JESSIE CHAU M.SC. THESIS

THE EFFECT OF PRENATAL STRESS ON A MOUSE MODEL OF ALLERGIC AIRWAY INFLAMMATION

BY JESSIE CHAU, B.Sc.

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AUTHOR: Jessie Chau, B.Sc.
SUPERVISOR: Paul Forsythe Ph.D.
COMMITTEE: Khalil Karimi Ph.D., Boris Sakic Ph.D. Mark Inman Ph.D

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ABSTRACT

Prenatal life events have been long observed to be able to influence disease into adulthood in both epidemiological and animal studies. Prenatal stress (maternal stress during gestation) is one of such factors that has been shown to impact cognition and behaviour of the offspring. However, the effects of prenatal stress on the immune system are not understood. This study has evaluated the effects of prenatal stress on a murine model. Prenatal stress increased allergic airway inflammation in male, but not female offspring following sensitization and challenge with cockroach extract. This corresponded with stress-induced changes in the immune environment of non-sensitized animals. These changes included a decrease in regulatory T cells at baseline in males compared to non-stressed controls and increased splenic dendritic cell percentage and cytokine, particularly IFN-γ, secretion compared to prenatally stressed females. In females, prenatal stress decreased allergic inflammation, which corresponded to a decreased percentage of dendritic cells in the lung and mesenteric lymph node. Prenatal stress did not affect dendritic cell antigen presentation in ether male or female offspring. There was no evidence to suggest a prenatal stress induced change in glucocorticoid sensitivity of dendritic cells. In order to explore the possibility of prenatal stress induced decrease of parasympathetic output, a vagotomy model was used as a proof of concept in naïve animals not exposed to prenatal stress. Vagal modulation of dendritic cell phenotype and function was assessed. While there was some evidence that vagotomy may indirectly modulate dendritic cell function, its effects on the immune system were different then the changes caused by prenatal stress and thus it is a role of reduced parasympathetic output was not supported. Overall this

data indicates a role of prenatal stress on the immune system with clear sex differences, but the mechanism for how this occurs is currently unknown. Further research is needed to investigate the role of TLRs and IFN-γ in this model, as well as other possible mediators of prenatal stress such as the changes to the parasympathetic nervous system that may in turn mediate alterations to the immune system. Differences in when the effects of prenatal stress are expressed during postnatal life are discussed.

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ACh	Acetylcholine
AChR	Acetycholine Receptor
BAL	Bronchoalveolar Lavage
BALF	Bronchoalveolar Lavage Fluid
CpG	Cytosine-Phosphate-Guanine oligodeoxynucleotides
CR	Cockroach Protein
DC	Dendritic Cell
EAE	Experimental Allergic Encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
GC	Glucocorticoid
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Element
HPA	Hypothalamus-Pituitary-Adrenal Axis
ACTH	Adrenocorticotropic Hormone / Corticotropin
CRH	Corticotropin Releasing Hormone
SNS	Sympathetic Nervous System
LPS	Lipopolysaccharide
MCh	Methacholine
Poly I:C	Polyinosinic:polycytidylic acid
PSNS	Para-Sympathetic Nervous System
PVN	Paraventricular Nucleus
Th2	T-helper 2
Th1	T-helper 1
TLR	Toll Like Receptor
T-reg	Regulatory T cell
IL-	Interleukin-
CD-	Cluster of Differentiation-
IgE	Immunoglobin E
IgG	Immunoglobin G
Vx	Vagotomy

Introduction

1.1 Asthma

Asthma is a respiratory disease affecting over 200 million people worldwide (Organization, 2011). In Canada, it affects 8.5% of the population and is estimated to cost the Canadian healthcare system $600 million dollars annually (Canada, 2012). Not only does it remain a socioeconomic burden, it is a continually growing problem, where its incidence has nearly doubled since the 1980's (Umetsu et al., 2002). It is a prominent health condition that is still not well understood and more research is needed for the development of new therapeutics and preventative measures.

Allergic asthma is a chronic, heterogeneous disease characterized by reversible airway inflammation, obstruction and airway hyper-reactivity to stimuli (Buc et al., 2009). While genetics contribute to atopy, in epidemiological studies it was discovered that maternal atopy is a much stronger indicator of allergic diseases in the offspring, than paternal atopy (Knackstedt et al., 2005; Pincus-Knackstedt et al., 2006). As such, genetic predisposition alone is insufficient for understanding the underlying cause of asthma. Environmental interactions also play a large role in mediating asthma progression, potentially through epigenetic modifications, which may very well be the missing component in the understanding of disease development and progression (Knackstedt et al., 2005).

Allergic asthma is a T-helper 2 (Th2) cell associated inflammatory disease, where Th2-type cytokines and chemokines play key roles in directing the pathological characteristics of the disease. Interleukin (IL)-4 and IL-13 stimulates B cells synthesis of immunoglobulin E (Gray et al.). As IgE crosslinks the allergen with mast cells, it causes mast cell degranulation and release of histamine and leukotrienes. Both are mediators of asthma symptoms and cause edema, smooth muscle contraction and mucus production. IL-9 stimulates mast cell proliferation. In late phase asthma, IL-5 recruits eosinophils, releasing ganule protieins, which causes further edema and smooth muscle contraction (Chen and Miller, 2007; Lambrecht and Hammad, 2009).

1.2 Intrauterine Programming Hypothesis for Disease

Most children that develop atopy and/or allergic asthma may have a Th2 polarization of their immune system early in life, which requires investigation of potential influences in early life. Environmental stimuli during early life, critical periods in development, can predispose individuals to disease development later in life. This theory by Barker was dubbed 'intrauterine programming' and stems from the fetal origins hypothesis, where a strong correlation between low birth weights and increasing rates of coronary heart disease were observed (Barker, 1995). Through other epidemiological studies, low birth weight has been linked to subsequent increase in the development of hypertension, insulin resistance, type 2 diabetes and cardiovascular disease (Barker, 2004). Although much of 'intrauterine programming' focuses on the effect of malnutrition leading to greater tendency of encountering disease in later life, subsequent studies have shown that factors such as glucocorticoids, psychological stress, prenatal environmental tobacco smoke, folic acid supplements, diesel exhaust particles and allergens may also affect intrauterine programming (Barker, 2004; Kuriakose and Miller, 2010; Miller and Chen, 2006). It has been postulated that prenatal plasticity helps the unborn animal in preparing for environmental conditions after birth (Harris and Seckl, 2011).

In agreement with Barker, Holt has hypothesized that various developmental factors affect postnatal maturation of immune and respiratory systems, which ultimately contributes to the development of asthma (Holt et al., 2005). In particular, developmental factors which delay maturation of Th1 immune response and increase Th2 type immune response may increase susceptibility to asthma and is consistently observed in children at high genetic risk of asthma (Holt et al., 2005).

The mechanism of 'intrauterine programming' is not well known but some researchers have alluded to the role of epigenetics. Epigenetic programming has been found in studies looking at the effect of maternal caregiving on the offspring, where increased licking and grooming by rat mothers can alter offspring epigenome of the glucocorticoid receptor gene, increasing glucocorticoid receptor expression and exhibit a more modest HPA response (Weaver et al., 2004). If perinatal environmental conditions can cause changes in epigenetics, then it is likely epigenetics may also mediate the effects of intrauterine programming.

1.3 Stress and HPA Axis

The process of "achieving stability through change or maintaining homeostasis" is known as allostasis (McEwen and Wingfield, 2010). Stressors are stimuli that activate the hypothalamic-pituitary-adrenal (HPA) axis and/or the sympathetic nervous system

(SNS) in order to help the organism adapt physiologically to cope with a threat for a short term (Glaser and Kiecolt-Glaser, 2005). Over time during allostasis, repeated or frequent adaptations are made in response to stressors cause the physiological systems to operate at a higher or lower level which can come at a physiological cost, known as the allostatic load (McEwen and Wingfield, 2010; Vig et al., 2006). It has been widely recognized that acute stress may have beneficial effects, while chronic stress can have detrimental effects on both the brain (McEwen, 2006) and immunity (Forsythe et al., 2004).

During stress, the hypothalamic-pituitary-adrenal (HPA) axis is activated. The paraventricular nucleus (PVN) of the hypothalamus secrets corticotrophin releasing hormone (CRH), which stimulates the anterior pituitary to produce adrenocorticotropin hormone (ACTH) causing the release of glucocorticoids from the adrenal glands. In humans, this glucocorticoid is cortisol and in mice, it is corticosteroid. Glucocorticoids act on a variety of cells, include immune cells, through the intracellular glucocorticoid receptor (GR), as well as providing negative feedback to the HPA axis by acting on the hypothalamus and pituitary gland (Chen and Miller, 2007).

Glucocorticoids (GCs) are known to have an anti-inflammatory effect on the immune system and are currently used in the treatment for various inflammatory diseases, including asthma. GCs mediate most of their effects through binding to glucocorticoid receptors (GRs), which causes nuclear localization of the complex and recruitment of co-factors, acting as a transcription factor. This process is known as transactivation, where this complex can then bind to glucocorticoid response elements (GRE) on the genome, upregulating anti-inflammatory cytokines such as IL-10 and TGF β (De Bosscher and

Haegeman, 2009). Simultaneously, transrepression also occurs where it inhibits inflammatory cytokine production by interfering with other transcription factors such as NFκB and AP-1 (De Bosscher and Haegeman, 2009). Various aspects of the immune system are affected by glucocorticoids. GCs suppress a number of cytokines (including IL-1β, TNFα, IL-6, etc.) and chemokines. GCs are also able to reduce B cell proliferation, prevent IgE mediated mast cell release and inhibit mast cell survival factors (Franchimont, 2004).

At the same time, GCs can upregulate anti-inflammatory cytokines IL-10 and TGF-β. Dendritic cells are the most potent antigen presenting cells, but have a decreased ability to present antigen and elicit a T cell response after GC exposure, as GCs prevent MHCII and co-stimulatory molecule expression. Taken together, GCs can partake in the differentiation of IL-10 producing regulatory T cells, as shown by Barrat and colleagues (Barrat et al., 2002). GC and vitamin D3 induced regulatory T cells were shown to have *in vivo* regulatory properties in a model of experimental allergic encephalomyelitis (EAE) (Barrat et al., 2002). The resultant effect of glucocorticoid administration is a dampened immune response (Franchimont, 2004).

1.4 Prenatal Stress

Early life is a particularly vulnerable window of time, where there is more susceptibility to programming. In humans, pregnant women who experience stress or nervousness have been reported to have higher cord blood IgE and increased proinflammatory cytokines, which are both suspected factors for higher allergy risk for the infant (Merlot et al., 2008).

Prenatal stress has been shown in mouse and rat models to have permanent and serious implications on their offspring including sexual behaviour, heightened anxiety and cognition (Welberg and Seckl, 2001). Results have varied and are sometimes contradictory dependent on strain, type of stressor, gestational timing, duration and sex (Welberg and Seckl, 2001). In a rat model, it was found that prenatally stressed females have greater basal corticosterone and ACTH levels when compared to males. One of the proposed mechanisms of prenatal stress is a decrease in density of adult hippocampal corticosteroid receptors, thus decreased sensitivity to the negative feedback from corticosterone (Welberg and Seckl, 2001).

Prenatal exposure to stress during pregnancy was also found to cause alterations in growth factors, cytokines and hormones, potentially leading to dysregulation of fetal immune development (Knackstedt et al., 2005; Wright, 2007). As prenatal stress is known to have pleiotropic programming effects on the offspring, it is not surprising that it may also cause an allostatic load on the immune system.

Although glucocorticoids have immunosuppressive properties in their ability to regulate expression of cytokines in T cells and inhibit mast cell release of allergic mediators, prolonged exposure to glucocorticoids may cause glucocorticoid resistance (Chen and Miller, 2007). High levels of glucocorticoid could cause lymphocytes to adapt by down regulating the expression of glucocorticoid receptors, which in turn makes them more resistant to anti-inflammatory signals and exacerbate asthma (Miller and Chen, 2006). Chronic stress has been shown to impair glucocorticoid responsiveness and IL-6 suppression in humans, which could ultimately lead to persistent activation of inflammation (Miller et al., 2002; Miller and Ho, 2008).

Maternal stress causes elevation of glucocorticoid levels, where 10-20% is passed into the fetus, causing overactivation of the fetal hypothalamic-pituitary-adrenal (HPA) axis (Knackstedt et al., 2005). In addition, maternal stress causes an increase in glucocorticoid production due to placental CRH, which directly affects the fetus. Corticotrophin- releasing hormone (CRH) is decreased in fetal the paraventricular nucleus (PVN), as a result of maternal stress and elevated glucocorticoids (Pincus-Knackstedt et al., 2006). The negative feedback from glucocorticoids may suppress the release of growth hormones important for growth and organ development (Knackstedt et al., 2005). In addition to causing stunted fetal growth in pups, it may cause a delay in naturally occurring shifts from Th2 to Th1 immunity in neonates, which predisposes them for atopic diseases (Knackstedt et al., 2005).

In a longitudinal study by Wright, chronic parental stress was found to be a predictor of wheezing in infancy (Wright et al., 2002) and an atopic profile (i.e. higher IgE) in childhood (Wright et al., 2004). Similarly, other studies have shown prenatal negative life events were associated with elevated cord blood IgE (Peters et al., 2012), wheezing (Reyes et al., 2011) and atopic dermatitis (Wen et al., 2011).

In animal models, prenatally stressed offspring were found to have increased susceptibility to airway inflammation and hyper-responsiveness, as well as a greater Th2 adaptive immune response (Pincus-Knackstedt et al., 2006). In the same study, it was also found that antigen-presenting cells (APCs) from adult offspring are able to encourage Th2 clonal expansion *in vitro* (Pincus-Knackstedt et al., 2006). The exact mechanism is currently not understood, but some studies suggest that blunted HPA functioning in pregnant women increase maternal IgE, which enhances fetal sensitization to allergens (Knackstedt et al., 2005).

Studies using a social disruption stressor (SDR) model, in addition to demonstrating similar results with enhanced allergic airway responses, also show reduced glucocorticoid sensitivity of Th2 cytokine released by immune cells *in vitro* (Haczku and Panettieri, 2010).

1.4 Glucocorticoid Resistance Model in Mediating Allergic Asthma

Inhaled corticosteroids have been shown to be ineffective in the treatment of asthma and a potential explanation is glucocorticoid insensitivity (Haczku and Panettieri, 2010). In an animal model of social disruptive stress and allergen exposure, increased susceptibility to airway inflammation and responsiveness was observed when compared to unstressed controls (Bailey et al., 2009). These findings can be attributed to corticosteroid insensitivity of LPS stimulated splenocytes and the enhanced ability of the splenocytes to produce Th2 cytokines and chemokines (Bailey et al., 2009). Other research has previously shown that this is closely linked to the inability of the intracellular glucocorticoid receptor (GR) to translocate into the nucleus and affect gene expression (Haczku and Panettieri, 2010). It was further demonstrated that there was a significant decrease of GR mRNA and protein expression which is in agreement with findings indicating a reduction in GR expression in asthmatic patients (Bailey et al., 2009). However, the mechanism of glucocorticoid receptor expression is not understood but might be attributed to alterations in transcriptional expression.

1.5 Other Markers of Maternal Stress

Stress can activate the sympathetic nervous system (SNS) inducing epinephrine release from the adrenal gland (Agarwal and Marshall, 2000). Catecholamines during SNS activation can bind to lymphocytes through adrenergic receptors and alter immune responses by modulating interleukin (IL)-4, IL-5, and IL-13, mast cell degranulation and eosinophil recruitment. However, in the same manner as glucocorticoid resistance occurs, down regulation of β2-adrenergic receptors (B2AR) could occur after prolonged catecholamine exposure (Chen and Miller, 2007). This has been previously observed in asthmatic children where there was a decrease in B2AR mRNA after experiencing a chronic stressor (Miller and Chen, 2006).

In a longitudinal study by Keicolt-Glaser *et al*. (2003), the relationship between chronic stress in individuals caring for a spouse suffering from dementia and interleukin-6 (IL-6) was examined over six years (Kiecolt-Glaser et al., 2003). The study showed that chronically stressed individuals had four times the amount of IL-6 in plasma than controls (Kiecolt-Glaser et al., 2003). More interestingly, increases in IL-6 have also been observed in individuals suffering allergic asthma so it is a possibility that stress increases asthma susceptibility through this mechanism. IL-6 has been observed in experimental asthma to both direct Th2 differentiation and inhibit regulatory T cell (Treg) differentiation in the lungs (Doganci et al., 2005).

1.6 Parasympathetic Nervous System Output

The autonomic nervous system contains the sympathetic, parasympathetic and enteric nervous systems, where both work towards regulating the internal environment of our bodies to maintain homeostasis (Porges, 1995). While the sympathetic nervous system functions to optimize an organism's ability to cope with the external environment (increase metabolic output), the parasympathetic nervous system acts to optimize the visceral environment (restoration and conservation of energy) to maintain homeostasis. In general, the sympathetic and parasympathetic nervous system acts reciprocally (Porges, 1995).

Stress is defined by the inability of the organism to cope with the demands and disruption of homeostasis, which in turn can be assessed through shifts in autonomic activity disrupting homeostasis (Porges, 1995). During stress, in order to better cope with challenges, an organism may have increase sympathetic activity and a reciprocal decrease in parasympathetic activity. In turn stressed can be measured by a depressed parasympathetic output or specifically vagal tone and decreased cardiovascular output (Porges, 1995).

The anti-inflammatory effects of the vagus nerve known as the cholinergic anti-inflammatory pathway further highlight the significance of this finding. Just as the nervous system plays a role in controlling vital organs, it plays a role in regulating inflammation (Pavlov et al., 2003). An initial trigger (i.e. pathogen, injury) causes an onset of inflammation, which is propagated by macrophage activation in order to neutralize potential challenges to the immune system. However, an over active inflammatory response can be harmful to the body and needs to be controlled. TNF-α and IL-1 are capable of activating neuroendocrine immunomodulatory responses through the HPA axis and SNS; more recently an alternate pathway – the cholinergic anti-inflammatory pathway – was proposed to contribute to this, and is activated through the afferent vagus (Pavlov et al., 2003). Once activated and relayed to the brain, anti-inflammatory effects are carried out by the efferent vagus nerve release of acetylcholine (ACh), interacting with α7 subunit of nicotinic receptors (ACh receptors) on macrophages (Pavlov et al., 2003). This is ultimately a form of neuroimmunomodulation to inhibit inflammation and macrophage release of inflammatory mediators such as TNF and IL-1 (Pavlov et al., 2003). With a depressed vagal (parasympathetic) output during stress, a lack of activity in the cholinergic anti-inflammatory pathway may contribute to the effects of increased hypersensitivity and asthma.

1.7 Regulatory T cells

While the role of Th2 type cells is well characterized in asthma, less is known about the regulatory mechanisms that protect against allergic disease. Regulatory T cells (T-regs) have been shown in both animal models and epidemiological studies to be affected in allergic airway disease. T-regs are T cells that can actively suppress the function of other cells and are important for tolerance against innocuous antigen.

Natural regulatory T cells (T-regs) are CD4+CD25+ cells with activation of the forkhead box P3 (*foxp3*) gene and develop in the thymus. T-regs can also be induced in the periphery by dendritic cells in conjunction with immunosuppressive cytokines (TGF-β, IL-10). FOXP3 is a transcription factor that is essential for establishing T-reg lineage and function (Beissert et al., 2006). T-regs work to regulate inflammation primarily through release of anti-inflammatory cytokines, such as TGF-β, IL-10, and direct interaction with dendritic cells (Beissert et al., 2006). They have also been shown to induce apoptosis and metabolic disruption in effector T cells. They are currently investigated for their role in immune disease, which may aid in the investigation of autoimmune disease pathogenesis and development of new therapeutics.

