of stress or different adaptation mechanisms depend on the stressor and may have diverse effects on asthma symptoms.

The effects of long-term stress on airway inflammation have also been replicated in a rat (73) and a guinea pig model (74). Studies in the rat model also showed an increase in allergen-induced plasma extravasation in the paw of stressed animals (73), while another study showed the opposite effect of repeated stress in plasma extravasation in the synovium (75). This discrepancy may indicate tissue specific responses to stress, or possibly the result of different stress protocols.

These animal model studies have confirmed predictions from observational studies in humans on the effects of stress on asthma. However, the main focus of future studies should be the identification of the pathways mediating the effects of stress on airway inflammation. The enhanced airway inflammation in long term stress, as opposed to depressed inflammation in short term stress models indicate that different mechanisms are at play. Our group showed that corticosteroids are not a major component in the effects of long term stress on airway inflammation, whereas they are involved in the effects of short term stress (66). Joachim *et al.* have shown that the mechanisms of the effects of long term stress likely involve the neurokinin-1 (NK-1) receptor present in human lung and submucosal glands, in addition to its presence on mast cells, lymphocytes and macrophages in rodents (74). Substance P is the primary tachykinin that acts on the NK-1 receptor. Inhibition of the latter in a sound stress model in guinea pig asthma ablates effects of long-term stress in the lung (74).

Discovering how chronic and acute stress exerts their effects on asthma is important: as thus far they are shown to employ different effects using different mechanisms. By discerning the differences in these mechanisms between effects of stress that are beneficial versus harmful to asthmatics, it may be possible to use this information to work towards therapies for the effects of stress.

VIII. Early-life Stress

Unlike stress which occurs during adult life, early life stress has the potential to cause more chronic effects that last into adulthood in contrast to the acute and chronic stressors described above. The reason for this is that unlike late life stress, early life stress has the ability to alter normal development of the nervous, immune, and endocrine systems thereby inducing permanent effects on the individual. This can lead to significant physiological and psychological changes that alter the susceptibility and progression of disease. These types of effects make early-life stress and its mechanism important to study.

a) Current Models of Early-life Stress

There are differential effects of early-life stress that depend upon the quality and duration of the stress. Due to the difficulty in carrying out this research in controlled settings in humans and the difficulty in quantifying stress early in life, animal models, especially rodent models, are commonly used to identify mechanisms. There is variation in timing, intensity, and quality among protocols for early life stress: some stresses are applied prenatally (76), whereas others are given as late as 4 weeks post-birth (77). The lack of standardization in protocols

and methodologies often make it difficult to explain differences in the published literature, however these approaches may also be beneficial to elucidate the mechanism by using different protocols.

Depending on when the stress is applied, different methods of stresses are also used. Stresses applied prenatally involve direct stress of the dam, such as injections with corticosteroids or restraint stress. While these types of stress can induce strong stress responses in the fetus, it is a result of physical stress that also impacts the dam. Changes in the dam's physiology can impact development of the fetus in ways that are indirect to the stress of the fetus. Additionally, this methodology of stress looks at physical stress, not psychological stress that may be more relevant. Other stresses done later (greater than 2 weeks of age in animals) can use stresses applied to adults such as restraint stress and electroshock stress. However, these stresses may miss the time period where much of the development of the lungs, nervous, and immune system take place. Thus, in order to study the effects of early life stress, the ideal stress is one that can take place immediately after birth, but within the crucial period when the development of the pups may be altered by the stress. This would allow the greatest chance of successfully identifying changes as a result of early-life stress, without introducing other stresses from the dam that can impact results.

One of the most commonly used methodologies for early life stresses that obey these rules is maternal separation (78). This involves separating the offspring from the mother for a period of time, usually repeatedly. This is commonly done in rat and mouse pups in the first 10 – 21 days post-parturition,

for as short as 15 minutes to as long as 24 hours. The wide variation of stresses employed probably results in variation of results.

b) Effects of Early Life Stress on the HPA Axis

As described above, one of the primary pathways of stress propagation to the immune system involves the HPA axis. The use of maternal separation or early-life handling has opposite effects depending on if the stress is acute or chronic in rats and mice. The differential effects are partly due to the length of the separation, as this had been shown to be proportional to the increasing levels of glucocorticoid secretion in the blood (79).

Significant changes in the development of the hippocampus, an important portion of the brain involved in memory formation, are associated with maternal separation (a minimum of 3 hours daily) (80). In clinical studies involving depression, similar effects were seen as a result of glucocorticoids (81) and HPA hyperactivity (82). This begins to explain the wealth of data of changes in psychological responses such as accentuated stress reactions, exploratory behavior, anxiety, and fear behavior in mouse models (79, 83, 84). Additionally, other neurological changes are present, for example selective suppression of TGF-α mRNA in the mouse pre-frontal cortex (85). TGF-α can act as a growth factor in the brain that may impact the psychological changes. Additionally, there are reports of alterations in reproductive behavior (86). These behavioral changes are a result of the separation, rather than a result of nutritional deficits (87). Interestingly, one aspect that makes early-life stress so potent is its ability to enhance stress reactions, including increased corticosteroid release in adult

life (83). This is termed stress hypersensitivity: the organism responds to stressors more easily and more vigorously (83, 88).

However, in contrast to maternal separation, acute handling of the pups, which involves picking up and stroking the pups for up to 15 minutes, has been shown to have long lasting protective effects on the pups physiology much like acute stress in the adult (79). Unlike chronic separation, acute handling of the pups can reduce the neuroendocrine and behavioral effects of stress later in life in rats, at least up to 26 months of age (79). Additionally, acute handling can reduce age related disturbances of spatial learning and cell loss in the hippocampus, and leads to blunted corticosteroid, ACTH, and CRH release upon stress, likely due to an increase in the number of glucocorticosteroid receptors in the hippocampus and frontal lobe (79, 86). In this way, acute and chronic stresses early in life appear to be similar to the effects later in life, with chronic maternal separation inducing later life depression, anxiety, worsening stress responses, and disease, whereas acute early-life stress has the opposite effect.

c) Metabolic Effects of Early Life Stress

In addition to the psychological effects of maternal separation, there are also some changes in the consumption habits and metabolism of the pups. Maternally separated rats and their dams both increase their consumption of fluids and sucrose solutions later in life compared to unseparated ones (89). In addition to being a result of psychological trauma, the higher levels of corticosteroids induced by maternal separation can induce hyperglycemia, hyperketonemia, increased expression of gluconeogenic and fatty acid oxidation genes in the liver,

and insulin resistance (90). This significantly alters the availability of necessary nutrients to support growth, and contributes to many of the physical effects induced by maternal separation.

d) The Role of the Mother

Not all of these effects from maternal separation are a direct result of the psychological stresses to the pups during the separation. In addition to the pup stress, stress of the mother likely plays an important role in causing permanent physiological changes in the pups, either through alterations in grooming behavior by the dam (87), or through transmission of maternal cytokines and hormones to the pups through milk (91), or in studies that use prenatal stress, *in utero* across the placenta.

The importance of how maternal grooming behavior can be beneficial has been indirectly demonstrated using early-life handling of pups (87). More conclusively however, other studies have shown that with a 3 hour stress period of maternal separation, the effects of separation can be partially inhibited using tactile stimulation of the pups during the separation period (88, 92-96). In mouse models, these studies of tactile stimulation confirm that corticosteroid levels, ACTH levels, and corticosteroid receptor expression revert to levels seen in unseparated mice (88). In addition to showing the importance maternal care has on the well being of the pup, this evidence suggests that the impact of maternal separation stress involves lack of maternal care, and not secondary factors such as loss of nutrition (87). The understanding of the importance of early-life handling has been adapted to clinical settings where it is now used to help

promote growth of isolated premature neonates, stimulating marked gains in weight, behavioral development, and maturation of their sympathetic-adrenal system (97). This also provides a method to revert changes in maternal separation experiments.

The dam can also influence the effects of maternal separation through hormone transmission through feeding and *in utero*. Generally, it is believed that in mothers that are stressed, the HPA axis stimulates the release of various hormones, including CRH and corticosteroids that can inhibit progesterone production. Since progesterone is required to promote a T_H2 response (and inhibit the T_H1 response) required for pregnancy, stress in the mother can inhibit T_H2 cytokines and cells (98). Across the placenta, the presence of T_H1 cytokines and hormones like TNF induce a compensatory stimulation of T_H2 cytokines and cells in the fetus, which has been confirmed in several studies in mice, rhesus monkeys, and humans (98, 99). In addition to this shift towards an allergic T_H2 phenotype in the offspring, stress also inhibits development of the immune system, as well as inhibiting T_H1 responses (98). Additionally, elevated corticosteroids during the final week of gestation can cause permanent increases in baseline corticosteroid concentration later in life (100, 101).

Since activation of the HPA axis and production of corticosteroids from the mother can induce the physiological equivalent of a stress response in the fetus, some models of early-life stress involve stressing the dam (such as providing her with foreign smells or restraint stress) (102, 103), or directly injecting her with synthetic corticosteroids (100). The negative effects of corticosteroids early in

life, inducing stress responses in the pups and leading to premature births and smaller body weights (104, 105), are among the reasons why it is not recommended to give synthetic corticosteroids like dexamethasone to young children, and pregnant or nursing mothers.

IX. Maternal Separation Effects on Disease

Maternal separation has been widely used in models of various diseases to identify the effects of early-life stress and due to the psychological effects that it can induce, in studies of depression.

a) Early-life Stress in Gut-related Diseases

Recent studies have shown that individuals with inflammatory bowel disease (IBD) have rates of depression three fold higher than the general population (106). Rat models of colitis have confirmed that early life stress can induce changes in permeability and ion secretion of gut epithelium that in turn enhance bacterial adherence to the colon (107). This results in bacterial invasion, increased neutrophil and mast cell infiltration into the mucosa, and increased vulnerability to colitis (108). Interestingly, as with models of colitis associated with stress later in life (108), CRH antagonists can inhibit changes induced by maternal separation (107). Additionally, the antidepressant desipramine given daily for 2 weeks after the separation period also inhibits these changes in a mouse model (109).

A common model of inducing gastrointestinal hemorrhage involves restraint stress in rats to stimulate gastric erosions. Rat pups separated from their mothers

by being weaned at day 15 (110) become hypothermic from restraint stress given on day 30 (showing an increase in the stress response) with a five-fold increase in gastric ulcers (91, 111). Interestingly, this is seen when the separation is at day 15, but not at day 22, indicating the importance of early-life in this response.

b) Early-life Stress in Asthma

Unlike gastrointestinal disease, research into the effects of early-life stress on lung diseases is limited. However, clinical observations point towards a detrimental effect of early-life stress. Klinnert *et al.* (1994) studied the effects of parenting and home environment and showed surprising correlations between household stress and susceptibility to asthma in 150 families with a genetic susceptibility (112). While 5% of the children in a low stress household had asthma, this doubled in high stress families and increased five-fold in high stress families with bad parenting (112). This study implies that increasing the household stress experienced by young children can drastically increase their development of asthma. Additional to increasing susceptibility to asthma, early-life stress also can worsen its severity. Wright *et al.* showed that in children 6 to 18 months of age, higher stress levels correlated with increased total IgE levels (113). Another study conducted by Wright *et al.* also showed increased caregiver stress correlated with increased wheezing in infants (114). This and other evidence has led to a call for early treatment of childhood depression because of correlations between stress and asthma severity (115). However the evidence is not unequivocal: there are many studies that have not found a relationship between stress and asthma (116).

Other allergic disorders like atopic eczema, which are associated with asthma (117), have also been shown to be differentially affected by early-life stress. In one study, maternal separation reduced the prevalence of atopic eczema by inhibiting the development of atopy (98), while other studies have shown that early-life stress may increase the prevalence of atopy (118).

No studies indicate that early-life stress is beneficial for human asthma later in life and the majority of studies with children provide evidence that early-life stress is detrimental. To better understand the role and the mechanisms that early-life stress impacts asthma, interventional studies in animals are needed. Accordingly, it is important to develop a model of early-life stress in asthma, so that we may be able to better understand what may lessen, and what may enhance the development and severity of atopy and asthma in children.

X. Early-life Development

Early-life stress may affect asthma through changes in the functionality or responsiveness of the immune system, changes in the development and innervation of the nervous system, or also anatomical changes in lung development. This is because during the crucial period of development, stress can result in changed physiology in these areas. Thus, to understand some of the mechanisms of early-life stress in asthma, it is important to first recognize some of the mechanisms underlying the effects of stress on neonatal development.

a) Immune System

The immune system undergoes a significant shift *in utero* and during early-life that shapes a fetus' immune system throughout its life. Normally, during pregnancy, the mother develops a T_H2 skewed environment in the uterus that inhibits T_H1 cellular immune responses to prevent rejection of the fetus. As explained before, stress can alter this environment towards a T_H1 phenotype. The presence of T_H1 cytokines that are shared across the placenta induce the fetus to begin shifting to a T_H2 skewed environment. This is one of the proposed mechanisms of how maternal stress can impact immune development (119). T_H2 cytokines and cells have been found in the fetus during the third trimester of pregnancy, and remain the dominant immune phenotype for the first couple years after birth. Contact with bacteria, such as those that colonize the gut, in addition to immunological factors in the mother's milk help the immune system to mature and achieve a T_H2 / T_H1 balance (120). In mouse models, it takes about 3 weeks after birth to begin developing an appropriate immune response to *Helicobacter pylori* (121).

