ABSTRACT

Neuroendocrine stimulation can impact disease states by regulating immune function. The purpose of our studies was to define the functional role of stress-induced neuroendocrine factors, catecholamines and corticotropin-releasing hormone (CRH) on immune responses involved in the pathogenesis of *Streptococcus pneumoniae* (*S. pneumoniae*) infection implementing both *in vitro* and *in vivo* methodology.

Dendritic cells play a pivotal role in antigen presentation and cytokine production, influencing both innate and adaptive immunity. Initial studies examined the potential immunomodulatory effect of epinephrine and CRH on DC cytokine production in response to the bacterial pathogenic ligand, lipopolysaccharide (LPS). In addition, the ability of DC to dictate $CD4^+$ T cell activation as a consequence of CRH or epinephrine pre-treatment was examined using an in vitro co-culture system. Epinephrine and CRH pre-treatment resulted in a preferential increase in IL-23 and IL-10 cytokine production. In contrast, IL-12p70 was significantly attenuated in response to epinephrine and CRH pre-treatment. Preferences in IL-23 and IL-10 cytokine production by DC pre-treated with epinephrine and CRH corresponded with an increase in IL-4 and IL-17A, but not IFN-γ cytokine production by $CD4^+$ T cells. These results suggest that exposure to stress-derived epinephrine/CRH dictates dendritic cells to generate a dominant Th2/Th17 phenotype in the context of subsequent exposure to a pathogen.

Our second study examined the functional properties of IL-23 during pulmonary *S. pneumoniae* infection. IL-23 plays a crucial role in establishing host defenses against extracellular pathogens. Further investigation is still required to define the impact of IL-23 on acute pulmonary *S. pneumoniae* infection. Utilizing IL-23p19 genetic deficient mice, we determined bacterial load, cytokine production and the contribution of neutrophils against *S. pneumoniae* infection using monoclonal antibody-mediated systemic neutrophil depletion. The absence of IL-23 induced a higher bacterial load in lung and blood as compared to IL-23 competent counterparts. In the absence of IL-23, production of proinflammatory cytokines such as IL-6, IL-12p70 as well as IL-17A and IFN-γ were dampened as compared to wild type mice. In addition, neutrophil distribution was also altered in IL-23-deficient mice, suggesting impaired neutrophil recruitment into lung. Interestingly, neutrophil depletion did not impact bacterial load in lung and blood in both IL-23 competent and deficient mice. These findings, suggest a novel role of IL-23 in pulmonary *S. pneumoniae* infection, potentially independent of neutrophil function.

We next examined the possible impact of CRH and catecholamines as regulators of immune function against acute bacterial infection in response to stress. Utilizing a murine model of acute pulmonary *S. pneumoniae* infection and restraint stress, we selectively blocked CRH receptors (CRHR1 and CRHR2) as well as the β2 adrenergic receptor prior to restraint stress followed by intranasal pulmonary *S. pneumoniae* infection. Antagonist administration did not impact restraint stress-induced physiological responses as compared to restraint stressed mice, which did not receive receptor antagonists. However, following *S. pneumoniae* infection, physiological changes including weight and temperature were altered in response to administration of selective CRH receptor and β2 adrenergic receptor antagonists. Survival rate,

bacterial load and cytokine production corresponded with physiological differences observed in response to selective CRH receptor and β2 adrenergic receptor antagonists. Importantly, preferential differences in bacterial colonization and survival corresponded with distinct differences in inflammatory cytokine production and immune cell distribution along pulmonary airways. In particular, opposing effects in IL-17A and neutrophil accumulation was found among mice administered the CRHR1 versus the CRHR2 antagonists. Together, these findings indicate that activation of each receptor can influence immune responses against *S. pneumoniae* infection. Thus, our findings provide further understanding of how stress-derived neuroendocrine factors directly impact immune responses related to immunopathology and immunoprotection.

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DIVERSE IMMUNE RESPONSES MEDIATED BY BETA-ADRENERGIC AND CORTICOTROPIN-RELEASING HORMONE RECEPTORS IN A MODEL OF PNEUMOCOCCAL SEPSIS

DISSERTATION

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CHAPTER I

PART I

INTRODUCTION TO THE STUDY

DEFINITION OF STRESS

Stress exposures, both physical and perceived, elicit a continuum of responses that influence not only the individual itself but also the surrounding environment (1). Based on these bi- or multi-directional communications, stress can be a factor of a neutral, negative, or positive experience dictated by differences among individuals (1). In many cases however, stress frequently refers to an adverse experience resulting in an inappropriate physiological response against non-specific or perceived experiences (stressors) resulting in emotional and physical failure (2). Thus, stress has been accepted as an important barometer of human health (3).