In a study by Dr. Hartl, there was a decrease of $CD4+CD25^{hi}$ T-regs in the bronchoalveolar lavage fluid of asthmatic children and for which there was dysfunction in suppressing proliferation of CD4+CD25- responder T cells (Hartl et al., 2007). Other studies on allergic diseases have similar findings where Tregs were less effective in suppressing T cell proliferation (Ling et al., 2004) . It is clear that changes in T-regs quantity or function can mediate the effects of increased asthma severity in prenatally stressed offspring.

1.8 Dendritic Cells

As allergic asthma is a Th2 polarized response, antigen-presenting dendritic cells (DCs) play a pivotal role in polarizing the T-cell response and increasing susceptibility to asthma and allergic diseases (Bharadwaj et al., 2007; Lambrecht and Hammad, 2009).

DCs bridge the gap between innate and adaptive immunity. They have the ability to process antigens and migrate to the draining lymph node in order to present antigen to T cells, directing the appropriate adaptive immune response.

Immature dendritic cells reside throughout lung tissue and capture antigen that they present to draining mediastinal lymph nodes within 12 hours (Vermaelen et al., 2001). In the case of harmless antigen, tolerance is usually obtained. However, in allergic asthma dendritic cells fully mature and induce antigen-specific T cell proliferation, sensitizing the individual for a specific antigen. Antigen-specific T cells are then capable of recirculation to the lungs and re-encounter the antigen on dendritic cells, and become effector T cells, propagating the disease. On the other hand, memory T cells remain and subsequent encounter with antigen through dendritic cells would lead to more T cell proliferation, with increased effector T cells whenever the individual is challenged with the antigen (Lambrecht and Hammad, 2003; Lambrecht et al., 1998). Depletion of dendritic cells prior to antigen challenge in transgenic mice can completely abrogate allergic airway disease in mice (Lambrecht et al., 1998; van Rijt et al., 2005). These considerations highlight a role for dendritic cells not only in initiation but continual propagation and maintenance of allergic airway disease.

Prenatal stress has been previously shown to cause adult offsprings to have an increased susceptibility to airway inflammation and hyper-responsiveness, where the isolated antigen presenting cells (APCs) were able to encourage Th2 clonal expansion *in vitro* (Pincus-Knackstedt et al., 2006).

Recently, Fedulov and Kobzik (2010) were able to isolate dendritic cells from neonates of allergic mouse mothers, which induced allergic susceptibility through adoptive transfer experiments and have enhanced antigen presenting function *in vitro* (Fedulov and Kobzik, 2011). Furthermore, through genome wide analysis, there were detectable congenital methylations in the pro-allergic CD11c+ dendritic cells (Bharadwaj et al., 2007; Fedulov and Kobzik, 2011). This suggests that epigenetic modifications in dendritic cells may play a significant role in polarizing Th2 response and increasing allergy risk.

1.9 Epigenetics

With the emergence of epigenetics in research, the mechanism of environmental influence on disease onset and progression can be more fully elucidated. Epigenetics was originally defined by Waddington, as 'the causal interactions between genes and their products, which bring the phenotype into being' (Kuriakose and Miller, 2010). Ultimately, epigenetic mechanisms including DNA methylation, histone modifications and micro-RNAs lead to heritable changes in gene expression without alterations in DNA (Miller and Ho, 2008).

Recent advances have illustrated the role of epigenetics in asthma, through differential environmental exposure and development of asthma at the molecular level (Miller and Ho, 2008). Epigenetic changes in various immune components that encourage Th2 polarization have been observed, including IFN-γ promoter methylation, IL-4 promoter demethylation, IL-13 promoter hypomethylation and region rad50 hypersensitive site 7 demethylation (involved in regulating expression of IL-4, IL-5 & IL-13) (Kuriakose and Miller, 2010).

Epigenetic modifications are thought to be more susceptible to changes during prenatal, early childhood and adolescence periods (Miller and Ho, 2008). In agreement with these findings, Barker hypothesizes that development programming *in utero* may predetermine physiological conditions of an individual into adulthood (Barker, 2004). Although much of 'intrauterine programming' focuses on the effect of malnutrition leading to greater tendency of encountering diseases in later life, subsequent studies have shown that factors such as glucocorticoids, psychological stress, prenatal environmental tobacco smoke (ETS), folic acid supplements, diesel exhaust particles and allergens may also affect intrauterine programming (Barker, 2004; Kuriakose and Miller, 2010; Miller and Ho, 2008).

Regulatory T cells, which play a role in suppressing effector cells, are found to have epigenetic mechanisms involved in controlling their development (Kuriakose and Miller, 2010). More specifically, epigenetic changes allow for the expression of transcription factor, FOXP3, and ultimately differentiation into regulatory T cells (Kuriakose and Miller, 2010). It should come as no surprise, that epigenetic modifications likely play a role in the function of other immune cells, such as dendritic cells.

The most promising aspect of epigenetic involvement in disease is the potential to reverse these modifications. Studies by Weaver *et al.* (2006) have demonstrated reversal of early-life epigenetic modifications of glucocorticoid receptor expression using histone deacetylase inhibitor trichostatin A (TSA) and methionine (Weaver et al., 2006). Through understanding the role of epigenetics in asthma, the possibility for effective therapeutic treatments and disease prevention strategies will follow.

1.11 Hypothesis

Differences in allergy susceptibility caused by intrauterine programming can be attributed to changes in dendritic cell functions and glucocorticoid responsiveness, caused by epigenetic mechanisms. Alternatively, other function alterations such as loss of parasympathetic output in the offspring, may also contribute to increase asthma severity.

1.12 Objectives

I. Characterize effects of intrauterine programming by stress on allergy severity of offspring to cockroach allergen

In order to address the hypothesis, a paradigm of prenatal stress affecting allergic airway disease needs to be established, where physiological outcomes need to be assessed for the severity of allergic airway disease. Characteristics of allergic airway disease including airway inflammation, lung histology, and cockroach specific antibodies (IgG, IgE) levels were assessed in normal healthy animals compared to prenatally stressed animals.

II. Determine if the effects of prenatal stress can be attributed to changes in cell population

Subsequently, we proposed that the effects of prenatal stress on allergic airway disease could be better understood through distinguishing the prenatal stress effects on the immune system and characterizing changes to dendritic cells and Foxp3+ regulatory T cells in the spleen and mesenteric lymph node.

Dendritic cells play an important role in determining whether to initiate a protective immune response or tolerance. Their interaction with naive T cells can polarize a Th2 type response and sensitize as well as propagate an allergic immune response to subsequent allergen exposure. The maturation status or the expression of maturation markers on dendritic cells is closely linked with its ability to effectively mount an allergic immune response and present antigen. While the ability of dendritic cells to polarize a Th2 type immune response is better understood, less is known about its ability to induce regulatory T cells.

Regulatory T cells have been shown in epidemiological studies to be affected in asthmatics, as well as in animal models of allergic airway disease. Their function is becoming more apparent in protecting against immunological diseases, including asthma. In addition, T-cell regulatory functions as well as induction require close interaction with dendritic cells.

III. Identify changes in immune cell function

While extracellular markers such as co-stimulatory molecules (i.e. CD80, CD86) on dendritic cells can allude to dendritic cell ability to present antigen to T cells, changes in function is a much stronger indicator for its role in propagating the effects of prenatal stress. Dendritic cell functions including cytokine release, antigen specific T cell proliferation and glucocorticoid responsiveness was assessed. Regulatory T cell ability to suppress antigen specific T cell proliferation was also assessed.

IV. Identify changes in DNA methylation

Finally, the methylation status of CpG island upstream of selected DNA segments were compared for differences leading to changes in gene expression and function.

Methods

2.1 Animals

BALB/c mice were purchased from Harlan Laboratory, but later purchased from Charles River Laboratories as a result of an enteric protozoan contamination at the Harlan facility. They were maintained in ventilated specific pathogen free conditions with a 12-h light/dark cycle and allowed to acclimatize for 1 week prior to experimentation. Animal care and experimental procedures were reviewed and approved by the Animal Research Ethics Board at McMaster University.

2.2 Prenatal Sound Stress

A sound stress protocol was adapted from a study by Pincus-Knackstedt *et al*. (2006). 6 to 8 week-old BALB/c females were mated with 6 to 8 week-old BALB/c male mice. Successful mating was assumed upon the appearance of a vaginal plug. Pregnant females were segregated and divided into two different subgroups. One group served as a control and was left undisturbed during pregnancy. The other group was exposed to stress on days 12 and 14 of gestation.

Exposure to sound stress was applied for the duration of 24 h similar to that described by Pincus- Knackstedt *et al*. (2006). A rodent repellent device (Conrad Electronic, Germany) emitted sound at 70 dB at a frequency of 300 Hz in intervals of 15 s. The stress device was placed into the mouse cage so that the mice could not escape the sound perception and lights were left on.

Delivery of litters was documented, and females with their respective litter were kept in individual cages and left undisturbed for a period of 21 days. Offspring were weaned and gender distribution was documented. Male and female offspring were separated for subsequent experiments.

2.3 Cockroach Model of Allergic Airway Disease

Lyophilized frozen whole body extracts from German cockroach (*Blattella germanica*) were obtained from Greer Laboratories, (Lenoir, NC, USA) and were resuspended in sterile normal saline and stored at 4°C. Mice were sensitized with cockroach protein (1mg/mL) intranasally every day from day 2 to day 7 after birth (day 0). The litters were otherwise left undisturbed during and after the weaning period of 19 to 21 days and challenged on day 49 to day 52 (4 consecutive days at 6 to 8 weeks old) with either saline or cockroach protein (1mg/mL). Adult offspring were assessed for characteristics of allergic airway disease 24 hours after final challenge (Figure 1).

2.4 SCIREQ Flexivent Pulmonary Function

Airway responsiveness was measured as a total respiratory resistance (R_{RS}) after intravenous injections of saline and then 100ug/kg of methacholine (MCh). R_{RS} was measured using the forced oscillation technique, as modified for use with mice adapted from Dr. Inman's group (Inman *et al*., 1999).

Mice were sedated with Xylazine/Rompun (Bayer Healthcare) at 10mg/kg and anesthetised with Sodium Pentobarbital (McMaster University) at 30mg/kg, both administered intraperitoneally. Once the mouse has been attached to the ventilator, Pancuronium Bromide at 20mg/kg was administered intravenously to prevent respiratory effort from the mouse during measurement.

Mechanics were assessed on a flexiVent v5.2 rodent ventilator (Scireq Scientific Respiratory Equipment Inc, Montreal, Canada). Mice were ventilated at a rate of 150 breaths/min through an 18-gauge ET tube. Lung volume history was normalized before each dose of MCh (Sigma) by inflating the lungs to a pressure of 25 cmH_2O and maintaining this pressure for 3 s, followed by 30 s of normal ventilation. Total respiratory system resistance (R_{RS}) was measured using a single-compartment, standardized breath signal, which allows measurement of resistance, dynamic compliance, and elastance (SnapShot-150 perturbation). Measurements were obtained at baseline and following intravenous (IV) MCh challenge using a dose of 100 μg/kg. Resistance measurements were obtained for 1 min following injection of MCh. To ensure that animals were adequately ventilated and not experiencing distress, heart rate and oxygen saturation were monitored throughout the procedures using infrared pulse oxymetry (Biox 3700; Ohmeda, Boulder, CO) with a standard ear probe placed on the proximal portion of the mouse's hindlimb. After the last dose, mice were removed from the ventilator, killed via terminal exsanguination, and further processed for tissue collection.

2.5 Bronchial Alveolar Lavage

Following sacrifice of the mice, the trachea was exposed and cannulated using an 18-gauge blunted needle. One injection of 1mL phosphate buffered saline (PBS) was injected and recovered through the needle. The bronchial alveolar lavage fluid was centrifuged for 10 minutes at 150G and 4°C. The cell pellet was resuspended and total cell count was confirmed using a hemocytometer. Cytocentrifuge slides were prepared with $5x10^4$ cells per 100uL (Cytospin 3; Shandon Scientific, Sewickley, PA) and stained with Diff-Quik, and cell differential counts were performed based on morphologic and histologic criteria.

2.9 Flow Cytometry

Spleens, lungs, bronchial lymph nodes and mesenteric lymph nodes of prenatally stress and control adult offspring were harvested. Mechanical disruption of the organs in collagenase type IV (Sigma, Oakville, ON, Canada) was completed and incubated for 30 minutes at 37°C with 5% CO_2 to obtain single cell suspensions. Red blood cell lysis (eBioscience, San Diego, CA, USA) was performed on all splenocyte samples. $1\text{-}2x10^6$ cells per sample were stained and analyzed through flow cytometry.

I. Regulatory T Cells

Spleens and mesenteric lymph nodes of prenatally stressed and control adult offspring were harvested. Mechanical disruption of the organs was performed to obtain single cell suspension. $1x10^6$ cells per sample were analyzed through flow cytometry for CD3, CD4, CD25 and intracellular Foxp3 measurement.

II. Dendritic Cells

Spleens, lungs (pulmonary perfusion was performed) and mesenteric lymph nodes of prenatally stressed and control adult offspring were harvested. Mechanical disruption of the organs in collagenase type IV (Sigma, Oakville, ON, Canada) or collagenase D (Roche, Mississauga, ON, Canada) was completed and incubated for 30 minutes at 37°C with 5% CO_2 to obtain single cell suspensions. Red blood cell lysis was performed on all splenocyte and lung samples. $1x10^6$ cells per sample were analyzed with flow cytometry for the extracellular marker CD11c and DC maturation markers (MHC II, CD86, CD40, CD80).

3.0 Regulatory T Cell Epigenetics

T cells were purified from spleenocytes for epigenetic analysis with CD4+ microbeads and MACS LS Columns (Miltenyi Biotec, Cambridge, MA, USA) according to the manufacturer's instructions. DNA was extracted from purified cells with the DNAEasy kit (Qiagen, Toronto, ON, Canada) and quantified using absorbance measures at 260nm. Bisulfite conversion was performed and samples were stored at -80°C prior to being sent to EpigenDX for CpG methylation analysis of two regions upstreams of the foxp3 gene.

3.1 Inflammatory Cytokine Cytometric Bead Array

Spleens of prenatally stressed and control adult offspring were harvested. Mechanical disruption of the organs in collagenase type IV (Sigma, Oakville, ON, Canada) was completed and incubated for 30 minutes at 37°C with 5% CO_2 to obtain single cell suspensions. Red blood cell lysis was performed on all samples.

Dendritic cells were purified from spleenocytes from 3 animals (for each sample) using CD11c+ microbeads and MACS LS columns (Miltenyi Biotec, Cambridge, MA, USA). They were stimulated with either 1μg/ml lipopolysaccharide (Sigma, Oakville, ON, Canada), 100μg/ml Poly I:C (Sigma, Oakville, ON, Canada) or 4μg/ml CpG (Mobix McMaster Univeristy, Hamilton, ON, Canada) for 18 hours and the supernatant was collected to measure cytokine release using flex sets (BD™ Cytometric Bead Array) measuring levels of IL-6, IL-10, TNFα and IL-12. Supernatant was assessed according to the manufacturer's protocol, where standards were provided. Samples were run using FACSArray bioanalyzer (Becton Dikinson, Mississauga, ON) and analyzed with FCAP Array™ Software.

In order to determine whether sex differences existed in the model, males and females from the two groups, prenatally stressed and control mice, were kept separate. Prenatally stressed animals were naive (not exposed to allergen). Sample size varied as a result of the number of animals available and difficulties with the FACSArray analysis with some samples - control female (n=21), prenatally stressed female (n=24), control male (n=12) and prenatally stressed male (n=18).

3.0 Antigen specific T cell proliferation assay

Dendritic cells were isolated from spleens of mice according to manufacturer's protocol using MACS microbead mouse CD11c+ cell kit (Miltenyi Biotec, Cambridge, MA, USA). DCs were incubated in OVA at 1mg/mL for 18-20 hours.

$CD4^+$ T cells from spleens of transgenic D011.10 mice were isolated according to manufacturer's protocol ex vivo using MACS microbead mouse CD4+ T cell isolation kit (Miltenyi Biotec, Cambridge, MA, USA). $CD4^+$ cells were labeled with CFSE.

DCs and CD4+ T cells were counted and plated at varying DC to T cell ratios (1:10, 1:20, 1:50), where T cells remained at a concentration of $2x10^5$ cells per well. The co-culture was left to incubate for 3 days at 37°C with 5% CO_2.

3.2 Catecholamines ELISA

After decapitation, trunk blood was collected in epitubes containing 50uL of 50mM EDTA and inverted 8-10 times before centrifugation at 1500 rpm 15 minutes at 4°C. The top plasma layer was transferred into new epitubes and stored at -80°C prior to running ELISA. Epineprine and norepinephrine in plasma from mice collected after decapitation was assessed using 2-CAT ELISA Fast Track (Rocky Mountain Diagnostics Inc, USA), following the manufacturer's protocol.

3.3 Glucocorticoid Responsiveness

Dendritic cells were purified from splenocytes using CD11c+ microbeads and MACS columns (Miltenyi Biotec, Cambridge, MA, USA). $2x10^5$ cells were stimulated with 1μg/ml of lipopolysaccharide (Sigma, Oakville, ON, Canada, E. coli 0111:B4) and varying concentrations of corticosterone (Sigma, Oakville, ON, Canada) – 0μM, 0.005μM, 0.05μM, 0.1μM, 0.5μM and 5μM. After 48 incubation at 37°C with 5% CO_2, Cell titer 96 aqueous non-radioactive proliferation (Promega, Madison, WI, USA) was used according to manufacturer's protocol to determine cell viability within cultures. 20μL of tetrazolium substrate solution was added to each 100μL sample and incubated at 37°C with 5% CO_2 and measured optical density (OD) at 492nm on an ELISA plate reader, every hour for 3 hours. To account for differences in background activity, the mean OD of three wells containing medium and 0.2% ethanol were subtracted from all values. Percentage of cell survival was also calculated for each sample, where 100% cell survival was cell sample stimulated with LPS and 0μM of corticosterone.

3.4 Vagotomy Surgical Procedure

Vagotomy was performed as previously described (Karimi et al., 2010). Briefly, mice were given water supplemented with Septra for 5 days prior to surgery and 5 days post surgery to minimize the opportunity for infection to occur. Mice were anesthetized with temegesic and the skin and abdominal wall were incised along the ventral midline and the intestine retracted to allow access to the left lateral side of the stomach. The left lateral lobe of the liver was retracted and a ligature placed around the esophagus at its entrance to the stomach, to allow gentle retraction to clearly expose both vagal trunks. These were dissected and all neural and connective tissue surrounding the esophagus below the diaphragm was removed to transect all small vagal branches. For 5 days post-surgery mice were given saline and paracetamol as needed and carefully monitored. A two-week recovery period was allowed.

3.5 Assessment of vagotomy

Successful vagotomy was assessed as previously described using cholecystokinin-octapeptide (CCK-8) (Sigma, Oakville, ON, Canada) (Karimi et al., 2010). CCK-8 induced satiety mediated by the afferent vagus nerve (Lorenz and Goldman, 1982). After 20h food deprivation, mice that were successfully vagotomised are unaffected by CCK-8 i.p. administration (8 μg/kg bodyweight). Their intake of food did not decrease significantly, as it would in controls that exhibit satiety.