In mice, airway hyperresponsiveness and inflammation changes as a result of the age of initial sensitization. As this age increases, there is a gradual shift towards T_H1 responses, marked by increasing levels of IFN-γ and reduced IL-4 and IL-10 secretion in response to airway challenge (99). This correlates with a decrease in airway hyperreactivity and inflammation in these mice over time. Thus since the immune system is under development early in life, disregulation

of the immune response during this time may cause permanent changes in the development of atopy and asthma.

b) Lung Development

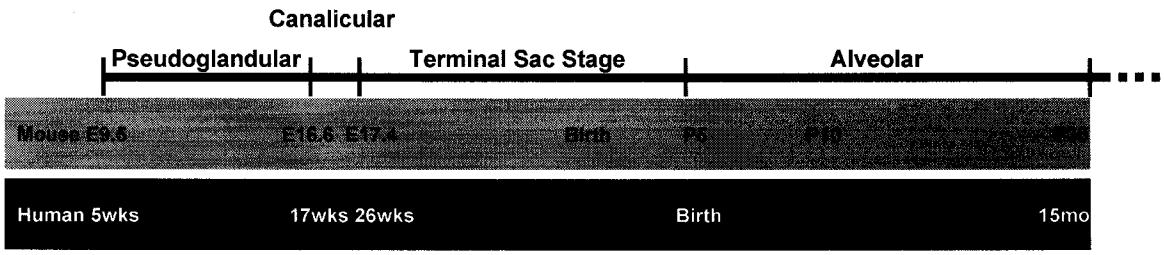
Traditionally, the development of the lung is divided into four main stages based on morphological characteristics (Figure 1-2). The first stage, the pseudoglandular stage, begins at about 5 weeks in gestation in the human or embryonic day (E) 9.5 in the mouse. During this stage, the bronchial and respiratory tree begins to develop and a primordial system begins to form. At about 17 weeks in the human, and E16.6 in the mouse, the canalicular stage evolves where terminal sacs and vascularization develop until 26 weeks in the human and E17.4 in the mouse. During the terminal sac stage (up to birth in the human and postnatal (P) day 5 in the mouse), terminal sacs and vascularization increase and type I and II pneumocytes begin to differentiate. In the final alveolar stage, the terminal sacs mature into alveolar ducts and alveoli. This lasts until P30 in the mouse and about 15 months after birth in the human (122, 123). In the human, this final stage is when 90% of all alveoli are formed after birth (123).

In the alveolar stage in humans and rodents, significant lung growth occurs and it is not surprising that stresses during this period of time can have severe impact on the airways. Glucocorticoids can inhibit epithelial cell division required for alveolar septation and fibroblast production (124). In addition, steroids can reduce capillary growth, which may also inhibit lung development (124). Studies in a rat model have found that dexamethasone injections of the

mother for 4 days postparturition suppresses cell proliferation and impairs alveolar formation for at least the first 2 weeks of life (125). In monkey studies, dexamethasone injections in the mother for 2 weeks, 1 month before term, reduces lung capacity later in life in the offspring (126). Another study has shown maternally separated female rats have greater resting tidal volumes but attenuated hypoxic ventilatory responses, whereas males have no change in tidal volumes at rest, but respond more strongly to hypoxia than unstressed controls (127). This observation identifies a possible role of gender in the physiological effects of maternal separation stress.

Since development of the lungs and alveoli occur up to day 30 in mice or 15 months in humans (histologically), disrupting the development during this period of time may lead to chronic effects that can affect disease later in life.

Figure 1-2: Stages of Lung Development.



Although lung development in the mouse and human are similar, the timelines of the histological stages of development vary.

XI. Hypothesis and Objectives

There is evidence that early-life stress can induce permanent harmful effects on asthma later in life. Thus, it is important to develop a model to study the effects of early-life stress on asthma. Since there are mouse models of asthma, and models of early-life stress using maternal separation, we chose to use these together. In this model, mice would be stressed early in life (postnatal days 1 – 10), and then sensitized to a protein as soon as lung development is completed and the immune system is able to give an optimal response based on the literature above (Day 30). The animals would then be challenged later in life, and tested for airway inflammation, airway hyperresponsiveness, and cytokine as well as corticosteroid levels would also be determined. Additionally, lung morphometry would be conducted to determine if there are any changes in alveolar septation. Thus, we hypothesized that animals stressed early in life will have *increased severity of airway hyperresponsiveness and inflammation.* Additionally, the elevated corticosteroid levels in maternal separated pups (128) will *result in reduced alveolar septation* of the lung. To test these hypotheses, our specific objectives were:

a) Specific Objectives

1. To **develop a model of early-life stress and airway inflammation** in mice. This involved two experiments. First, we confirmed that maternal separation (MS) stress produced measurable changes in weaning weight, a hallmark of early-life stress. Second, we confirmed that 30 day old

mice can be successfully sensitized to an allergen using our protocols, and that this results in increases in airway hyperresponsiveness and eosinophilic inflammation.

2. To **determine the effects of MS stress on a model of airway inflammation** in mice. To do this, we investigated airway inflammation (total and differential cell counts in the lung), airway hyperresponsiveness, serum corticosterone levels, cytokine levels in the airways, and lung morphometry. Our hypothesis was that this stress would cause an increase in airway hyperresponsiveness and inflammation, with an increase in corticosterone levels and a shift to a T_H2 cytokine profile. Additionally, we expected to see a reduction in alveolar septation.
3. To **determine how quality of stress can alter airway inflammation** in mice. For this, an increased severity of maternal separation stress was used, and the same outcomes were measured as in objective #2. We hypothesized that increasing the level of stress would increase airway inflammation and hyperresponsiveness.

Chapter 2: Materials and Methods

I. Animal Handling

Pregnant Balb/c mice that were timed to give birth on the same day were obtained from HSLAS (Health Sciences Laboratory Animal Services) breeding facility at the University of Alberta, on Day 14 of gestation. Locally bred mice were chosen to minimize stress due to shipping of pregnant animals. Mice were housed at one dam per cage in a VAF environment which is maintained on a 12 hour light/dark cycle (lights on from 6:00 am to 6:00 pm), and supplied with food and water *ad libitum*. All mice were housed in plastic cages with a layer of wooden chip nesting material, and a plastic tube for environmental enrichment. Dams were checked daily until birth of pups, usually between Day 19 – 20 of gestation. All experiments and procedures described were approved by the University of Alberta Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care.

a) Maternal Separation

At birth, litters (with dams) were randomly assigned to one of two groups: Maternal Separation (MS) or unhandled controls (CON). MS dams were removed from their cages for 3 hours daily for the first 10 days after birth (day 1–10). The separation was done between 1:00 pm and 4:00 pm. MS was performed at the same time each day to ensure that diurnal cycles, which fluctuate based upon time of day, do not impact results. During separation the dams were placed individually in separate, clean cages with water and food *ad libitum*. These cages were placed in a separate room away from their pups for the

duration of the separation. The original cages with the pups were placed on a circulating water heating pad maintained at a constant 38°C. CON animals (both dams and pups) were undisturbed during this period. For consistency between experiments and to minimize stress from handling, all cages were changed only on day 10 after birth immediately after the final MS. CON animals were also only changed on day 10.

b) Enhanced Maternal Separation

A different maternal separation protocol (called MS+) was used for certain experiments. This protocol involved MS dams being removed from their cages for a daily period of 3 hours for the first 10 days after birth (day 1–10). During the separation, they were housed in separate, clean cages with water and food *ad libitum*. It was noted that if the pups were moved as a group, they often became active whereas if the pups were moved one pup at a time from one cage to another, they usually remained docile. Accordingly, the second method was chosen to minimize disruption. So the pups were individually removed from their home cage and placed together into a separate clean cage on a circulating water heating pad maintained at 38°C for the duration of the separation. After the 3 hour period was complete, the pups were returned individually into their home cage nest, followed by the return of the dam immediately afterwards. As above, all cages were changed on day 10.

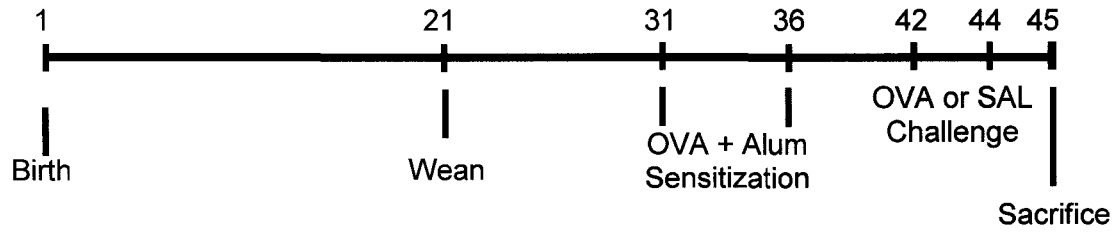
c) Weaning

Weaning was done by animal facility staff when the pups were 21 days old. All mice were weighed, sexed, and randomly assigned to cages within their MS, MS+, or CON group. Mice were housed at a density of 5 per cage in sex segregated rooms. From this point onwards, all animals were checked and their cages were opened for ventilation daily. Animals received food and water *ad libitum*, and cages were changed weekly.

d) Immunization and Airway Challenge with an Antigen

Since lung development is histologically complete as of Day 30, Day 31 was used as the start date for immunization. On days 31 and 36, animals were sensitized via intra-peritoneal (IP) injection of chicken egg ovalbumin (OVA; 10 μg) and $Al(OH)_3$ (Alum; 2 mg) suspended in 500 μl of 0.9% saline. On days 42 and 44, mice were challenged intranasally with either 50 μg OVA in 25 μl of 0.9% saline or 25 μl of saline as a negative control. This was done by first anaesthetizing the animals IP with 7.5% ketamine (100 mg/ml) and 2.5% acepromazine (10 mg/ml) in a total volume of 200 μl 0.9% saline.

Control Group (CON)



Maternal Separation Group (MS)

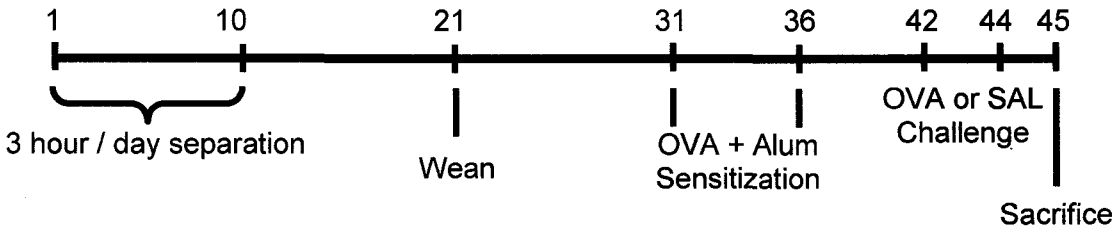


Figure 2-1: Protocol for Maternal Separation, Immunization, and Allergen Challenge.

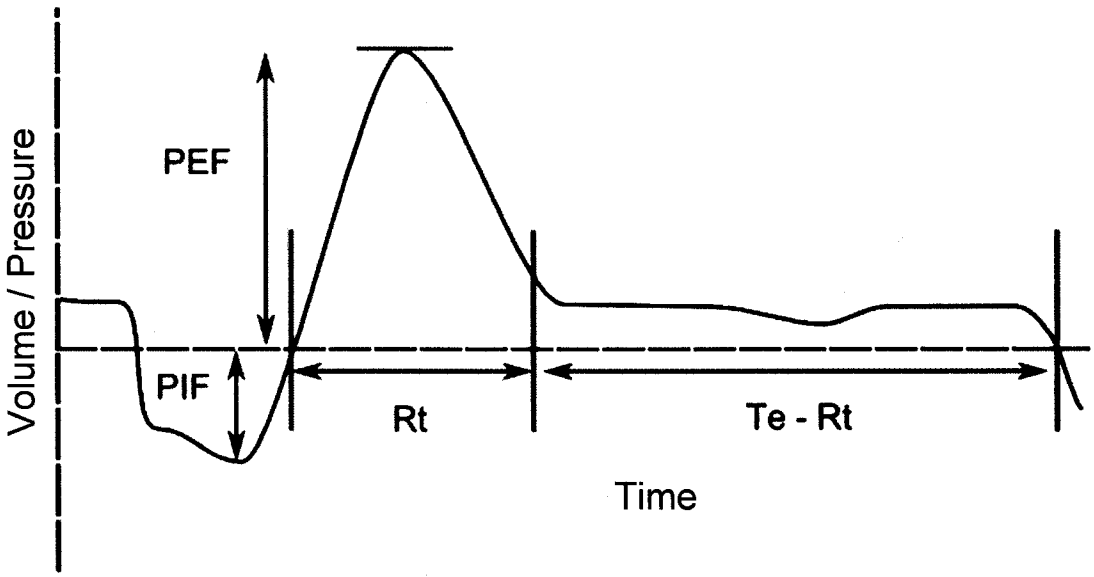
During day 1 – 10 after birth, "stress" group moms are removed from their pups for 3 hours daily. Unstressed controls are left untouched. All animals (stress group and unstressed controls) are weaned at day 21. OVA and Aluminum hydroxide (alum) sensitization by IP injection occurred at day 31 and 36. Intranasal challenges with OVA or saline occurred at day 42 and 44. Animals were sacrificed at day 45 after measurement of airway hyperresponsiveness.

II. Outcomes

a) Airway Hyperresponsiveness

On day 45 (the day after the final challenge with OVA or Saline (SAL)), airway hyperresponsiveness was determined using the BUXCO whole body plethysmography (WBP) (Buxco Electronics, NY, USA) system that calculates a dimensionless variable Penh (enhanced Pause, Figure 2-2) (129).

In Balb/c mice, Penh correlates strongly with airway resistance in OVA sensitization-challenge models and changes in Penh are good markers for AHR (130). Additionally, this methodology allows for relatively free movement of the mice in the chambers, which likely reduces stress compared to restraint based methods. Increasing doses of methacholine (0 – 32 mg/ml) were nebulized into the closed chamber system with the mice inside, and a dose-response curve of methacholine on Penh was generated. Prior to starting the experiment, the BUXCO chambers (where the mice are placed) were flushed for 30 minutes with saline to remove any traces of methacholine in the system. Additionally, prior to each dose of methacholine, a 10 minute period of nebulized saline was used to allow Penh values of the mice to return to baseline values.



$$Penh = \frac{PEF}{PIF} \times \left(\frac{Te}{Rt} - 1 \right)$$

Figure 2-2: Enhanced Pause.