STRESS AND HEALTH PROBLEMS

It is known that stress exposure is associated with a disruption of physiological homeostasis that can trigger and/or perpetuate diseases such as allergic asthma, autoimmue diseases, and infectious diseases.

Asthma for example, is a chronic lung disease with symptoms of cough, wheezing, and dyspnea accompanied with reversible obstruction of airflow caused by inflammation and hyper-responsiveness of airways (4). Nowadays, onset and severity of asthma is predicted by psychosocial influences including maternal distress, psychological illness and stress (5-9). Marin et al, also demonstrate that acute negative life events have a particularly strong impact among subgroups of children with asthma who are under high chronic family stress (10). In an experimental animal model of allergic bronchial asthma, stress was associated with increased airway reactivity, increased allergy-induced airway inflammation, and higher eosinophils levels (11). In support, Deshmukh et al., also showed that controllability against external stress can attenuate Th2-domiant allergic airway inflammatory responses and reduce pulmonary pathogenesis (12).

Autoimmune diseases such as rheumatoid arthritis (RA), multiple sclerosis (MS) and Crohn's disease are caused by an unwarranted recognition of self-antigen normally presenting in the body by the immune system (13-15). Several findings support that perceived stress provides a higher risk for exacerbation of these autoimmune pathologies. Walker et al., suggested that the type of stressor and individual differences in stress appraisal and reactivity may prove to be important prognostic factors in disease onset and /or progression for RA patients (16). Liu et al., also suggested that psychosocial factors are closely linked to the pathogenesis, pathophysiology and clinical symptoms of MS (17). In support, Brown et al demonstrated that multiple stressors rather than severity of stressors are most important in relation to MS relapse risk (311, 312). Maunder and Levenstein also provided evidence that psychological stress contributes in the inflammatory process in ulcerative colitis and Crohn's disease (18).

Infectious diseases are caused by invading of microbial pathogens such as viruses, bacteria, fungi and multicellular parasites to host individuals (19, 20). Cohen et al suggested that stress is associated with increases in illness behaviors and may be similarly associated with increased onset and reactivation of verified infectious disease (21). In addition to clinical observations, there have been several experimental approaches such as restraint stress, cold/heat shock and electric foot shock to investigate influences of stress on the pathophysiological control of infectious diseases. In particular, several animal viral infection models displaying human symptoms have been used to evaluate influences of stress. For example, Cohen et al. demonstrated that a higher intensity of stress induced greater susceptibility to rhinovirus infection reflected by lower neutralizing antibody titers and greater cold symptoms (22). In additional studies, both acute and chronic stress induced impaired antibody titers following vaccination with Hepatitis B or influenza in humans (23-25). In additional animal stress models, followed by influenza infection, restraint stress altered the immune response to viral infection. This involved alteration in the kinetics of antibody titer and suppression of both proinflammatory and anti inflammatory cytokine production. Furthermore, trafficking of mononuclear cells to virus-infected lung was significantly reduced in stressed animals. Interestingly, virus-specific cytokine responses of T cells in restraint-stressed mice were restored in the lymph nodes by blockade of the glucocorticoid receptor with antagonist RU486 (26, 27). Recently, Gonzales et al found that restraint stress also induced impaired bacterial clearance of *S. pneumoniae* in lung (28). Consistent with the above reports of viral disease, this result also demonstrated alteration in the initiation of immune responses against bacterial infection mediated by stress. In their report, this influence was accompanied with differential CRH receptor (CRHR) 1 and CRHR2 mRNA transcription level in two APC cell types, $CD11c^{+}$ $MHCII^{+}$ and $CD11c^{-}$ $MHCII^{+}$ cells, suggesting

a cell-type specific response on stress-induced CRH (28). Yet, survival rate of the restraint-stressed group was significantly impaired (~50 %) in restraint-stressed animals after secondary challenge of pulmonary *S. pneumoniae*. These findings strongly implicate that restraint stress significantly alters the development of adaptive immunity and results in a failure to generate a proper defense system against secondary invading *S. pneumoniae*. Based on these findings, there is a need to establish a direct interaction between neuroendocrine stress factors and cellular immune function.

