CORTICOTROPIN RELEASING HORMONE'S EFFECT ON DENDRITIC CELL FUNCTION

THESIS

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas

Health Science Center at Fort Worth

In Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

Kay T. Kayembe, B.S.

Fort Worth, Texas

May 2010

TABLE OF CONTENTS

ACKNOWLEDGMENTS

I would like to begin by acknowledging my mentor, Dr. Harlan Jones. I have gained a deeper appreciation for scientific research under your mentorship. I thank you for your patience with me, your guidance and more importantly wise council. Your uniqueness in being able to perform research at such a high level while maintaining a positive atmosphere in the lab and reminding me to always enjoy myself is truly commendable. I could not have done it without you.

Thank you to Dr. Rance Berg, Dr. Jozef Borvak and Dr. Robert Routh for being on my committee and providing me with valuable insight for my project. The constructive criticism you provided at every meeting only made me a better scientist.

In addition, I would like acknowledge my lab mate and friend Byung-Jin Kim who was also instrumental in my success. Thank you for taking time to teach me and for not giving up on me. I truly admire your commitment to science. To my other lab mates, Britney Mott and Daniel Tallent, I also thank you for all your support. I also would like to thank Timothy Break for being there with me from day one. Your friendship has truly been a blessing.

I would also like thank my entire family for all of your immeasurable support. To my father Anthony Kazadi Kayembe and mother Judith Ngwashi Kayembe, I thank you both for going the extra mile for me and for always knowing what to say and when to say it. To my brothers Jethy Kayembe, Glenn Kayembe, Aron Kayembe and his wife Yollande Kayembe and to my sister Tabitha Kayembe, you guys are the reason why I

strive for success. I am inspired by each and everyone one of you and could not wish for a better family.

I thank you all for sowing seeds in me that are life's foundations that I get to keep with me for the rest of my life. I love you all and wish you the very best in your future endeavors.

Last but certainly not least, I thank my father God without whom none of this would be possible.

Sincerely

Kay T. Kayembe

CHAPTER I

INTRODUCTION:

I. Does Stress Hold The Key to Disease Susceptibility?

Anecdotal evidence suggests a role for stress as a trigger for disease onset and progression, fostering the belief that exposure to environmental factors (social, economic, occupational) are significant determinants for the risk of disease (Agelaki, Tsatsanis, Gravanis, & Margioris, 2002). Toward defining causal relationships, experimental studies have highlighted a link between central nervous system (CNS) and immune system networks associated with host protection against disease (Agelaki et al., 2002). Specifically, research has shown that a disharmony or threat in body homeostasis by specific stress factors can influence immune activity (Shi et al., 2003). The resultant of such findings has developed into the field of psychoneuroimmunology (Black, 1994), with an objective of understanding the influences of the CNS on immune system function. To date, there is still further need to define the underlying mechanistic pathways to address whether stress factors hold the key to disease susceptibility.

The immune and central nervous systems represent numerous cellular and biochemical processes, which by themselves have very complex regulatory networks. Hence, this study's focus was to investigate the influence of a single neuroendocrine factor, corticotropin releasing hormone (CRH) on an immune cell constituent, the dendritic cell (DC), both of which are

considered master regulators of CNS and host immunity, respectively. The paragraphs that follow in this introduction provide a brief overview of the concepts linking the CNS and immune system with the goal of setting a foundation for the reader's understanding of this study.

II. The Dendritic Cell: A Major Cellular Regulator of The Immune System

The most common characteristic of the immune system is to provide protection against the invasion of pathogenic species, through a coordinated interplay between specialized immune cell populations. Fundamentally, these specialized cells are comprised of two major arms. The "innate" constituency functions as the non-specific responder to initial pathogenic exposure. This includes mainly macrophages which are known to engage in phagocytosis of infectious microbes or killing via respiratory burst. Granulocytes also respond to initial pathogen exposure by respiratory burst, release of free radicals, while dendritic cells initiate adaptive immunity through antigen presentation. In contrast, the "adaptive" arm participates in the generation of a specific and lasting defense. The main cells of the adaptive response are B cells, important for humoral immunity, and T cells involved in cell mediated immune responses. Importantly, while separate, both function in an interdependent manner, and in many recent studies are believed to be less distinct. Immune responses are well orchestrated in most cases, producing an appropriate defense against opportunistic pathogens. However, under certain circumstances improper immune function occurs, resulting in hyperactivation or hypoactivation of cellular immune function (Lemos et al., 2009; Ma et al., 2010). Over the years, researchers have begun to explain this dysfunction by defining the alterations in cell-mediated actions involving diverse cell-types and their function including cytokine, chemokine and complement activity related to a number of disorders including cardiovascular disease (Aggarwal, Shishodia, Sandur, Pandey, & Sethi,

2006), Asthma (Chen, 2006) as well as mycoplasma associated respiratory disease (Jones, Tabor, Sun, Woolard, & Simecka, 2002). Findings from these and other studies have been beneficial in the development of strategies to tailor immune responses for the benefit of host protection against disease as well as prevent immune-mediated inflammatory conditions associated with many non-pathogenic disease states.

Dendritic cells (DCs) are major antigen presenting cells responsible for linking innate and adaptive immunity through antigen uptake, processing and presentation to T cells. Dendritic cells primed by an antigen will undergo a maturation process associated with the up regulation of major histocompatibility complex II (MHC II) along with co-stimulatory molecules (CD80 and CD86) and secrete cytokines to efficiently prime naïve CD4+ T cells. In turn, these T cells will differentiate into several subpopulations, which through the release of specific cytokines impart very tailored function against pathogens (Baril et al., 2006; Rabquer, Shriner, Smithson, & Westerink, 2007; Trzcinski et al., 2008) such as induction of humoral antibody production (Rabquer et al., 2007), cytolytic function, and maintain phagocytic uptake of antigen (Colino, Shen, & Snapper, 2002). DCs can also activate CD8+ T cells and more recently have been implicated in the regulation of innate responses by augmentation of natural killer cells (Castillo, Stonier, Frasca, & Schluns, 2009) and neutrophil functioning (Happel et al., 2005). In total, DCs play a pivotal role in immune defenses underscoring the importance in disease susceptibility.

Taking advantage of primary cells from various tissues and the use of DC cell lines facilitates our understanding of DC function (Adams, O'Neill, & Bhardwaj, 2005). In humans, peripheral blood leukocytes provide a source for DC isolation. However, human blood dendritic cells are heterogeneous and scarce, accounting for just about 1% of circulating peripheral blood

mononuclear cells (PBMCs) (Kassianos, Jongbloed, Hart, & Radford, 2010). Thus, isolating a sufficient population of DCs for functional studies can pose a significant challenge. Established protocols involve extensive depletion steps to ensure purity of isolated population (Lee et al., 2009). Therefore, taking advantage of mouse bone marrow-derived dendritic cell provides a straightforward valuable approach for evaluating functionality of a homogenous dendritic cell population.

III. Corticotropin Releasing Hormone: A Trigger for Altered Immunity

The central nervous system (CNS), made up of the brain and spinal cord, controls many bodily functions. Evidence shows that events which take place in the brain, specifically at the hypothalamic-pituitary-adrenal (HPA) axis in response to stress can regulate downstream activity of immune cells (Shi et al., 2003). Under conditions of stress, a rapid increase of a wide variety of stress-associated neuroendocrine peptides such as epinephrine (catecholamine), cortisol, urocortin and CRH occurs to subsequently have a direct or indirect effect on immune function (Iwamoto, Ishida, Takahashi, Takeda, & Miyazaki, 2005). Importantly, the nature and duration of stressor can lead to either immunosuppression or immunoenhancement driven by preferences in catecholamine and corticosteroid production, suggesting a specificity of control (Black, 1994; Shi et al., 2003).

(CRH) is one, if not the major, stress response factor of the CNS. Its prominence is believed to be due to its global impact on downstream stress response pathways through its confined release in the HPA (Agelaki et al., 2002; Fukudo, 2007; Lee et al., 2009). CRH is a 41 amino acid peptide. CRH is historically secreted via the paraventricular nucleus (PVN) of the hypothalamus and is known to act on the pituitary to release ACTH (Adrenocorticotropic Hormone) that subsequently leads to cortisol secretion via the adrenal cortex (figure 1). In

addition to being produced in the hypothalamus, CRH has also been found to be synthesized in peripheral tissues (Baigent, 2001). Patients with osteoarthritis and rheumatoid arthritis have been shown to express CRH in synovial fluids and tissues (Langenkamp, Messi, Lanzavecchia, & Sallusto, 2000). It has been hypothesized that peripheral CRH acting on immune cells via autocrine and possibly paracrine signaling, may modulate overall immune outcome (figure 2). Specifically, CRH is known to impart its function on immune cells through ligation with two cellular receptors, CRH Receptor 1 (CRH R1) and CRH Receptor 2 (CRH R2). CRH R1 is the most prevalent isoform of the two receptors (Chen, 2006) and has an affinity for CRH ten times higher than that of CRH R2 (Tsatsanis et al., 2007). In this regard, it has also been reported of macrophages, mast cells and T cells to express CRH receptors (Feng et al., 2009) and most recently, in our study, by pulmonary-associated dendritic cells (Gonzalez, 2008) and others (Fukudo, 2007). To date, it remains unknown how peripheral CRH activity impacts disease susceptibility. Understanding the functional significance of CRH and its specificity on immune cells will be an important finding relevant to the stress-immune axis and provide further insight of the role that stress factors portray in disease susceptibility.

IV. Stress-induced CRH regulates Dendritic cell function against *S. pneumoniae*

As a first step in understanding the role of CRH on immune function, our laboratory has begun research using a respiratory pathogen model. *Streptococcus pneumoniae* (*S. pneumoniae*) infection is a leading cause of community acquired respiratory illness and mortality in the United States (Paterson & Mitchell, 2006). *S. pneumoniae* is a gram-positive, alpha-hemolytic bacterium responsible for a variety of diseases including respiratory pneumonias, bacterial meningitis, otitis media and sepsis (Paterson & Mitchell, 2006). Pneumococcus pathogenicity requires adherence

to host cells; typically *S. pneumoniae* will colonize upper respiratory airways and gain access to the lung. Mucosal invasion increases the risk for systemic spread. Populations at risk include: immunocompromised, elderly and infants (Speert, 2006; Zhang et al., 2007). The lack of an effective vaccine in addition to the emergence of drug resistant strains makes protection against *S. pneumoniae* mainly dependent on host natural immunity.

Natural protection against *S. pneumoniae* infection requires the involvement of both innate and adaptive compartments of the immune defense system (Gonzales, Deshmukh, Pulse, Johnson, & Jones, 2008). The proper activation of innate mediators such as complement protein, C-reactive protein (CRP) and pro-inflammatory cytokines (IL-1β, IL-6, IL-12, TNF-α, etc) can contribute to bacterial clearance (Walport, 2001a; Walport, 2001b). Macrophages and neutrophils have also been shown to recognize bacterial pathogen using pattern recognition receptors and induce phagocytosis and intracellular destruction. In addition, Cell-mediated immunity has also been shown to be crucial against *S. pneumoniae* (Rabquer et al., 2007). Antigen specific CD4+ T helper cells are the known main cell-type that provides protection against pneumococcal colonization of the lung (Trzcinski et al., 2008) and during systemic spreading (Baril et al., 2006). It is therefore likely that stress-associated alterations of the natural immune response can have a significant influence on host vulnerability.

We have previously demonstrated that stress exposure can impact the generation of protective immunity. Our previous published work demonstrated that stressed mice have a significant elevation in CRH compared to the non-stressed counterparts. Secondly, stressed mice were unable to generate protective immunity against *streptococcus pneumoniae* (Fig 3). Furthermore, the mRNA detection of CRH in total lung cells and of CRH receptors by dendritic

cells suggest an association between stress-induced impairment of adaptive immunity and CRH activity on APC (Fukudo, 2007). "I therefore hypothesize that neuroendocrine factors have regulatory effects on antigen presenting cell's (APC) function, and is a cause of impaired resistance against *S. pneumoniae*." The purpose of our current research is to define mechanisms by which stress-associated neuroendocrine factors modulate host immune responses. In particular, my study focused on CRH's potential to impact DC function.