Penh **is enhanced pause,** ***PIF*** **is the peak inspiratory volume,** ***PEF*** **is the peak expiratory volume,** ***Te*** **is the expiration time, and** ***Rt*** **is the time to expire 65% of total breath volume. The above diagram is an approximation of data obtained through the BUXCO whole body plethysmography system during a single breath recorded by pressure electrodes attached to a closed chamber where the animal resides.**

b) Airway Inflammation

Twenty-four hours after the last allergen challenge (Figure 2-1) mice were euthanized with an overdose of 50% v/v ketamine (100 mg/ml) and 50% rompin v/v (20 mg/ml). After cardiac puncture to remove blood and ensure death of each mouse, the trachea was exposed via dissection and was catheterized using a polyethylene tube attached to a 21 gauge needle. Bronchoalveolar lavage (BAL) was performed using 5 aliquots of 0.8 ml and 1 aliquot of 1.0 ml totaling 5 ml of cold sterile PBS (137 mM NaCl, 3.6 mM KCl, 2.0 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 897 μM $CaCl_2$, 491 μM $MgCl_2$, pH 7.2). Once the BAL fluid was collected, the cells were spun down at 300 x g for 15 minutes and 1.0 ml of BAL supernatant is saved for cytokine analysis. The cells were washed with PBS, spun again at 300 x g, and resuspended into a final volume of 1.0 ml of PBS. The total numbers of viable cells were determined by staining with trypan blue and counting using a hemocytometer. Slides were prepared from these cells using a Cytospin (Thermo Shandon, PA, USA) and stained using DiffQuick stain (Biochemical Sciences, NJ, USA) that fixed the cells and stained them to allow for identification based on morphological characteristics (131). Differential cell counts were performed by counting 100 cells per slide, one slide per BAL sample. In these cell counts alveolar macrophages, lymphocytes, neutrophils, and eosinophils are counted based on morphology.

c) BAL Cytokine Determinations

IL-4, 5, 6, 9, 13 and IFN-γ from BAL fluid were analyzed by Dr. John Gordon (Department of Veterinary Microbiology, University of Saskatchewan) using commercially available ELISA kits (BD Biosciences, San Jose, United States) (66, 132) which have a detection sensitivity of 2 – 5 pg/ml.

d) Determining Corticosterone Concentrations

To determine corticosterone concentrations, serum was first extracted from blood collected by cardiac puncture on Day 45, and spun down at 8000 x g for 10 minutes. The collected serum was used in a Radioimmunosorbent assay (MP Biomedicals, Solon, United States).

e) Determining OVA Specific IgE concentrations

OVA-specific IgE levels were determined by Dr. John Gordon (Department of Veterinary Microbiology, University of Saskatchewan) from the same serum samples as the corticosterone assays above. The assay involved coating 96-well plates with anti-IgE, then a blocking solution of Phosphate buffered saline with 10% fetal calf serum. This was followed by adding biotinylated OVA and the serum samples. After a 90min incubation, streptavidin-horse radish peroxidase (HRP) was added and incubated for 30min. 2-2'-azino-di[3-ethyl-benzthiazoline sulfonate], the substrate for HRP was added and absorbance was determined by a plate reader at 405 nm.

f) Lung Morphometry

On day 30, some MS and CON animals were euthanized by an intra-peritoneal overdose of 200 µl of a 50% v/v ketamine (100 mg/ml) and rompin (20 mg/ml) solution. Dissection of the abdomen was carried out to puncture the diaphragm to deflate the lungs and sever the hepatic veins to drain blood. Using catheterization as described for BAL, a tube was inserted into the trachea, and a suture used to form a tight seal. The lungs were filled with 10% formalin under 25 cm H_2O pressure maintained for 5 minutes to allow fixation. The trachea was tied off, the lungs removed from the chest cavity via dissection, and then the lungs were immersed in 10% formalin for at least 24 hours (maximum 36 hours). Afterwards, the left lobe of the lung was removed, embedded in paraffin, sectioned, and stained with hematoxylin & eosin staining. A G4 Macintosh computer preloaded with Adobe Photoshop CS (Adobe Systems, Inc.) was used to scan the sections and generate 100 random images per lung section where 156 µm lines were placed in the center of each image. The number of alveolar wall (septa) intersections per line was counted. When the length of the line was divided by the number of intersections per line, a mean linear intercept value was obtained (133).

g) Statistical Analyses

Statistical analyses were performed using the Prism 3 software suite (GraphPad Software Inc). When two groups were compared, the student's t-test was used. For comparing more than two groups, one way ANOVA was used for

statistical comparisons. To compensate for inter-experimental variability, experiments involving counting total inflammatory cells were normalized. The total number of cells in each mouse was divided by the average inflammatory cells present in the CON OVA/OVA group within each individual experiment. This allowed for the standardization of each experiment to minimize variability between experiments without losing intraexperimental variability.

Additionally, two-way ANOVA was used for comparing the effects of stress on total cell inflammation taking into account different experiments. All values are reported as mean (± standard error); a p-value < 0.05 was considered statistically significant.

Chapter 3: Developing the Models

I. Introduction

Since asthma is a disease that impacts many aspects of the physiology of an individual, it is imperative to study it holistically in a complete organism. In an effort to do that, we used a mouse model of airway inflammation and airway hyperresponsiveness that is used widely to study asthma in animals. The first goal of this project was to develop a general model of early-life stress in asthma using an animal model. Several possible models that have been used were described in the first chapter, for both the stress component and the allergen challenge component.

Maternal separation stress was chosen as it is well characterized in animal models, and is one of the best characterized models of early life stress in mice. Additionally, unlike other models of stress that occur either prenatally or several days or weeks after birth in mice, the stress as a result of maternal separation for days 1-10 occurs during the time when lung development is equivalent to late-pregnancy and the first several months of life in humans (Figure 1-2). The stress during this period of time has been shown to induce significant physical, psychological, and immunological effects which in human studies appear to lead to the increased risk and severity of asthma.

The OVA challenge model is a common and effective model in the field of asthma to induce hyperresponsiveness and inflammation in rodent airways. In essence, it involves sensitizing mice to an allergen (usually chicken egg ovalbumin, OVA) via an IP injection along with a T_H2 stimulating adjuvant. This results in a T_H2 immune response. When the animal is challenged, usually

at least a week later intranasally, an eosinophilic immune response is generated against the protein. With lung development complete by Day 30, and literature suggesting a strong T_H2 immune response against the OVA protein in mice sensitized 1 week after birth, day 31 was chosen to begin sensitization of the mice.

II. Results

a) Maternal Separation

Several studies, using maternal separation, have shown that one interesting effect is the reduction in weight of the animals (98, 134). This is believed to be a result of the stress-induced chronically increased levels of corticosterone, and the detrimental effect that the corticosteroid has on tissue growth. Accordingly, to ensure that our model of maternal separation was successful in inducing early-life stress, animals were weighed at weaning (day 21). In the sample experiment shown, weights at weaning remained significantly lower in MS group mice in comparison to CON mice in both males ($p = 0.0008$) and females ($p = 0.024$). In addition, we found that the reduction in weight remained when reweighed about 20 days later (males: $p < 0.0001$; females: $p = 0.001$; Figure 3-1). Overall, across all experiments conducted, the average drop in weight for MS mice at weaning was a significant $7.8 \pm 3.2\%$ (3 male, 7 female experiments, Tables 4-1 and 4-2). The enhanced maternal separation (MS+) protocol that will be used in chapter 5 also noted a statistically significant drop in weaning weight ($11.34\ g \pm 0.19\ g$ in CON to $10.54\ g \pm 0.22\ g$ in MS+ mice; $p = 0.013$; $n = 19$).

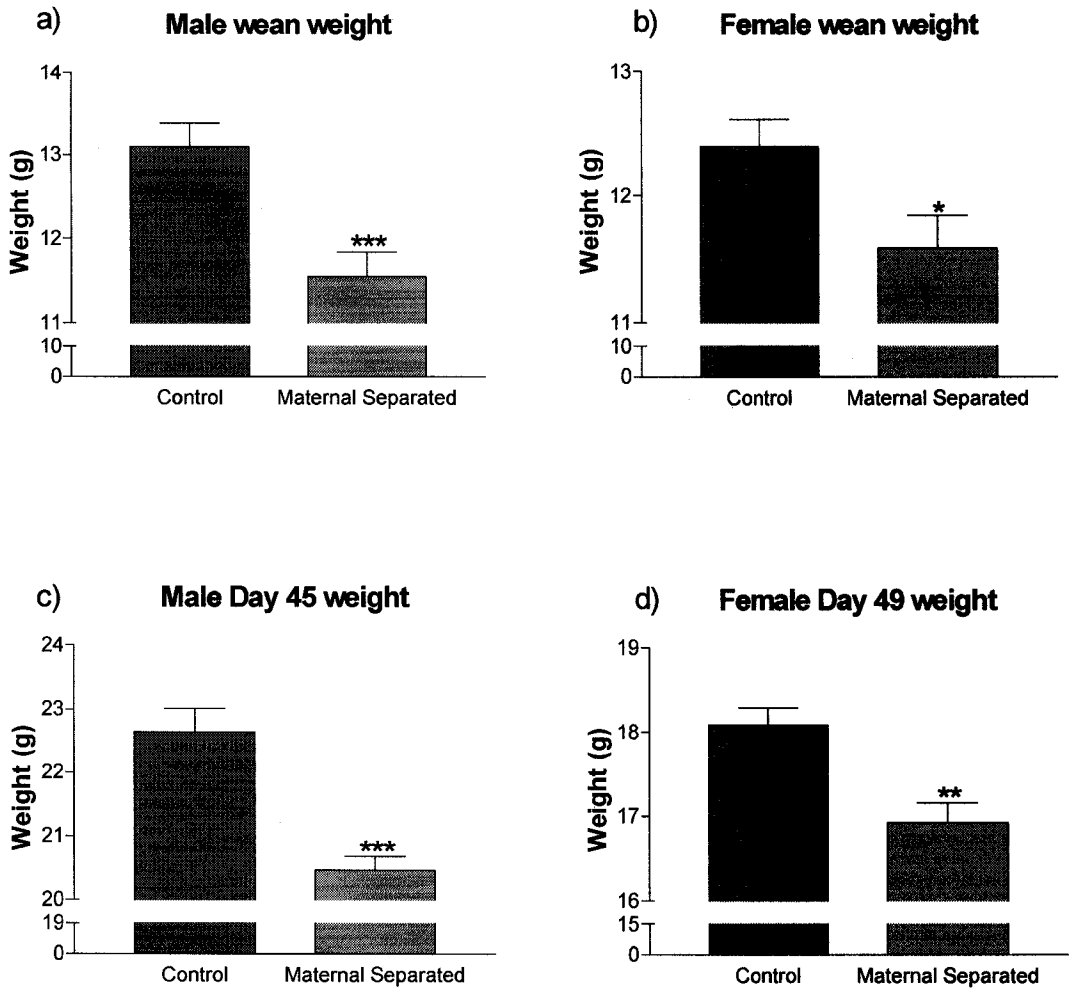


Figure 3-1: Representative Sample of Weights from One Experiment.

Weights were taken at weaning in males (a) and females (b). The Maternal separation group in both cases had lower weaning weights (n = 14). Additionally, weights upon sacrifice remained significantly lower in MS mice in both males (c) and females (d). One star indicates $p < 0.05$, two stars indicate $p < 0.01$, and three stars indicate $p < 0.001$ via student's T-test.

b) OVA-Dependent Inflammation in Young Mice

We wanted to make sure that young mice, 31 days old when sensitized, would be able to be sensitized and challenged to OVA and generate a classic immune response with airway inflammation and hyperresponsiveness. Thus, young Balb/c mice were ordered and the protocol was carried out as described, beginning the first sensitization at day 31. The outcomes that were looked at were airway hyperresponsiveness and inflammation in the BAL fluid. As expected, the mice that were challenged with OVA experienced an influx of eosinophils ($p < 0.01$) and an increase in airway responsiveness ($p < 0.05$) (Figure 3-2).