STRESS AND NERVOUS SYSTEM; STRESS-DERIVED NEUROENDOCRINE FACTORS

Physiological mal-adaptations to stress is mediated by two major nervous pathways, the hypothalamus-pituitary-adrenal (HPA) axis in central nervous system (CNS) and sympathetic nervous system (SNS) (29, 30). Stressful situations result in the stimulation of these two pathways and, as a consequence, various neuroendocrine factors are produced and directly or indirectly influence compartments of the body's physiological system such as the immune system (3, 31, 32).

1. Corticotrophin-releasing hormone (CRH): a novel stress-induced immunomodulator in the periphery.

Corticotrophin-releasing hormone (CRH), also known as corticotrophin-releasing factor (CRF), is a neuroendocrine factor composed of 41-amino acids (33). CRH was originally known

as a stress-induced factor produced in paraventricular nucleus (PVN) of the hypothalamus that triggers production of adrenocorticotropic hormone (ACTH) in pituitary, and consequently, induces production of glucocorticoids (34, 35). Thus, CRH has been considered as a major regulator of the hypothalamic-pituitary-adrenal (HPA) axis. Two receptor isotypes (CRHR1 and CRHR2) have been reported to mediate its biological effects, which belong to G protein-coupled receptor (GPCR) superfamily (36, 37). In addition to its production and location in the central nervous system (CNS), CRH and its receptors have been identified in several other sites such as placenta, fetal membranes and spleen (38-41). In particular, several immune cell types such as mast cells, peripheral blood monocytes, macrophage, and lymphocytes including Th2 cells have been identified as a potential target of CRH (41-45). Interestingly, CRH-mediated enhancement of immunological responses corresponding with specific receptor expression has been reported. Agelaki et al showed that CRH stimulation induces proinflammatory cytokine production in the macrophage cell line RAW264.7 (46). In this study, they also showed that LPS-induced systemic shock was suppressed by administration of CRHR1 antagonist. Wang et al also reported CRH-mediated modulation of LPS-induced inflammatory cytokines in human trophoblast (47). In addition, killing activity of natural killer (NK) cell was enhanced by direct CRH treatment (48). These findings implicate that free CRH released from the location of its production has direct effects on immune responses separate from HPA axis. In support, several ectopic production of CRH from extra hypothalamic tissues such as placenta and T cells has been reported (39, 40). Gonzales et al reported that transcriptional activity of CRH in mouse lung was significantly enhanced after restraint stress paradigm (28). In addition, Kalantaridou et al also showed that embryonic trophoblast and maternal decidual cells produce CRH (38-40). This peripheral CRH plays crucial roles in implantation, as well as in the anti-rejection process that protects the fetus

from the maternal immune system, primarily through apoptotic effects on activated T cells. These findings suggest diverse roles of peripheral CRH different from CNS-derived stress responses. In short, contribution of central and peripheral CRH may have an influence on several biological activities including immune responses and therefore, they provide a potential mechanism for homeostatic maintenance against stress-induced disorders.

2. Catecholaminergic neurotransmitters, epinephrine and norepinephrine: common stress-induced immunomodulators.

The peripheral sympathetic nervous system (SNS) is also a major neuronal axis regulating stress that innervates numerous sites of body and is commonly associated with the "Flight or Fight" response (49, 50). Two neurotransmitters, epinephrine and norepinephrine are synthesized from the amino acid tyrosine and released from synaptic end of SNS in response to stress. Innervation of SNS is found in several tissues, including blood vessels, liver, kidney, intestines, lung, heart and brain (51). Major immune organs such as lymph node, bone marrow and spleen also have SNS innervations (52, 53). In addtion, Flierl et al reported autocrine production of epinephrine and norepinephrine from macrophages. This finding suggests a novel role of catecholaminergic neurotransmitters as an autocrine regulator immune cell functions (54). The expression of adrenergic receptor subtypes is also detected on several immune cells including NK cells, Th1 cells, macrophages and dendritic cells (55-59). These adrenergic receptors mediate the functional effects of these neurotransmitters. Catecholamine neurotransmitter-associated modulation in Th1 and Th2 adaptive immunity has been suggested as possible mechanisms influencing the exacerbation or restriction of pathologic responses mediated by the immune system (56).