T cells express CRH receptors (Feng et al., 2009), and we now know that DC can express CRH and both CRH R1 and CRH R2 (Chen, 2006; Fukudo, 2007; Gonzales et al., 2008). However, it is still unclear how CRH through preferential interaction with CRH receptor-types impacts DC function. Specifically, the type of cytokines secreted by DC can dictate subsequent differentiation and effector T cell function. Mainly, IL-12 secretion supports IFN-γ production by T helper-1 and cytotoxic T cells. In contrast, IL-10, TGF-β, and IL-23 facilitate the differentiation of T helper-2, T regulatory and Th17 cellular response respectively. **Consequently, I hypothesized that CRH can act preferentially via CRH R1 and CRH R2 expression on DC, promoting maturation and inducing preferences in cytokine production.** The following specific aims were developed to address the hypothesis.

Specific aim 1: To determine if *in vitro* BMDCs are practical to examine CRH's role on dendritic cell function. In establishing a model to investigate the functional role of CRH and CRH receptor expression on DCs, this aim determined how bone marrow-derived dendritic cells (BMDCs) as an in-vitro model system, could be used to test CRH-mediated activity on DCs. First, I determined if BMDCs express CRH and/or CRH receptors in the basal state or in response to lipopolysacharides (LPS). LPS is known to simulate antigen presenting cell by

engaging toll like receptor 4 on BMDC (Iwamoto et al., 2005). I also determined whether LPS stimulation would influence CRH expression as well as promote selectivity in receptor expression.

Specific aim 2: To determine the influence of CRH on dendritic cell function in response to lipopolysaccharide and *S. pneumoniae*. Dendritic cells that mature in the presence of LPS or *S. pneumoniae* will have a cytokine profile indicative of their activation. The purpose of this aim was to determine whether supplementing BMDC cultures with CRH prior to LPS or *S. pneumoniae*-induced BMDC production of IL-10, IL-12p70 and IL-23 cytokines. In following, I began to investigate the role of CRH upstream of cytokine production. Activated DC express MHC II in addition to the up-regulation of co-stimulatory molecules B7-1 (CD80) and B7-2 (CD86) (Colino et al., 2002). I obtained preliminary results indicating the potential role of CRH in BMDC maturation.

Specific aim 3: To demonstrate whether preferences in CRH receptor activity dictates dendritic function. CRH is known to act on immune cells through its receptors. To further address our hypothesis that CRH acts preferentially through selectivity of CRH receptors and promote preferences in cytokine production, I used antagonists for CRH R1 and CRH R2. Antalarmin was used to block CRH R1 and astressin$_2$B was used to block CRH R2.

CHAPTER II

EXPERIMENTAL DESIGN AND METHODS:

Mice:

Female (4-12 weeks old) CD1 mice were used in all studies. Mice were maintained in a pathogen-free environment under a 12 hour light-dark cycle. Mice were housed 5 per cage under optimal temperature and humidity and provided appropriate care in accordance with the institutional animal care and usage committee. All animals were given an acclimation period prior to experiment.

Derivation of Bone Marrow-Derived Dendritic Cells:

Bone marrow-derived dendritic cells were derived from bone marrow pre-cursor cells of the long bones of naïve mice. Specifically, mice were anesthetized using a cocktail of ketamine (10ng/ml) and xylazine (8ng/ml) combination and euthanized by cervical dislocation. Bone marrow was flushed from both femurs and tibia with RPMI-1640 (1% FBS and 1% penicillin/streptomycin) using a 25G1½ needle attached to a 10mL syringe. Eluent was collected and centrifuged at 200 X g for 10 minutes at 4°C. The supernatant was decanted away from the cell pellet, which was subsequently treated with ACK lysis buffer (Ammonium chloride solution) for 10 minutes on ice to remove contaminating red blood cells. Following, incubation, RPMI-1640 wash media was added to the ACK lysis solution, centrifuged at 200 X g for 10

minutes at 4°C. The cell pellet was then resuspended in RPMI-1640 media. Viable lymphocytes from cell suspension were isolated using Lympholyte®-M (cedarlane laboratories, Burlington NC) and washed with RPMI-1640 media. Bone marrow pre-cursor mononuclear cells were seeded in T75 culture flasks and cultured for seven days in culture media containing 10% FBS, 1% penicillin/streptomycin, 2mM L-glutamine, 50uM β-mercaptoethanol and 10mM Hepes. Differentiation of bone marrow pre-cursors cells into Bone marrow derived dendritic cells (BMDCs) was initiated by supplementing the media with 10ng/ml granulocyte-macrophage colony stimulating factor (GM-CSF) and 10ng/ml interleukin IL-4 on days 0, 3 and 6. Floating and loosely adherent cells were removed on day 3 and replaced with fresh media. Additional fresh media was added to the cell culture on day 6.

On day 7, cells were transferred to a 48 well plate ($5X10^5$ cells per well). All cultures used in study were checked for dendritic cell (DC) purity (Figure 4).

Preparation of the Bacterial Stimuli:

Streptococcus pneumoniae (*S. pneumoniae*) strain #6301 (ATCC, Manassas, VA) was grown overnight to achieve mid-log phase cultures on Blood Agar plates. The bacteria were suspended in Todd Hewitt Broth and adjusted to give an optical density absorbance of 1 (600 nm) which corresponded to $5X10^8$ CFUs (colony forming units). After PBS wash, *S. pneumoniae* suspension was adjusted to achieve appropriate ratio of CFU per DC used for stimulation. All *S. pneumoniae* preparations were treated with antibiotic cocktail to render them non-viable for *in vitro* cultures.

In vitro Stimulation of BMDCs:

An *in vitro* culture system was established to demonstrate corticotropin releasing hormone CRH's influence on BMDCs' function in response to lipopolysaccharide (LPS) and anti-biotic-treated *S. pneumoniae*. Prior to stimulation of BMDCs with the TLR-4 agonist LPS and *S. pneumoniae*, BMDCs were pre-exposed to (CRH) at a pre-determined optimal concentrations (10^{-8}M) for 2 hours followed by 24 hours stimulation using LPS (1µg/ml) or killed *S. pneumoniae* (300 and 500 CFUs per DCs). For comparisons, additional cells were cultured with or without CRH pretreatment for the entire culture period. All conditions were compared to the negative control in which BMDCs were not exposed to CRH or stimulation (LPS or *S. pneumoniae*).

The determination of CRH function and preferences in CRH receptor ligation was demonstrated using selective CRH R1 antagonist, Antalarmin, and CRH R2 antagonist, Astressin$_2$B. Specifically, each antagonist was added to the culture 30 minutes at various concentrations (10^{-6},$^{-7}$,$^{-8}$M) prior to the introduction of CRH.

BMDCs were stimulated with LPS or *S. pneumoniae* for 2 and 6 hours to assess peak time point(s) at which to measure BMDC function. Based on our findings, a single time point was used in all subsequent studies (data presented in thesis). Figure (5) outlines the in vitro culture protocol. All supernatants were collected and stored at -80°C until analyzed.

Real Time PCR:

Total RNA from Trizol (Trizol® reagent, invitrogen) isolated unstimulated BMDC were reverse transcribed using Molony murine leukemia virus reverse transcriptase (Promega,

Madison, WI). RT-PCR was done using SYBR green master mix with CRH R1/CRH R2 as the target gene primers (SuperArray Bioscience, Frederick, MD). The threshold of the growth curve (CT) was set at a value of 35. The expression of the housekeeping gene glycerolaldehyde 3-phosphate dehydrogenase (GAPDH) was used to normalize target gene expression between samples. Differences in target gene expression were calculated using the following formula: $\Delta\Delta^{CT} = \Delta^{CT}$ (target gene) $- \Delta^{CT}$ (GAPDH). The $\Delta\Delta^{CT}$ value of cDNA amplification from the unstimulated BMDC was considered the calibrator for baseline levels of mRNA expression. Data were expressed as the ratio of target gene expression of Stimulated BMDCs to the target gene expression of the unstimulated BMDCs, resulting in fold difference in target gene mRNA levels.

Flow Cytometry:

Two color flow cytometric technique was utilized to demonstrate CRH's influence on the expression of BMDC-associated $CD80^+$ surface co-stimulatory molecule expression and major histocompatibility class II surface molecule expression ($MHC\ II^+$). Following in vitro culture, BMDCs were collected using 1 X PBS with 5mM EDTA and transferred to 96 well plates for staining. The cells were suspended in staining buffer (1 X PBS, 1% FBS) containing 2mM EDTA to prevent cellular aggregation and incubated with Fc blocker (clone 2.4G2) (BD Pharmigen, San Diego, CA) for 10 minutes to inhibit non-specific binding to FcRs. Subsequently, cells (5×10^5) were stained with Phycoerythrin (PE)-labeled anti-$CD11c^+$ antibody, Fluroscein isothiacyanate (FITC) anti-$MHC\ II^+$, or FITC anti-$CD80^+$ antibody for 30 minutes in the dark at 4°C. Following incubation, cells were washed with staining buffer and fixed with 2% paraformaldehyde prior to analysis using the FC500 flow cytometer (Beckman-Coulter, Miami, FL).

ELISA:

Enzyme-Linked Immunosorbent Assays (ELISAs) were performed using OptEIA kit for mouse Interleukin 10 (IL-10) from BD Bioscience (Franklin Lakes, NJ) and Interleukin 12p70 (IL-12p70), Interleukin 23 (IL-23) from eBiosciences (San Diego, CA). Briefly, 96 micro-well plates were coated with 100 µl per well capture antibody (IL-10 diluted 1:125 in sodium phosphate pH 6.5, IL-12 diluted 1:250 in phosphate buffer pH 7.2, IL-23 diluted 1:250 in phosphate buffer pH 7.4). The plates were sealed and incubated overnight at 4°C. The plates were then washed, 3 times for IL-10 and 5 times for IL-12 and IL-23, with ≥ 250 µl/well wash buffer (1 X PBS with 0.05% Tween-20). Following the last wash the plate were inverted and blotted on a paper towel to remove residual buffer. The wash conditions remained constant throughout the course of the protocol unless otherwise noted. The plates were blocked with 200 µl/well assay diluents (1 X PBS with 10% FBS) following the wash step and incubated overnight at 4°C. The plates were washed and 100 µl/ well of samples were added to the appropriate wells. Using assay diluents, the standards were diluted in 2-fold serial dilutions starting from the top standard and 100 µl/ well of each dilution were added to the appropriate wells to generate the standard curve. The plates were incubated overnight at 4°C. After washing IL-12 and IL-23 plates, 100 µl of detection antibody diluted 1:250 in assay diluent were added, and the plates sealed and incubated 1 hour at room temperature (RT). Following an additional wash, 100 µl/well enzyme reagent (Streptavidin-horseradish peroxide conjugate) diluted 1:250 in assay diluent was added to IL-12 and IL-23 plate before being sealed and incubated 30 minutes RT. IL-10 plate; following the blocking step was washed and incubated one hour with 100 µl detection antibody with streptavidin-horeseradish peroxide conjugate both diluted 1:250. For the final plate wash, all three plates were washed 7 times with wash buffer, letting the buffer sit in

the wells for ≥ 30 seconds per rinse. 100 µl/well TMB (Tetramethylbenzidine, BD pharmigen) were added and the plates were incubated for approximately 20 minutes at RT in dark. 50 µl/well stop solution (0.25 M HCl) was added to stop the reaction and colorimetric absorbance of the plate was read on an ELISA plate reader at 450nm.

Statistical Analysis:

Statistical analysis was performed using GraphPad prism Version 4.0 for Windows (GraphPad Software, San Diego, USA). For multi-experimental group analysis, data were subjected to analysis of Variance (Univariant ANOVA) followed by Newman-Keuls Multiple Comparison Test. Data represent the mean ± standard error ($n \geq 3$) per experimental conditions. Each asterisks (*), (**) and (***) indicates significant difference ($p \leq 0.05$), ($p \leq 0.01$), and ($p \leq 0.001$) respectively between individual condition.