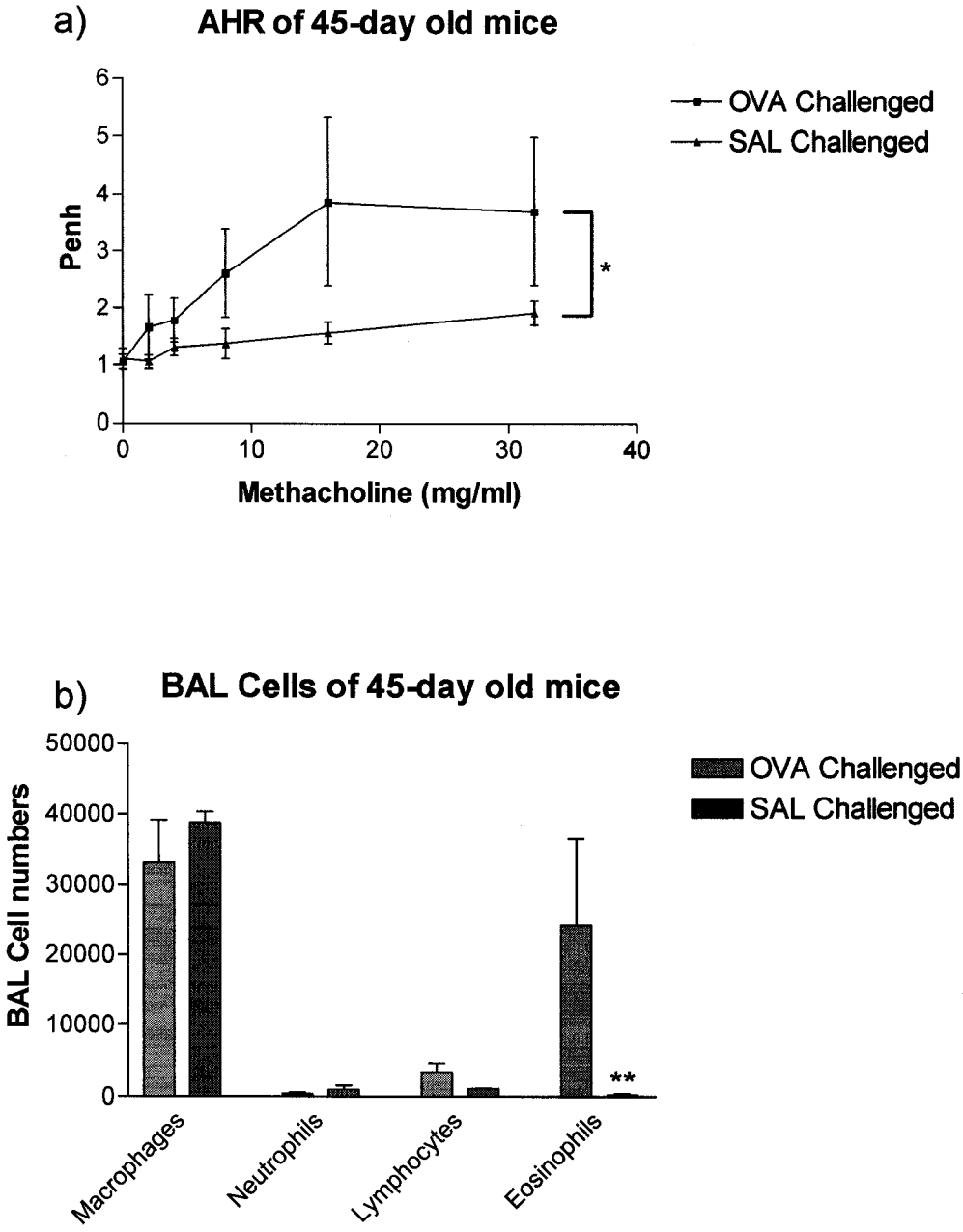


Figure 3-2: Airway Hyperresponsiveness and Inflammation in BAL Fluid in 45 Day Old OVA Sensitized and Challenged Mice.

Penh (a) and total BAL cell numbers (b) in mice sensitized to OVA and challenged with OVA or SAL. Penh increased as a result of allergen challenge due to a methacholine challenge ($p < 0.05$, $n = 4$, ANOVA). Also, there was an increase in eosinophils that entered the BAL fluid ($p < 0.01$, $n = 4$, ANOVA) as a result of OVA challenge.

Chapter 4: The Effect of Maternal Separation on Airway Inflammation in Mice

I. Introduction

In an effort to study asthma in the complete organism, we used a mouse model of airway inflammation and airway hyperresponsiveness that is used widely to study asthma in animals. We sensitized mice to OVA in the presence of a T_H2 adjuvant, aluminum hydroxide, and challenged with the same allergen intranasally. This produces eosinophilic airway inflammation, airway hyperresponsiveness, and in long term experiments airway remodeling, which are all characteristics of chronic asthma in humans.

To look at how early-life stress can impact asthma, we used maternal separation from the pups for 3 hours / day to investigate how early-life stress can impact the psychology and physiology of rodents. As explained above, the maternal separation stress period (birth to day 10) for mouse lung development correlates with similar development that occurs in late pregnancy to the first few months after birth in the human (122, 123). Since mouse lung development is complete at day 30 (122) and allergen sensitization can begin even earlier (99), using mice in maternal separation allows us to develop a model that may mimic the effects of early-life stress in human asthma.

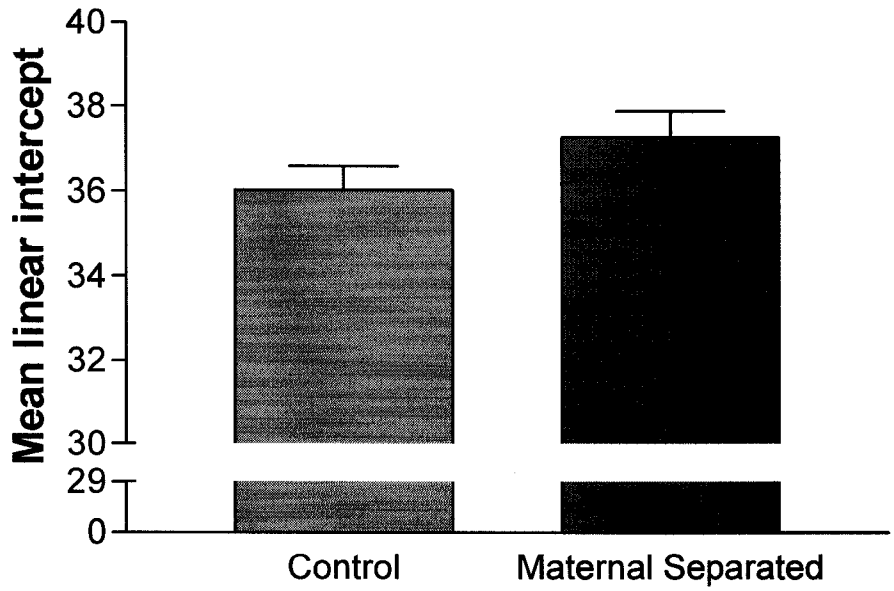
First, we looked at the effects of stress on lung morphometry by looking at mean linear intercept, a measure of alveolar septation in the lung. We then studied the effects of the MS protocol on airway inflammatory cells, airway hyperresponsiveness, corticosterone levels in the blood, and cytokine levels.

II. Results

a) Lung morphometry

In order to determine if the MS protocol alters development of the lung, we first decided to look at any effects to alveolar structure. Thus morphometry was done. Thirty day old mice, 4 males and 5 females in each group, were euthanized and their lungs were fixed. Sections were stained using hematoxylin and eosin, and alveolar septation was determined using mean linear intercept (MLI) via a semi automated method (135). Although there appeared to be a slight increase in MLI in MS mice, it was not statistically different (Figure 4-1).

Figure 4-1: Effect of Maternal Separation on Mean Linear Intercept.



Lung morphometry was determined using mean linear intercept (μm). Bars represent mean values ± SE (n = 9). Although there is a trend towards a higher MLI in MS mice, the difference was not statistically significant using a student's T-test.

b) Airway Hyperresponsiveness

One of the primary measures used in this mouse model is airway reactivity, or hyperresponsiveness (AHR). This is a measure of the airway smooth muscle sensitivity in response to a bronchoconstrictor. Humans with asthma have greater sensitivity to bronchoconstriction agents compared to normal individuals, hence they are hyperresponsive. Twenty-four hours after the second allergen challenge (Figure 2-1), AHR was determined using whole body-plethysmography (WBP). In short, saline (SAL) or chicken egg ovalbumin (OVA) challenged animals were placed inside closed chambers while increasing doses of aerosolized methacholine were pumped into the chambers. Changes in the breathing of the animals detected by a transducer were converted into a dimensionless outcome measure, enhanced pause (Penh).

Both male and female control (CON) animals sensitized and challenged with OVA showed a significant increase in Penh in comparison to SAL challenged animals ($p = 0.0075$ in males, $p = 0.0006$ in females; Figure 4-2). The pattern of increased Penh in OVA sensitized and challenged animals was also seen in the maternally separated (MS) animals ($p = 0.0030$ in males, $p = 0.0048$ in females; Figure 4-2). However, there were no statistical differences between MS and CON animals within each stress group. Also, at 0 mg/ml of methacholine, the Penh between all groups were similar, indicating that OVA challenge did not influence baseline airway tone.

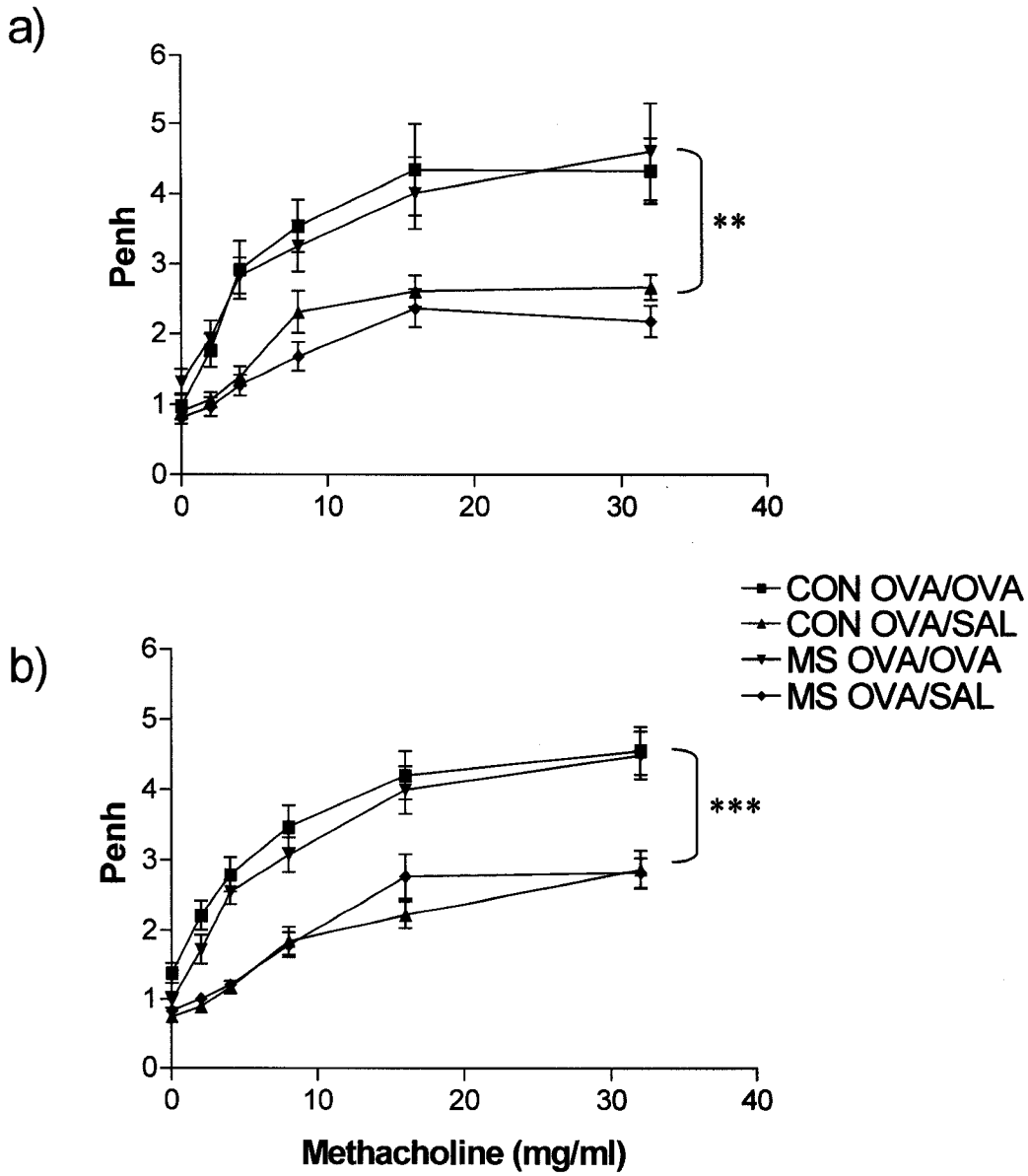


Figure 4-2: The Effect of Early-life Stress on Airway Hyperresponsiveness in MS and CON mice sensitized with OVA and challenged with OVA or SAL.

Penh response of male (a) and female (b) Balb/c mice to increasing levels of methacholine challenge in MS and CON mice. Mice were sensitized with OVA and challenged with OVA or SAL. Each point represents the mean ± SE (n = 15). Two stars indicate a statistical difference of $p < 0.01$, three stars indicate $p < 0.001$.

c) Airway Inflammation

Another characteristic of this mouse asthma model is the influx of inflammatory cells, especially eosinophils, into the airways. Immediately after determining AHR, mice were euthanized and bronchoalveolar lavage (BAL) was performed to obtain cells from the airways. Since there was variability in the total numbers of cells obtained between experiments, the numbers of cells were normalized to the average number of cells obtained in the CON OVA sensitized and challenged mice. Thus the average CON OVA was set to 100% (Tables 4-1 and 4-2).

OVA sensitized, SAL challenged controls were used as comparisons to the OVA sensitized and challenged mice. In all cases, the numbers of cells in OVA sensitized and challenged animals were significantly higher than SAL challenged animals (Tables 4-1 and 4-2). However, in MS males sensitized and challenged with OVA, the total number of cells recovered was 58% (± 11%) of the control values, whereas in females cell recovery was 67% (± 7%) of the number of cells in control animals (Figure 4-3). Thus there were significantly less cells in BAL in both males ($p = 0.012$) and females ($p = 0.015$) that had experienced MS as pups compared to CON. Additionally, two-way ANOVA analysis was performed using the total BAL cell numbers (Tables 4-1 and 4-2) which additionally accounted for variations in cell numbers as a result of the date of each experiment. With this statistical analysis, significant reductions in cell numbers were also found in MS males ($p = 0.0008$) and females ($p = 0.0082$).