3.6 Nicotine receptor antagonist treatment

The nicotinic antagonists, mecamylamine (1 mg/kg), and methyllycaconitine (5 mg/kg) were administered twice daily through interperitoneal injections for 5 days.

3.7 Cytokine ELISA

Cytokine ELISAs for IL-6, IL-12 and TNF-α (BD Bioscience, Mississauga, ON, Canada) were performed according to manufacturer's protocol.

3.8 Statistical Analysis

Results are expressed as means ± the standard errors of the means. Statistical analyses were performed by means of one-way or two-way analysis of variance (ANOVA), followed by the Bonferroni test for comparing all pairs of groups. Significant differences between two groups were determined using the unpaired Student's t test. The statistical software package GraphPad PRISM™ version 5.0 was used for the analysis. A P value of less than 0.05 was considered statistically significant.

Results

4.0 Effects of Prenatal Stress on Allergic Airway Disease

I. Prenatal stress increases allergic airway inflammation in males, but decreases allergic airway inflammation in females

Allergic airway inflammation was assessed through total cell count of bronchoalveolar lavage fluid (BALF) of sensitized animals challenged intranasally with either cockroach protein (CR) or PBS. The effects of the CR challenge were evident in both male (P<0.001) and female offspring (P<0.001), when compared with PBS challenged animals. In male offspring, the effects of prenatal stress was evident (P<0.05), Bonferroni's post-hoc test found prenatal stress increased BAL total cell numbers compared to controls in CR challenged animals (51.73±7.57 vs. 31.47±4.26, Bonferroni P<0.01) (Figure 2A). In females, the effect of prenatal stress (P<0.01) decreased total BAL total cell numbers compared to controls in CR challenged animals (18.40±1.47 vs. 30.40±3.92, Bonferroni P<0.01) (Figure 2B). In both sexes, there was an interaction between prenatal stress and use of CR challenge (P<0.05).

Differential cell count was performed on the BALF, where the percentage of eosinophils was examined. Samples of each of the groups are provided in Figure 3. In both sexes, the effect of CR challenge was evident (P<0.001), compared to PBS challenges animals. In male offspring, prenatal stress increased the percentage of eosinophils in the BALF compared to control animals (64.92±3.79 vs. 47.04±6.62, Bonferroni P<0.05) (Figure 4A). However, the effect of prenatal stress on percentage of eosinophils in the BALF was not detected in female offspring (Figure 4B).

II. Prenatally stressed female offspring exhibit airway hyporesponsiveness

Monitoring airway resistance in response to methacholine, acts as a measure of airway responsiveness. In order to normalize the data, total airway resistance of control or prenatally stressed animals that were challenged with cockroach protein (CR) was calculated as a fold increase of saline challenged animals. The effectiveness of CR challenge was seen in both male ($P<0.001$) and female offspring ($P<0.01$) compared their saline challenged controls. There was no significant effect of prenatal stress on either male (Figure 5A) or female offspring (Figure 5B). A sample demonstrating how airway resistance was calculated is shown in Figure 5C.

4.1 Changes in Cell Population

I. Lower percentage of regulatory T cells in prenatally stressed animals

The effect of prenatal stress on the regulatory T cell population of the spleen and mesenteric lymph node was assessed. The percentage of T-regs in the total cell population was assessed through flow cytometry and characterized for $CD4^+CD25^+Foxp3^+$. Sample gating is shown in Figure 6.

In the spleen of males, a t-test revealed significantly decreased percentage of regulatory T cells in prenatally stressed animals compared to controls (35.17±3.83 vs. 50.87±2.55, $P<0.01$) (Figure 7A). This was also seen in the mesenteric lymph node where prenatally stressed animals had a lower percentage of $CD4^+CD25^+Foxp3^+$ T-regs

(32±6 vs. 58±3, P<0.01) (Figure 7B). In females, no differences were found in either spleen or mesenteric lymph node (data not shown).

Next we sought to determine if these differences were mediated through DNA methylation. As the *foxp3* gene is x-linked all data are from male animals only in order to avoid mixed results from x-inactivation found in female animals. Two CpG rich regions in the promoter were analyzed for methylation through bisulfite conversion of DNA samples obtained from mouse spleens. An example of bisulfite conversion is shown in Figure 8. The two regions are distal from the promoter; region A657 is -12435bp to -12225bp from ATG with 25 CpG sites and region A569 is -12211bp to -12060bp from ATG with 30 CpG sites (Figure 9C). Methylation was decreased in both region A657 (2.12±0.12 vs. 18.02±6.78, P<0.01) and region A569 (11.7±0.61 vs. 33.14±9.54, P<0.01) in prenatally stressed animals compared to controls (Figure 9AB).

II. Sex differences and effects of prenatal stress were observed in DC phenotype

The effect of prenatal stress on the DCs of lungs, spleen and mesenteric lymph nodes in otherwise naive (non-sensitized) animals were assessed. DCs were assessed on the basis of extracellular maturation markers CD86, CD40 and CD80, using flow cytometry. DCs were characterized on the basis of $CD11c^{+}MHCII^{+}$ expression. Sample gating of total spleen cells is provided in Figure 11. Corresponding receptors on T cells are shown in Figure 10.

In the spleen, we first examined the percentage of $CD11c^{+}MHCII^{+}$ expression by 2-way ANOVA and found a main effect for sex (P<0.05) but not prenatal stress, with the males showing an increased expression compared to the females collapsed across prenatal stress status. With post-hoc tests, we found a specific increase in prenatally stressed males compared to prenatally stressed females (13.82±1.68 vs. 9.25±0.71, Bonferroni $P<0.05$) (Figure 12A). There were no differences on any of the DC maturation markers analyzed (Figure 12B-C).

Prenatal stress decreased the percentage of CD11c$^+$MHCII$^+$ cells in the mesenteric lymph node (4.44±0.47 vs. 7.63±1.32, Bonferroni $P<0.05$) (Figure 13A) and lungs (5±1.17 vs. 9.37±1.52, Bonferroni $P<0.05$) (Figure 14A) compared to control female offspring but had no effect on males. However, the effect of prenatal stress or sex was not seen in the expression of maturation markers CD86, CD40 and CD80 in both mesenteric lymph node (Figure 13B-D) and lungs (Figure 14B-D).

4.2 Identify Changes in Dendritic Cell Function

I. Sex differences and effect of prenatal stress were seen in DC cytokine release in response to TLR ligation

Isolated spleen DCs were assessed for cytokine (IL-6, IFN-γ, TNF-α) release in response to 18h TLR stimulation with LPS, PolyI:C or CpG (Figure 15).

In LPS stimulated DCs, there was no effect on IL-6 release (Figure 15A). There was a main effect of prenatal stress with increased IFN-γ in prenatally stressed animals of both sexes compared to controls ($P<0.05$). The post-hoc test shows prenatal stress specifically increased DC IFN-γ secretion in males (7.46±1.92 vs. 1.52±1.09, Bonferroni $P<0.05$) (Figure 15B). There was a main effect of sex, but not prenatal stress, with greater TNF-α secretion in males than in females collapsed across prenatal stress status ($P<0.05$). Post-hoc test shows increased TNF-α in prenatally stressed males compared to prenatally stressed females (125.20±10.67 vs. 77.34±9.29, Bonferroni $P<0.05$) (Figure 15C).

In Poly I:C stimulated DCs, there was no effect on IL-6 (Figure 15D) or TNF-α (Figure 15F) release. There was a main effect of sex, but not prenatal stress, with increased IFN-γ secretion in males compared to the females collapsed across prenatal stress status ($P<0.01$). Post-hoc test shows prenatally stressed males secrete more IFN-γ than prenatally stressed females (5.24±1.04 vs. 1.24±0.50, Bonferroni $P<0.01$) (Figure 15E).

In CpG stimulated DCs, there was a main effect of sex on IL6, with a greater production in males than in females ($P<0.05$). Post-hoc test shows increased IL-6 in prenatally stressed males than in prenatally stressed females (591±73.02 vs. 350.5±50.09, Bonferroni $P<0.01$) (Figure 15G). There was a main effect of both sex and prenatal stress on IFN-γ ($P<0.01$), specifically where prenatally stressed males produced a greater amount of IFN-γ than prenatally stressed females, as revealed by the Bonferroni post-hoc test (333.6±36.67 vs. 135.5±24.6, Bonferroni $P<0.001$) (Figure 15H). In addition, there was an interaction where males and females would respond differently to prenatal stress ($P<0.01$) (Figure 15H). There was also a sex different in TNF-α release, with increased release from males compared to females ($P<0.01$). Post-hoc test revealed that prenatally stress males produced higher TNF-α compared to prenatally stressed females (1164±144.1 vs. 736.3±56.18, Bonferroni $P<0.01$) (Figure 15I).

The same experiment was performed on isolated DCs from control animals in order to determine the source of IFN- γ. Isolated DCS were stimulated with CpG for 18 hours and analyzed using flow cytometry. Sample gating is provided in Figure 16. Cells were gated for size (FSC, SSC), and then gated for expression of CD11c+ and MHCII+. They were then excluded for B cells by gating for CD19- population. The resulting population was analyzed for intracellular expression of IFN-γ and F4/80. 15.99% of resulting population expressed IFN- γ and 11% also expressed F4/80 (Figure 16).

II. No effect of prenatal stress on dendritic cell glucocorticoid sensitivity

Isolated spleen DCs were assessed for responsiveness to corticosterone in the presence of TLR stimulus. The effects of varying GC concentrations were evident both in males ($P<0.001$) and females ($P<0.05$). However, effects of prenatal stress were not seen in either males (Figure 17A) or females (Figure 17B).

III. No effect of prenatal stress on dendritic cell induced antigen specific T cell proliferation

DCs were loaded with 1mg/mL of OVA for 18-20 hours prior to co-culture with CD4+ T cells from transgenic D011.10 mice. CD4+ T cells were stained with CFSE prior to co-culture. Subsequent proliferation induced by DCs can be analyzed through assessing the % of CD4+ T cells with decreased CFSE intensity. Sample gating is provided in figure 18.

The effect of prenatal stress was not seen in either male (Figure 19A) or female offspring (Figure 18B). The effect of varying DC to T cell ratio was apparent in both experiments ($P<0.001$).

4.3 Proof of Concept: Effect of Parasympathetic Modulation on Dendritic Cell Function

During stress, the balance between sympathetic and parasympathetic nervous system is altered. In addition to elevated basal and stress-induced plasma corticosterone levels, prenatally stressed offspring have also been reported to have elevated and prolonged stress-induced catecholamines (Weinstock *et al*., 1998). In order to assess the effects changes in the autonomic nervous system on the immune system, particularly on dendritic cells, sub-diaphragm vagotomy was performed to inhibit the parasympathetic pathway.

I. Dendritic cells of vagotomised animals have decreased CD40, but not CD86 expression

Vagotomized animals were assessed for changes in dendritic cells extracellular maturation markers using flow cytometry. DCs were identified as $CD11c^{+}MHCII^{+}$ cell population and subsequently gated for CD40 and CD86 expression. Vagotomised animals expressed lower CD40 than sham operated animals (51.42 ± 4.07 vs. 63.02 ± 1.36, $P<0.01$) (Figure 20A), but no effect was seen on CD86 expression (Figure 20B).

II. Dendritic cells of vagotomised animals have enhanced IL-6 and IL-12 expression in splenocyte population, but not after CD11c isolation

Single cell suspension from the spleens of vagotomised and sham operated animals were stimulated for 18 hours with CpG, with golgi plug and $CD11c^{+}MHCII^{+}$ cells were assess for intracellular expression of IL6, IL-10, IL-12 and TNF-α (Figure 21). IL-6 expression was increased in vagotomised animals ($P<0.001$) in both unstimulated (9.91±1.07 vs. 4.77±0.51, Bonferroni $P<0.05$) and CpG stimulated cells (12.91±2.14 vs. 5.73±0.44, Bonferroni $P<0.01$) compared to controls (Figure 21A). IL-12 expression was also increased in vagotomised animals ($P<0.001$) in both unstimulated (11.01±0.98 vs. 6.94±0.27, Bonferroni $P<0.05$) and CpG stimulated cells (15.01±1.84 vs. 7.58±0.25, Bonferroni $P<0.05$) compared to controls (Figure 21C). The effect of vagotomy was not observed in IL-10 (Figure 21B) or TNF-α expression (Figure 21D).

Dendritic cells were isolated from the spleen based on extracellular marker CD11c using MACS Microbeads. DCs were stimulated for 18 hours with CpG, with golgi plug and assess for intracellular expression of IL6, IL-10, IL-12 and TNF-α (Figure 22). The effect of CpG stimulation was evident in all 4 cytokines compared to unstimulated cells ($P<0.001$). However, there was no effect of vagotomy in any of the four cytokines.

In a similar experiment, dendritic cells stimulated for 18 hours with CpG and supernatant was assessed for IL-6, IL-12 and TNF-α using ELISA. The effects of CpG stimulation was evident in all 3 cytokines compared to unstimulated cells ($P<0.001$), but no effect of vagotomy was observed (Figure 23).

III. No effect of vagotomy on dendritic cell induced antigen specific T cell proliferation

Splenic DCs were loaded with 1mg/mL of OVA for 18-20 hours prior to co-culture with CD4+ T cells from transgenic D011.10 mice. CD4+ T cells were stained with CFSE prior to co-culture. Subsequent proliferation induced by DCs can be analyzed through assessing the % of CD4+ T cells with decreased CFSE intensity. Sample gating is provided in figure 18.

Vagotomy had no apparent effect (Figure 22). The effect of varying DC to T cell ratio was apparent ($P<0.001$).

IV. Nicotinic receptor antagonist

As previous reports have suggested a role for nicotinic receptors in mediating vagal regulation of other cell types, nicotinic receptor antagonists were used to mimic the results found in vagotomised animals. Mecamylamine, a general nicotinic receptor antagonist, and methyllycaconitine, nicotinic $\alpha 7$ specific receptor antagonist, were both used. The effects of nicotinic receptor antagonist were not observed in DC CD40 and CD86 expression (Figure 25AB), DC cytokine (IL-6, IL-12, TNFα, IL-10) expression in splenocyte (Figure 26A-D) or after isolation (Figure 27A-D) and cytokine (IL-6, IL-12, TNFα) release from isolated DCs measured using ELISA (Figure 28). Both nicotinic receptor antagonists failed to have any detectable effects on dendritic cell induced antigen specific T cell proliferation (Figure 29).

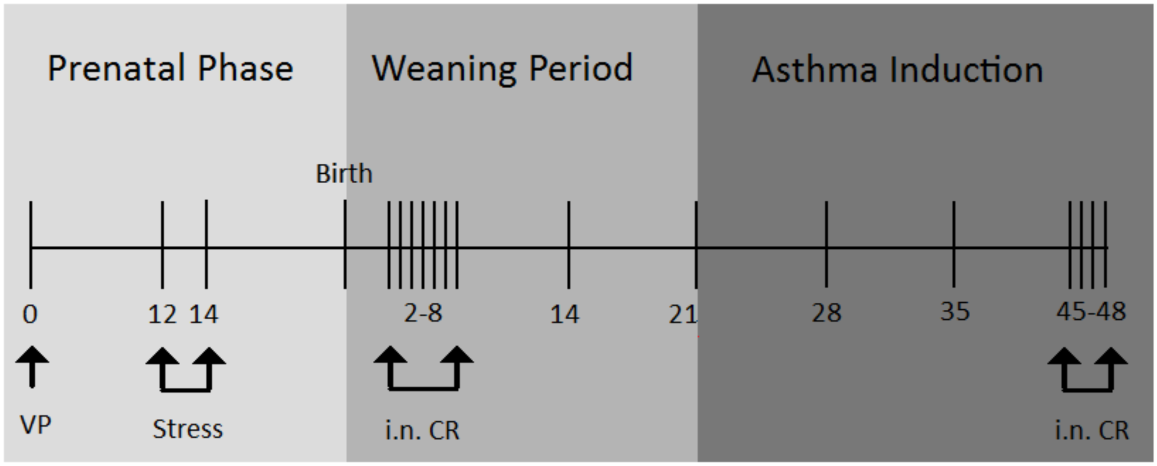

Figure 1. Model of Prenatal Stress and Allergic Airway Disease.

Pregnant dams are stressed on gestation day 12 and 14. Pups are sensitized intranasally (i.n.) with 10uL of 1mg/mL cockroach protein and challenged i.n. with 25uL of 1mg/mL cockroach protein at 6 to 8 weeks old. Prenatal stress model adapted from (Pincus-Knackstedt et al., 2006).

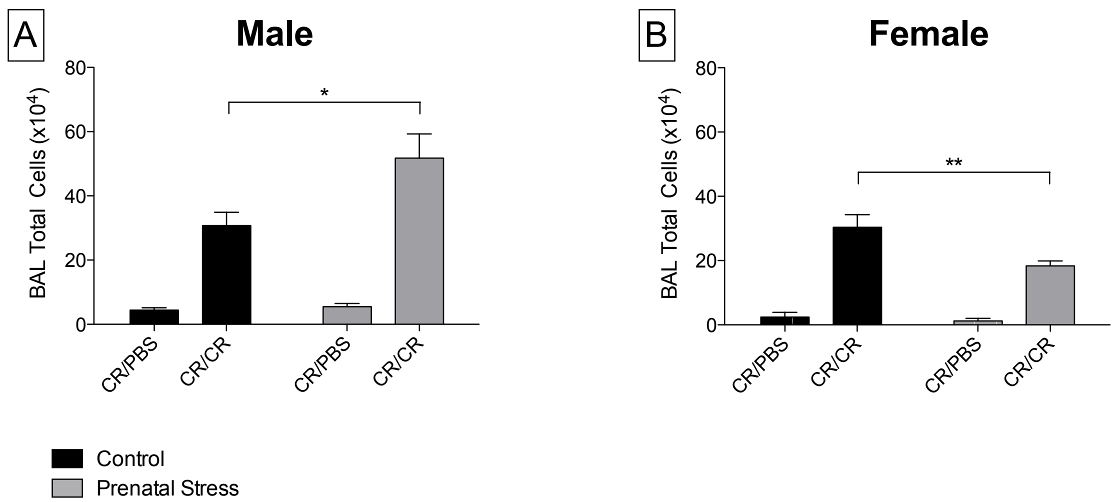

Figure 2. Cell infiltrates in the bronchaveolar lavage fluid (BALF) were increased in prenatally stressed males but decreased in prenatally stressed females.