CHAPTER III

RESULTS

I. BMDCs express CRH receptors 1 and 2.

Lipopolysaccharide (LPS) results in a preference in CRH receptor 1 by BMDCs.

We previously published data demonstrating that pulmonary antigen presenting cells express CRH receptors (Gonzales et al., 2008). BMDCs provide an excellent tool for investigation of DC function. Thus, initial studies were performed to determine whether CRH receptors were expressed on these cells, and hence provide a useful model to investigate CRH's role on DC function. The results from this study demonstrated mRNA expression of both CRH receptors by unstimulated BMDCs (Figure 6A) using quantitative Real Time PCR (qRT-PCR) and confirmed by gel visualization of PCR products (Figure 6B). Analysis of mRNA levels did not reveal significant quantitative differences between CRH receptor 1 and receptor 2.

LPS is known to simulate BMDCs in a Toll-like receptor-4 mediated (TLR-4) pathway (Iwamoto et al., 2005). I determined whether activation of BMDCs would promote gene expression of either CRH receptor. Both receptors were up regulated in response to LPS (1µg/mL). A 1.4 fold higher mRNA expression of CRH R1 was found compared to CRH R2 mRNA (Figure 6A).

II. Influence of CRH on BMDC cytokine production in response to LPS and *Streptococcus pneumoniae*

A) Kinetics of cytokine production by BMDCs in response to LPS and *S. pneumoniae* stimulation

Taking advantage of LPS as a well-established stimulant of BMDCs, I performed a time dependent stimulation of BMDCs using LPS and measured cytokine production as a functional output. BMDCs were stimulated with LPS (1ug/ml) for 2 hours and 6 hours. Production of Interleukin-10 (IL-10) did not change but Interleukin-12 (IL-12) and Interleukin-23 (IL-23) production were significantly increased after 6 hours of LPS stimulation (Figure 7).

I also optimized our *in-vitro* stimulation with antibiotic-treated *S. pneumoniae*. BMDCs were cultured in 48 well plates at a cell density of 5 X 10^5 DCs per well in the presence of either 300 colony forming units (CFUs) or 500 CFUs per DC. Cytokine production was also used to measure BMDC function. BMDCs responded in the presence of 300 and 500 CFUs (Figure 8). Subsequently, I measured cytokine kinetics at two and six hours using 300 CFUs/DCs of *S. pneumoniae* and recorded no change in levels of IL-10, IL-12 and IL-23 between untreated and treated at 2 hours, but demonstrated a significant increase at 6 hours for all three cytokines (Figure 9). Based on the above findings, all subsequent experimental measures in response to LPS and 300 CFUs of antibiotic-treated *S. pneumoniae* were performed for 6 hours.

B) The effect of CRH on BMDC cytokine production

Cytokine production by DCs is an important predictor of subsequent T cell differentiation and effector function. IL-10 typically promotes differentiation of $CD4^+$ T helper-2

cells and potentially $CD4^+$ T regulatory cells while IL-12 and IL-23 promote $CD4^+$ T helper-1 (Colino et al., 2002; Lemos et al., 2009) and $CD4^+$ T helper-17 (Lemos et al., 2009; Ma et al., 2010) cellular responses respectively.

CRH alone significantly inhibited basal level of IL-10 but did not have an effect on IL-12 and IL-23 production. LPS treated BMDCs responded by secreting significantly more IL-12 and IL-23 than unstimulated BMDCs, but induced no changes in IL-10. During CRH pre-treatment followed by LPS stimulation (CRH+LPS), IL-10 and IL-12 production were both significantly ($P \leq 0.05$) inhibited while production of IL-23 remained unchanged (Figure 10).

In response to *S. pneumoniae* stimulation, BMDCs responded by secreting significantly more IL-10, IL-12 and IL-23 than the untreated control. Following CRH pretreatment, *S. pneumoniae* stimulation did not change IL-10 while slightly inhibiting IL-12 production. In the case IL-23, exposure of BMDCs to *S. pneumoniae* following CRH pre-treatment resulted in a significant ($P \leq 0.05$) inhibition in IL-23 (Figure 11).

III. Investigating whether selective CRH receptor antagonist dictate LPS and *S. pneumoniae*-induced responses

To further demonstrate CRH influence on DC function, which I believe to be mediated through CRH receptor 1 (CRH R1) and CRH receptor 2 (CRH R2) expressed on BMDC, I used antagonist for both receptors. Antalarmin is a selective CRH receptor 1 antagonist and astressin$_2$B is specific to CRH R2. I measured cytokine production in LPS and *S. pneumoniae* stimulated BMDCs.

Blocking CRH receptor 1 before LPS stimulation with specific antagonist, antalarmin (antalarmin+CRH+LPS), inhibited the LPS-induced IL-10 production but showed no difference when compared to the CRH pre-treatment followed by LPS stimulation (CRH+LPS). Antalarmin addition also blocked both LPS-induced and CRH+LPS-induced IL-23 production. IL-12 production however, was significantly higher than CRH+LPS stimulation and similar to LPS only stimulation when receptor 1 was blocked (Figure 12).

Addition of CRH R2 antagonist astressin$_2$B (astressin$_2$B+CRH+LPS) significantly inhibited LPS-induced IL-10 production but showed no difference when compared to CRH+LPS. Production of IL-12 following astressin$_2$B+CRH+LPS was significantly inhibited compared to LPS and CRH+LPS stimulation. Astressin$_2$B+CRH+LPS showed no changes in LPS and CRH+LPS-induced IL-23 production (Figure 12).

In response to *S. pneumoniae*, production of IL-10 was significantly increased when antalarmin was added (antalarmin+CRH+*S. pneumoniae*) and compared to *S. pneumoniae* only. CRH+*S. pneumoniae* compared to antalarmin+CRH+*S. pneumoniae*-induced IL-10 production, showed no difference. IL-12 production following antalarmin+CRH+*S. pneumoniae* showed no difference compared to *S. pneumoniae* only but was significantly higher than CRH+*S. pneumoniae*. In the case of IL-23, antalarmin+CRH+*S. pneumoniae* showed no significant differences compared to *S. pneumoniae* only and CRH+ *S. pneumoniae* (Figure 13).

CRH R2 blockade mediated by astressin$_2$B addition, did not affect *S. pneumoniae* and CRH+ *S. pneumoniae*-induced production of IL-10, IL-12 and IL-23 (Figure 13).

IV. PRELIMINARY FINDINGS:

The effect of CRH on BMDC MHC II and CD80 surface molecule expression following LPS and *Streptococcus pneumoniae* stimulation.

In response to antigen, DCs convey various signals indicating their level of maturation and activation status. MHC II^+ expression in complex with antigen peptide presented on the surface of DCs is essential for T cell receptor (TCR)-mediated recognition and its subsequent activation. Furthermore, the induction of the co-stimulatory molecule CD80 on the surface of DCs provides essential secondary signals that regulate DC-T cells interactions. Using flow cytometry methods, I examined the expression of MHC II^+ and $CD80^+$ on $CD11c^+$ BMDCs. The level of expression was expressed as the mean fluorescence intensity (MFI) surface staining MHC II^+ and $CD80^+$ from the total $CD11c^+$ cell population. Exposure to CRH alone showed no effects for both MHC II^+ and $CD80^+$ expression by BMDCs (Figure 14). BMDCs stimulated with LPS resulted in no change in expression, which remained constant in the presence of CRH (CRH+LPS).

In response to *S. pneumoniae*–induced activation, BMDCs showed no change in expression of both MHC II^+ and $CD80^+$ when compared to their unstimulated counterpart (Figure 15). In addition, CRH pre treatment (CRH+*S.pneumoniae*) did not modulate expression of MHC II^+ and $CD80^+$ but rather it simply maintained it.

CHAPTER IV

DISCUSSION:

The central nervous system's response to stressors in the form of neuroendocrine activation is known to alter multiple biological functions and is believed to promote disease. Such evidence is found in studies, which demonstrate how stressors (e.g perceived or physical) modulate immune function in the context of infectious and non-infectious disease (Kiecolt-Glaser et al., 2005; Segerstrom & Miller, 2004). To increase our understanding of the relationships between stress and immunity, the purpose of this study was to examine the impact of CRH in modulating DC function. The basis for this study is attributed to our previous published work whereby mice pre-exposed to chronic restrain stressed were unable to develop resistance against re-exposure to the respiratory pathogen, *Streptococcus pneumoniae* (Figure 3). We attribute this mal-adaptation of the immune system in part to the significant increase in CRH expression, in total lung tissue of mice exposed to restrain stress and primary infection. Secondly, in conjunction with increased CRH gene expression, pulmonary-associated $CD11c^{+}$ MHC II+ DCs were found to express CRH receptors. Furthermore, ongoing studies also indicate that *in vivo* blockade of CRH receptors can influence disease susceptibility among stressed mice. Together, these findings suggest a role for CRH regulation of DC-associated induction of adaptive immunity, providing novel insight toward defining the relationships between stress and disease.

Our initial studies sought to determine the feasibility of using BMDCs to study CRH-effects on DC functioning. Granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin-4-generated BMDCs phenotypically and functionally share significant similarities with conventional or myeloid DCs from secondary lymphoid organs such as lymph nodes (Adams et al., 2005; Lutz et al., 1999; Shortman & Liu, 2002). Furthermore, similarities in cytokine production have also been proven between BMDCs and primary DC populations (Boonstra et al., 2003; Morelli et al., 2001). To our knowledge, Lee et al., was first to show both transcription and protein expression of CRH receptors on human monocyte-derived dendritic cells (Lee et al., 2009). In mice, bone marrow precursors in addition to other tissues (e.g. spleen and peripherial blood leukocytes) provide a resource for DC differentiation. To initiate our studies, I examined whether BMDCs were capable of expressing CRH receptors. Indeed, both CRH receptors mRNA were expressed by unstimulated BMDCs determined by quantitative RT-PCR (Figure 6). In addition, LPS-induced activation of BMDCs resulted in a significant induction of both receptors. Furthermore, a 2200 and 1700 fold increase in mRNA expression of CRH R1 and CRH R2 respectively was determined by BMDCs in response to LPS. In support, previous studies have shown that CRH R1 is the prevalent isoform of the two receptors (Lee et al., 2009) with 10 times the affinity for CRH than that of CRH R2 (Tsatsanis et al., 2007). This finding was also consistent with our previous studies, in which CRH R1 was preferentially expressed by pulmonary DCs (Gonzales et al., 2008). Thus, given the confirmation of CRH receptor expression by BMDCs and their preferences in response to LPS-induced BMDC activation, one might consider whether CRH binding to a specific receptor could impact DC function. An important limitation to our findings however is the lack of correlative CRH receptor expression at the protein level. Although previous studies have confirmed CRH receptor

expression at the mRNA level to be convincing (Lee et al., 2009), these results do not demonstrate CRH receptor protein on the cell surface of BMDCs. Therefore, more conclusive data of CRH receptor expression at the protein level would benefit interpretation of our results. In addition to confirming the CRH receptor expression by BMDCs in response to LPS, it will be significant to test the effect of *S. pneumoniae* exposure on CRH receptor expression. This is of particular relevance based on the known TLR-4 specificity for LPS versus TLR-2 specificity of *S. pneumoniae* cellular epitopes. I anticipate that results from future studies have the potential for predicting how antigens dictate CRH receptor expression and in turn impact DC function (e.g cytokine production, maturation and phagocytosis).