Table 4-1: BAL Cell Numbers of Individual Male Balb/c mice

	CON OVA/OVA	MS OVA/OVA	CON OVA/SAL	MS OVA/SAL	CON OVA/OVA (%)	MS OVA/OVA (%)	CON OVA/SAL (%)	MS OVA/SAL (%)
Experiment #1	280,000	145,000	25,000	10,000	92.8%	48.1%	8.3%	3.3%
	200,000	300,000	80,000	40,000	66.3%	99.5%	26.5%	13.3%
	425,000	440,000	70,000	10,000	140.9%	145.9%	23.2%	3.3%
Mean	**301,667**	**295,000**	**58,333**	**20,000**	**100.0%**	**97.8%**	**19.3%**	**6.6%**
SE	**65,849**	**85,196**	**16,915**	**10,000**	**21.8%**	**28.2%**	**5.6%**	**3.3%**
Experiment #2	470,000	295,000	85,000	70,000	68.5%	43.0%	12.4%	10.2%
	635,000	510,000	30,000	55,000	92.5%	74.3%	4.4%	8.0%
	955,000	70,000	135,000	55,000	139.1%	10.2%	19.7%	8.0%
Mean	**686,667**	**291,667**	**83,333**	**60,000**	**100.0%**	**42.5%**	**12.1%**	**8.7%**
SE	**142,371**	**127,028**	**30,322**	**5,000**	**20.7%**	**18.5%**	**4.4%**	**0.7%**
Experiment #3	115,000	80,000	35,000	35,000	49.5%	34.5%	15.1%	15.1%
	115,000	45,000	25,000	50,000	49.5%	19.4%	10.8%	21.5%
	350,000	90,000	20,000	25,000	150.8%	38.8%	8.6%	10.8%
	315,000	170,000	20,000	55,000	135.7%	73.2%	8.6%	23.7%
	320,000	200,000	15,000	30,000	137.9%	86.2%	6.5%	12.9%
	195,000	80,000	20,000	30,000	84.0%	34.5%	8.6%	12.9%
	215,000		50,000	35,000	92.6%		21.5%	15.1%
Mean	**232,143**	**110,833**	**26,429**	**37,143**	**100.0%**	**47.7%**	**11.4%**	**16.0%**
SE	**37,031**	**24,577**	**4,592**	**4,206**	**18.8%**	**10.6%**	**2.0%**	**1.8%**
OVERALL MEAN	**353,077**	**202,083**	**46,923**	**38,462**	**100.0%**	**58.9%**	**13.4%**	**12.2%**
OVERALL SE	**69,015**	**44,246**	**9,994**	**5,012**	**10.2%**	**11.2%**	**7.1%**	**6.0%**

Total cell counts in BAL from MS and CON Males. Each value represents BAL from a single mouse, with means and standard error (SE) provided for each individual experiment conducted. Overall mean and standard error are for all mice in each column. Percentages are in reference to the average CON OVA/OVA cell number in each experiment.

Table 4-2: BAL Cell Numbers of Individual Female Balb/c mice

	CON OVA/OVA	MS OVA/OVA	CON OVA/SAL	MS OVA/SAL	CON OVA/OVA (%)	MS OVA/OVA (%)	CON OVA/SAL (%)	MS OVA/SAL (%)
Experiment #1	1,010,000	205,000	25,000	25,000	270.5%	54.9%	6.7%	6.7%
	180,000	475,000	45,000	35,000	48.2%	127.2%	12.1%	9.4%
	185,000	275,000	45,000	70,000	49.6%	73.7%	12.1%	18.8%
	335,000	145,000	45,000	25,000	89.7%	38.8%	12.1%	6.7%
	290,000	150,000	35,000	25,000	77.7%	40.2%	9.4%	6.7%
	240,000	475,000	25,000	75,000	64.3%	127.2%	6.7%	20.1%
Mean	**373,333**	**287,500**	**36,667**	**42,500**	**100.0%**	**77.0%**	**9.8%**	**11.4%**
SE	**129,664**	**62,313**	**4,014**	**9,639**	**34.7%**	**16.7%**	**1.1%**	**2.6%**
Experiment #2	185,000	105,000	85,000	85,000	89.2%	50.6%	41.0%	41.0%
	230,000				110.8%			
Mean	**207,500**	**105,000**	**85,000**	**85,000**	**100.0%**	**50.6%**	**41.0%**	**41.0%**
SE	**22,500**	**N/A**	**N/A**	**N/A**	**10.8%**	**N/A**	**N/A**	**N/A**
Experiment #3	1,520,000	655,000	220,000	60,000	107.7%	46.4%	15.6%	4.3%
	1,160,000	955,000	345,000	200,000	82.2%	67.7%	24.4%	14.2%
	1,555,000	1,010,000	355,000	175,000	110.2%	71.6%	25.2%	12.4%
Mean	**1,411,667**	**873,333**	**306,667**	**145,000**	**100.0%**	**61.9%**	**21.7%**	**10.3%**
SE	**126,238**	**110,315**	**43,429**	**43,108**	**8.9%**	**7.8%**	**3.1%**	**3.1%**
Experiment #4	895,000	345,000	90,000	80,000	113.3%	43.7%	11.4%	10.1%
	620,000	245,000	100,000	30,000	78.5%	31.0%	12.7%	3.8%
	855,000			295,000	108.2%			37.3%
Mean	**790,000**	**295,000**	**95,000**	**135,000**	**100.0%**	**37.3%**	**12.0%**	**17.1%**
SE	**85,781**	**50,000**	**5,000**	**81,292**	**10.9%**	**6.3%**	**0.6%**	**10.3%**
Experiment #5	155,000	85,000	10,000	15,000	54.8%	30.1%	3.5%	5.3%
	220,000	205,000	30,000	5,000	77.8%	72.5%	10.6%	1.8%
	75,000	275,000	15,000	75,000	26.5%	97.2%	5.3%	26.5%
	130,000	445,000	20,000	55,000	46.0%	157.3%	7.1%	19.4%
	635,000	310,000	20,000	40,000	224.5%	109.6%	7.1%	14.1%
	565,000	95,000	60,000	55,000	199.8%	33.6%	21.2%	19.4%
	200,000		10,000	20,000	70.7%		3.5%	7.1%
Mean	**282,857**	**235,833**	**23,571**	**37,857**	**100.0%**	**83.4%**	**8.3%**	**13.4%**
SE	**84,140**	**56,072**	**6,611**	**9,627**	**34.7%**	**19.8%**	**2.3%**	**3.4%**
Experiment #6	350,000	380,000	15,000	35,000	111.1%	120.6%	4.8%	11.1%
	135,000	225,000	35,000	10,000	42.9%	71.4%	11.1%	3.2%
	605,000	115,000	20,000	15,000	192.1%	36.5%	6.4%	4.8%
	170,000	210,000	35,000	25,000	54.0%	66.7%	11.1%	7.9%
Mean	**315,000**	**232,500**	**26,250**	**21,250**	**100.0%**	**73.8%**	**8.3%**	**6.8%**
SE	**107,529**	**54,867**	**5,154**	**5,543**	**34.1%**	**17.4%**	**1.6%**	**1.8%**
Experiment #7	90,000	80,000	15,000	15,000	46.2%	41.0%	7.7%	7.7%
	295,000	120,000	45,000	15,000	151.3%	61.5%	23.1%	7.7%
	220,000	90,000			112.8%	46.2%		
	175,000	65,000			89.7%	33.3%		
Mean	**195,000**	**88,750**	**30,000**	**15,000**	**100.0%**	**45.5%**	**15.4%**	**7.7%**
SE	**42,866**	**11,614**	**15,000**	**0**	**22.0%**	**6.0%**	**7.7%**	**0.0%**
OVERALL MEAN	**457,931**	**297,692**	**69,800**	**60,769**	**100.0%**	**67.3%**	**12.5%**	**11.6%**
OVERALL SE	**77,715**	**49,194**	**18,630**	**13,228**	**10.8%**	**6.9%**	**1.7%**	**1.7%**

(For Table 4-2) Total cell counts in BAL from MS and CON Females. Each value represents BAL from a single mouse, with means and standard error (SE) provided for each individual experiment conducted. Overall mean and standard error are for all mice in each column. Percentages are in reference to the average CON OVA/OVA cell number in each experiment.

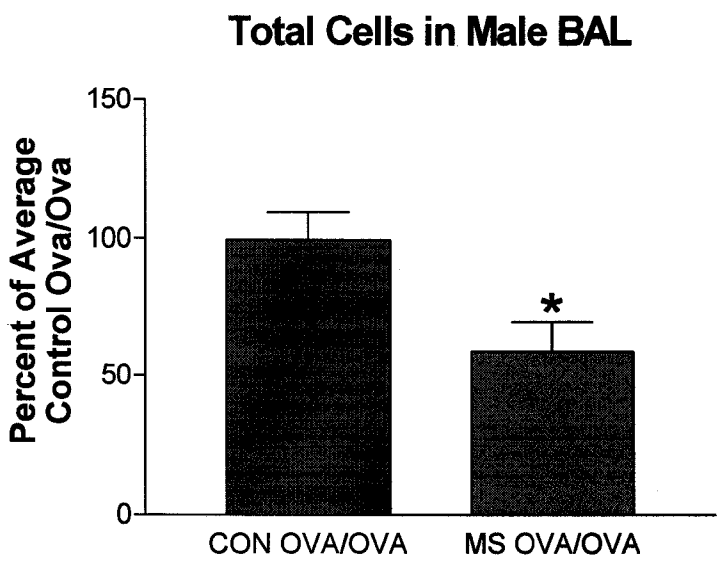
Table 4-3: Summary of Total Inflammatory Cell Count in Sensitized and Challenged Mouse BAL.

Stress	Gender	Mean total cells	Standard error
Control (n = 13)	Male	353,077	69,015
Maternal Separation (n = 12)	Male	202,083	44,246
Control (n = 29)	Female	457,941	77,715
Maternal Separation (n = 26)	Female	297,692	49,194

Table 4-4: Summary of Differential Inflammatory Cell Count in BAL (male and female; n = 11 each)

Stress	Macrophages	Neutrophils	Lymphocytes	Eosinophils
Control OVA/OVA	119,478 ± 26,706	18,361 ± 6,345	3,003 ± 1,371	310,364 ± 56,970
Maternal Separation OVA/OVA	102,282 ± 17,046	14,848 ± 5,892	3,588 ± 1,001	224,056 ± 37,945
Control OVA/SAL	79,708 ± 15,835	4,569 ± 2,222	755 ± 318	7,259 ± 1,895
Maternal Separation OVA/SAL	111,520 ± 40,362	1,738 ± 475	3,255 ± 3,072	1,986 ± 789

a)



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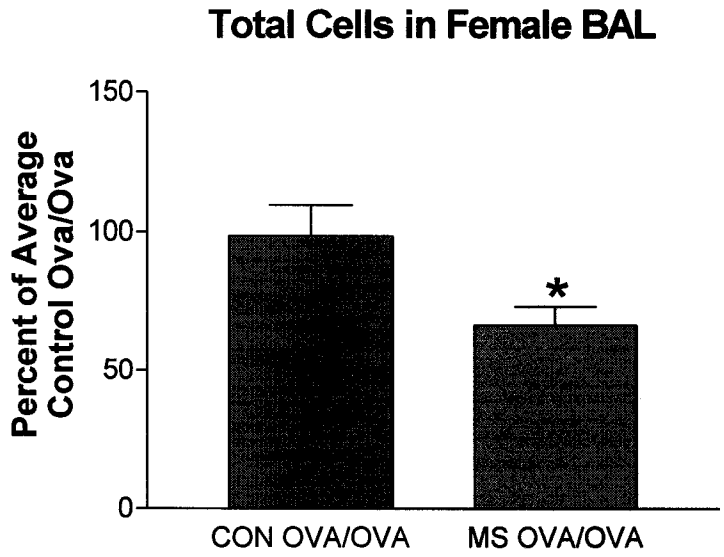


Figure 4-3: The Effect of Early-life Stress on Total Cells in BAL of OVA Sensitized and Challenged Mice.

MS animals, both male (a) and female (b) showed decreased number of cells obtained from BAL 24 hours after OVA challenge compared to CON. Each bar represents the mean ± SE (males: n = 12; females: n = 26). MS animals showed a significant ($p < 0.05$, one star) decrease in total BAL cells in comparison to controls in both males and females using a student's T-test.

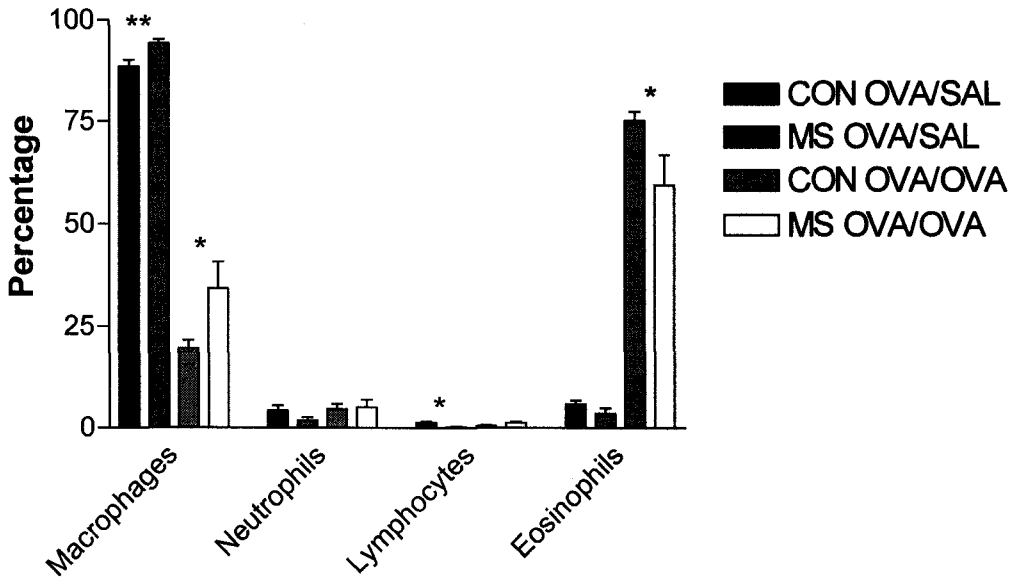
Differential cell counts were also performed on the BAL fluid. In all cases, challenging with OVA following OVA sensitization caused a significant increase in the percentage of eosinophils, and a proportional drop in the percentage of alveolar macrophages (Figure 4-4). What was interesting however was that MS mice had a reduction in the percentage of eosinophils in comparison to control animals and a proportional increase in the percentage of macrophages in their BAL fluid as a result of OVA challenge (Figure 4-4). However, as expected, due to the reduction in total cells in the MS mice, this change in ratios resulted in little change in the total macrophages present in the BAL fluid (Table 4-4). Additionally, the absolute numbers of eosinophils showed a trend to lower numbers in MS mice (Table 4-4).

d) Cytokines and OVA-Specific IgE

The change in cell recruitment to the lungs in MS animals could be a result of changes in cytokine profile. Thus, we investigated the cytokine profile in BAL fluid. A broad range of cytokines that could potentially have impact on airway inflammation were studied, looking specifically at a potential shift in a T_H1 vs. T_H2 profile. If such alterations are present in the cytokine profile, they may explain the reduction in eosinophils in the lungs. Accordingly, ELISAs were performed on IL-4, 5, 6, 9, 13, and IFN-γ. Interestingly, there was a significant drop in IL-4 and IFN-γ levels in MS mice sensitized and challenged with OVA in comparison to similarly challenged CON mice, but no change in IL-5, 6, 9, and 13 between the same groups (Figure 4-5).