The total number of cells in the BALF was counted in control and prenatally stressed animals challenged with either saline (CR/PBS) or cockroach protein (CR/CR). (A) In males, prenatal stress increased total cell count in CR challenged animals compared to controls (n=12 per group). (B) In females, there was a significant decrease in total cells of prenatally stressed animals challenged with cockroach protein (CR/CR) than control females. In both sexes, cockroach challenged animals had a significantly higher total cell count than saline challenged animals (n=5 per group). Graphs show mean + SEM. ** $P < 0.01$

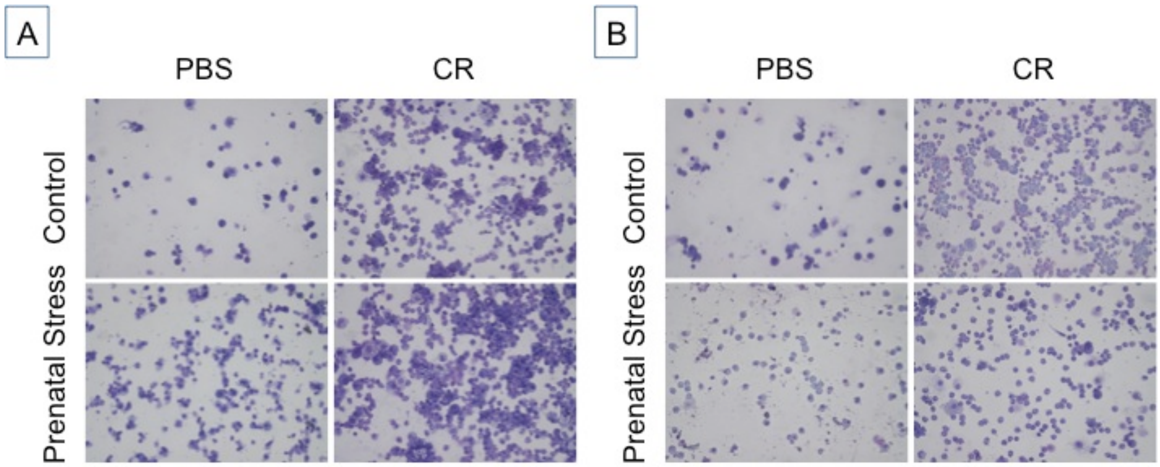

Figure 3. Cell infiltrates in the bronchaveolar lavage fluid (BALF) were increased in prenatally stressed males but decreased in prenatally stressed females.

Cells of the BALF were collected from CR-sensitized offspring where cytospins were prepared and stained for leukocyte differentiation. Representative examples of BALF cytospins of control (prenatally non-stressed) and prenatally stressed, CR-challenged and PBS-challenged (A) male offspring and (B) female offspring.

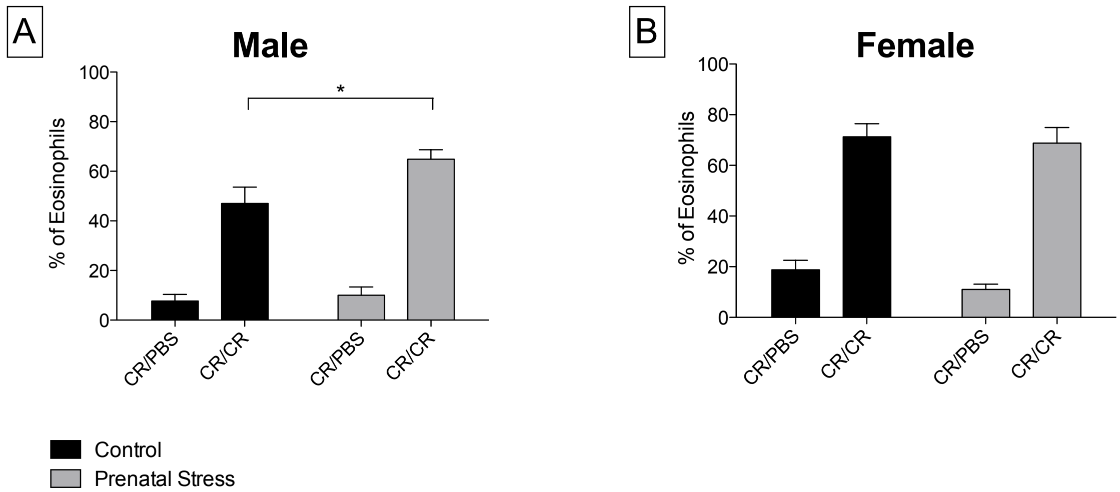

Figure 4. Percentage of eosinophils were increased in prenatally stress male offspring but not in females.

Cytospins of the BALF was prepared and stained for leukocyte differentiation and counted for percentage of eosinophils in (A) male and (B) female offspring (n=5-10 per group). Graphs show mean + SEM. * $P < 0.05$

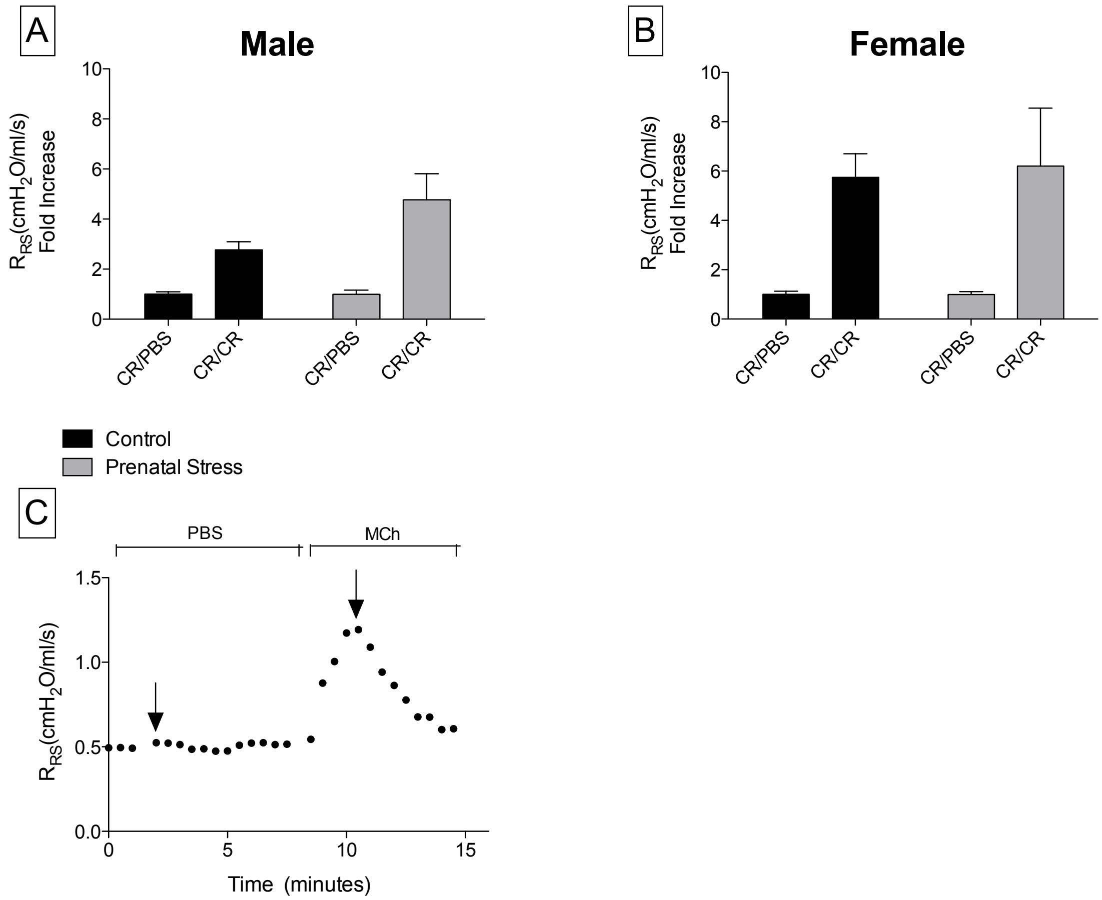

Figure 5. Prenatal stress does not affect airway hyporesponsiveness in male or female offspring.

Fold difference was calculated where each group was compared against the saline challenged (CR/PBS) control. There was no significant difference due to prenatal stress in males (A) (n= 6-10 per group) or females (B) (n=5-8 per group). In both sexes, cockroach challenged animals had a significantly higher respiratory resistance than saline challenged animals ($P<0.05$). (C) Total respiratory resistance (R_{RS}) was measured every 30 seconds before and after intravenous injection of saline (PBS) followed by 100ug/kg of methacholine (MCh). The difference between the peak measurements (indicated by ↓) was used as an indicator of respiratory resistance. . Graphs show mean + SEM.

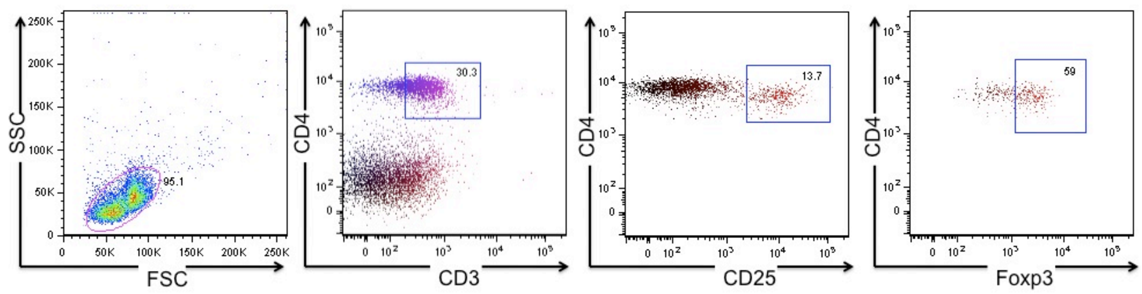

Figure 6. Sample gating for regulatory T cells of the mesenteric lymph node.

Consistent population is gated and analyzed for CD3+CD4+ population. This resulting population is gated for CD25, followed by intracellular expression of Foxp3. The resultant population is CD3+CD4+CD25+Foxp3+.

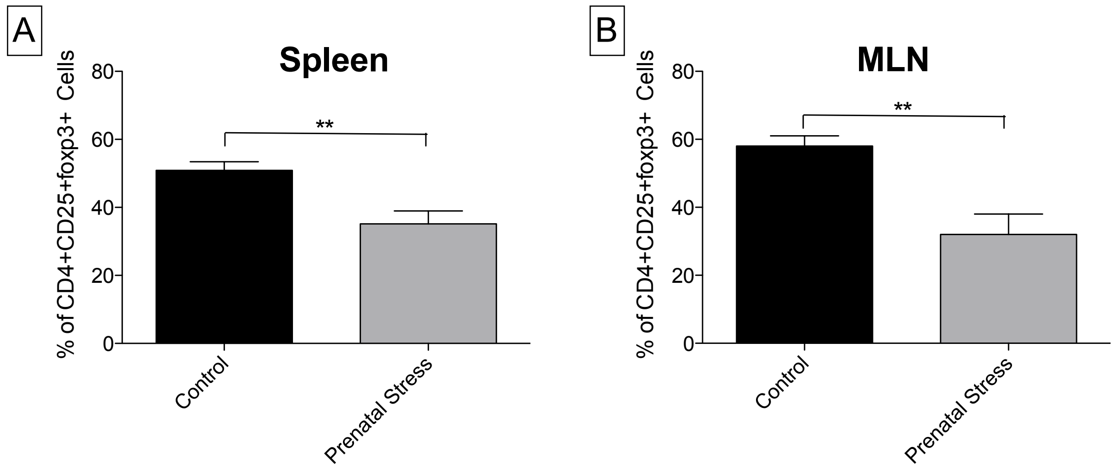

Figure 7. Prenatal stress decreased the percentage of Tregs ($CD4^{+}CD25^{+}Foxp3^{+}$) in the mesenteric lymph node but not the spleen.

(A) Prenatally stressed animals do not have a significantly different percentage of Tregs in the spleen (n=8 per group). (B) Prenatally stressed animals have a lower percentage of Tregs in the mesenteric lymph node (n=5 per group). Graphs show mean + SEM. ** $P < 0.01$

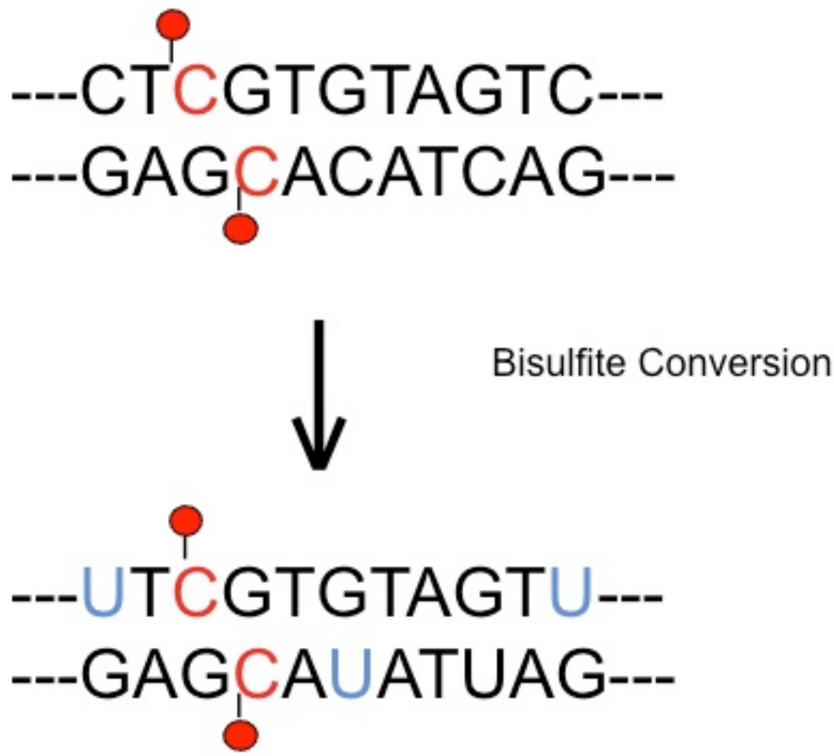

Figure 8. Process of bisulfite conversion.

DNA is treated with bisulfite converting all unmethylated cytosine residues into uracil. Methylated cytokine residues are protected from the reaction. Subsequent sequencing of the DNA will allow for accurate analysis of methylation of cytosine in the DNA segment.

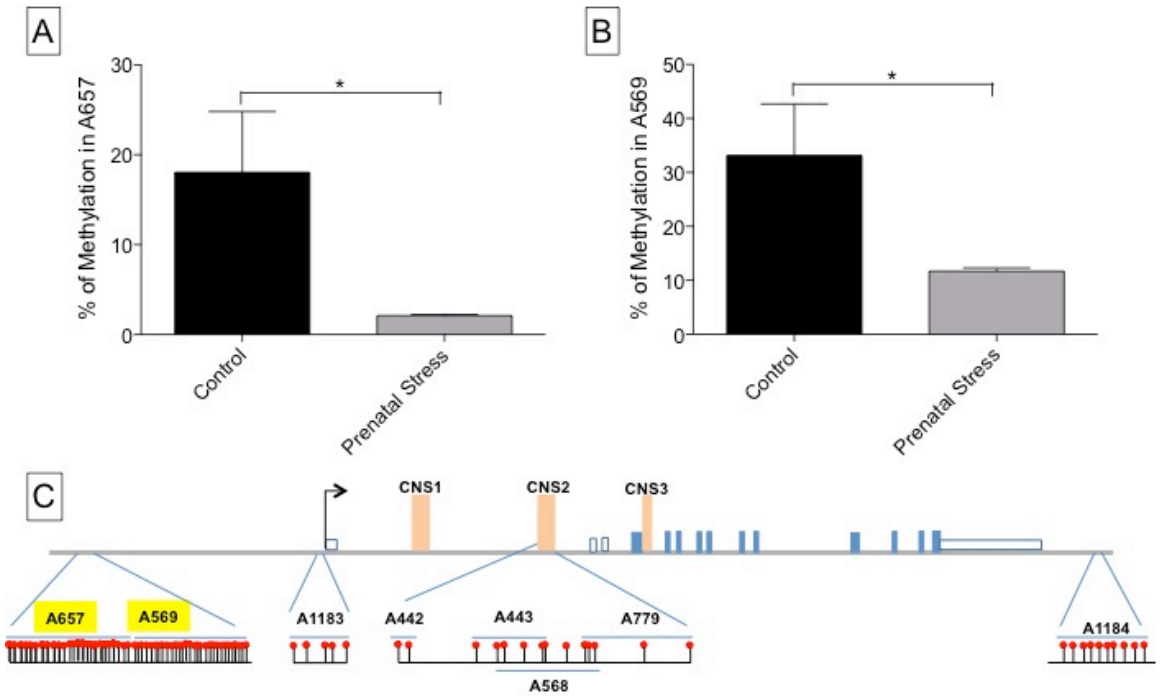

Figure 9. Prenatal stress decreases methylation in regions A657 and A569.

(A) Prenatally stressed animals had decreased percentage of methylation in region A657, with 25 CpG islands (n=5). (B) Prenatally stress animals had decreased percentage of methylation in region A569, with 30 CpG islands (n=5). (C) CpG rich regions are shown on the x-chromosome near the *foxp3* gene. Distal promoter regions analyzed are highlighted. Graphs show mean + SEM. * $P < 0.05$

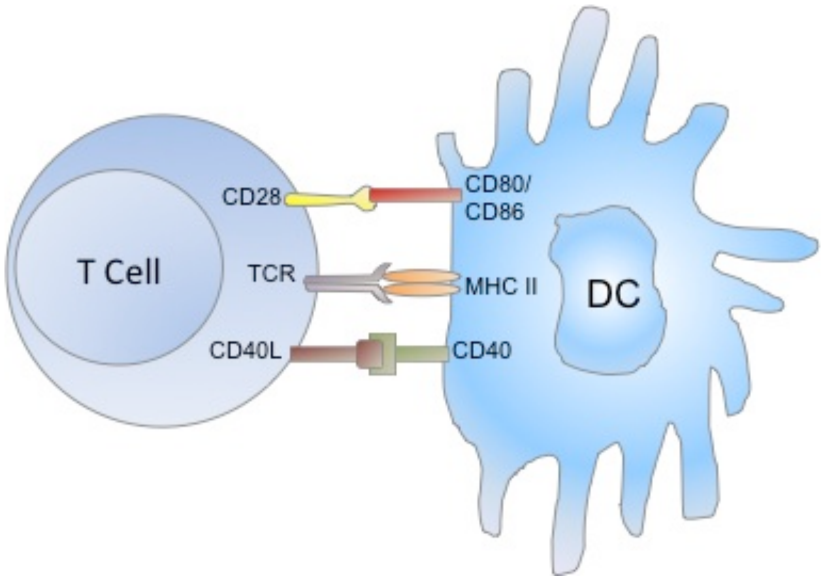

Figure 10. Dendritic cells co-stimulatory molecules.

Dendritic cells were characterized through flow cytometry for MHCII+CD11c+ expression. Subsequent analysis of maturation/co-stimulatory molecules CD80, CD40 and CD86 were analyzed, as they can directly affect DC ability to present antigen to T cells, as shown in the figure above.