Cytokine secretion by DCs is an important predictor of subsequent T cell differentiation and effector function. In response to LPS-mediated activation, BMDCs did not secrete anti-inflammatory cytokine IL-10 but were capable of secreting both pro-inflammatory-associated cytokines, IL-12 and IL-23 with distinctly different kinetics. I hypothesized that CRH could alter the type and quality of IL-12, IL-23 and IL-10 cytokine production by BMDCs. CRH treatment resulted in a significant inhibition of IL-10 and IL-12 in response to LPS stimulation, suggesting that CRH has the potential to influence T helper-2 and T helper-1 differentiation, respectively. Interestingly, CRH did not influence LPS-induced IL-23 production, which is important for T helper-17 differentiation. Our findings have both similarities and differences between previous reports. For example in human models, Lee et al demonstrated that while CRH induced no changes in IL-6, CCL17, CCL18 and CCL22 production in human monocyte-derived DCs (MoDCs), CRH decreased IL-18 production. More importantly, CRH suppressive effect was more significant in MoDCs from atopic dermatitis patient than non-atopic healthy controls (Lee et al., 2009) suggesting anti-inflammatory activity by CRH. In another study Benou et.al,

demonstrated that CRH exerted a pro-inflammatory effect in a model of experimental autoimmune encephalomyelitis (EAE) with a selective increase in Th-1 phenotype (Benou et al., 2005). Such differences are likely due to cellular phenotype, stimulation and experimental paradigms. A major parameter to consider in the interpretation of these findings, with that of others, is the use of LPS as an activator of DC function. LPS typically activates DCs through TLR-4 signaling pathways and as a consequence preferentially elicits cytokine activation through the IL-12/IL-23 pathway as well as IL-1. Thus, how CRH may potentially influence TLR-4 signaling as compared to other pathways of DC activation may dictate the functional outcomes including cytokine/chemokine production, co-stimulatory molecule expression adhesion and antigen presentation.

As mentioned above, other factors such as MHC II and co-stimulatory molecule expression are key events needed for DCs-associated cytokine production instrumental in priming and maintenance of adaptive immune responses, through regulation of T cell activation. Based on the current literature, no one has determined the direct effect of CRH on dendritic cells expression of MHC II and expression of the co-stimulatory surface molecules CD80 and CD86. This study investigated the role of CRH on MHC class II expression. Our results demonstrated that BMDCs treated with recombinant CRH alone (10^{-8} M) for a total of 8 hours resulted in a no change in MHC II^{+} as compared to untreated BMDCs (Figure 14). This suggests that CRH alone is capable of maintaining DC maturation. Secondly, in the presence of LPS alone, no significant induction of MHC II^{+} was observed by BMDCs. Finally, pre-exposure of BMDCs to CRH prior to introducing LPS did not inhibit nor decrease MHC II^{+} expression but rather maintained its expression (Figure 14). To date, we are the first to investigate the direct interaction of CRH with BMDC maturation by way of MHC II expression. Secondly, I decided to investigate whether

CRH would play a role in co-stimulatory molecule CD80 expression. Again BMDCs were treated with CRH and $CD80^+$ expression measured. Our experiment revealed that CRH treatment alone also maintained CD80 expression by BMDCs. When BMDCs were activated using LPS, CRH pretreatment did not change the LPS-induced expression of CD80. CRH maintains BMDC maturation by supporting both MHC II and CD80 expression.

CRH effect on *S. pneumoniae* stimulated BMDC and correlation with *in-vivo* observation

The results above provide relevant insight of the potential modulatory effect of CRH on cytokine production at least in response to LPS and how it may impact downstream adaptive immunity. Based on our ongoing studies, an investigation of the impact of CRH on BMDC's response to *S. pneumoniae* would provide insight related to the role of DCs action *in vivo*. Thus, a series of experiments were performed in which non-viable *S. pneumoniae* was introduced to BMDC cultures in the presence of CRH. I observed that CRH treatment showed a slight increase in *S. pneumoniae*–induced IL-10 production. Yet, an attenuation of IL-12 was observed, suggesting that CRH could potentially inhibit $CD4^+$ T helper-1 cells. T helper-1 cells are producers of IFNγ and have been reported to play an important role in pneumococcal clearance (Blair, Naclerio, Yu, Thompson, & Sperling, 2005). In addition, I also notice that During *S. pneumoniae* stimulation CRH treatment significantly inhibited IL-23. As mentioned previously, IL-23 production is important for T helper-17 differentiations and neutrophils recruitment is facilitated by IL-17 produced by T helper-17 cells (Dubin & Kolls, 2007; Happel et al., 2005; Wu et al., 2007). The following is of particular importance because recruited neutrophils provide protection against many encapsulated bacteria (Ferretti, Bonneau, Dubois, Jones, & Trifilieff, 2003; Wu et al., 2007) and are specifically believed to play an important role for effective

clearance against pneumococcal challenge by phagocytosis and intracellular destruction (Paterson & Mitchell, 2006; Walport, 2001a). Thus, inhibition of IL-23 by CRH, resulting in potential impairment of neutrophil recruitment could account for the lack of protection seen in our *in vivo* model.

The next question to address was whether CRH modulating effects would influence BMDC MHC II and co-stimulatory expression in the presence of *S. pneumoniae* antigen. When treated with our bacterial stimuli, non-viable *S. pneumoniae*, BMDCs simply maintained their MHC II^+ expression to a level relatively close to the untreated group. Untreated BMDCs expressing good basal level MHC II could indicate already matured BMDCs not needing to further express MHC II. To investigate whether CRH had an effect on the *S. pneumoniae* induced expression of MHC II^+; I pre-treated BMDCs with CRH prior to them being stimulated with *S. pneumoniae* and noticed MHC II^+ expression being maintained (Figure 15). The investigation of CRH influence on secondary signal CD80 during *S. pneumoniae* stimulation, revealed no effect. This observation was consistent with our LPS model in which CRH also maintained the LPS-induced expression of CD80. Our results suggest that CRH does not seem to be impairing DC maturation and thus this interaction is probably not responsible for the increase mortality of stress mice during secondary challenge with *S. pneumoniae*.

Linking CRH receptor-specific activity is likely to provide a clearer picture of CRH's function on BMDC. Antalarmin is a potent CRH R1 antagonist both *in vitro* and *in vivo* (Webster, Torpy, Elenkov, & Chrousos, 1998). Addition of Antalarmin prior to LPS stimulation further inhibited IL-10 and IL-23 but allowed for the CRH-inhibited IL-12 production to be restored. On the other hand blocking with CRH receptor 2 antagonists, $Astressin_2B$, resulted in

IL-12 inhibition which suggest that CRH's ability to inhibit IL-12 is mediated by CRH receptor 1. Interestingly, our discovery of Antalarmin antagonizing CRH R1 on BMDCs and influencing their cytokine profile may have specific therapeutic potential during inflammation. *In vivo* administration of Antalarmin has been shown to significantly antagonize both central and peripheral actions of CRH in rats (Webster et al., 1998).

Similarly, I tested the effects of CRH receptor antagonist on BMDC function in response to *S. pneumoniae*. *In vitro* administration of Antalarmin prior to stimulation with *S. pneumoniae*; resulted in a significant increasing in IL-10 while also increasing IL-12 production. More importantly, Antalarmin addition restored IL-23 production. Astressin$_2$B showed no effect in cytokine production. All together, I notice that CRH acting through CRH receptor 2 does seem to mediate some changes in BMDC function following *S. pneumoniae* stimulation. Conversely, it does inhibit IL-23 and IL-12 which are both involved in the differentiation of important CD4 helper T cells required for protection against pneumococcal infection.

Concluding Remarks

Resistance against disease requires involvement of both innate and adaptive components of the immune response. Complement activation in conjunction with phagocytosis and oxidative killing by macrophages and neutrophils are part of the non-specific innate response reported to contribute to bacterial clearance (Paterson & Mitchell, 2006; Walport, 2001a; Walport, 2001b). In addition, cell-mediated and humoral antibody response has also been shown to be critical in the generation of an adaptive immune response against *S. pneumoniae* (Rabquer et al., 2007). Specifically, antigen specific $CD4^+$ T helper cells are the known main cell-type that provides protection against pneumococcal colonization of the lung (Trzcinski et al., 2008) and during

systemic spreading (Baril et al., 2006). Therefore it was important for us to understand the impact of stress-associated factors on natural immunity during pneumococcal infection. I focused on the involvement of dendritic cells, as they are the major cell type responsible for initiating and directing adaptive immune responses against foreign pathogen such as *Streptococcus pneumoniae*, and the influences of CRH during adaptive immunity. DCs act as sentinels in peripheral tissues where their main function is to capture pneumococcal antigen at sites of infection and process the bacterial antigen for major histocompatibility complex class II (MHC II) to bind with TCR (T cell receptors) present on naïve T cells, creating a receptor complex that following cytokine production will induce differentiation of $CD4^+$ T helper cells which are required for protection (Colino et al., 2002; Elftman, Norbury, Bonneau, & Truckenmiller, 2007; Gonzales et al., 2008). Kadioglu et al demonstrated in a mouse model that wild-type mice inoculated intranasally with a strain of pneumococcus rapidly cleared bacteria from the lungs and blood within 2 days. Whereas Major histocompatibility complex class II-knockout mice in the same experiment, showed persistent infection in both lungs and blood over 3 days, suggesting a nonspecific role for $CD4^+$ T cells in early host defense (Kadioglu, Coward, Colston, Hewitt, & Andrew, 2004). $CD4^+$ T helper cells are key mediators of protection against pneumococcal challenge and I now know that CRH does not promote a T helper-17 response or T helper-1. It is therefore likely that potential impairment in $CD4^+$ T cells differentiation will have a significant influence on host vulnerability.

Numerous evidence now suggest that neuroendocrine peptides which were once believed to be solely of brain and pituitary origin are, in fact, involved in a large number of paracrine and autocrine actions in the periphery (Baigent, 2001). Peripheral CRH acting directing on DCs determined the overall immune outcome. The results of this project showed the following;

BMDCs do express CRH receptors 1 and 2 at a basal level and engagement of the cells using an inflammatory mediator induced increase in receptor expression. BMDCs responded to CRH pretreatment by maintaining their maturation profile along with preferentially inhibiting IL-23 cytokine production. Also, the ability of CRH to mediate these changes was confirmed using CRH receptor antagonist. Consequently, I was able to demonstrate in an *in-vitro* model, the ability of CRH to impact dendritic cell function.

Based on all these findings, I conclude that CRH can potentially influence CD4+ T cell differentiation by influencing their cytokine production. In addition, it is important to note that CRH can have distinct effect on DCs depending on the type of inflammatory mediator used. In summary, our study provides further convincing scientific evidence that stress hormone, CRH, can dictate immune response. It is becoming more evident that stress does not exclusively suppress all aspects of immunity as it was once believe, but rather can effect wide changes in immunocompetence and even exaggerated responsiveness of specific inflammatory components of the immune system.

CHAPTER V

FIGURES:

FIG. 1

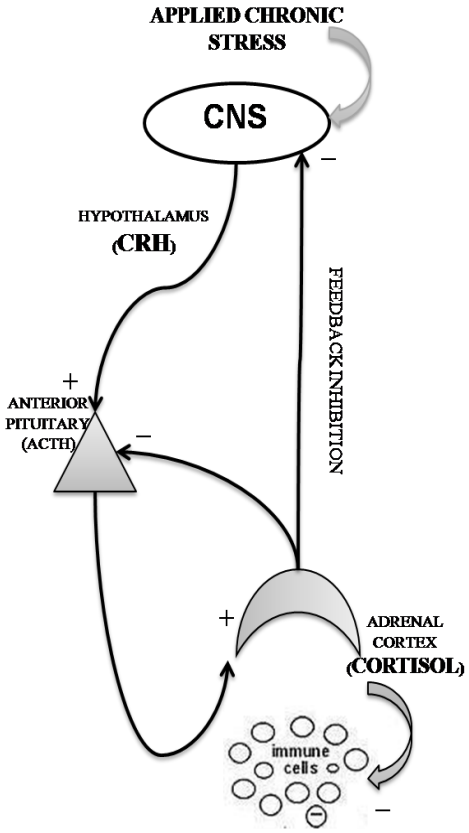


FIGURE 1 A schematic representation of the HPA axis. A major neuropeptide produced by the hypothalamus of the central nervous system that mediates physiological responses.