Adrenergic receptors are included in the GPCR (G protein-coupled receptor) family, and therefore, their biological effect is mediated by coupling with different G-protein-associated signaling (60). Adrenergic receptors are subdivided into 9 isotypes; 3 $\alpha 1$ types ($\alpha 1_A$, $\alpha 1_B$ and $\alpha 1_C$), 3 $\alpha 2$ types ($\alpha 2_A$, $\alpha 2_B$ and $\alpha 2_C$) and 3 β types ($\beta 1$, $\beta 2$ and $\beta 3$) (55). Notably, it is widely known that the expression of $\beta 2$ adrenergic receptors on immune cells plays an important role in regulating their biological activity (55, 56, 61, 62). In particular, $\beta 2$ adrenergic receptor expression and binding to epinephrine and norepinephrine results in decreased IFN-γ production in Th1 type helper T cells whereas no effects are discerned in Th2 type cell, because of the absence of receptor expression (63, 64). In addition, Maestroni et al showed that $\beta 2$ adrenergic receptor antagonist regulates production of Th1 cytokines IL-2 and IFN-γ but not IL-4 in lymph nodes (65). In support, Panina-Bordignon et al showed $\beta 2$ agonists prevent Th1 development by selective inhibition of IL-12, while promoting Th2 cell phenotype (56). Norepinephrine is rapidly oxidized and turned into epinephrine (66), more biologically stable form. Furthermore, pharmacologically, epinephrine possesses higher affinity than norepinephrine for the $\beta 2$ adrenergic receptor (67). Therefore, as a potent ligand for $\beta 2$ adrenergic receptor, epinephrine may be a key regulator for the function of $\beta 2$ adrenergic receptor expressing immune cell types.

STRESS AND IMMUNE SYSTEM

Numerous studies have investigated direct effects of stress-derived neuroendocrine factors on various immune cells (68). Several immune cells including macrophages/monocytes, dendritic cells, NK cells, neutrophils, T cells and B cells express receptors for several neuroendocrine factors such as glucocorticoid, substance P, corticotropin releasing hormone

(CRH), and catecholamines (norepinephrine and epinephrine) (69-75). Therefore, cellular components of the immune system are able to sense and respond to neuroendocrine-mediated stress responses. Conversely, it has been shown that immunological components such as proinflammatory cytokines, IL-1β and IL-6 play an important role in psychological and neuronal disorders including depression, autism and schizophrenia (76-80). Thus, the nervous and immune systems are mediated by a complex network of bi-directional signals.

The peripheral immune system is composed of two major compartments, innate and adaptive immunity. Innate immunity is a first defense line against external invading pathologic agents such as bacteria, parasites and viruses (81, 82). The innate immune system recognizes non-specific, but stable pattern molecules of these invaders through clusters of receptors named pattern recognition receptors such as toll-like receptors (TLR), macrophage mannose receptors and scavenger receptors (82-86). Several cell types such as macrophages, neutrophils and NK cells play a pivotal role in innate immunity (87-92). While the innate immune system is activated, another immune cell population, antigen presenting cells (APC), process and present antigen on major histocompatibility complex (MHC) molecules. These processes initiate adaptive immunity characterized as memory and specificity. Adaptive immunity is composed of cellular and humoral immunity regulated by T and B cells (93, 94). T cells have two major subpopulations, $CD8^+$ cytotoxic T cells and $CD4^+$ helper T cells. $CD4^+$ T cells are also subdivided into at least 4 different types including Th1, Th2, Th17 and regulatory T cells after activation and differentiation. Activation of T cells requires several signals including antigen presentation on MHC molecule (MHC I for $CD8^+$ T cells and MHC II for $CD4^+$ T cells), costimulatory molecule and cytokines. B cell activation is generated by Th2 type T cells and triggers production of antibody or activation of other immune cells such as eosinophils (93, 94).

PART II

INTRODUCTION TO NEUROENDOCRINE FACTORS

AND DENDRITIC CELLS

DENDRITIC CELLS

Dendritic cells (DCs) were first identified from lymphoid organs based on morphological differences among other cells (94). DCs are known to consist of heterogeneous groups according to their phenotypes and functions; however, high surface expression of CD11c and MHCII are typical cellular markers of DCs (94, 95). They are also located in heterogeneous tissues such as bone marrow, thymus, lymph node, skin, intestine, kidney and liver (94).