Additionally, OVA-specific IgE antibody titres (measured as OD_{450}) were looked at in the serum using ELISA; however no significant differences were seen between the groups (n = 14; CON OVA/OVA: 0.234 ± 0.007, MS OVA/OVA: 0.237 ± 0.005, p = 0.784).

Figure 4-4: Differential BAL Counts of Cells in MS and CON Animals after Challenge with OVA or SAL as a Percentage of Total BAL Cells.



Bars represent the mean ± SE percentage of cells in BAL fluid of male and female Balb/c mice (n = 11). One star indicates statistical significance with $p < 0.05$, and two stars $p < 0.01$ between CON and MS of each challenge group using ANOVA.

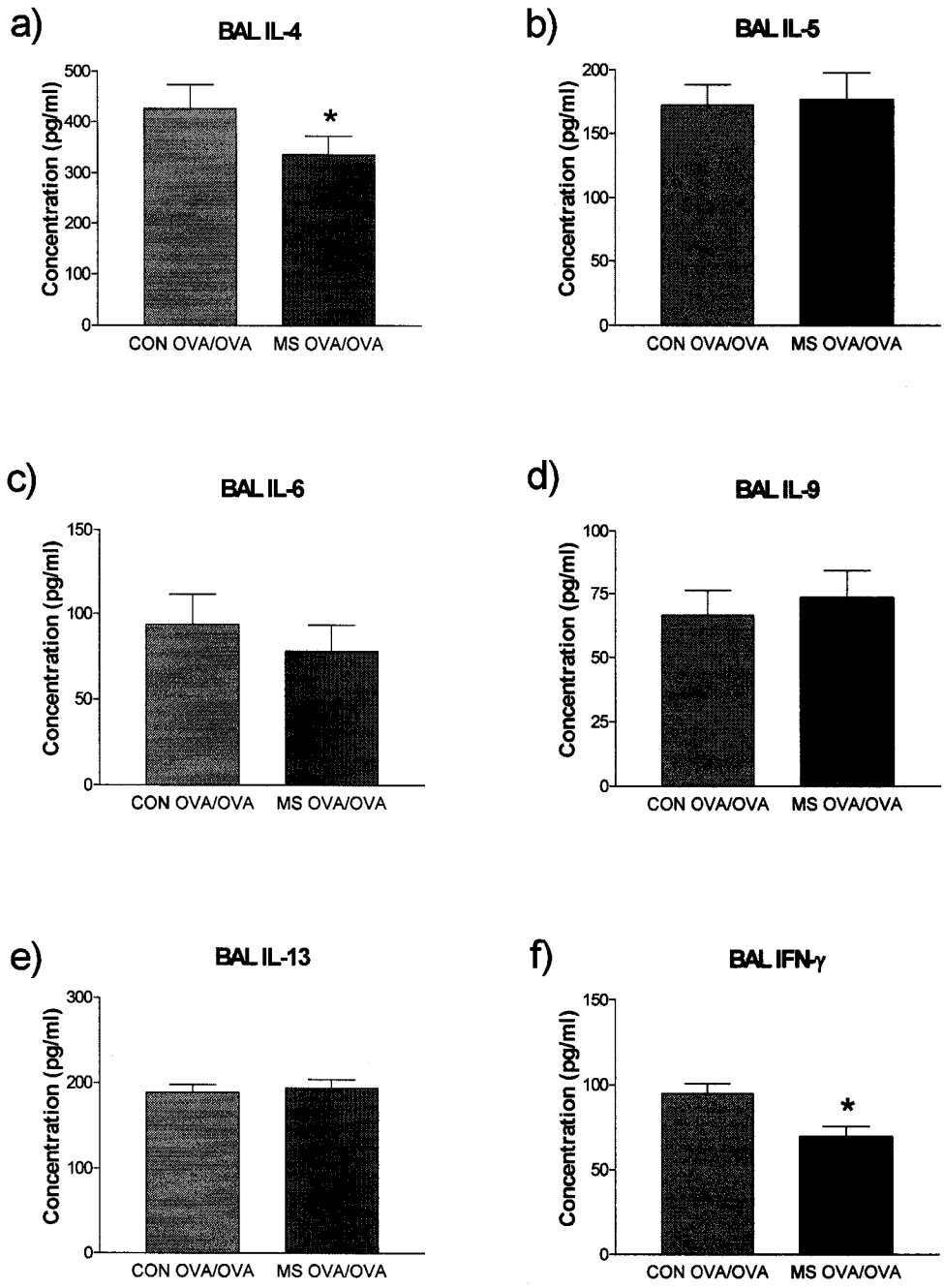


Figure 4-5: BAL Cytokine Levels in OVA Sensitized and Challenged MS and CON Mice.

Cytokine levels in the BAL of mice sensitized and challenged with OVA. Data are pooled with males and females. Bars represent mean values ± SE (n = 14). One star represents a significant difference of $p < 0.05$ using a T-test.

e) Corticosterone

Immediately prior to BAL, a cardiac puncture was done and serum was obtained. Corticosterone levels in the serum were quantified using a radio-immunosorbant assay. The corticosterone levels were higher in mice challenged with OVA relative to the SAL controls. Additionally, there was a tendency towards higher corticosterone levels in MS OVA mice than CON OVA mice, although this increase was not statistically significant (Figure 4-6).

III. Summary

Using maternal separation for the first 10 days as a model for early-life stress, we were able to characterize its effects on several characteristics. First, we identified that this model of early-life stress does not cause significant changes to alveolar septation as measured by mean linear intercept. This implies that there were no significant structural changes in the lung, or lung development as a result of this stress. When the MS mice were sensitized and challenged with OVA, we noted significant reductions in total airway inflammation including a reduction in the percentage of eosinophils present compared to CON animals, although not in AHR. Additionally, we showed that certain cytokines, IFN-γ and IL-4 were present in reduced levels in the BAL of MS mice, although others tested remained constant. As well, levels of corticosterone, tested after 24 hours, were not significantly different in MS mice.

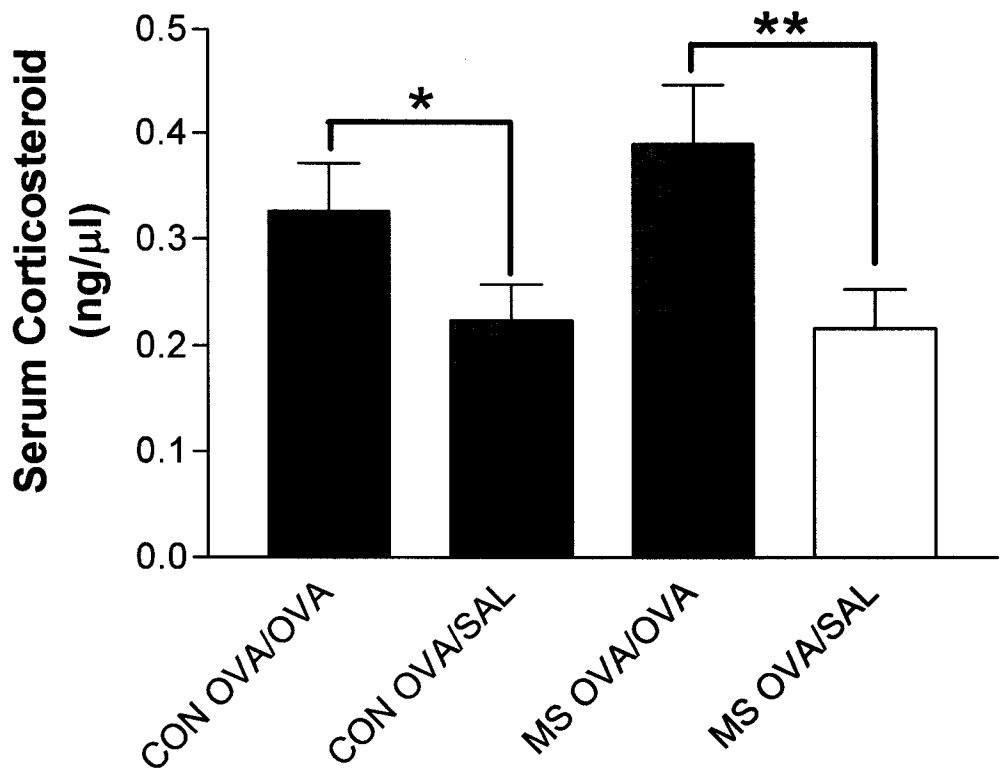


Figure 4-6: Corticosterone Levels in Serum in MS and CON Balb/c Mice.

Corticosterone concentration in the serum of CON and MS mice sensitized to OVA and challenged with OVA or SAL. Data are pooled for males and females. Bars represent the mean ± SE of male and female Balb/c mice (n = 30). SAL challenged mice had statistically lower CCS levels than their OVA challenged counterparts (p = 0.043 in CON, 0.0085 in MS).

Chapter 5: The Effects of an Enhanced Model of Early-life Stress on Airway Inflammation in Mice

I. Introduction

In the previous chapter, I described a model of maternal separation that was used to evaluate the effects of early-life stress on airway inflammation. Surprisingly, this model showed effects on airway inflammation, such as a reduction in inflammatory cells, especially eosinophils, and a reduction in some inflammatory cytokines which may be beneficial to the animal. However, these results are not consistent with much of the described effects of stress on asthma. As described above, clinical studies suggest that early-life stress has a detrimental effect on asthma. Is it possible that our model of early-life stress did not imitate stress in a fashion applicable to human disease?

The literature shows that early-life stress can induce different effects depending on the quality of the stressor. In most cases, 15 minutes of stress (acute) is considered beneficial, however 3 hours (chronic) usually leads to negative effects. To investigate what effect the quality of early-life stress might have on our allergen challenge model, we modified our maternal separation protocol. In an effort to increase the stress the pups faced, we used a graded way of increasing stress. Rather than simply removing the dam from her cage, to increase the stress, we also removed the pups into a clean cage to alter their environment. This would reduce the scent of the dam and potentially enhance stress of the maternal separation. We termed this form of stress "enhanced maternal separation" (MS+). Other groups have reported that this form of maternal separation increased corticosterone levels of the pups and ultrasonic vocalizations compared to unstressed controls, in addition to increased

behavioral sensitivity to dopaminergic agonists which indicate a more highly stressed state (102). When compared to the earlier protocol of only removing the dams from the cages, MS+ has been shown to induce more severe effects (83).

Since in the previous chapter males and females acted similarly in all the aspects tested, male and female data were pooled to increase statistical relevance in each experiment.

II. Results

a) Airway Hyperresponsiveness

AHR was determined using WBP. Similar to the results obtained in the previous chapter with MS mice, there was a significant increase in airway hyperresponsiveness as a result of challenging sensitized mice with OVA (CON: $p = 0.0101$, MS+: $p = 0.0063$, Figure 5-1) compared to SAL challenge. However, there were no significant differences with regards to hyperresponsiveness between CON and MS+ mice, as was seen with the MS mice in the previous chapter.

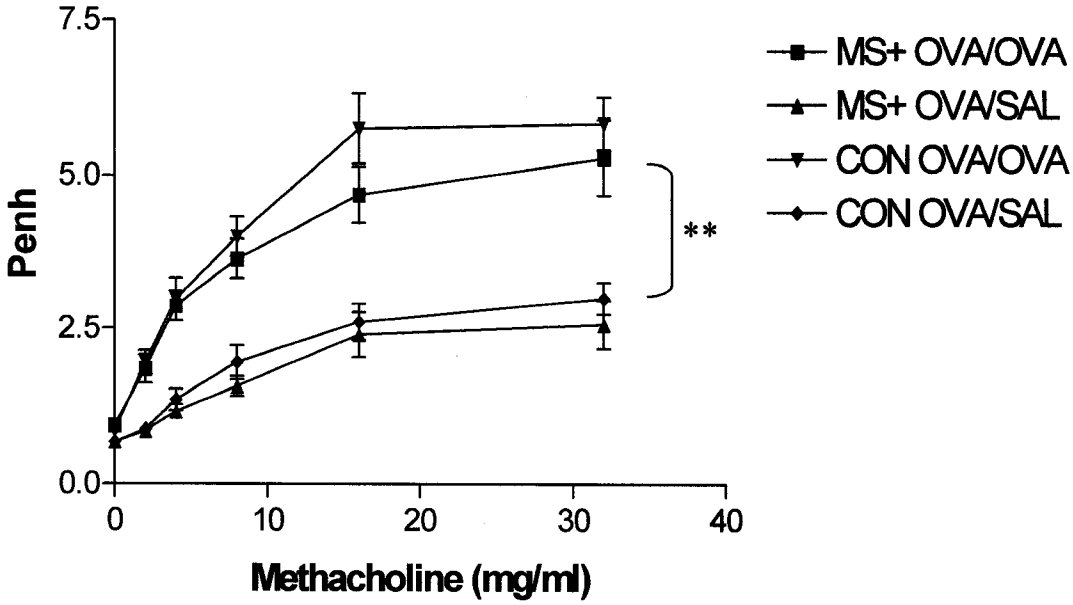


Figure 5-1: The Effect of Enhanced Early-life Stress on Airway Hyperresponsiveness in Balb/c Mice.