FIG. 2

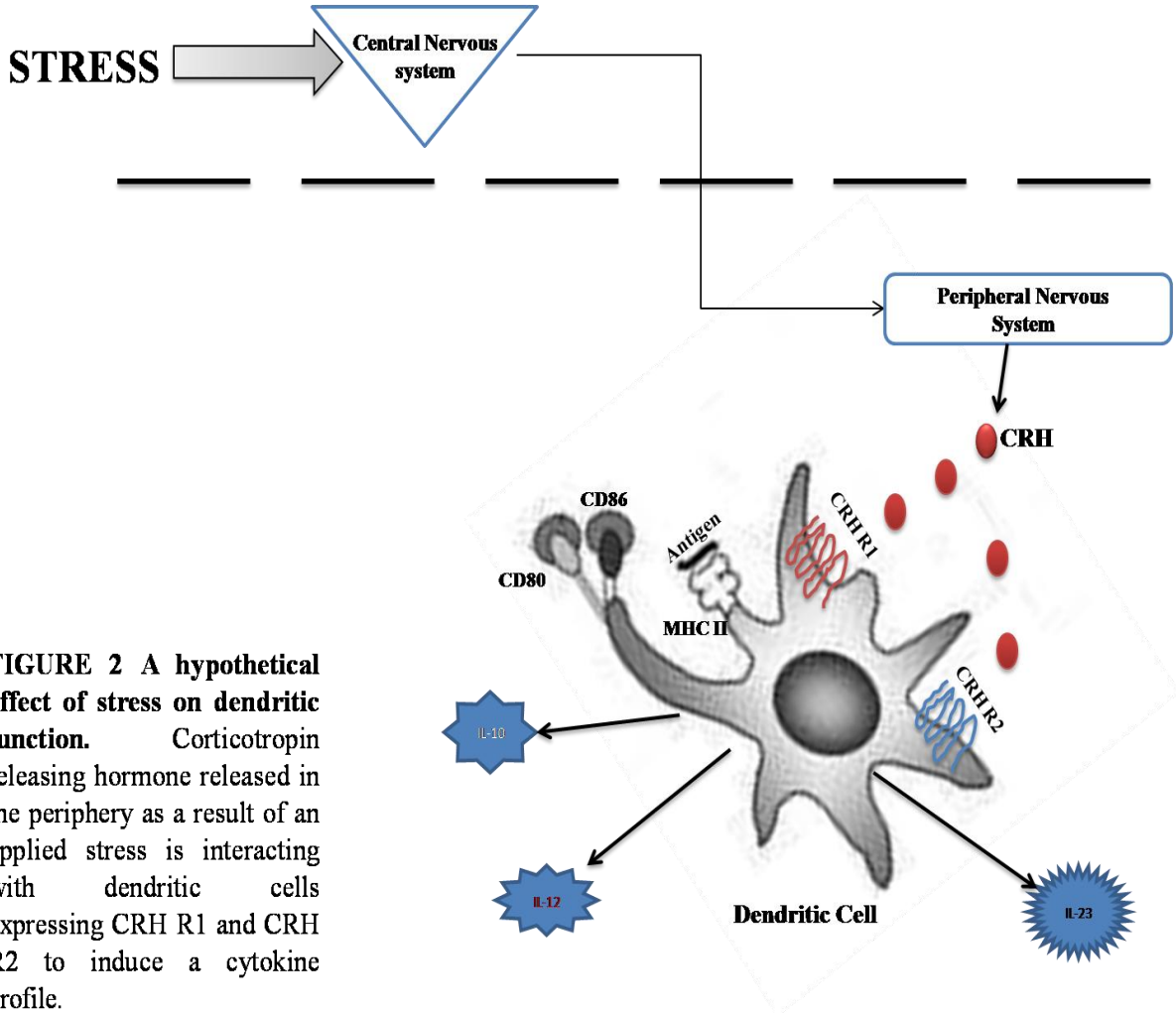


FIGURE 2 A hypothetical effect of stress on dendritic function. Corticotropin releasing hormone released in the periphery as a result of an applied stress is interacting with dendritic cells expressing CRH R1 and CRH R2 to induce a cytokine profile.

FIG. 3

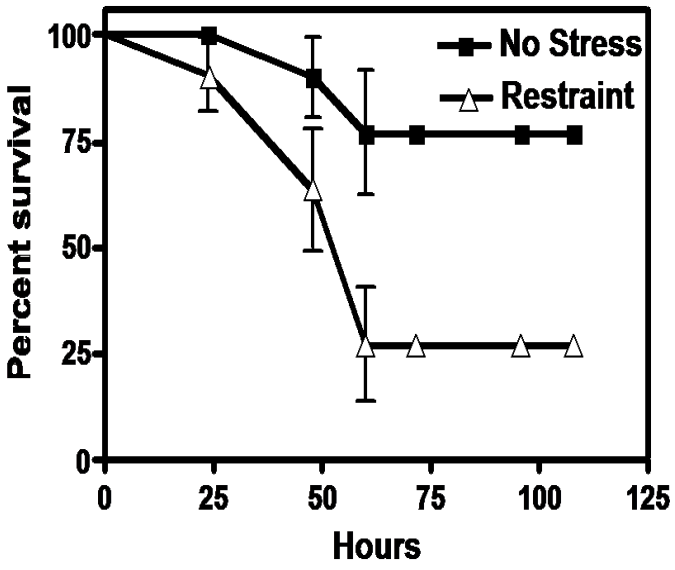


FIGURE 3 Potential role of stress involved in the regulation of host defense against *S. pneumonaie.* Survival among subjects exposed to stress (restraint) and secondary challenge of *streptococcus pneumonaie.* Results are the means ± SE of n = 20 per group.

Reference: RS. Gonzalez et. al. Brain, Behavior and immunity (2008).

FIG. 4

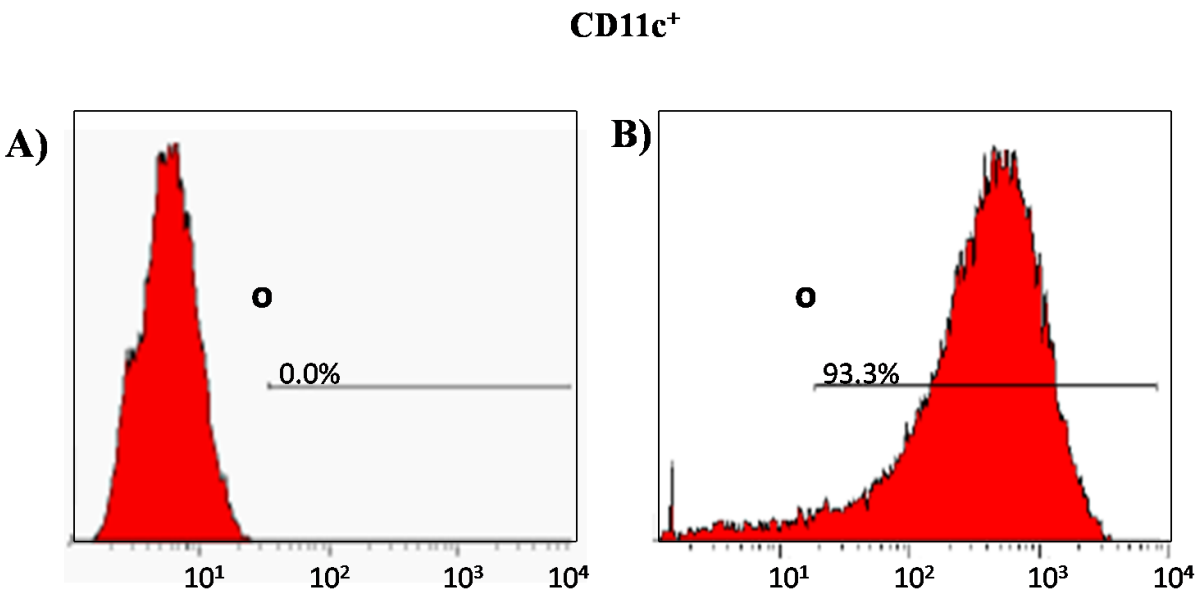


FIGURE 4 Derivation of Bone Marrow Derived Dendritic cell (BMDC). Bone marrow precursor cells cultured for 7days in culture media containing granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin IL-4 (IL-4). Flow cytometry analysis of BMDC showing percentage $CD11c^+$ cells. No stain (A) and $CD11c^+$ (B). Procedure generated 80% average $CD11c^+$ cells.

FIG. 5

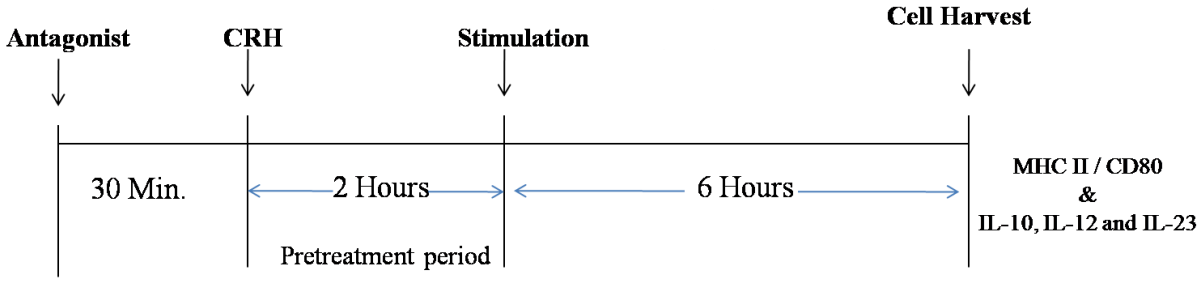


FIGURE 5 Experimental design for maturation profile and cytokine production of BMDC. In all experiment the above conditions were used. BMDCs were pre-exposed to corticotropin releasing hormone (CRH) at a 10^{-8}M concentrations for 2 hours followed by 6 hours stimulation using LPS (1μg/ml) or killed *S. pneumoniae* (300 CFUs per DCs). Additional cells were cultured with or without CRH pretreatment for the entire culture period. The determination of CRH function and preferences in CRH receptor ligation was demonstrated using selective CRH R1 antagonist, Antalarmin, and CRH R2 antagonist, $Astressin_2B$. Each antagonist was added to the culture 30 minutes (10^{-6}M) prior to the introduction of CRH.

FIG. 6

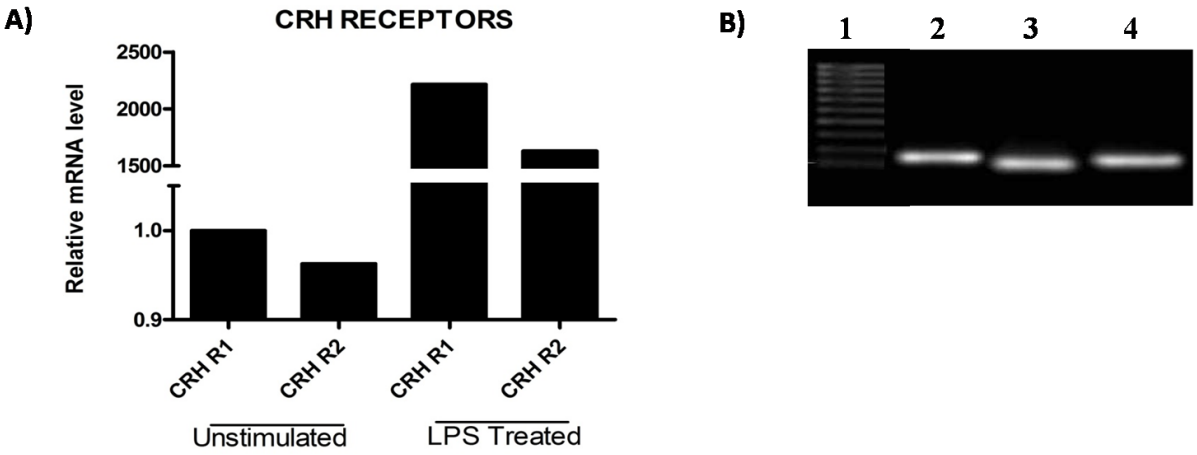


FIGURE 6 Lipopolysaccharide (LPS) stimulation of BMDCs results in a preference in CRH receptor 1. RT-PCR analysis of mRNA of CRH Receptors in BMDC. For activation, BMDCs were stimulated for 24 hours in culture medium with lipopolysaccharide and representated quantitatively (A). RT-PCR product were ran on 1% agarose gel. Lane 1: 100bp ladder, lane 2: Unstimulated (CRH R1), lane 3: Unstimulated (CRH R2), lane 4: GAPDH (B).