DCs are the most efficient antigen presenting cell (APC) population, which facilitate the generation of adaptive immunity through processing of extracellular and intracellular antigens on MHC, resulting in the recognition, activation and differentiation of naïve T cells (94). These functional benefits are mediated by high-level expression of co-stimulatory molecules such as CD80, CD86 and CD40 (94, 95). Furthermore, dendritic cells provide additional fate-deciding signals to naïve T cells by production of cytokines and chemokines, binding to their receptors on target cells (95-97). DCs also produce several pro- and anti-inflammatory cytokines important in regulation of innate immunity (82, 84, 96). In addition, recent investigations have shown that DCs plays important roles in the induction of T cell tolerance, generation of regulatory T (Treg) cells and activation of NK cells (98-100). Thus, activation and maturation of dendritic cells is an important requirement to establish both innate and adaptive immunity.

1. Dendritic cells and helper T cells (CD4+ T cells) differentiation

As mentioned earlier, DCs provide cytokine signals in addition to antigen presentation and co-stimulatory signals to promote the differentiation of $CD4^+$ T cells toward Th (T helper) 1, Th2, Th17 and Treg subsets (101-103). The Th1 phenotype is mediated by IL-12p70 from DCs. They produce IFN-γ to activate and promote cellular immunity against intracellular pathogens and tumor cells (104). The Th2 phenotype is promoted by DC in the absence of IL-12p70 and presence of IL-10 (104-106). Th2 type T cells produce IL-4, which is required for B cell activation and efficient elimination of extracellular pathogens (106). Differentiation of Th17 phenotype is primarily promoted by DC-derived IL-6 and TGF-β, and this phenotype is further maintained by DC-derived IL-23 (103, 107, 108). Th17 cells are known to promote protection against extracellular bacteria (103). DC-derived TGF-β can also facilitate differentiation of Treg cells, which produce IL-10 and TGF-β, suppressing several functions of effector T cells (109). Thus, facilitating appropriate immune response is dependent on proper differentiation of $CD4^+$ T cells controlled by DC activations. By the contrast, failure of functional $CD4^+$ T cell generation may results in pathogenesis such as autoimmune disease (4, 110-113). For instance, hyper activation of Th2-mediated immune responses promotes allergic airway hypersensitivity, including asthma (4, 110, 114). Furthermore, several investigations have provided evidence that Th17 is also extensively associated with autoimmune diseases (111-113, 115). Thus, molecular environment influencing DC activation is key factor to decide the direction of $CD4^+$ differentiation, which will be important for immune response to protective or pathogenic.

2. Dendritic cells and functional regulation by neuroendocrine factors: CRH and epineprhine

Interestingly, DCs express receptors for several neurotransmitters. Pacheco et al., showed expression of glutamate receptor on DC (116, 117). Dopamine D1 receptor is expressed on DC (118). In addition, serotonin receptor (5-HTR) and α7-nicotinic acetylcholine receptor (AChR) are also expressed on DCs (119, 120). These finding suggest that neurotransmitters may directly influence DCs.

As previously mentioned, CRH directly influences inflammatory responses, cytokine production and cytotoxic activity in several immune cells such as macrophage cell line and NK cells (46, 48). As previously shown, Gonzales et al first showed that mRNA transcription level of CRHR1 and CRHR2 in pulmonary $CD11c^{+}$ $MHCII^{+}$ cells, a predominantly dendritc cell type, are significantly influenced by restraint stress (RS) (28). Interestingly, regulation of these two different receptor types showed opposite induction with significantly increased transcription level of CRHR1 and significantly decreased transcriptional regulation of CRHR2. In particular, CRHR1 is known as a receptor isotype associated with promoting proinflammatory responses (28). It is also known that CRHR1 has 10 times higher affinity for CRH than CRHR2 (121). This finding suggested that dendritic cells induce changes in receptor expression as a response against stress input and serve as a sensor for processing stress responses at the cellular level. However, influences of CRH associated with expression of CRHR on DC function remain uncertain.