Combined Penh of male and female mice in response to increasing concentrations of methacholine. Enhanced early-life stressed (MS+) and unstressed control (CON) Balb/c mice were sensitized with OVA and challenged with OVA or SAL. Each point represents the mean ± SE (n = 14, 7 males and 7 females). Two stars indicate a statistical difference of $p < 0.01$ using ANOVA.

b) Airway Inflammation

Unlike AHR, the influx of inflammatory cells in MS+ BAL showed significant deviations from that of MS mice. MS+ did not show any differences in inflammation compared to CON mice. However, in the previous chapter MS stress significantly reduced total BAL cell numbers. Thus although inflammation dropped to 60% (± 10%) of the CON OVA/OVA inflammation in MS mice ($p < 0.05$), it is 108% (± 23%) in MS+ mice ($p < 0.05$ in comparison to CON animals) (Figure 5-2). These experiments were done with $n = 8$ in all OVA challenged groups and $n = 6$ in SAL challenged groups spread over 2 experiments, one with males and one with females. Challenging with SAL, as a negative control had low levels of inflammatory cell influx relative to the OVA challenged animals. CON SAL challenged animals had 11% (± 6.5%), MS SAL challenged animals were at 5.4% (± 4.1%), and MS+ SAL was at 6.5% (± 2.0%). In all cases, these were statistically different from the values in OVA challenged mice ($p < 0.001$ for CON and MS+, $p < 0.05$ for MS), but not significant between each other.

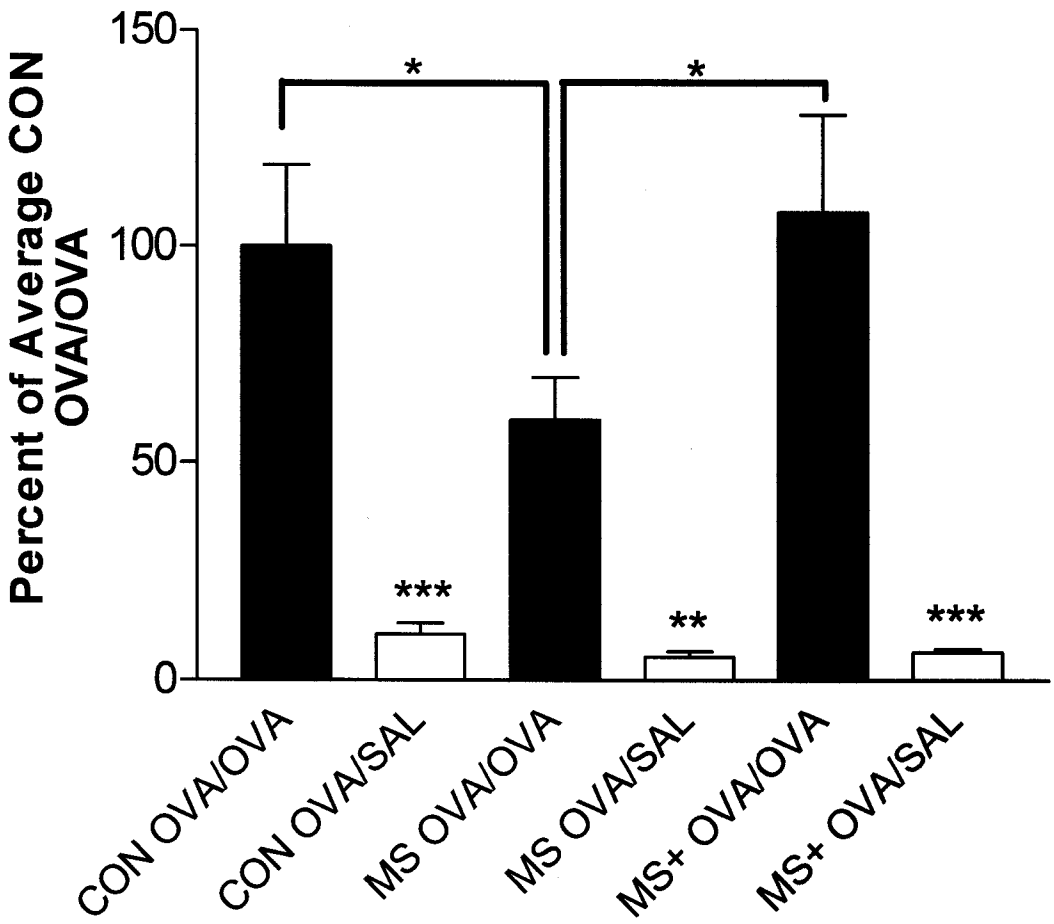


Figure 5-2: The Effect of Early-life Stress and Enhanced Early-life Stress on Total BAL Cell Count.

MS animals showed decreased number of cells obtained from BAL 24 hours after OVA challenge ($p < 0.05$). MS+ animals showed no significant change. In each case, the SAL challenged animals had significantly less inflammation in comparison to equivalent OVA challenged mice. Each bar represents the mean ± SE (OVA: n = 8; SAL: n = 6). One star indicates $p < 0.05$. Comparing OVA/SAL and OVA/OVA of each stress group, two stars indicate $p < 0.01$, and three stars indicate $p < 0.001$ using ANOVA.

Table 5-1: Total Inflammatory Cell Count of OVA Sensitized Maternally Stressed and Unstressed Balb/c Mice (OVA n = 8, SAL n = 6)

Stress	Challenged	Mean total cells	Standard error
Control	SAL	27,500	5,123
Maternal Separation	SAL	19,170	3,745
Maternal Separation +	SAL	17,500	2,814
Control	OVA	255,000	58,190
Maternal Separation	OVA	160,600	37,580
Maternal Separation +	OVA	286,900	66,750

c) Cytokines

With the absence of reduction in airway inflammation as a result of the MS+ protocol, it was important to investigate if the changes noticed in corticosterone and BAL cytokines as a result of MS stress were also lost.

With the cytokines, there were no significant differences between cytokine levels in controls versus the MS+ group ($p > 0.05$ in all cases; Figure 5-3). BAL from MS done along side the MS+ protocol showed a drop with IL-4 and IFN-γ, similar to the differences shown in the previous chapter. Interestingly however, IL-5 was also significantly lower in the MS control mice BAL relative to CON mice which was not seen previously. Reasons for this decrease are unknown.

d) Corticosterone

MS mice, in the previous chapter, showed a slight but non-significant increase compared to CON mice in corticosterone levels after OVA challenge. In the MS+ protocol however, a slight but non-significant decrease was seen in the corticosterone levels relative to CON animals (Figure 5-4).

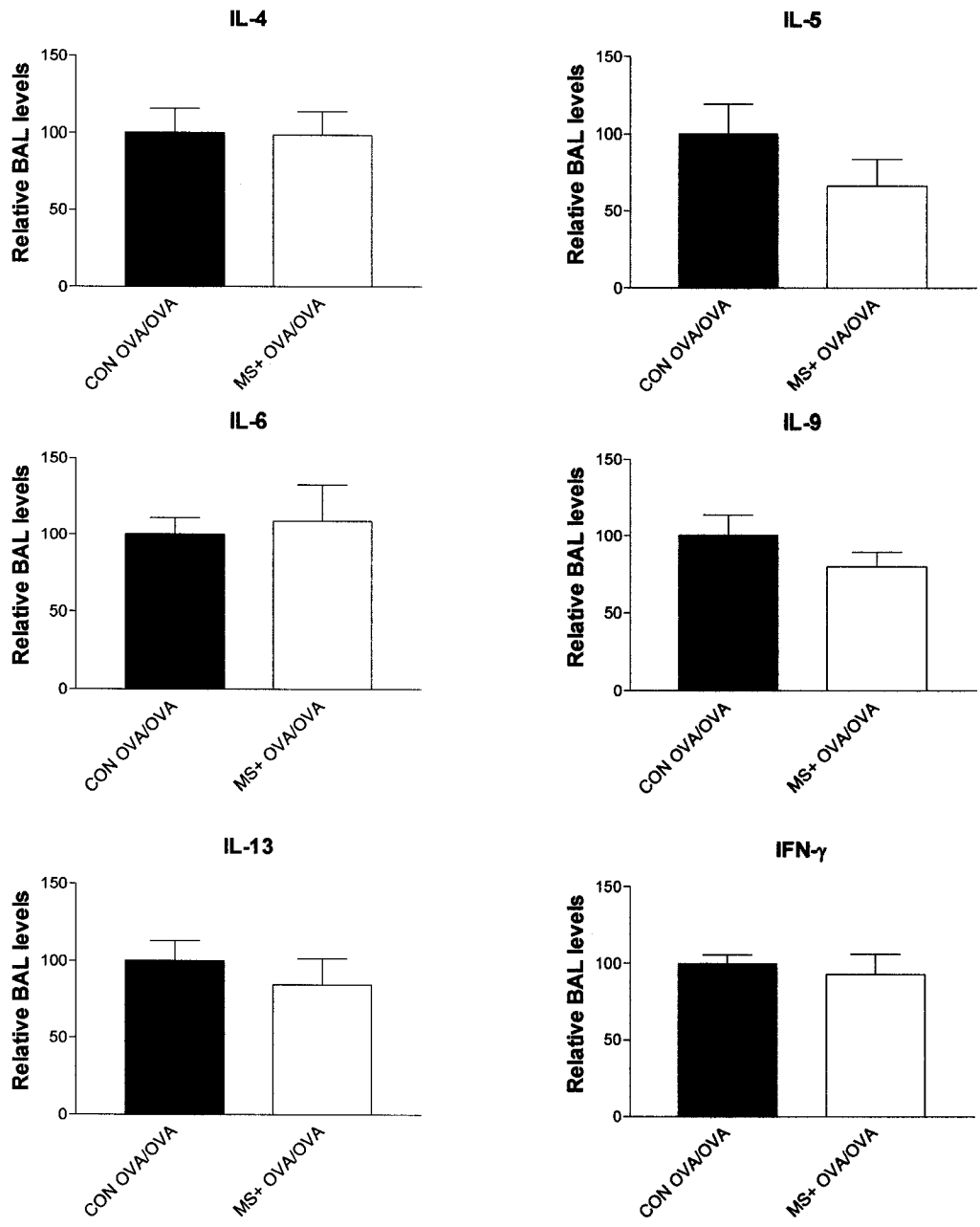


Figure 5-3: BAL Cytokine Levels in OVA Challenged CON and MS+ mice.

Cytokine levels in the BAL of mice sensitized and challenged with OVA. Data are pooled with males and females. Bars represent mean values ± SE (n = 12), no statistical differences were noted ($p > 0.05$) using T-tests.

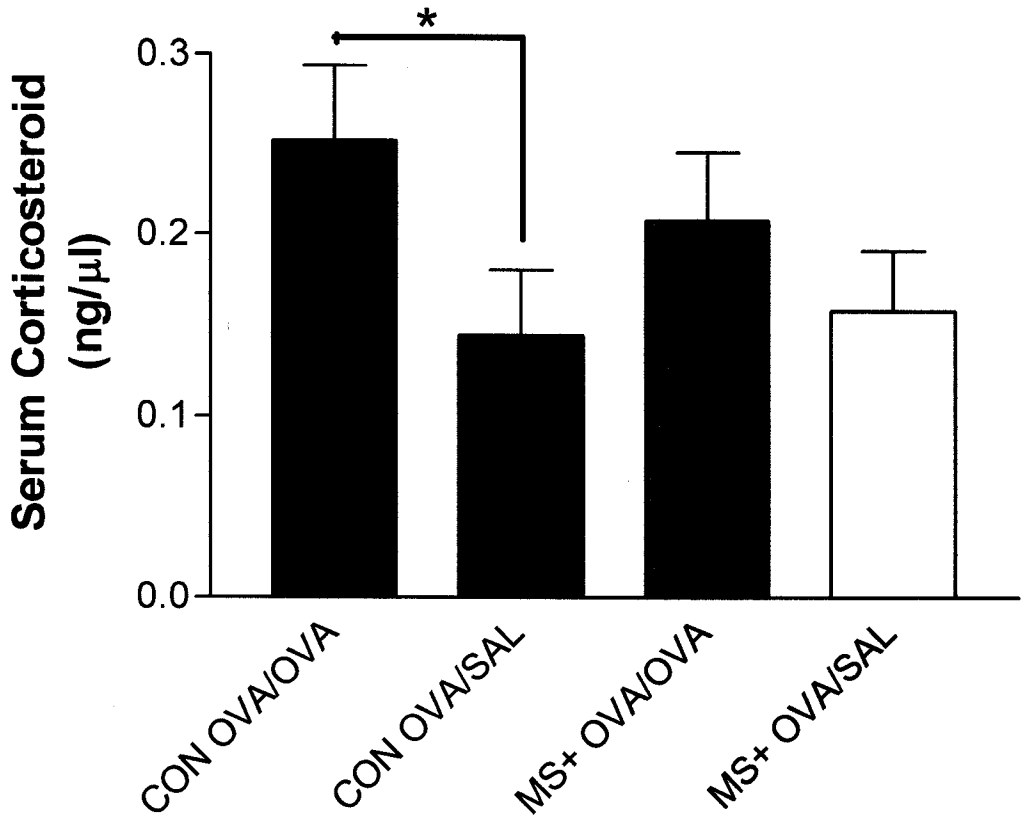


Figure 5-4: Corticosterone Levels in CON and MS+ Serum.