FIG.7

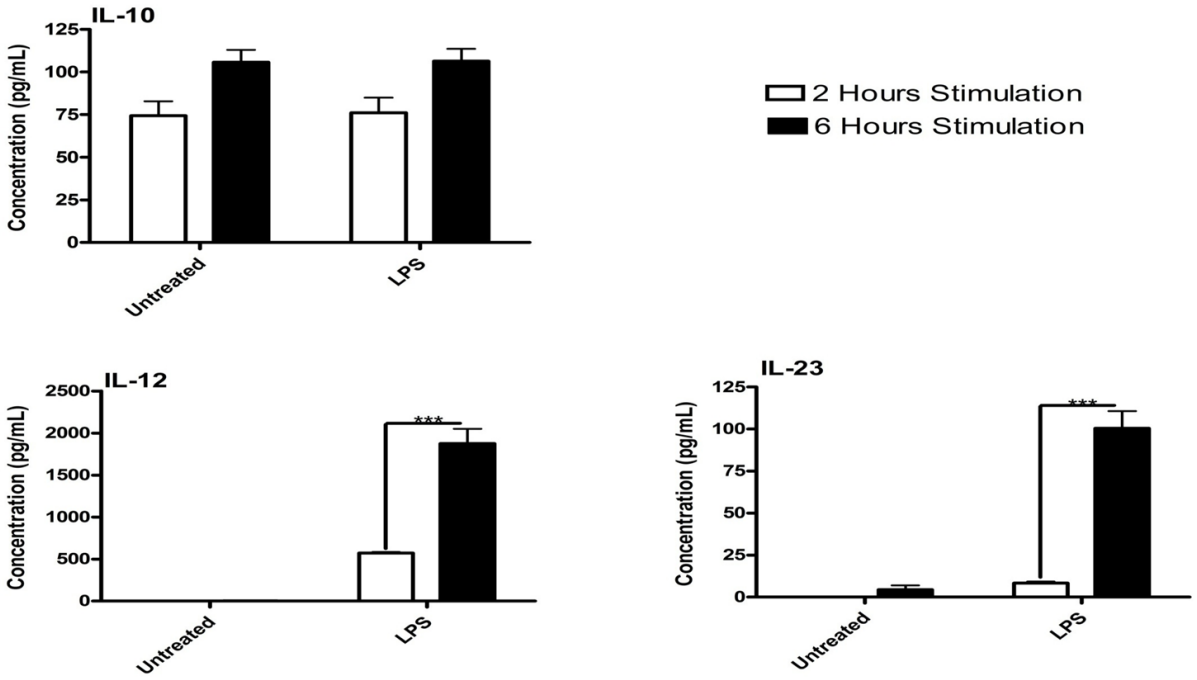


FIGURE 7 Cytokine production by BMDCs in response to LPS. Interleukin10 (IL-10), Interleukin 12p70 (IL-12p70) and Interleukin 23 (IL-23) production was evaluated in culture supernatants after LPS (1μg/ml) stimulation for 2 hours, and 6 hours. Protein secretion measured by standard enzyme-linked immunoabsorbant assay (ELISA). Data represent the mean ± standard error (n=3) per experimental conditions. Each asterisks (*), (**) and (***) indicates significant difference ($p \leq 0.05$), ($p \leq 0.01$) and ($p \leq 0.001$) respectively between individual condition.

FIG. 8

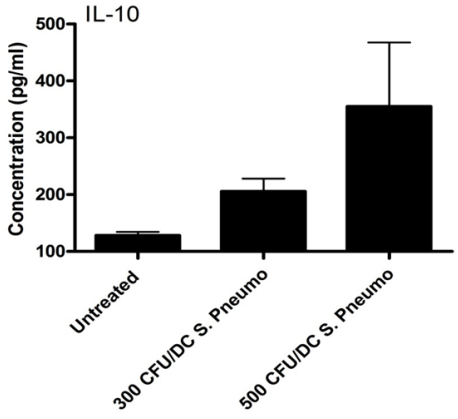
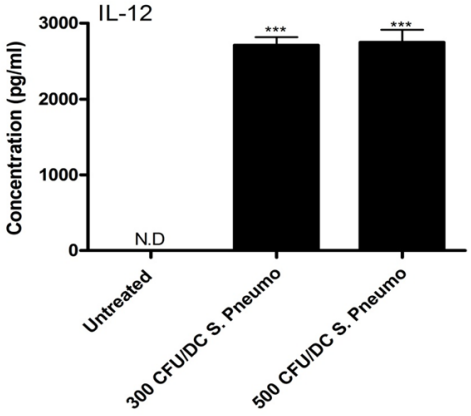


FIGURE 8 Optimization of in vitro S. pneumoniae stimulation. IL-10, IL-12 and IL-23 production was evaluated in culture supernatants after *S. pneumoniae* (300 CFUs and 500 CFU/DC) stimulation for 6 hours. Protein secretion measured by standard enzyme-linked immunoabsorbant assay (ELISA). Data represent the mean ± standard error (n=3) per experimental conditions. Each asterisks (*),(**) and (***) indicates significant difference ($p \leq 0.05$), ($p \leq 0.01$), and ($p \leq 0.001$) respectively between individual condition.



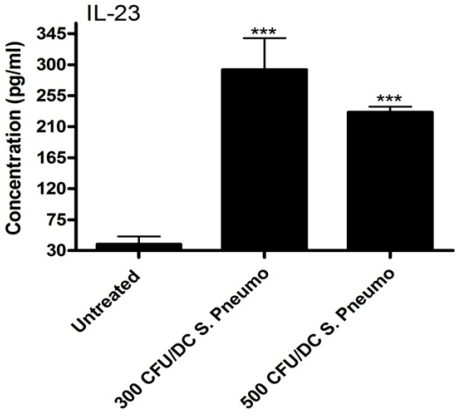


FIG. 9

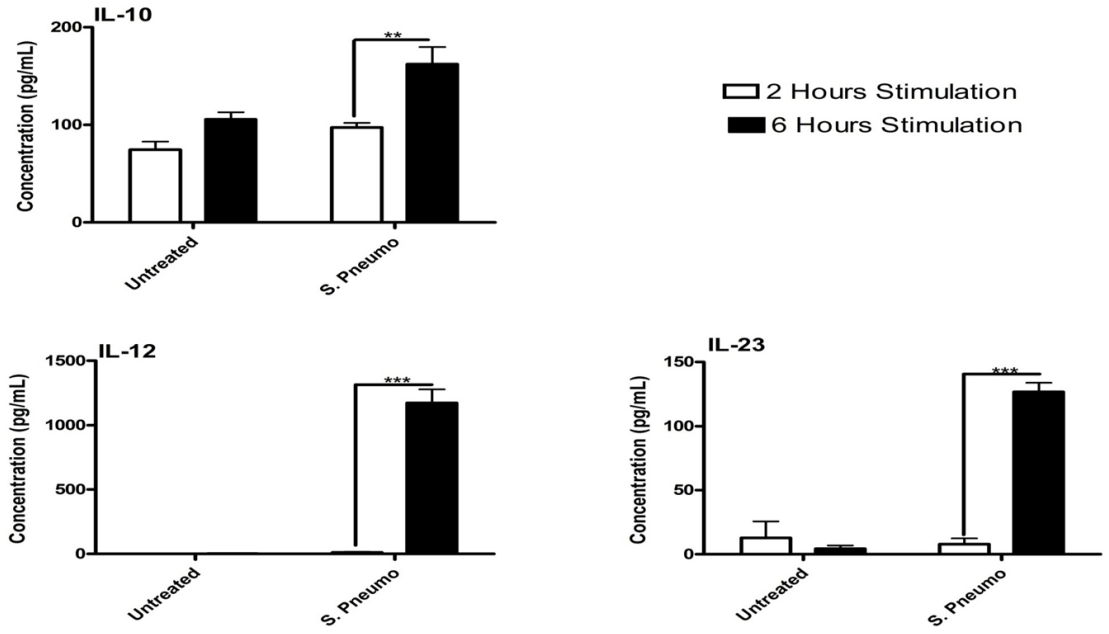


FIGURE 9 Effect of CRH on *S. pneumoniae*-induced BMDC cytokine production. BMDC pre-treated with CRH followed by *S. Pneumoniae* stimulation (300 CFUs/DC) for 2 hours and 6 hours. Data represent the mean ± standard error (n=3) per experimental conditions. Each asterisks (*),(**) and (***) indicates significant difference ($p \leq 0.05$), ($p \leq 0.01$), and ($p \leq 0.001$) respectively between individual condition.

FIG. 10

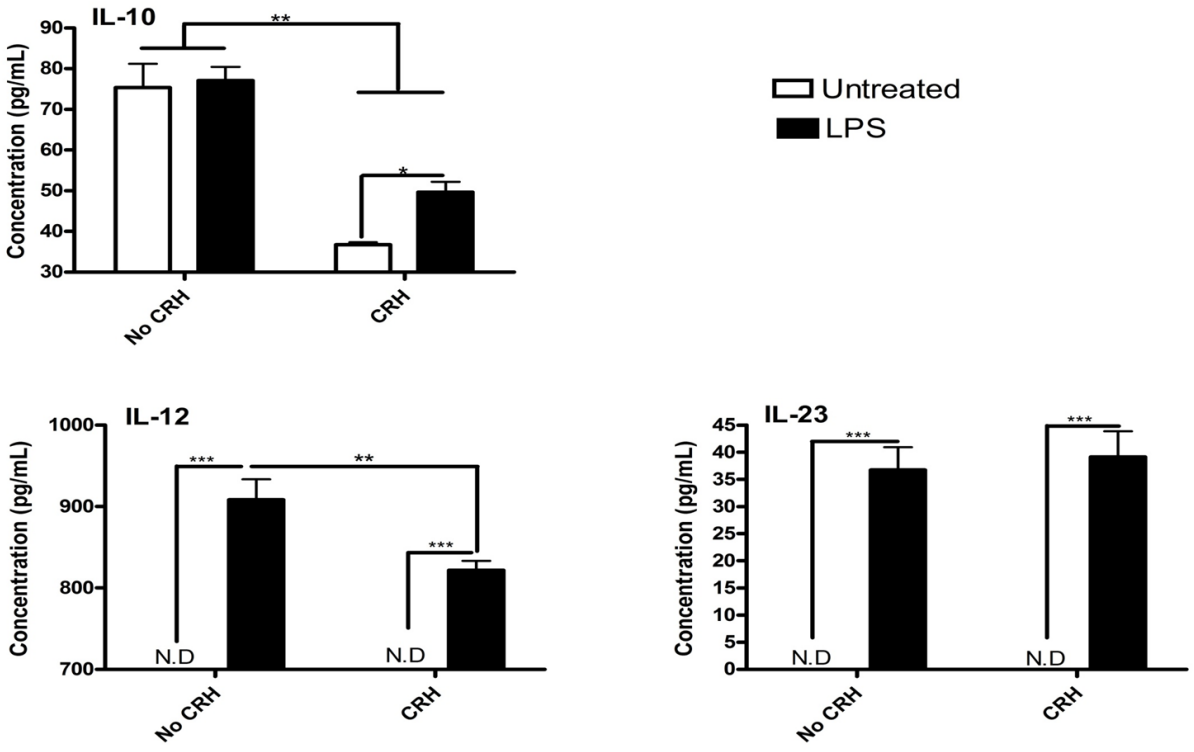


Figure 10 Effect of CRH on LPS-induced BMDC cytokine production. BMDC pre-treated with CRH followed by LPS stimulation. Data represent the mean ± standard error (n=3) per experimental conditions. Each asterisks (*), (**) and (***) indicates significant difference ($p \leq 0.05$), ($p \leq 0.01$) and ($p \leq 0.001$) respectively between individual condition.