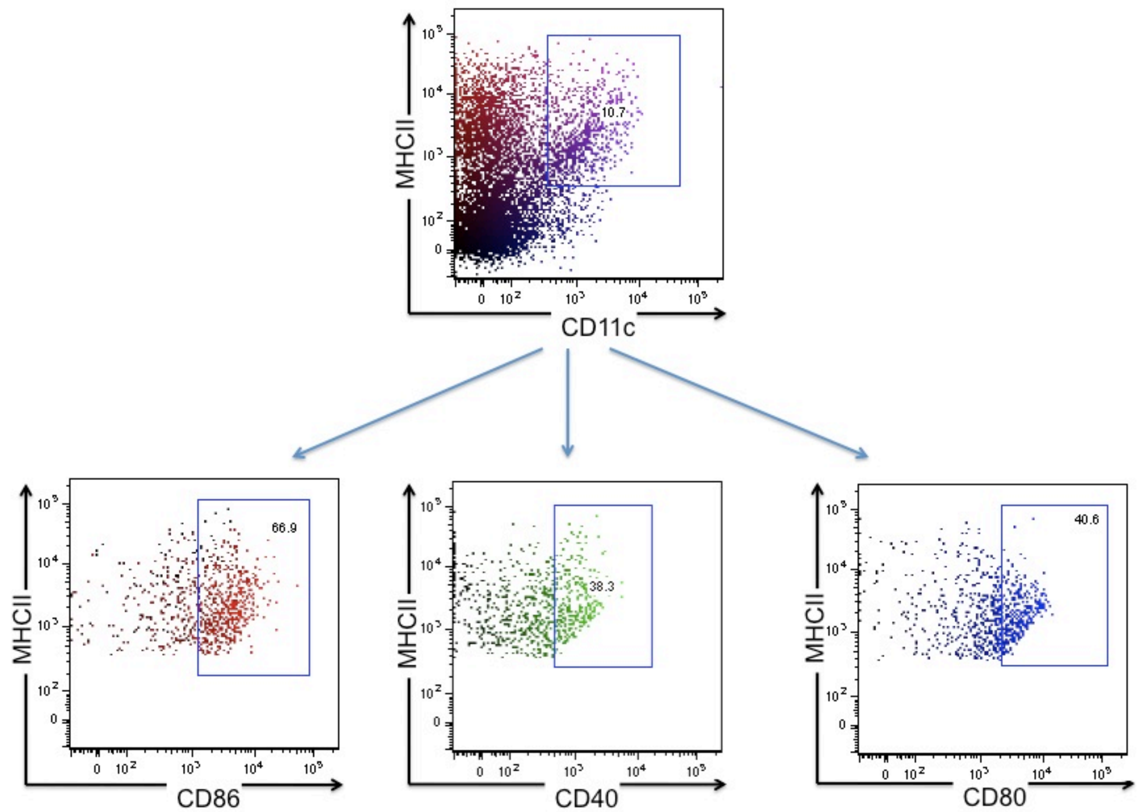

Figure 11. Sample gating for dendritic cells in the spleen.

The population is first analyzed for CD11c+MHCII+ population. Subsequently this population is analyzed for percentage of CD86, CD40 and CD80 expression, separately. Three resulting populations are analyzed between groups – CD11c+MHCII+CD86+, CD11c+MHCII+CD40+ and CD11c+MHCII+CD80+.

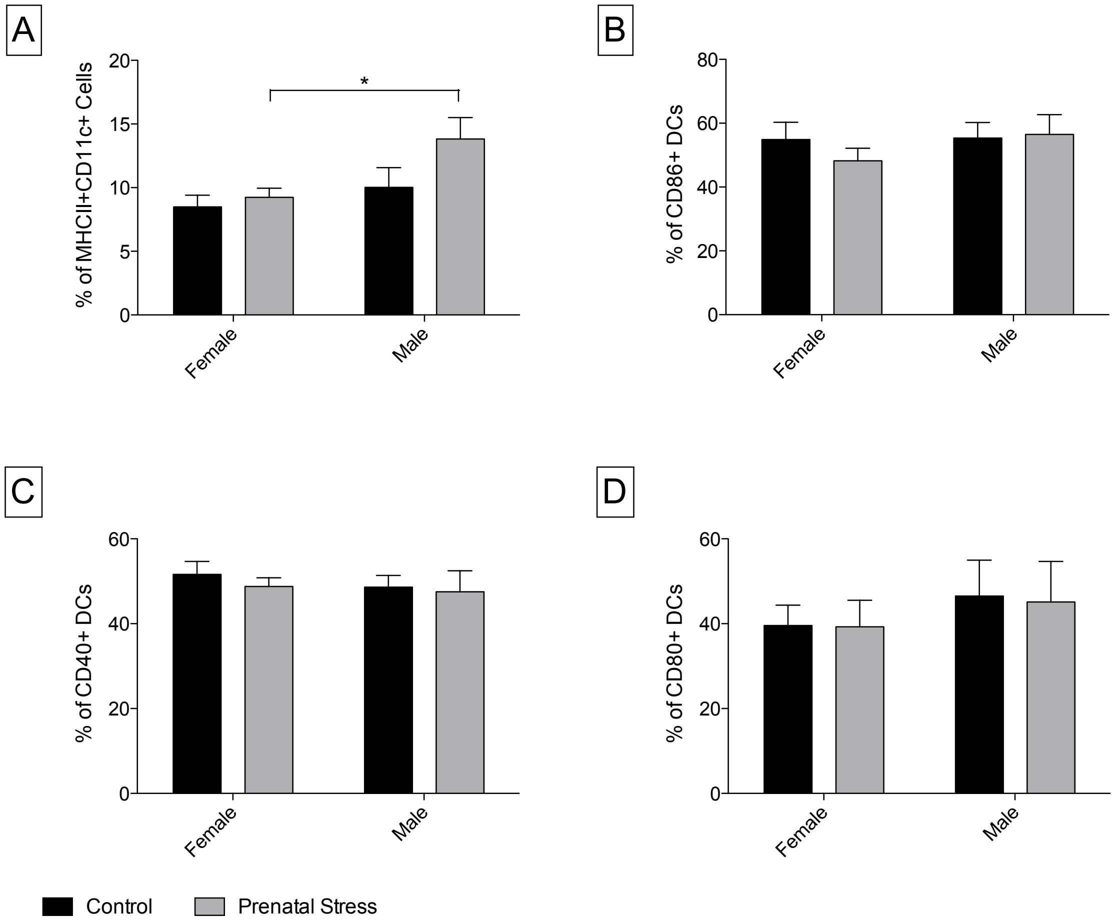

Figure 12. Stress induced differences between male and female animals.

Spleen DCs were characterized as MHCII$^+$CD11c$^+$. Co-stimulatory molecules CD86, CD40 and CD80 were also analyzed through flow cytometry. (A) Prenatally stressed males had a higher percentage of MHCII$^+$CD11c$^+$ cells then prenatally stressed females. No differences were observed in co-stimulatory molecules CD86 (B), CD40 (C) and CD80 (D). Graphs show mean + SEM, n = 12 per group, pooled from 3 separate experiment, * $P < 0.05$

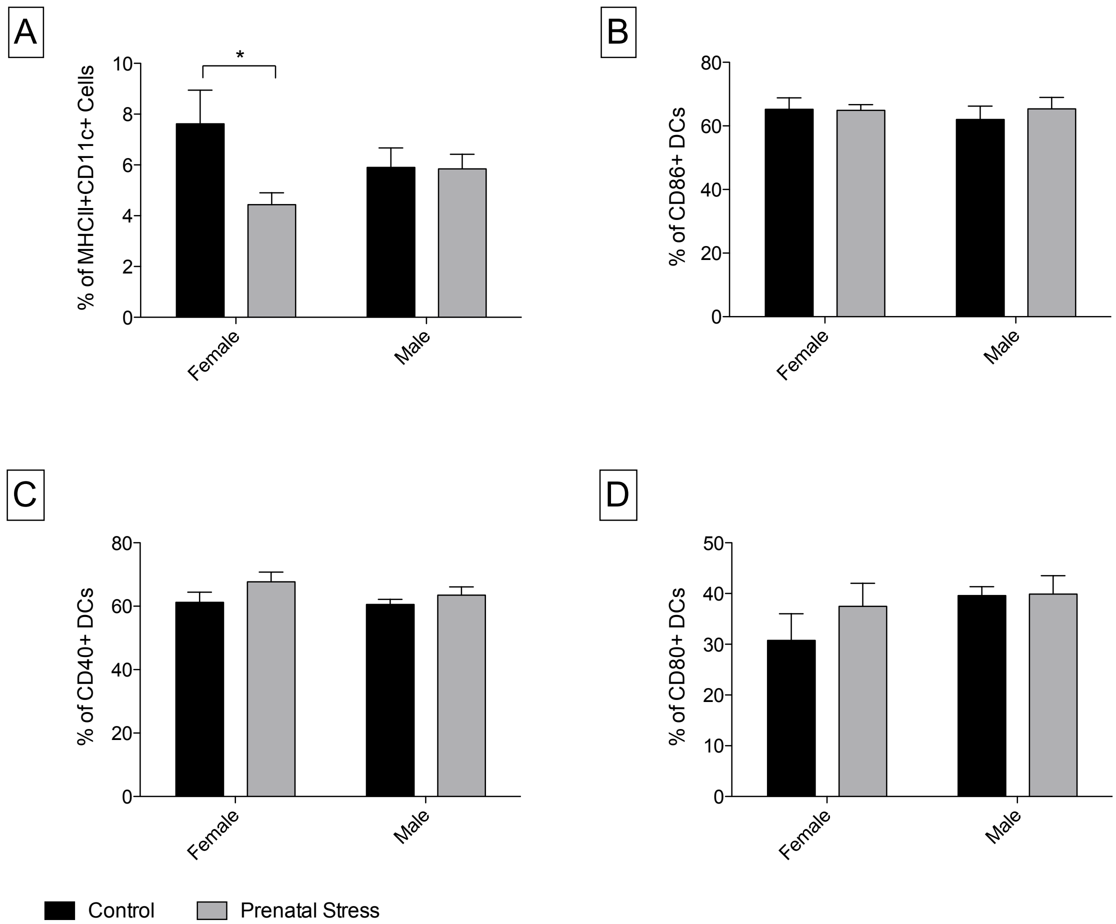

Figure 13. The effects of prenatal stress were seen in mesenteric lymph node DC of female offspring, but not male offspring.

Mesenteric lymph node DCs were characterized as $MHCII^{+}CD11c^{+}$. Co-stimulatory molecules CD86, CD40 and CD80 were also analyzed through flow cytometry. No differences were observed in the percentage of $MHCII^{+}CD11c^{+}$ (A) or expression of co-stimulatory molecules CD86 (B), CD40 (C) and CD80 (D). Graphs show mean + SEM, n = 12 per group, pooled from 3 separate experiments. * $P < 0.05$

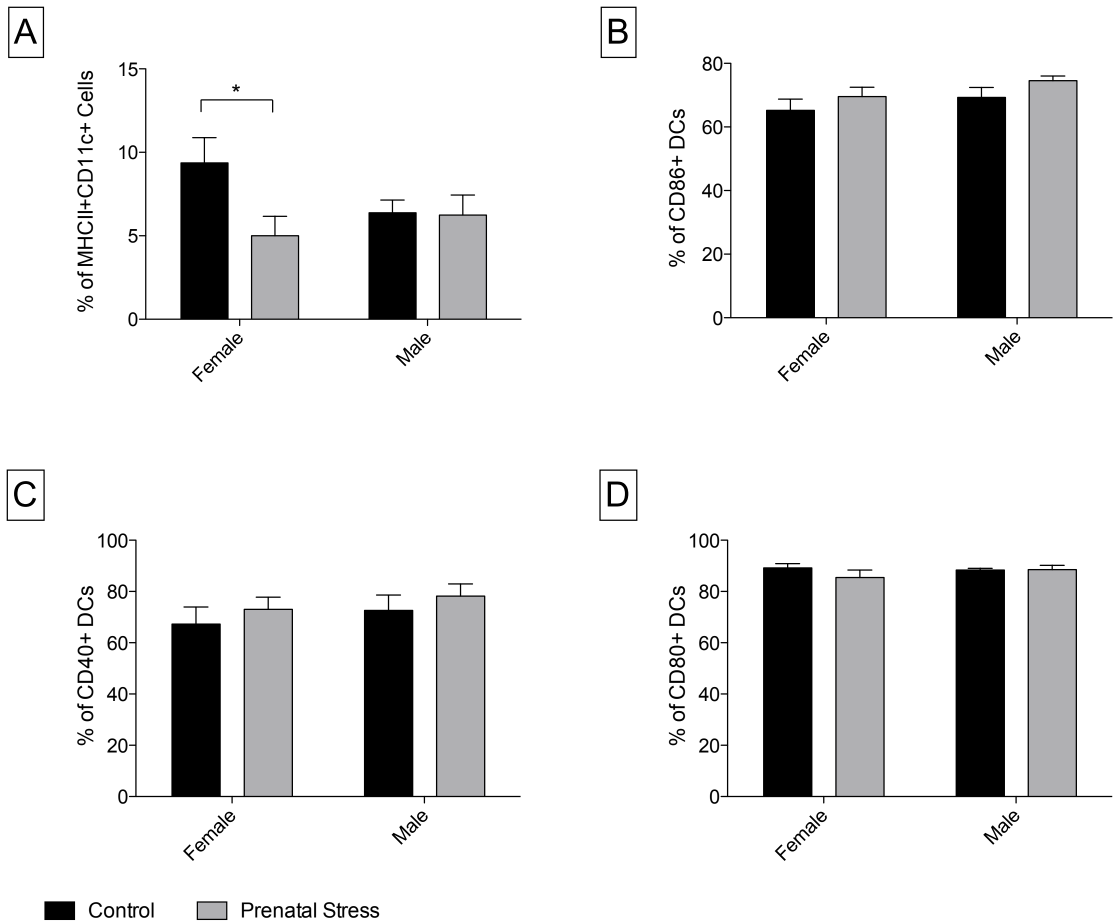

Figure 14. The effects of prenatal stress were seen in lung DC of female offspring, but not male offspring.

Lung DCs were characterized as $MHCII^{+}CD11c^{+}$. Co-stimulatory molecules CD86, CD40 and CD80 were also analyzed through flow cytometry. No differences were observed in the percentage of $MHCII^{+}CD11c^{+}$ (A) or expression of co-stimulatory molecules CD86 (B), CD40 (C) and CD80 (D). Graphs show mean + SEM, n = 12 per group, pooled from 3 separate experiments. * $P < 0.05$

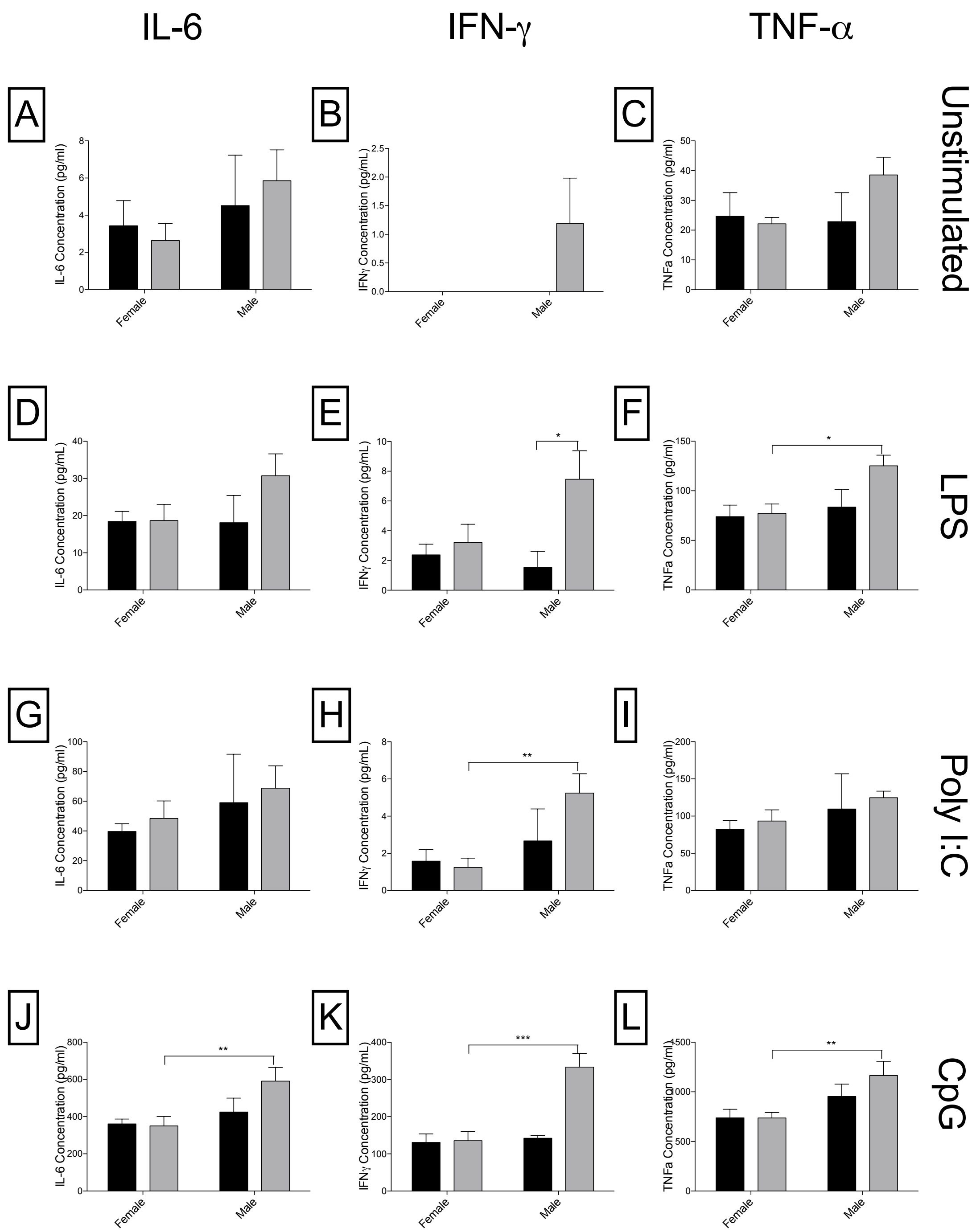

Figure 15. Sex dependent effects of treatment were observed in isolated DCs cytokine response 18h following TLR ligation, were prenatally stressed males produced more cytokines than prenatally stressed females.

Isolated DCs were either unstimulated (A-C), stimulated with LPS (D-F), Poly I:C (G-I) and CpG (J-L). IL-6 (A, D, G, J), IFN-γ (D, E, H, K) and TNF-α (C, F, I, L) were analyzed using the cytokine bead array. Graphs show mean + SEM, n = 6 per group, * P < 0.05, ** P <0.01, *** P<0.001

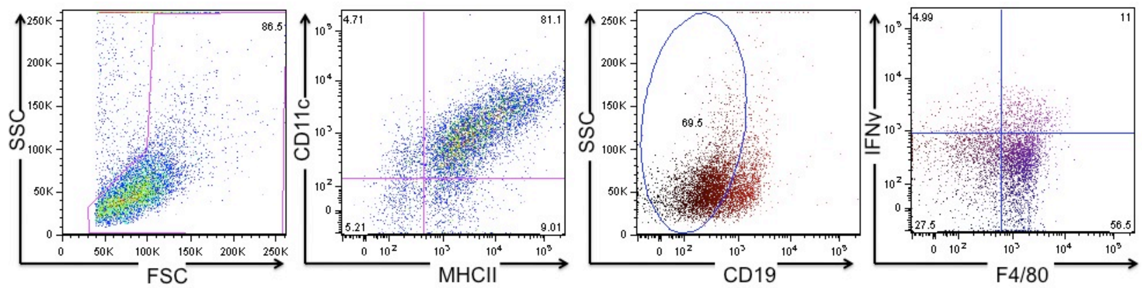

Figure 16. IFNγ production found in F4/80+ cells.

Isolated DCs were stimulated with CpG and treated with golgi plug. Through flow cytometry, DCs were gated as CD11c+MHCII+. Population was gated for CD19-, excluding B cells. The resulting population was gated for IFNγ and F4/80 expression. The majority of IFNγ expressing cells also expressed F4/80.