Serum Corticosterone levels in Balb/c mice. Bars represent the mean ± SE of male and female Balb/c mice (n = 14). CON SAL challenged mice had statistically smaller CCS levels than their OVA challenged counterparts (p = 0.02).

III. Summary

In summary, we identified the effects of MS+ during the first 10 days of life on asthma characteristics in this mouse model. MS+ did not induce the same changes that MS had on our asthma model. Overall, there were no differences in airway inflammation, and no change in cytokine levels between CON and MS+ animals. Additionally, there appeared to be a slight reduction in corticosterone levels.

Chapter 6: Discussion and Future Directions

I. Discussion

a) Summary of Data

First we showed that OVA and Aluminum hydroxide induces allergic airway inflammation and AHR when mice are sensitized at day 31. With the differences in weight at weaning, we were able to determine that maternal separation stress was successful in stressing the pups. Also, by determining lung morphometry, which only showed a small trend towards less alveolar septation, it appeared that alveolar septation was unaffected by the stress.

The primary objective of this project was to test the hypothesis that early-life stress negatively impacts asthma using an animal model. Although our hypothesis predicted an increase in severity of asthma in maternally stressed mice compared to controls, our results surprisingly showed the opposite effect. Using our initial model of maternal separation stress, where just the mother was removed from the cages for 3 hours per day for the first 10 days, we found a significant reduction in influx of inflammatory cells into BAL (especially eosinophils), and reduction of BAL levels of two key cytokines, IL-4 and IFN-γ. Serum corticosterone levels showed a trend towards higher levels in MS mice, and significant increases in levels in OVA challenged mice in contrast to SAL challenged mice. AHR, although increased in OVA challenged mice, showed no differences between the MS and CON groups.

However, a small modification in the protocol, removing the pups from their home cage during the separation period, gave a different result. In these

"enhanced stress mice", both the reduced inflammation and cytokine levels that were seen in the MS mice were not present and were at levels comparable to the unstressed group. Additionally, the corticosterone levels in MS+ OVA challenged mice was less pronounced in MS+ mice so that it was no longer significant in comparison to the SAL challenged animals.

Other outcomes that were assessed, including airway hyperreactivity and most cytokines tested, showed no differences between CON, MS, and MS+ mice.

b) Inflammation in BAL Fluid and Corticosterone in MS mice

The primary finding of this project is that maternal separation reduces airway inflammation in the pups evidenced by a reduction in total BAL cells, eosinophil numbers, and changes in the BAL cytokine profile. In some respects, this appears to be similar to the effects seen in adult OVA sensitized animals as a result of acute stress before OVA challenge. Several models of acute stress have shown that this often leads to beneficial effects in rodents, such as reduced airway inflammation (66) in a corticosteroid dependent manner (66, 68). The literature shows that acute laboratory stressors reliably increase corticosteroid levels (136). Our research shows that corticosteroid levels increase as a result of acute allergen challenge (which implies a stress). Other stressors that occurred during this period, such as a result of WBP or euthanasia are not responsible for this increase in corticosterone as these stressors were consistent between all groups. Additionally, the time required to increase or reduce cell influx into the lungs would be too short after euthanasia, and experiments where WBP was not

conducted also showed the significant reductions in cells. Unfortunately, although our research shows that corticosterone levels increase as a result of acute allergen challenge, they were not shown to differ significantly between maternally separated or control animals when studies in the pups 24 hours after the final challenge. This may be a result of the timing of obtaining corticosterone levels as acute laboratory stresses increase these levels to a peak approximately 20 to 40 minutes after the onset of the stress (136). In our experiments, to prevent inducing additional stresses in the animals, corticosterone data was obtained 24 hours after the OVA or SAL final challenge, during euthanasia. It is probable that had we assessed serum corticosterone levels 30 minutes after the final allergen challenge, we may have been able to show differences between MS and CON groups.

However, we did see an increase in corticosterone levels resulting from allergen challenge. An important characteristic of MS stress is stress hypersensitivity: its ability to increase sensitivity to stress and increase the severity of stress reactions (83, 88). For example, if acute stress, such as the last allergen challenge were present, stress hypersensitivity would induce a corticosterone response that is significantly greater and more sustained in MS mice. Since the acute stress response can depress airway inflammation in adult Balb/c mice (66), stress hypersensitivity could explain the reduced airway inflammation in our model of maternal separation. However, corticosterone data immediately after allergen challenge would be required to make a definite conclusion.

In addition to changes in corticosterone, there were also changes in inflammation in BAL fluid, both in total numbers and in ratios of cells. As a percentage, the reduction in eosinophils of the MS group compared to the CON group was equivalent to the gain in macrophages (Figure 4-4). However, due to the significantly less recovered total cells, the majority of the reduction in lung inflammation that we discovered was a reduction in infiltrating eosinophils. For example, although the number of macrophages dropped from 119,478 to 102,282 (a 14% reduction), and the number of neutrophils was reduced 20%, neither reduction was significant (Table 4-4). The number of eosinophils however, which originally constituted 69% of all the cells present in an allergen challenged lung, was reduced by 28% in the MS group. Thus, the reduction in cell numbers was a result of a reduction in the numbers of all cell types, although eosinophils were reduced the most. This would imply a general immuno-depressive effect that appears to have greater potency towards eosinophils.

However, the literature indicates that in the majority of cases of human asthma, early-life stress appears to worsen asthma (53). The majority of studies demonstrate a reduction in T_H1 disease (cell mediated), but an increase in T_H2 disease (which is involved in asthma) (117). In clinical studies, these chronic early-life stressors include parent-child interactions, caregiver stress, and lower socioeconomic status (112-114).

Other recent studies have shown that early-life stress does increase airway inflammation in animal models of asthma (77, 137). However, these studies utilize different protocols and definitions of early life stress. For example, in a

recent study by Chida *et al.* (2007), early life stress is able to increase airway inflammation, especially eosinophil influx into the lungs due to activation of the HPA axis (77). However, the stresses were given during the fourth week of life, at the tail end of lung development and while the immune system was relatively mature. Thus these stresses may not allow for permanent developmental changes to occur that would affect the nervous or immune systems later in life. In this way, this study is similar to the late life stress studies that usually stress animals between 6 - 8 weeks of age and see the same results. Another study by Pincus-Knackstedt *et al.* (2006), shows a similar result, but using a prolonged 24 hour prenatal restraint stress of the dam (137). Since this stress is occurring prenatally, it is more of a physical rather than psychological stress. Additionally, it is arguable whether the biological changes induced in the dam by this severe form of stress make this a credible form of early-life stress. These examples illustrate the disparities that exist with stress protocols that differ in timing and quality, and how distinct protocols may generate different results. Regarding my results, it also may explain why our data does not match with the current literature on early life stress using animal models.

c) Inflammation in BAL Fluid and Corticosterone in MS+ Mice

Since our results were similar to effects of an acute-stress in adults (66) where inflammation was reduced, we tested if altering the magnitude of the stress might alter the outcome. Thus we used a more severe model of maternal separation where the pups were also removed from their home cage. With this

protocol (MS+), the level of inflammation was similar to that in unstressed mice, but increased compared to the MS protocol.

Additionally, with OVA-challenged MS+ mice, the corticosterone levels after 24 hours were similar to the SAL challenged animals. This is similar to the findings of other early-life stress studies which suggest that there is a blunted or reduced corticosteroid response and downregulation of corticosteroid receptors on inflammatory cells (136). This would result in hyposensitivity to corticosteroids, or in other words a reduced effect as a result of a stress response. Thus when the acute stress of the second allergen challenge is given, the mice do not respond as severely to the stress as the control animals would. This would predict lower corticosterone levels and comparable inflammation between control and MS+ animals.

d) Cytokine Levels

Cytokine levels in BAL fluid 24 hours after the final challenge were assessed. BAL fluid was chosen as the source of these cytokines, as they represent closely the cytokine levels in the lung tissue, and more importantly, the source where inflammation is taking place. IL-4, 5, 6, 9, 13, and IFN-γ were chosen to assess a broad spectrum of cytokines and evaluate if there was a shift between T_H1 and T_H2 responses.

Although most of the cytokines tested showed no difference in levels (including IL-5, 6, 9, and 13) between MS and CON mice, there were significantly less IL-4 and IFN-γ in MS mice. This is an apparent paradox because IL-4 is considered to be a T_H2 cytokine, often correlated strongly with

asthma severity, whereas IFN-γ is deemed to be a T_H1 cytokine, both which normally are inversely related in asthmatics due to crossregulation (2, 3).

In periods of brief stress, the T_H1 response is generally inhibited, with a reduction in IFN-γ; however T_H2 cytokines, such as IL-4 are generally increased (136). Moreover, CRH deficient mice that cannot produce corticosteroids have increased IL-4 levels but reduced IFN-γ (67). Additionally, injections of dexamethasone, a corticosteroid analogue, inhibit production of T_H2 cytokines (136). However, in studies with chronic stressors, there is a general immunosuppressive effect as recorded by cytokine levels. For example, in chronic stress in a caregiver or sibling of a mother with breast cancer, IFN-γ and IL-4 are both reduced significantly (136, 138).

These results match the overall immuno-depression seen above, as all cell types appeared to be reduced in those mice that underwent MS stress. These cytokine data support the idea that with MS stress, there were acute beneficial effects by reducing T_H1 and T_H2 responses, in addition to reducing influx of inflammatory cells in the airway. In MS+ stress the airway inflammation and IL-4 and IFN-γ levels in the BAL fluid were similar to that seen in CON mice. This indicates that MS protocol induced a general immuno-depression that may be related to the reduction in cell numbers observed. Assuming that this is correct, this would imply that early-life stress in conjunction with an acute stress may be beneficial for an asthmatic possibly through a reduction in inflammatory cells and cytokines.

e) Other Considerations

To extend our analysis, several other factors were investigated that showed few differences. For example, to prevent effects from the female estrous cycle, male and female mice were segregated and data obtained from each were kept separate. Overall through our experiments, we found that although the total BAL cell counts varied between males and females (females consistently had higher cell numbers than males), the trends that were observed were present equally in both males and females (Table 4-3).

Additionally, airway hyperresponsiveness showed little change between the control and either of the maternally separated groups. This is not surprising, as other stress studies done in adult mice, although showing significant differences in airway inflammation and cytokines, were also unable to show differences in hyperresponsiveness (66). As pointed out previously, this may be a result of genetic differences in the mouse strain that we looked at as stresses in other models do show differences in AHR as a result of acute stress (25).

II. Conclusions

Overall, we have shown that early-life stress induces significant changes in the response to an allergen challenge. These responses depend not only on the length of the stressful stimulus, but also on the quality of the stressor, as in our hands a small change in protocol that was expected to affect the intensity of stress resulted in drastically different results. These changes were primarily in airway inflammation, especially in the number of eosinophils recruited to the

lung, and in two key cytokines. Changes in corticosteroids also may suggest general immuno-depression as a result of acute stress.

III. Future Directions

a) Determining the Effect of Corticosteroids

Despite being able to show some significant effects as a result of the two different maternal separation protocols, there is a still much that needs to be done to understand the results in this model. Although the stresses used reduce inflammation, the cytokine and corticosterone data only hint at a potential mechanism, via the HPA axis and corticosterone secretion, as is the case with acute stress in adults. It will be important to more thoroughly investigate corticosterone levels by looking at its levels during the maternal separation period, and 30 minutes after the final challenge to see if there are significant differences in serum corticosterone levels. If there are increases in corticosterone during the challenge period, this will confirm that early life stress does induce stress hypersensitivity, as has been described in this model.

Additionally, to ensure that corticosteroids are indeed responsible, the use of HPA axis blockers such as RU486 would be helpful. By giving RU486 at different timepoints especially immediately prior to allergen challenge, which prevents corticosteroid binding to its receptors, we can determine if the effects that we are seeing are a result of stresses occurring during the final challenge or a direct result of the early life stress.

b) Reversibility of Early-life Stress

Since the MS+ model is able to reverse changes seen in the MS model, it is apparent that the differences in inflammation are a direct result of stress rather than other physiological factors. However, it is imperative to use a model that is common in the literature to confirm this. Other researchers use anogenital stroking of the pups with a wet brush as a way to relieve stress during separation, to confirm that the stress is the inducer of the changes seen later in life. This approach could confirm that the reduction in inflammatory cells and cytokines are a result of separation stress.

c) Chronic Stress

One interesting observation in this research was that our results could be explained by a reset of the HPA axis which would induce stress hyperreactivity later in life. By giving the final challenge 24 hours prior to looking at inflammation, we believe that this acute stressor increased corticosterone levels, and thus is likely partially responsible for the reduction in inflammation. However, other studies have observed in adult mice that, although acute stressors have this effect, chronic stressors can induce significant increases in airway inflammation and in some cases airway hyperresponsiveness, in mouse models (66).

Accordingly, one of the reasons why our data does not fit the paradigm of clinical stress worsening asthma in children may be that early-life stress enhances the stress response later in life. Coupled with a chronic stress later in life, early-life stress may increase airway inflammation and hyperresponsiveness

beyond what a chronic stress can induce in mice that were not exposed to stress early in life. This is very relevant to clinical stress in humans, which is usually a chronic stressor. Thus, an important experiment that needs to be done is to determine if a chronic stress can increase inflammation in maternally separated mice. If so, this may provide an important mechanism by which asthma severity may be worsened in those children who have been stressed early in life.

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