FIG. 11

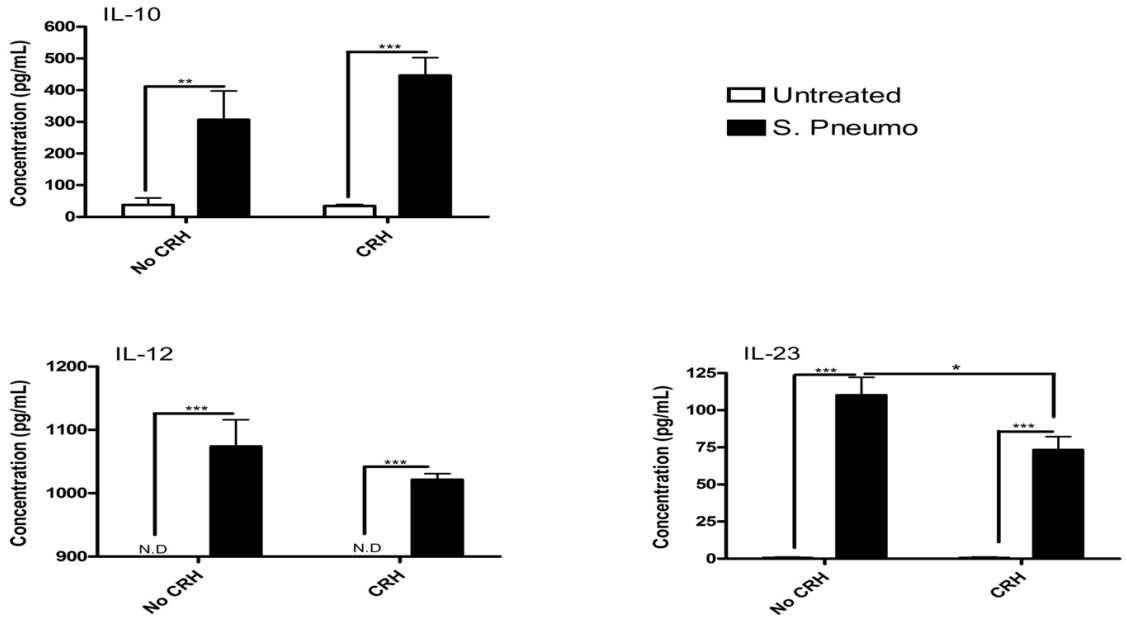


FIGURE 11 Effect of CRH on *S. pneumoniae*-induced BMDC cytokine production. BMDC pre-treated with CRH followed by *S. Pneumoniae* stimulation (300 CFUs/DC) for 6 hours. Data represent mean ± standard error of two experiments with n=3 per experimental conditions. Each asterisks (*),(**) and (***) indicates significant difference ($p \leq 0.05$), ($p \leq 0.01$), and ($p \leq 0.001$) respectively between individual condition.

FIG. 12

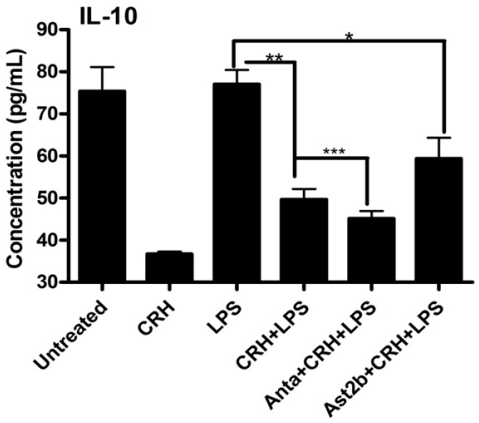
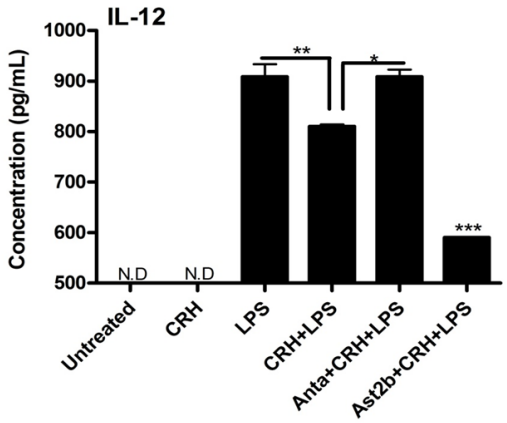


FIGURE 12 Effect of CRH on LPS-induced BMDC cytokine production. BMDC pre-treated with CRH and selective CRH receptor antagonist Antalarmin (CRH R1) and Astressin$_2$B (CRH R2) followed by LPS stimulation. Data represent the mean ± standard error (n=3) per experimental conditions. Each asterisks (*),(**) and (***) indicates significant difference ($p \leq 0.05$), ($p \leq 0.01$), and ($p \leq 0.001$) respectively between individual condition.



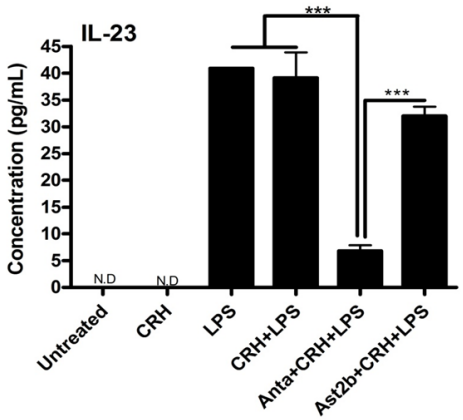


FIG. 13

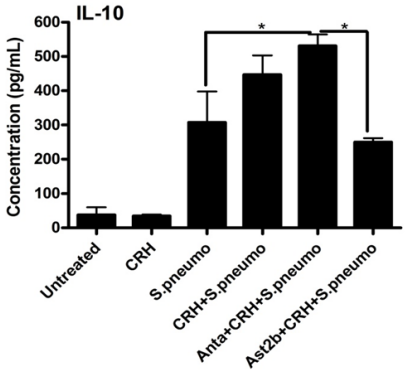
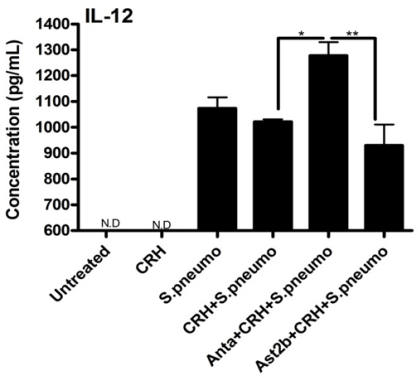


FIGURE 13 Effect of CRH on *S. pneumoniae*-induced BMDC cytokine production. BMDC pre-treated with CRH and selective CRH receptor antagonist Antalarmin (CRH R1) and Astressin$_2$B (CRH R2) followed by S. Pneumoniae stimulation (300 CFUs/DC). Data represent the mean ± standard error (n=3) per experimental conditions. Each asterisks (*),(**) and (***) indicates significant difference ($p \leq 0.05$), ($p \leq 0.01$), and ($p \leq 0.001$) respectively between individual condition.



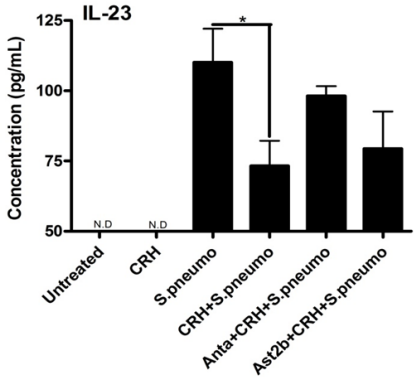


FIG. 14

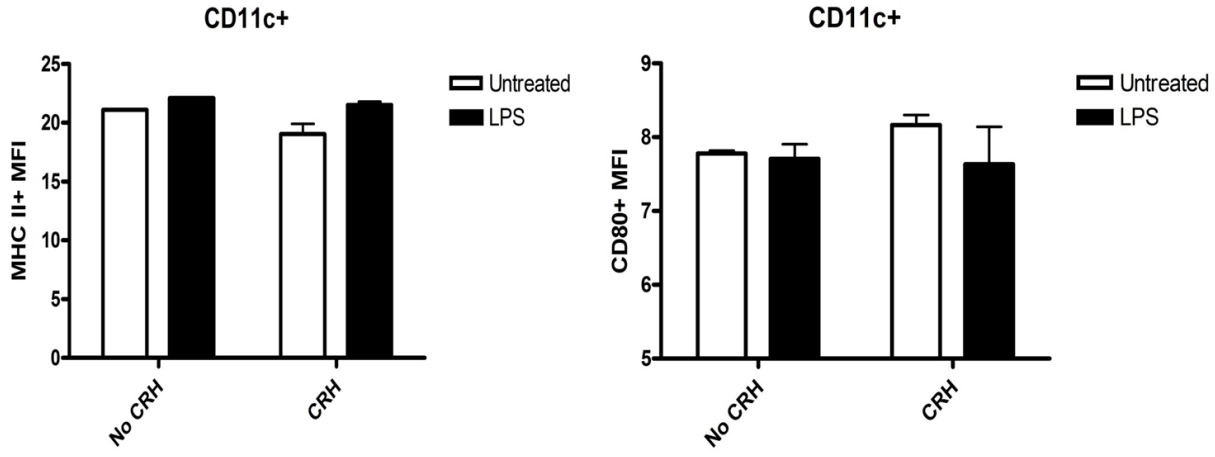


FIGURE 14 Effects of CRH on BMDC maturation-associated MHC II and CD80 surface expression. Flow cytometry analysis of MHC II^+ and $CD80^+$ MFI on $CD11c^+$ BMDC pre-treated with CRH followed with LPS Stimulation. (A) MHC II^+ and (B) $CD80^+$ expression on $CD11c^+$. Data represent the mean ± standard error (n=3) per experimental conditions. Each asterisks (*),(**) and (***) indicates significant difference ($p \leq 0.05$), ($p \leq 0.01$), and ($p \leq 0.001$) respectively between individual condition.

FIG. 15

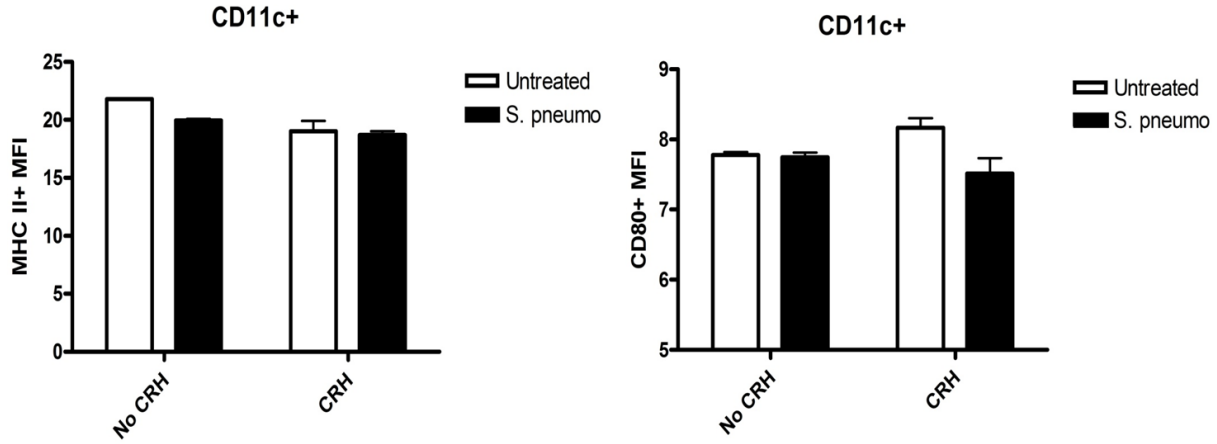


FIGURE 15 Effects of CRH on BMDC maturation-associated MHC II and CD80 surface expression. Flow cytometry analysis of MHC II^+ and $CD80^+$ MFI on $CD11c^+$ BMDC pre-treated with CRH followed with *S. pneumoniae* Stimulation (300 CFUs/DC). (A) MHC II^+ and (B) $CD80^+$ expression on $CD11c^+$. Data represent the mean ± standard error (n=3) per experimental conditions. Each asterisks (*),(**) and (***) indicates significant difference ($p \leq 0.05$), ($p \leq 0.01$), and ($p \leq 0.001$) respectively between individual condition.