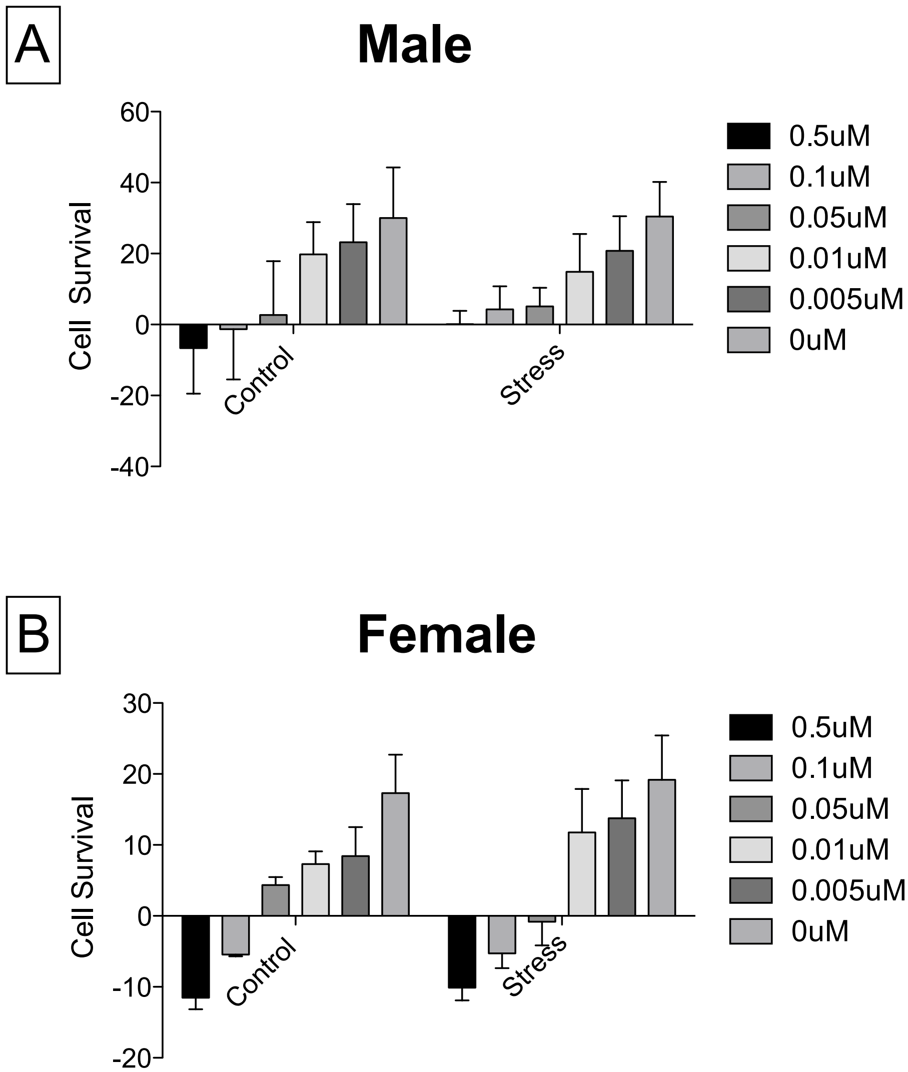

Figure 17. Glucocorticoid responsiveness of prenatally stressed male and females do not differ significantly from their respective controls.

Isolated DCs were stimulated by CpG with increasing increments of corticosterone was added (0µM, 0.005µM, 0.01µM, 0.05µM, 0.1µM and 0.5µM) to assess glucocorticoid responsiveness. 48h following culture, cell survival was measured. No significant differences were found between prenatally stress and controls animals, of either (A) female or (B) male offspring (n=5 per group). Data is shown as mean +SEM.

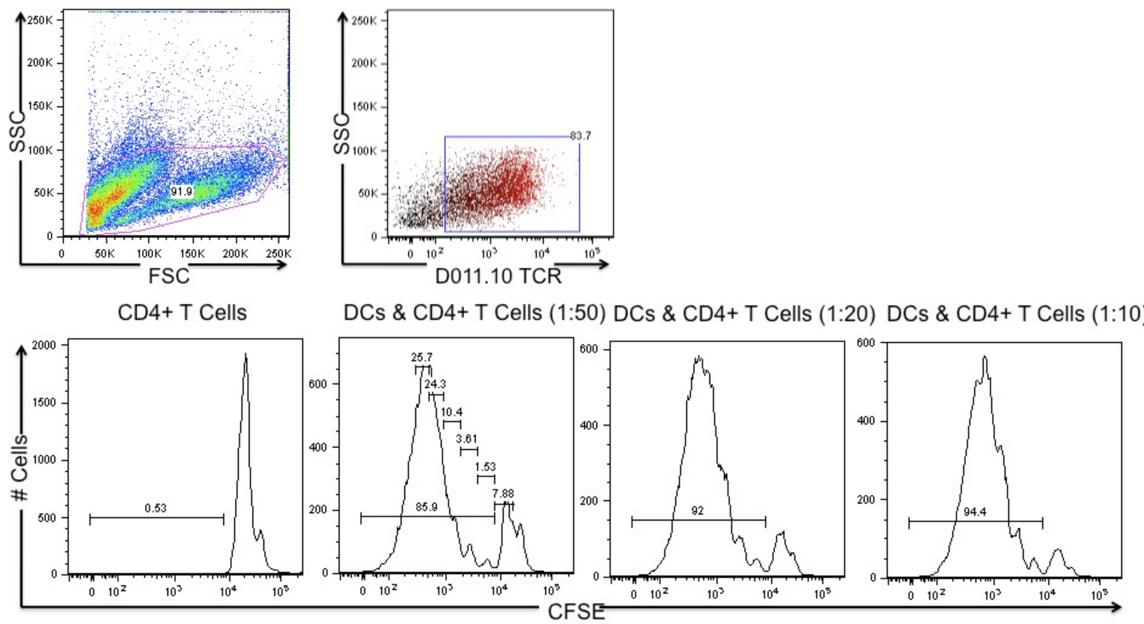

Figure 18. Sample gating of T cell proliferation.

Three days after DC and D011.10 CD4+ T cell co-culture, cells are analyzed for proliferation. Consistent population is selected and analyzed for D011.10 TCR expression. This population is then analyzed for CFSE expression. The original population of T cells was stained with CFSE, where any subsequent proliferation will decrease CFSE intensity. Sample gating of various DC to CD4+ T cell ratios are shown.

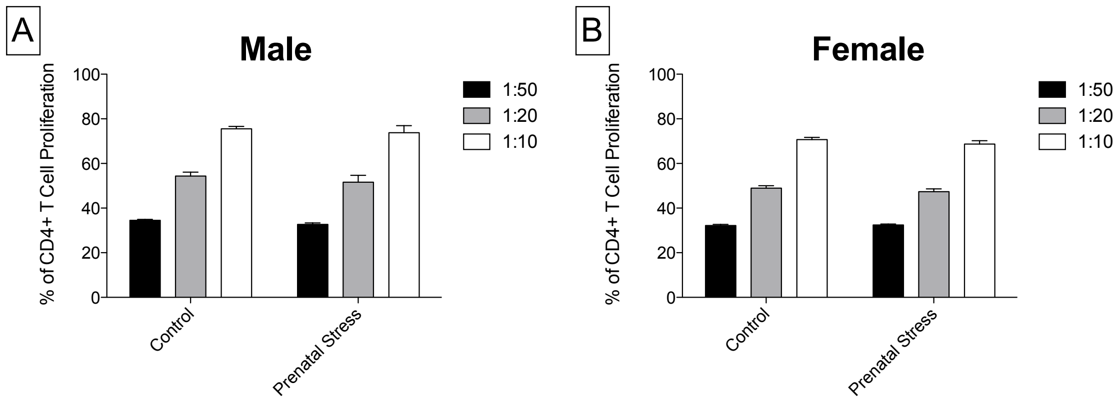

Figure 19. Prenatal stress did not affect the ability of DCs to induce antigen specific T cell proliferation in both male and female animals.

Isolated DCs ($CD11c^+$ splenocytes) were pulsed with OVA for 18-20 hours, prior to co-culture with CD4+ T cells from D011.10 transgenic mice to induce antigen specific T cell proliferation for 3 days. DCs were co-cultured at 3 different DC to T cell ratios, where the number of T cells remained consistent – 1:10, 1:20 and 1:50. Co-culture was analyzed for the % of CD4+ T cell proliferation in males (A) and females (n=6 per group). Data is shown as mean +SEM.

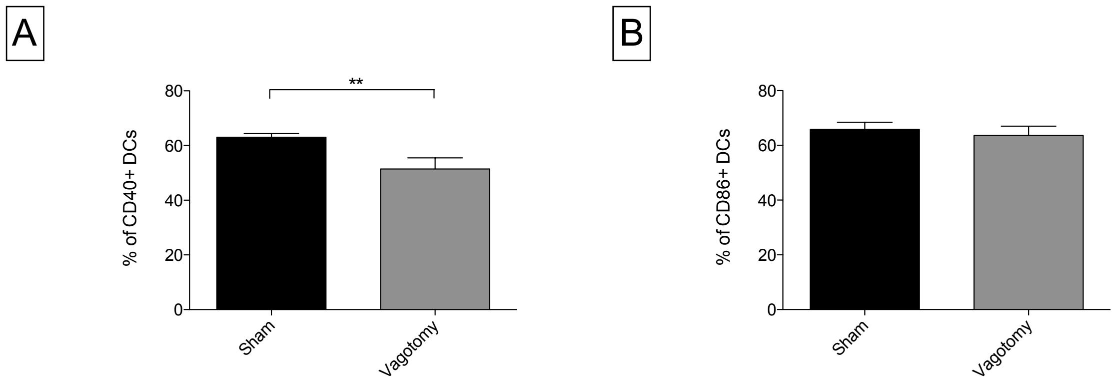

Figure 20. Splenic DCs from vagotomised mice express lower expression of CD40 but not CD86.

DCs were characterized as $MHCII^{+}CD11c^{+}$. Co-stimulatory molecules CD86, CD40 and CD80 were also analyzed through flow cytometry. The percentage of DCs expressing CD40 decreased in vagotomised animals (A) but did not differ in the percentage of DCs expressing CD86 (n=10-12 per group). Graphs show mean + SEM, **P<0.01

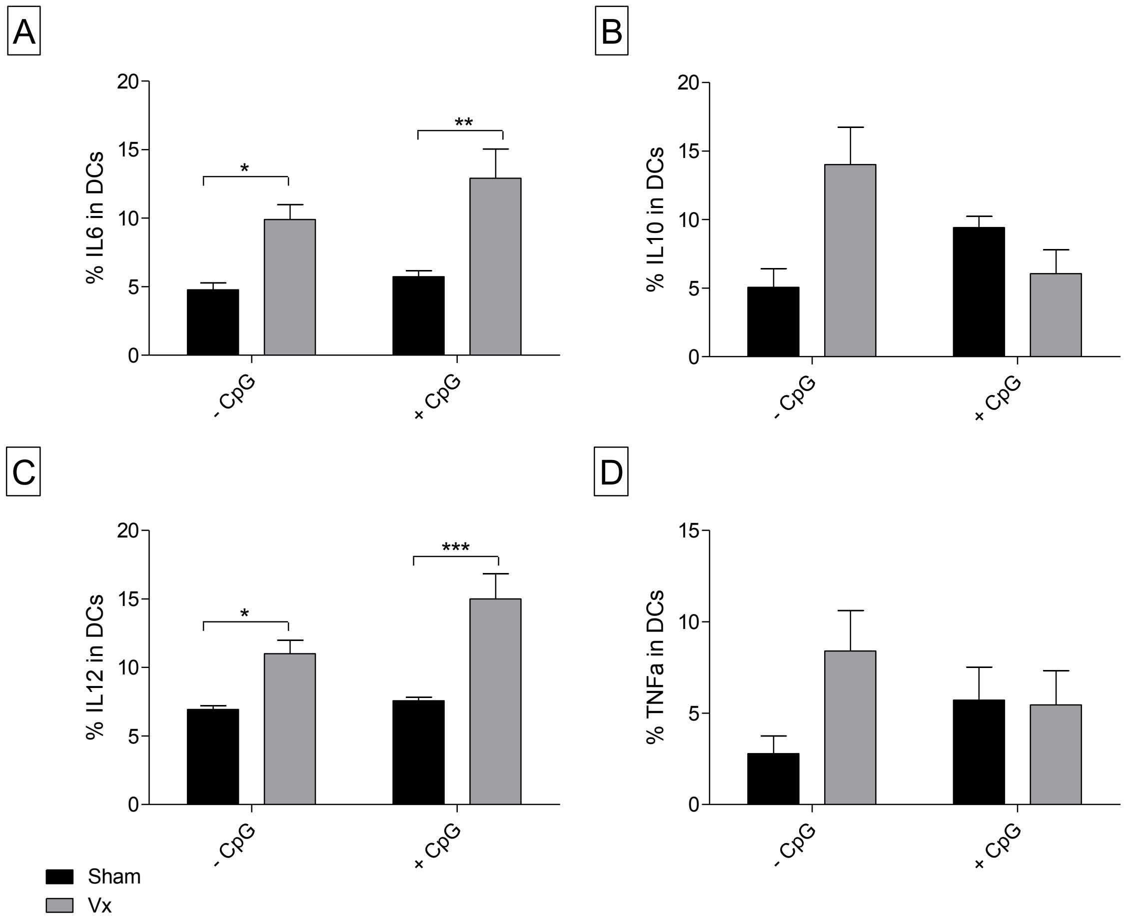

Figure 21. Vagotomised mouse spleenocyte produce more IL-6 and IL-12 than sham mice.

Spleenocytes were either left unstimulated or stimulated with CpG. 18h following incubation, DCs (CD11c+MHCII+) were assessed for intracellular cytokine production of (A) IL-6, (B) IL-10, (C) IL-12 and (D) TNF-α. (n=5 per group) Data is shown as mean +SEM, *P <0.05 ** P < 0.01 ***P<0.001

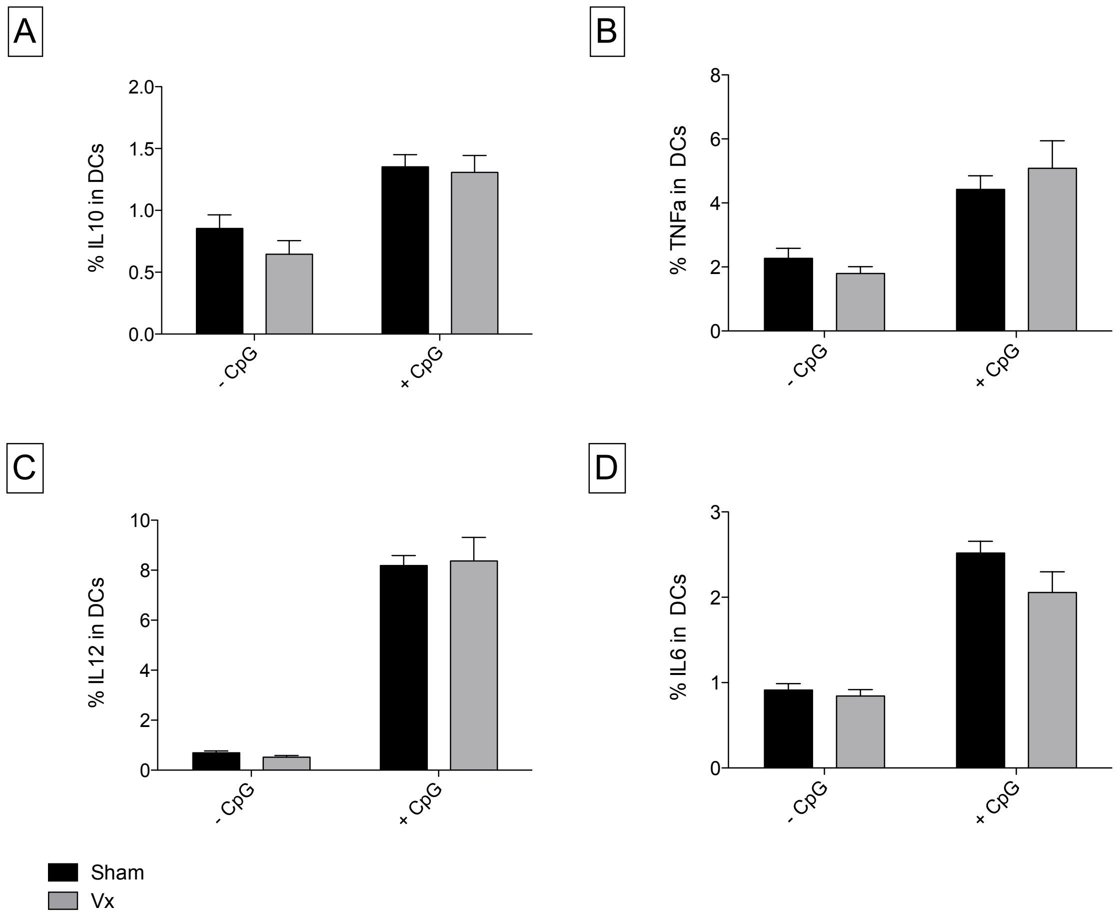

Figure 22. CD11c+ cells of vagotomised mice did not significantly differ from sham mice, in terms of intracellular cytokine production.

Isolated CD11c+ cells were either left unstimulated or stimulated with CpG. 18h following incubation, DCs (CD11c+MHCII+) were assessed for intracellular cytokine production of (A) IL-6, (B) IL-10, (C) IL-12 and (D) TNF-α (n=5 per group). Data is shown as mean +SEM.

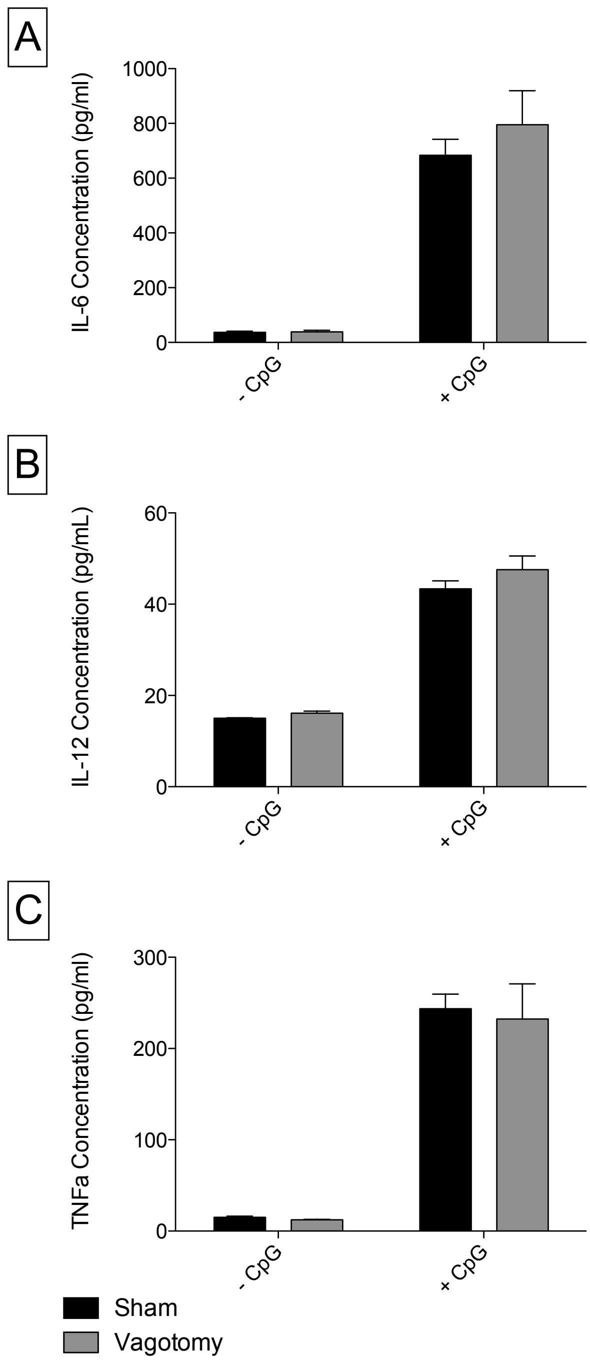

Figure 23. CD11c+ cells of vagotomised mice did not significantly differ from sham mice, in terms of cytokine release.

Isolated CD11c+ cells were either unstimulated with CpG. 18h following incubation, supernatant was assessed for IL-6 (A), Il-12 (B) and TNFa (C) concentration using ELISA. Data is shown as mean +SEM, n = 5 per group

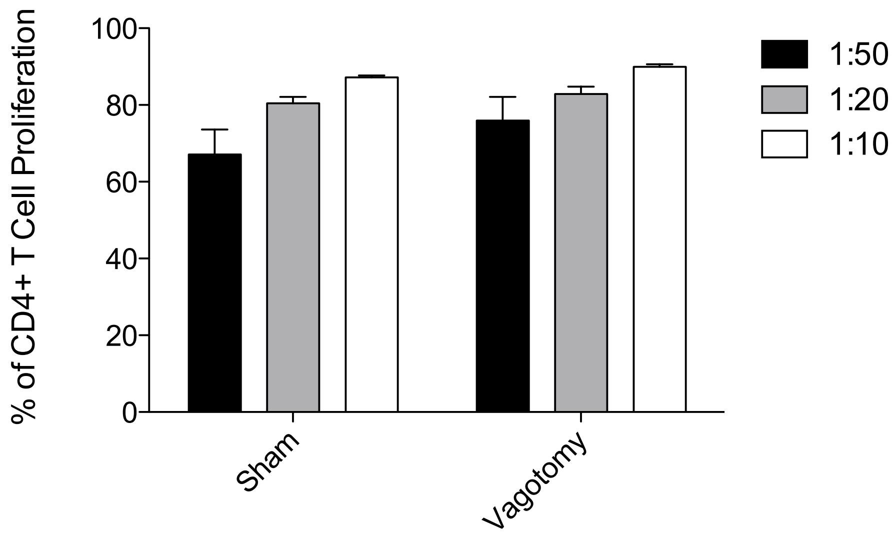

Figure 24. CD11c+ cells of vagotomised mice did not differ in its ability to induce antigen specific T cell proliferation.

Isolated DCs (CD11c^{+} splenocytes) were pulsed with OVA for 18-20 hours, prior to co-culture with CD4+ T cells from D011.10 transgenic mice to induce antigen specific T cell proliferation for 3 days. DCs were co-cultured at 3 different DC to T cell ratios, where the number of T cells remained consistent – 1:10, 1:20 and 1:50. Co-culture was analyzed for the % of CD4+ T cell proliferation (n=10 per group). Data is shown as mean +SEM.

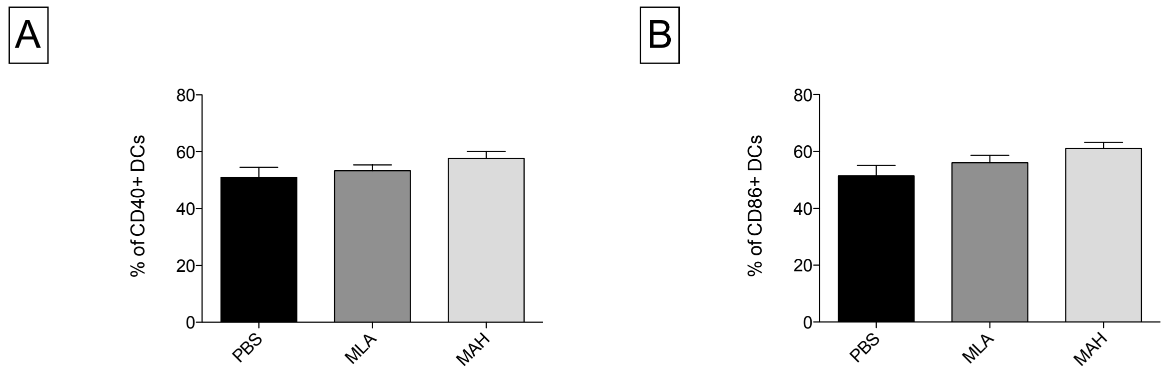

Figure 25. Splenic DCs from nicotine antagonist injected mice did not differ in expression of CD40 or CD86.

DCs were characterized as MHCII^{+}CD11c^{+}. Co-stimulatory molecules CD86, CD40 and CD80 were also analyzed through flow cytometry. The percentage of DCs expressing CD40 (A) or CD86 (B) did not differ in nicotine antagonist injected animals compared to controls (n=8-10 per group). Graphs show mean + SEM.

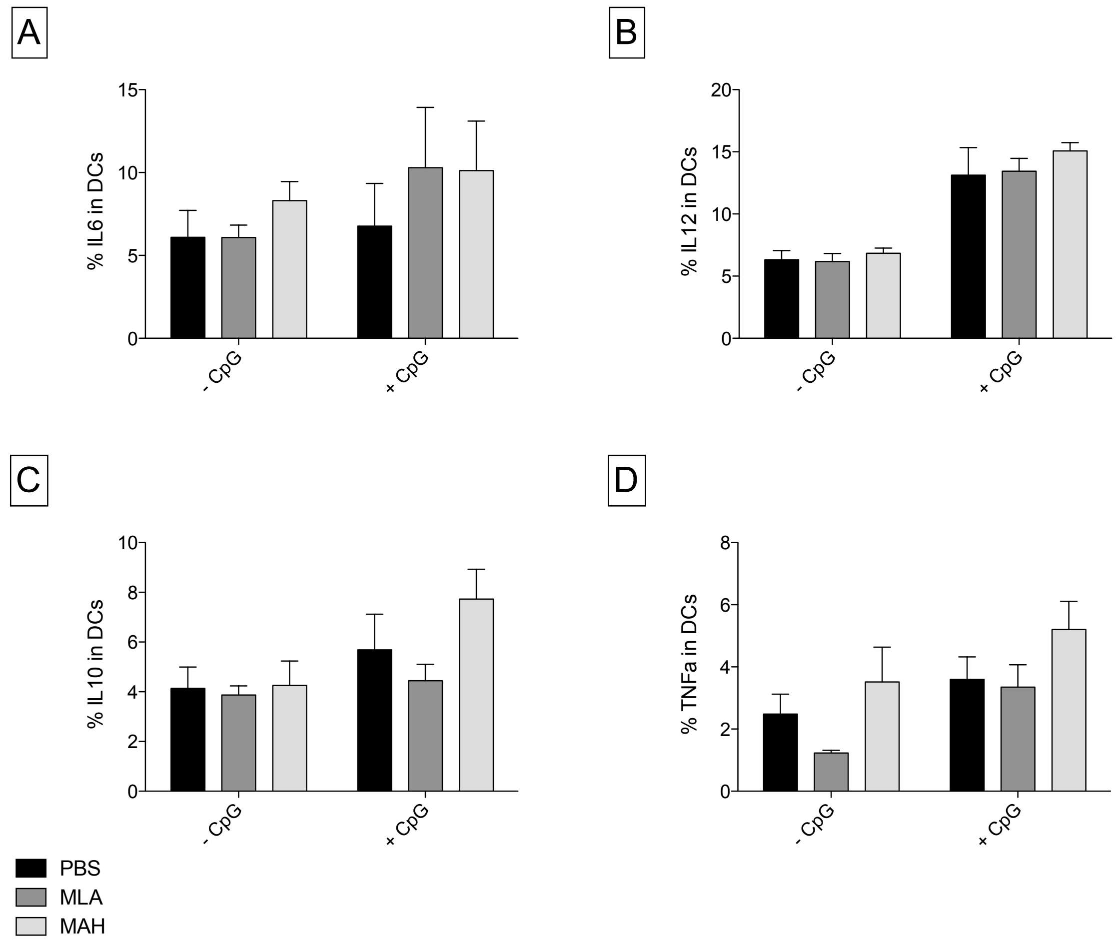

Figure 26. Spleenocytes from nicotine antagonist injected animals do not differ in cytokine production compared to control animals injected with PBS.

Spleenocytes were either left unstimulated or stimulated with CpG. 18h following incubation, DCs (CD11c+MHCII+) were assessed for intracellular cytokine production of (A) IL-6, (B) IL-10, (C) IL-12 and (D) TNF-α (n=5 per group). Data is shown as mean +SEM.

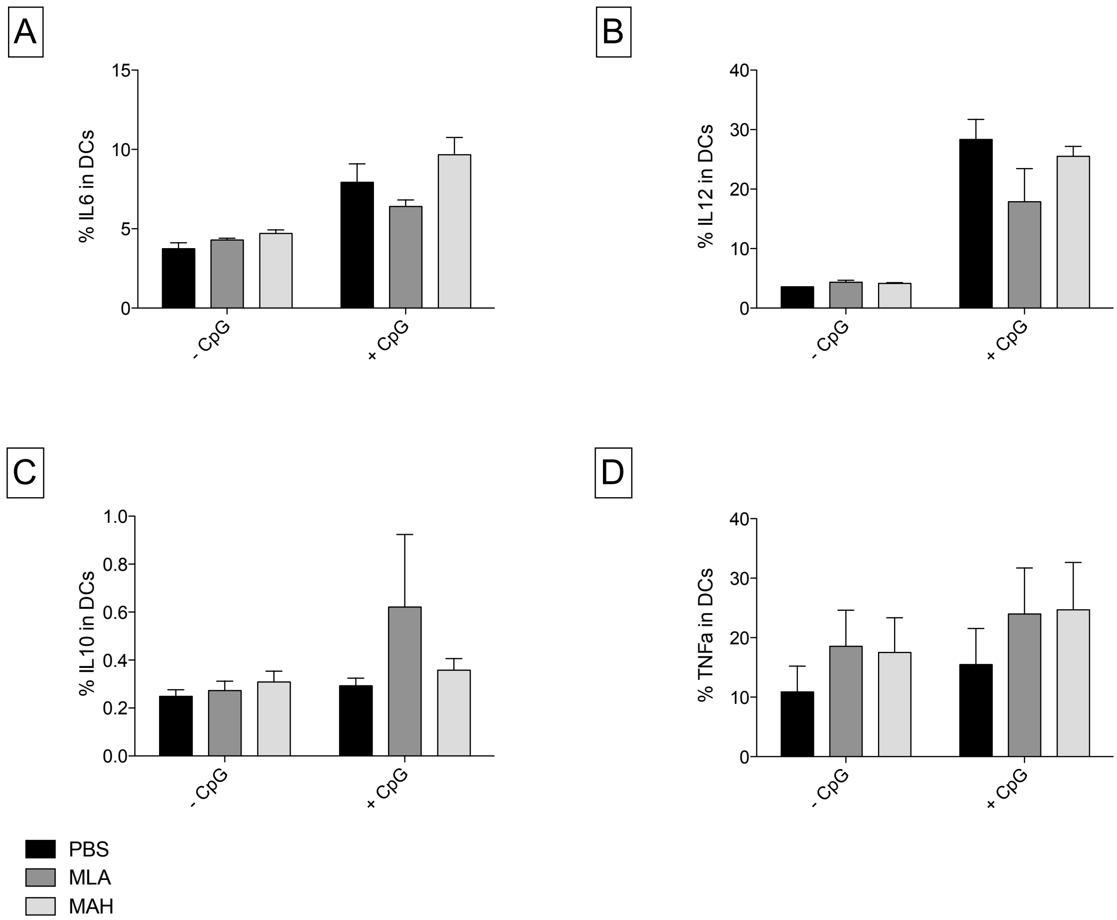

Figure 27. Spleenocytes from nicotine antagonist injected animals do not differ in cytokine production compared to control animals injected with PBS.

Splenocytes were either left unstimulated or stimulated with CpG. 18h following incubation, DCs (CD11c+MHCII+) were assessed for intracellular cytokine production of (A) IL-6, (B) IL-10, (C) IL-12 and (D) TNF-α (n=5 per group). Data is shown as mean + SEM.

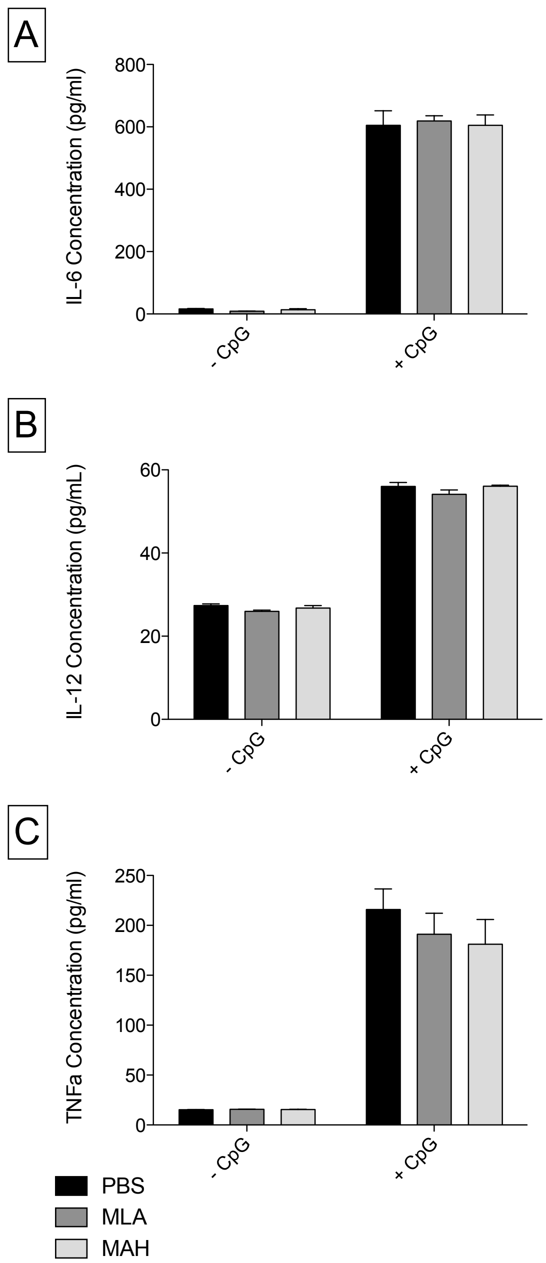

Figure 28. CD11c+ cells of nicotine antagonist injected mice did not significantly differ from control mice, in terms of cytokine release.

Isolated CD11c+ cells were either unstimulated with CpG. 18h following incubation, supernatant was assessed for IL-6 (A), Il-12 (B) and TNFa (C) concentration using ELISA (n=5 per group). Data is shown as mean +SEM.

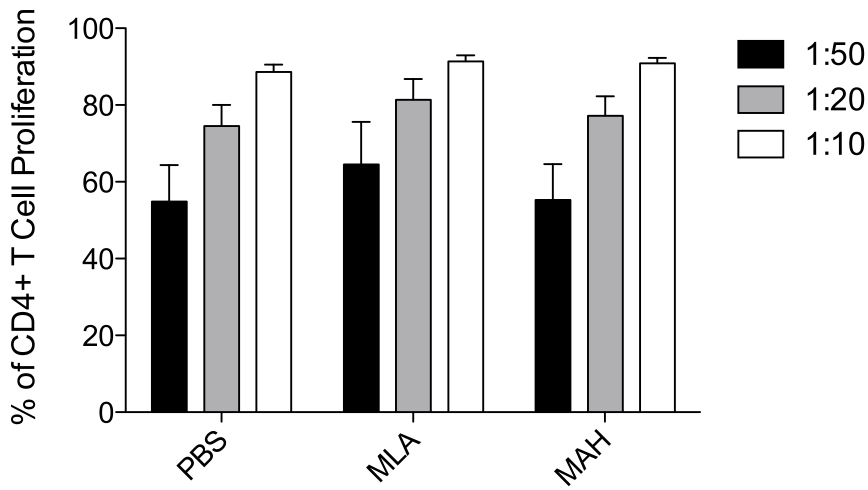

Figure 29. CD11c+ cells of nicotine antagonist injected mice did not differ in its ability to induce antigen specific T cell proliferation, compared to controls.

Isolated DCs ($CD11c^+$ splenocytes) were pulsed with OVA for 18-20 hours, prior to co-culture with CD4+ T cells from D011.10 transgenic mice to induce antigen specific T cell proliferation for 3 days. DCs were co-cultured at 3 different DC to T cell ratios, where the number of T cells remained consistent – 1:10, 1:20 and 1:50. Co-culture was analyzed for the % of CD4+ T cell proliferation (n=12 per group). Data is shown as mean +SEM.

Discussion

Fetal programming has been demonstrated in epidemiological and animal studies alike, for various factors to act prenatally but have long lasting effects on the offspring into adulthood for susceptibility to disease. Stress during maternal gestation is one of such factors. While prenatal stress has been long observed to have effects on the hypothalamus-pituitary-adrenocortical (HPA) axis and changes in behaviour, little notice has been given on its potential effects on immune development in the offspring. As such, there is a need for studies to fully understand the effects of prenatal stress on the offspring.

In this study, the effect of prenatal stress on the immune system was explored through a model of allergic airway disease. We found that prenatal stress heightens allergic airway inflammation in males but depresses allergic airway inflammation in females (Figure 2). Coinciding with the findings in males, the percentage of regulatory T cells was found to decrease in prenatally stress males compared to controls suggesting a lack of immunomodulation (Figure 7). Prenatally stress males were also found to have increased percentage of dendritic cells ($CD11c^{+}MHCII^{+}$) in spleen (Figure 12) and greater cytokine production upon TLR ligation (Figure 15) compared to prenatally stress females. As dendritic cells are a key player in sensitization and subsequent antigen challenge in allergic airway disease, the results highlight the effects of prenatal stress on dendritic cells. The possibility of changes in dendritic cell glucocorticoid responsiveness and depressed parasympathetic output were excluded as potential mechanisms mediating prenatal stress effects on the immune system. These findings are in agreement with another studying which found prenatal stress enhanced allergic airway disease in females (Pincus-Knackstedt et al., 2006).

In our model of allergic airway disease, cockroach (CR) protein was used to sensitize and challenge animals intranasally. The effectiveness of cockroach protein challenge compared to saline challenged animals was confirmed in measures of total airway responsiveness/resistance and airway inflammation of both sexes. We chose to use intranasal cockroach protein exposure in a model of allergic airway disease, as it is more physiologically relevant allergen than the more commonly used ovalbumin model of allergic disease and does not require the use of adjuvants. This model uses intranasal administration, which more closely mimics human sensitization than conventional allergic airway models involving intraperiontal injections. Cockroach allergy has been known for 30 years to prevalent in inner cities with a prevalence ranging from 17% to 41% in the United States (Arruda et al., 2001). In addition, cockroach allergen sensitization was found to be associated with increase asthma morbidity in inner city children (Arruda et al., 2001).

The prenatal stress model of sound stress was previously established by (Pincus-Knackstedt et al., 2006), where pregnant dams were stressed in later gestation (post-implantation) to avoid teratogenesis but still able to influence lung and immune development.

Characteristics of Allergic Airway Disease

Allergic airway disease is a model of asthma and is a chronic inflammatory disorder. Inflammation in conjunction with goblet cell hyperplasia and bronchoconstriction causes airway hyperreponsiveness (Jalusic-Gluncic, 2011). The effects of prenatal stress on a murine model of allergic airway disease were quantified through measures of inflammation and airway hyperresponsiveness.