CHAPTER VI

References

Adams, S., O'Neill, D. W., & Bhardwaj, N. (2005). Recent advances in dendritic cell biology. *Journal of Clinical Immunology, 25*(3), 177-188.

Agelaki, S., Tsatsanis, C., Gravanis, A., & Margioris, A. N. (2002). Corticotropin-releasing hormone augments proinflammatory cytokine production from macrophages in vitro and in lipopolysaccharide-induced endotoxin shock in mice. *Infection and Immunity, 70*(11), 6068-6074.

Aggarwal, B. B., Shishodia, S., Sandur, S. K., Pandey, M. K., & Sethi, G. (2006). Inflammation and cancer: How hot is the link? *Biochemical Pharmacology, 72*(11), 1605-1621.

Baigent, S. M. (2001). Peripheral corticotropin-releasing hormone and urocortin in the control of the immune response. *Peptides, 22*(5), 809-820.

Baril, L., Dietemann, J., Essevaz-Roulet, M., Beniguel, L., Coan, P., Briles, D. E., et al. (2006). Pneumococcal surface protein A (PspA) is effective at eliciting T cell-mediated responses during invasive pneumococcal disease in adults. *Clinical and Experimental Immunology, 145*(2), 277-286.

Benou, C., Wang, Y., Imitola, J., VanVlerken, L., Chandras, C., Karalis, K. P., et al. (2005). Corticotropin-releasing hormone contributes to the peripheral inflammatory response in experimental autoimmune encephalomyelitis. *Journal of Immunology (Baltimore, Md.: 1950), 174*(9), 5407-5413.

Black, P. H. (1994). Central nervous system-immune system interactions: Psychoneuroendocrinology of stress and its immune consequences. *Antimicrobial Agents and Chemotherapy, 38*(1), 1-6.

Blair, C., Naclerio, R. M., Yu, X., Thompson, K., & Sperling, A. (2005). Role of type 1 T helper cells in the resolution of acute streptococcus pneumoniae sinusitis: A mouse model. *The Journal of Infectious Diseases, 192*(7), 1237-1244.

Boonstra, A., Asselin-Paturel, C., Gilliet, M., Crain, C., Trinchieri, G., Liu, Y. J., et al. (2003). Flexibility of mouse classical and plasmacytoid-derived dendritic cells in directing T helper type 1 and 2 cell development: Dependency on antigen dose and differential toll-like receptor ligation. *The Journal of Experimental Medicine, 197*(1), 101-109.

Castillo, E. F., Stonier, S. W., Frasca, L., & Schluns, K. S. (2009). Dendritic cells support the in vivo development and maintenance of NK cells via IL-15 trans-presentation. *Journal of Immunology (Baltimore, Md.: 1950), 183*(8), 4948-4956.

Chen, C. (2006). Recent advances in small molecule antagonists of the corticotropin-releasing factor type-1 receptor-focus on pharmacology and pharmacokinetics. *Current Medicinal Chemistry, 13*(11), 1261-1282.

Colino, J., Shen, Y., & Snapper, C. M. (2002). Dendritic cells pulsed with intact streptococcus pneumoniae elicit both protein- and polysaccharide-specific immunoglobulin isotype responses in vivo through distinct mechanisms. *The Journal of Experimental Medicine, 195*(1), 1-13.

Dubin, P. J., & Kolls, J. K. (2007). IL-23 mediates inflammatory responses to mucoid pseudomonas aeruginosa lung infection in mice. *American Journal of Physiology.Lung Cellular and Molecular Physiology, 292*(2), L519-28.

Elftman, M. D., Norbury, C. C., Bonneau, R. H., & Truckenmiller, M. E. (2007). Corticosterone impairs dendritic cell maturation and function. *Immunology, 122*(2), 279-290.

Feng, Y., Johansson, J., Shao, R., Manneras, L., Fernandez-Rodriguez, J., Billig, H., et al. (2009). Hypothalamic neuroendocrine functions in rats with dihydrotestosterone-induced polycystic ovary syndrome: Effects of low-frequency electro-acupuncture. *PloS One, 4*(8), e6638.

Ferretti, S., Bonneau, O., Dubois, G. R., Jones, C. E., & Trifilieff, A. (2003). IL-17, produced by lymphocytes and neutrophils, is necessary for lipopolysaccharide-induced airway neutrophilia: IL-15 as a possible trigger. *Journal of Immunology (Baltimore, Md.: 1950), 170*(4), 2106-2112.

Fukudo, S. (2007). Role of corticotropin-releasing hormone in irritable bowel syndrome and intestinal inflammation. *Journal of Gastroenterology, 42 Suppl 17*, 48-51.

Gonzales, X. F., Deshmukh, A., Pulse, M., Johnson, K., & Jones, H. P. (2008). Stress-induced differences in primary and secondary resistance against bacterial sepsis corresponds with diverse corticotropin releasing hormone receptor expression by pulmonary CD11c+ MHC II+ and CD11c- MHC II+ APCs. *Brain, Behavior, and Immunity, 22*(4), 552-564.

Happel, K. I., Dubin, P. J., Zheng, M., Ghilardi, N., Lockhart, C., Quinton, L. J., et al. (2005). Divergent roles of IL-23 and IL-12 in host defense against klebsiella pneumoniae. *The Journal of Experimental Medicine, 202*(6), 761-769.

Iwamoto, S., Ishida, M., Takahashi, K., Takeda, K., & Miyazaki, A. (2005). Lipopolysaccharide stimulation converts vigorously washed dendritic cells (DCs) to nonexhausted DCs expressing CD70 and evoking long-lasting type 1 T cell responses. *Journal of Leukocyte Biology, 78*(2), 383-392.

Jones, H. P., Tabor, L., Sun, X., Woolard, M. D., & Simecka, J. W. (2002). Depletion of CD8+ T cells exacerbates CD4+ th cell-associated inflammatory lesions during murine mycoplasma respiratory disease. *Journal of Immunology (Baltimore, Md.: 1950), 168*(7), 3493-3501.

Kadioglu, A., Coward, W., Colston, M. J., Hewitt, C. R., & Andrew, P. W. (2004). CD4-T-lymphocyte interactions with pneumolysin and pneumococci suggest a crucial protective role in the host response to pneumococcal infection. *Infection and Immunity, 72*(5), 2689-2697.

Kassianos, A. J., Jongbloed, S. L., Hart, D. N., & Radford, K. J. (2010). Isolation of human blood DC subtypes. *Methods in Molecular Biology (Clifton, N.J.), 595*, 45-54.

Kiecolt-Glaser, J. K., Loving, T. J., Stowell, J. R., Malarkey, W. B., Lemeshow, S., Dickinson, S. L., et al. (2005). Hostile marital interactions, proinflammatory cytokine production, and wound healing. *Archives of General Psychiatry, 62*(12), 1377-1384.

Langenkamp, A., Messi, M., Lanzavecchia, A., & Sallusto, F. (2000). Kinetics of dendritic cell activation: Impact on priming of TH1, TH2 and nonpolarized T cells. *Nature Immunology, 1*(4), 311-316.

Lee, H. J., Kwon, Y. S., Park, C. O., Oh, S. H., Lee, J. H., Wu, W. H., et al. (2009). Corticotropin-releasing factor decreases IL-18 in the monocyte-derived dendritic cell. *Experimental Dermatology, 18*(3), 199-204.

Lemos, H. P., Grespan, R., Vieira, S. M., Cunha, T. M., Verri, W. A.,Jr, Fernandes, K. S., et al. (2009). Prostaglandin mediates IL-23/IL-17-induced neutrophil migration in inflammation by inhibiting IL-12 and IFNgamma production. *Proceedings of the National Academy of Sciences of the United States of America, 106*(14), 5954-5959.

Lutz, M. B., Kukutsch, N., Ogilvie, A. L., Rossner, S., Koch, F., Romani, N., et al. (1999). An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *Journal of Immunological Methods, 223*(1), 77-92.

Ma, J., Wang, J., Wan, J., Charboneau, R., Chang, Y., Barke, R. A., et al. (2010). Morphine disrupts interleukin-23 (IL-23)/IL-17-mediated pulmonary mucosal host defense against streptococcus pneumoniae infection. *Infection and Immunity, 78*(2), 830-837.

Morelli, A. E., Zahorchak, A. F., Larregina, A. T., Colvin, B. L., Logar, A. J., Takayama, T., et al. (2001). Cytokine production by mouse myeloid dendritic cells in relation to differentiation and terminal maturation induced by lipopolysaccharide or CD40 ligation. *Blood, 98*(5), 1512-1523.

Paterson, G. K., & Mitchell, T. J. (2006). Innate immunity and the pneumococcus. *Microbiology (Reading, England), 152*(Pt 2), 285-293.

Rabquer, B., Shriner, A. K., Smithson, S. L., & Westerink, M. A. (2007). B cell mediated priming following pneumococcal colonization. *Vaccine, 25*(11), 2036-2042.

Segerstrom, S. C., & Miller, G. E. (2004). Psychological stress and the human immune system: A meta-analytic study of 30 years of inquiry. *Psychological Bulletin, 130*(4), 601-630.

Shi, Y., Devadas, S., Greeneltch, K. M., Yin, D., Allan Mufson, R., & Zhou, J. N. (2003). Stressed to death: Implication of lymphocyte apoptosis for psychoneuroimmunology. *Brain, Behavior, and Immunity, 17 Suppl 1*, S18-26.

Shortman, K., & Liu, Y. J. (2002). Mouse and human dendritic cell subtypes. *Nature Reviews.Immunology, 2*(3), 151-161.

Speert, D. P. (2006). Bacterial infections of the lung in normal and immunodeficient patients. *Novartis Foundation Symposium, 279*, 42-51; disussion 51-5, 216-9.

Trzcinski, K., Thompson, C. M., Srivastava, A., Basset, A., Malley, R., & Lipsitch, M. (2008). Protection against nasopharyngeal colonization by streptococcus pneumoniae is mediated by antigen-specific CD4+ T cells. *Infection and Immunity, 76*(6), 2678-2684.

Tsatsanis, C., Dermitzaki, E., Venihaki, M., Chatzaki, E., Minas, V., Gravanis, A., et al. (2007). The corticotropin-releasing factor (CRF) family of peptides as local modulators of adrenal function. *Cellular and Molecular Life Sciences : CMLS, 64*(13), 1638-1655.

Walport, M. J. (2001a). Complement. first of two parts. *The New England Journal of Medicine, 344*(14), 1058-1066.

Walport, M. J. (2001b). Complement. second of two parts. *The New England Journal of Medicine, 344*(15), 1140-1144.

Webster, E. L., Torpy, D. J., Elenkov, I. J., & Chrousos, G. P. (1998). Corticotropin-releasing hormone and inflammation. *Annals of the New York Academy of Sciences, 840*, 21-32.

Wu, Q., Martin, R. J., Rino, J. G., Breed, R., Torres, R. M., & Chu, H. W. (2007). IL-23-dependent IL-17 production is essential in neutrophil recruitment and activity in mouse lung defense against respiratory mycoplasma pneumoniae infection. *Microbes and Infection / Institut Pasteur, 9*(1), 78-86.

Zhang, Q., Bagrade, L., Bernatoniene, J., Clarke, E., Paton, J. C., Mitchell, T. J., et al. (2007). Low CD4 T cell immunity to pneumolysin is associated with nasopharyngeal carriage of pneumococci in children. *The Journal of Infectious Diseases, 195*(8), 1194-1202.