Airway inflammation was determined using the total cells found in the bronchoalveolar lavage fluid (BALF) 24 hours after final CR challenge. The effect of prenatal stress on allergic inflammation was prominent in both sexes, but affected males and females differently. In males, prenatal stress enhanced the total cell count of the bronchoalveolar lavage fluid (BALF) (Figure 2A) and percentage of eosinophils (Figure 4A). In females, prenatal stress decreased the total cell count of the BALF (Figure 2B), but did not affect the percentage of eosinophils (Figure 4B).

Airway responsiveness to methacholine was expressed as a fold difference compared to control animals, which were not prenatally stressed and challenged with saline. No effect of prenatal stress was observed in airway responsiveness in either male or female offspring. However, it is important to consider the possibility that a greater sample size may be required to adequately assess whether prenatal stress can effect airway responsiveness because of the greater variability between animals.

While airway inflammation and the number of eosinophils inversely correlate with lung function, airway responsiveness is also affected by other factors such as airway remodelling thus causing a greater variability in outcome (Crimi et al., 1998). It is worth noting that the relationship between airway inflammation and airway hyperresponsiveness, does not have a simple correlation (Crimi et al., 1998). These findings confirm an effect of prenatal stress on the immune system, where prenatal stress increases allergic airway inflammation in males but decrease allergic airway inflammation in females.

Previous Studies on the effects of Prenatal stress

In a previous study where only female mice were used, prenatally stressed females were found to have increased airway responsiveness measured by whole-body plethysmograph and airway inflammation (Pincus-Knackstedt et al., 2006). These differences from our work can perhaps be attributed to the use of cockroach allergen instead of ovalbumin, and more importantly to the age of initial sensitization. The age of initial sensitization has been shown to affect the quantity and quality of immune response, where older mice (30-40 weeks old) sensitized in later life were unable to respond to allergen challenge (Gelfand et al., 2004). In our model, mice were sensitized intranasally daily from postnatal day 2 to day 8 while Pincus-Knackstedt and colleagues sensitized mice i.p. for 3 days during postnatal weeks 6-8. It is possible that the age of initial sensitization, route of administration and type of allergen affects immune response when challenged later in life, and this might explain the discrepancy we have examined here.

While there is only one other study examining prenatal stress and allergic airway disease in a mouse model, there are many studies that have attempted to clarify the effect of prenatal stress on the immune system through several different immune parameters.

The effects of prenatal stress have many inconsistencies, especially due to differences in timing, duration of stressor, type of stressor, species, strain, age of offspring and immune parameter investigated. In studies examining innate immunity prenatal stress has been shown to have an inhibitory effect in the majority of studies. In fact the study by Pincus-Knackstedt *et al*. (2006) is one of the few studies showing enhanced innate immunity in prenatally stressed animals. However, epidemiological findings support the hypothesis that prenatal stress can disrupt the immune system *in utero*. Wright has found that prenatal stress correlates with altered innate and adaptive immunity, which can then affect allergic disease (Wright et al., 2010). Nervousness during pregnancy is correlated with increase IgE (Lin et al., 2004) and stressed pregnant women have elevated serum pro-inflammtory cytokines which may affect their offspring (Coussons-Read et al., 2007).

In a prenatal stress studies with mice (daily foot shock from gestation day 15 to day 19), decreased macrophage spreading and phagocytosis were observed (Fonseca et al., 2002; Palermo Neto et al., 2001). In addition, other prenatal stress studies have found decreased cytotoxicity of NK cells, decreased splenic lymphocyte proliferation in response to pokeweed mitogen (Kay et al., 1998) and increased tumour growth (Kay et al., 1998; Palermo Neto et al., 2001). Another important parameter found in previous studies of prenatal stress is the variable timing of when the effects of prenatal stress become apparent. For example, decreased macrophage function was found in only adult mice but not juvenile mice (Fonseca et al., 2002). Unfortunately, there is currently no theory for how and why this may occur.

There are several difficulties to studying prenatal stress but the biggest problem is being able to compare different studies. There are critical differences between species in terms of length of gestation, developmental time line and physiology (Merlot et al., 2008). Although the length of gestation could be looked at as a percentage of total length of gestation, immune development is species specific so it may be difficult to compare results between species. In addition, strain specificity further complicates the picture. Different strains of rats have been observed to have different response to prenatal stress (Stohr et al., 1998). In mice, different strains have been shown to have a different response to stress, but I was unable to find studies, which have looked at different mouse strains and prenatal stress. This points to a role in genetics for varied susceptibility and response to prenatal stress.

Regulatory T cells Distribution & Methylation of Foxp3

Regulatory T cells (T-regs) have been shown to play a role in suppressing or controlling the function of immune cells and the development of autoimmunity and allergies (Akbari et al., 2003; Umetsu et al., 2003). T-regs in asthmatics have been found to be decreased in number and to be dysfunctional (Hartl et al., 2007; Ling et al., 2004). Adoptive transfer of IL-10 or TGF-β producing T cells was able to reverse allergic airway inflammation and hyperreactivity in mice (Hansen et al., 2000; Oh et al., 2002).

In our model of prenatal stress and allergic airway disease, prenatally stressed male animals were found to have decreased number of regulatory T cells (characterized through flow cytometry as a CD3+CD4+CD25+Foxp3+ population) in the mesenteric lymph node but not the spleen. This corresponds with increased allergic inflammation in prenatally stress males, where decreased regulatory T cells will results in a lack of immunoregulation and heightened inflammation.

T-regs are characterized through expression of transcription factor Foxp3, which is under regulation through methylation of CpG rich regions (Huehn et al., 2009; Lal et al., 2009). Previous studies examining methylation of T-regs have found that regions upstream of the promoter were unmethylated in T-regs (Lal et al., 2009).

To address whether prenatal stress affected the methylation status of foxp3, 2 regions upstream of the promoter were analyzed for methylation. As foxp3 is found on the X-chromosome, only males were used to avoid artefacts from x-inactivation in females. Contrary to flow cytometry data, where prenatal stress decreased the number of T-regs, prenatal stress appeared to increase methylation of the two regions analyzed.

The two regions analyzed are amongst many other CpG rich regions near foxp3. It is possible, that the two regions analyzed are not entirely representative of the methylation status of foxp3 and may not correlate with other data we have found. In addition, other studies have found that unmethylaton in naturally occurring T-regs but incomplete unmethylation in TGF-β induced T-regs that also express Foxp3 (Floess et al., 2007). It is also possible that the type of foxp3 induced by prenatal stress might not necessarily have to be unmethylated in the regions analyzed. There are also many pathways in which foxp3 expression can be induced and thus can be affected by prenatal stress. For example, chromatin remodelling enzymes are often recruited to methylated

CpG motifs could affect the expression of foxp3 or through other signalling pathways downstream of CD28 (Huehn et al., 2009).

Regulatory T cells mediate their suppressive effects through the secretion of anti-inflammatory cytokines such as IL-10 or TGF-β and also interaction with dendritic cells (Akbari et al., 2003; Beissert et al., 2006). In turn, dendritic cells have been found to induce tolerance through inducing T-regs (Akbari et al., 2003).

Dendritic Cells Distribution & Phenotype

As dendritic cells are important for allergen sensitization and subsequent challenge in allergic airway disease, dendritic cells of prenatally stressed naive animals were analyzed for distribution and phenotype in the spleen, mesenteric lymph node and lungs. The maturation of DCs is essential to their immunostimulatory ability to link the innate and adaptive immunity (Banchereau and Steinman, 1998). A significant effect of sex was observed. In the spleen, prenatally stressed males had a high percentage of dendritic cells ($MHCII^+CD11c^+$) present, demonstrating the effects of prenatal stress on another immune cell. Where increased dendritic cells could at least in part, cause enhanced allergic airway inflammation.

However in the lungs, the effect of prenatal stress was apparent only in females, where prenatal stress decreased the percentage of dendritic cells ($MHCII^+CD11c^+$) present. This may be an indicator of decreased dendritic cells in circulation or a lack of dendritic cell migration into lymphoid tissue in prenatally stressed females and account for suppressed allergic inflammation in allergic animals. Maturation/co-stimulatory molecules (CD86, CD40, CD80) were also assessed, but no differences were found between sexes or due to prenatal stress.

Dendritic Cell Cytokine Secretion

To explore the effects of prenatal stress on DC ($CD11c^+$) function, cytokine secretion was assessed in response to a bacterial CpG ODN (TLR9 agonist), LPS (TLR4 agonist) and Poly I:C (TLR3 agonist). After 18-hour stimulation, supernatants were assessed for a panel of inflammatory cytokines including IL-6, TNF-α and IFN-γ. Stimulation was successful with all three TLR agonists, with CpG being the most effective.

Sex differences were observed throughout this study. pPrenatally stressed males produced more cytokines in response to TLR agonist (more TNF-α in response to LPS, more IFN-γ in response to Poly I:C and more IL-6, TNF-α and IFN-γ in response to CpG) compared to prenatally stressed females. Prenatally stressed males, most prominently had higher secretion of IFN-γ to all three TLR agonist. Prenatal stress has not only affected the percentage of DCs found in the spleen, but also altered DC function in males which can attribute to enhanced inflammatory ability as found using a model of allergic airway disease.

To investigate the exact cell type secreting IFN-γ, $CD11c^+$ cells were stained intracellularly for IFN-γ and extracellular markers CD19 and F4/80, in order to exclude the presence of B cells and macrophages, respectively. While CD11c is an important marker of dendritic cells, there is currently no marker unique to dendritic cells alone.

Studies have even suggested that in order to specific a subset of dendritic cell population, 10 or more extracellular markers may be needed. CD11c can also be found on B cells and macrophages.

Using FACS analysis, CD11c purity was determined to be 80-90%. F4/80$^+$ cells were found to be the sole producers of IFN-γ in the CD11c$^+$ population, which indicates a role for macrophages or F4/80+ DCs. In our model prenatally stressed males had macrophages or F4/80+ DCs, which produce higher IFN-γ in response to TLR stimulation.

These findings are similar to those found in previous epidemiological studies looking where cord blood mononuclear cell from prenatally stressed offspring had increased cytokine responses (Wright et al., 2010), as well findings in mouse models where prenatally stressed offspring had a greater Th2 cytokine production (Pincus-Knackstedt et al., 2006).

The importance of this finding is highlighted by studies that have explored the role of toll like receptors (TLRs) in asthma. TLRs are evolutionary conserved innate immune receptors that can recognize an array of microbial or viral molecules, activation of transcription factors for proinflammatory cytokines and dendritic cell activation. TLRs are also able to influence subsequent adaptive immune response induced by dendritic cells. TLR2 stimulation has been found to induce a Th2 biased immune response and promoting allergic airway disease, which is not seen by TLR9 stimulation (Redecke et al., 2004). Epidemological studies have also revealed similar findings, where TLR2 genetic variability affects allergy susceptibility (Eder et al., 2004). On the other hand studies looking at TLR3 and TLR7 have been found to have suppressive effects on experimental asthma (Sel et al., 2007). Several TLR-related genes have been found to be associated to atopy using multifactor dimensionality reduction analysis of a large cohort of over 3000 children (Reijmerink et al., 2010). It could be possible that prenatal stress can affect TLRs or TLR-related molecules in male to increase their immune response in allergic inflammation, or decreased response in female offspring. TLRs are also found on macrophages, regulatory T cells and epithelial cells so it would be worthwhile investigating the extent of TLR involvement in my model of prenatal stress and allergic airway disease.

The effect of IFN-γ in my model of prenatal stress and allergic airway disease warrants further exploration. Spontaneous elevated IFN-γ production was observed in BAL cell cultures of asthmatics (Cembrzynska-Nowak et al., 1993) and IFN-γ polymorphisms correlate to atopic asthmatic individuals in the Indian (Nagarkatti et al., 2002) and Japanese population (Nakao et al., 2001). A transgenic IFN-γ knockout mice with constitutive lung specific IFN-γ production, revealed a role for IFN-γ in IL-5, IL-13 expression, and greater eosinophilia (Koch et al., 2006). The role of IFN-γ in allergic asthma is not completely understood, but would be interesting to explore whether this could be a mechanism whereby prenatal stress increased eosinophilia in male offspring.

Dendritic Cell Glucocorticoid Responsiveness

Stress had been found to decrease glucocorticoid responsiveness of splentocytes, which produce more Th2 cytokines in the presence of glucocorticoid and LPS compared to controls (Bailey et al., 2009). To investigate whether the impairment of glucocorticoid responsiveness due to prenatal stress was contributing to the increased allergic inflammation in males, isolated DCs were exposed to varying concentrations of cortcosterone in the presence of CpG. 48 hours later, cell viability was determined. No differences were observed between sexes or due to prenatal stress. This suggests changes of dendritic cell glucocorticoid responsiveness do not occur, but other cell types could be affected by prenatal stress.

Dendritic Cell Induced Antigen Specific T Cell Proliferation

Another key function of dendritic cells is its ability to present antigen to T cells and induce antigen specific T cell proliferation. This is important as its ability to present antigen, is important to sensitization and subsequent challenge of an individual with allergies and asthma. DCs were loaded with OVA and co-cultured with CD4+ T cells of transgenic D011.10 mice. No differences were observed between sexes or due to prenatal stress.

Proof of Principle: Role of Cholinergic Anti-inflammatory Pathway

Another possible avenue of neuroimmune modulation through prenatal stress is depressed parasympathetic output, indicated by decrease vagal tone. During stress, the sympathetic nervous system is heightened and parasympathetic output is depressed (Porges, 1995). More recently, the parasympathetic nervous system has been found to be able to regulate inflammation through the vagus nerve. This pathway is known as the cholinergic anti-inflammatory pathway. It utilizes release of acetylcholine and interacting with the α7 nicotinic receptor found on macrophages (Pavlov et al., 2003) and nicotinic receptor on T cells (Karimi et al., 2010), in response to afferent nerve detection of inflammation. This pathway will then prevent the release of inflammatory mediators by the immune cells.

Although there have been no reports of the vagus nerve regulation of dendritic cell function, these cells express multiple nicotinic receptor subunits with $\alpha 2$, $\alpha 5$, $\alpha 6$, $\alpha 7$, $\beta 2$ and $\beta 4$ being identified in the mouse (Skok et al., 2006). Human monocyte derived DC matured in the presence of nicotine exhibit lower endocytic and phagocytic activities produce decreased levels of IL-12 IL-1β, and TNF and display a markedly reduced ability to induce a Th1 response (Guinet et al., 2004; Nouri-Shirazi and Guinet, 2003). Similarly mouse bone marrow derived DC matured with nicotine have reduced capacity for antigen uptake produce less IL-12 and preferentially support the proliferation and differentiation of ovalbumin (OVA)-specific naïve T cells into Th2-like cells (Nouri-Shirazi et al., 2007). In contrast, other studies indicate that nicotine activates DC and enhances the cells capacity to perform endocytosis, stimulate T-cell proliferation, and produce IL-12 (Aicher et al., 2003). These apparently conflicting results may indicate that the influence of nicotinic signalling in DC depends on maturation and activation state of the cell at the time of exposure.

In order to explore the possibility of decreased vagal tone in our model, we have tested dendritic cell phenotype and function in vagotomised and nicotinic receptor antagonist injected mice. When splenocytes from vagotomised animals were stimulated with CpG, DCs showed a marked increase in IL-6 and IL-12 production compared to sham operated animals (Figure 21). However, no differences were found when DCs were isolated before stimulation with CpG (Figure 22 & 23). This suggests a role for DCs indirectly affected by the vagus nerve, potentially mediated through another cell type found in the spleen. Other changes in DC phenotype (Figure 20) and ability to induce antigen specific T cell proliferation were not found in vagotomised animals (Figure 24). The same assessments were conducted on splenic DCs from nicotinic receptor antagonist injected animals and no differences were found (Figure 25-29). These findings prove that DCs are not affected by the efferent vagal output through the nicotinic receptor antagonist and at most may be indirectly affected by another immune cell type such as macrophages or T-cells.

Sex Differences in Asthma

Sex differences were found throughout the study, which is noteworthy, as few studies on allergic airway disease in animal models have used both sexes. In adult balb/c mice (8-10 weeks old), females develop a more severe allergic airway inflammation in response to allergen compared to males with increased eosinophils, T cells, B cells, IgE and cytokines, but decreased regulatory T cells (Melgert et al., 2005). Another study with similar findings, found that castrated males mice had similar allergic airway diseases as females, attributing these effects in part to testosterone (Hayashi et al., 2003).

In epidemiological studies, it is clear that asthma and atopy is more prevalent in males prior to puberty (0 to 5 years old), equal during puberty (10 to 15 years old) but more prevalent in females after puberty (de Marco et al., 2000). It has been postulated that sex hormones may play a role in the sex reversal in the prevalence of asthma (de Marco et al., 2000). It would be worth exploring in our model, whether the differences that we have observed are true at other ages that may equate to before puberty, during puberty and after puberty.

Sex Differences in Prenatal Stress, Stress & Early Life Influences

What further complicates the link between stress and allergic airway disease is that prenatal stress itself has also been reported to be associated with sex differences. Prenatal stress has been previously found to have a more pronounced suppressive effect on the immune response of female rats (Kay et al., 1998). However, there were no changes in distribution of immune cells were found, suggesting that prenatal stress alters effector functions (Kay et al., 1998).

While there are limited studies examining prenatal stress and sex dimorphism, quite a few studies have examined sex differences in stress. A comprehensive review by Kudielka and Kirschbaum (2005), suggest adult men are more responsive to psychological stress than women and could in part be explained by differences in circulating sex steroids (Kudielka and Kirschbaum, 2005).

Prenatal stress studies have found males to exhibit depressive behaviour with decreased mobility in tail suspension and forced swim tests, while prenatally stressed females exhibited increased mobility (Mueller and Bale, 2008). Sex differences were also observed in other studies looking at the early life influence of germ free versus conventional mice, where males were found to be susceptible the influence of a germ free environment altering their brain-derived neutrotrophic factor, serotonin and plasma tryptophan levels but females remained unaffected (Clarke et al., 2012). While sex differences are consistently found throughout studies in prenatal stress, stress and early life influences, more studies are required in order to better understand the mechanisms of how this occurs and the relevance of these differences in the sex differences seen in autoimmune and inflammatory diseases.

Conclusion

Overall, the present results suggest that neuro-immune interactions exist since prenatal stress is able to influence regulatory T cells and dendritic cells. This also highlights the importance of understanding the effects of prenatal stress on the immune system. Prenatally stressed males have enhanced allergic airway inflammation, coinciding with decreased number of regulatory T cell compared to controls and increased dendritic cells and dendritic cell cytokine secretion compared to prenatally stress females. In females, decreased allergic airway inflammation was found, coinciding with a decreased number of dendritic cells in the mesenteric lymph node and lungs. However, prenatal stress did not affect the ability of DC to present antigen. In this study, I found no evidence to suggest changes in glucocorticoid sensitivity in dendritic cells as a potential mechanism. Based on a proof of principle study using vagotomy, it also seems unlikely that reduced parasympathetic input leads to the changes observed in dendritic cells The findings of increased dendritic cell cytokine production in prenatal stress requires further research to investigate the role of TLRs and also the role of IFN-γ in this model. Other potential mediators or mechanism of prenatal stress include changes to the sympathetic nervous system, which would be an interesting avenue to pursue in future studies.

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