As compared with CRH, the functional association of sympathetic nerve-derived catecholaminergic neurotransmitters with dendritic cells has been relatively well-known. Maestroni et al reported that β2 adrenergic receptor-mediated inhibition of IL-12 and stimulation of IL-10 in bone marrow-derived dendritic cells (BMDC) (55). They also showed that $\alpha 1_b$

adrenergic receptor mediates migration of immature bone-marrow dendritic cells from footpad to lymph node. These results demonstrated that catecholaminergic neurotransmitters, epinephrine or norepinephrine, are important factors that influence functional aspects of dendritic cells.

PART III

INTRODUCTION TO IL-23/IL-17 AND NEUTROPHILS

The studies outlined in chapter 2 will demonstrate that pre-exposure to CRH and epinephrine can alter cytokine production by dendritic cells (DC) and as a consequence shift dominant Th1 cellular responses toward a predominant Th17 and Th2 phenotype in response to a bacterial component, lipopolysaccharide (LPS). Based on these findings and in the interests of establishing a platform to define stress and susceptibility to sepsis due to *S. pneumoniae* infection, studies sought to investigate the potential relevance of IL-23 in regulation of immune defense against experimental murine *S. pneumoniae* infection. In this chapter, we review the importance of IL-23 and IL-23-related cytokine and cellular networks known to date, which impact disease pathogenesis.

IL-23/IL-17 AXIS IN PATHOGENESIS

IL-23 is a heterodimeric cytokine composed of the IL-12p40 subunit, shared with IL-12p70, and unique IL-23p19 subunit (122). Similar to IL-23, the IL-23 receptor is also a heterodimeric molecule composed of IL-12 receptor β1 subunit (IL-12Rβ1) and a unique IL-23

receptor subunit (IL-23R) (122). In conjunction with IL-6 and TGF-β, IL-23 plays pivotal a role in the differentiation of naïve CD4+ T cells into the IL-17A phenotype (Th17 T cells), which has been shown to be important in the protection against extracellular bacterial pathogens (123-125). In particular, IL-23 is essential to maintenance of the Th17 phenotype (107). Yet, IL-23 does not directly influence the initial differentiation of Th17 cells regulated by IL-6 and TGF-β (103, 123). Such selectivity of IL-23 on Th17 phenotype was defined by studies of Oppmann et al., which demonstrated that the presence of IL-23 during *in vitro* culture resulted in increased proliferation of activated T cells (126). Langrish et al., also provided evidence that adding IL-23 in T cell culture increased frequencies of IL-17^{+} cells (127). Furthermore, Elson et al., demonstrated that IL-23 is a survival factor for Th17 cells (128). IL-23 is also an important regulator of IL-22, a known inducer of anti-microbial peptide production (129-132). Munoz et al., showed that IL-23 mediates *Toxoplasma gondii* - induced immunopathology in the gut via matrixmetalloproteinase-2 and IL-22 (132). Kleinschek et al., also showed that pathogenesis against fungal infection of *Cryptococcus neoformans* was significantly decreased by systemic administration of IL-23 through regulation of antimicrobial peptide activation (130, 131). Therefore, these findings provide a central role for IL-23 consisting of effective cytokine cross-talk in defense against bacterial pathogenesis through two distinctive cytokines, IL-17 and IL-22.

In contrast, IL-23 also acts as a key cytokine in acute and chronic disease pathologies including allergenic asthma, inflammatory bowel disease (IBD) and experimental autoimmune encephalomyelitis (EAE) (133-138). For example, Brereton et al., showed that suppression of IL-23 expression attenuated pathogenesis in an animal model of EAE (139). Additional animal models and clinical research of autoimmune disease have also shown significant roles of IL-23 for severity of pathogenesis (133, 140-142). Thus, clarification of potential role of IL-23 in

disease pathogenesis is an important point of further investigation in understanding the dichotomous mechanisms of IL-23 as a determinant of immunoprotective and immunopathologic responses related to infectious and non-pathogenic disease.

IL-17 (or IL-17A) is a member of the IL-17 family of cytokines including: IL-17B, IL-17C, IL-17D and IL-17E (or IL-25) (143). IL-17 receptor on target cells such as epithelial cells and endothelial cells consists of IL-17 receptor A and C (IL-17RA and IL-17RC), which signal through intracellular NF-κB and MAPK signaling pathways (144). IL-17 is a proinflammatory mediator known to induce the production of cytokines such as IL-1β, IL-6, TNF-α, G-CSF and GM-CSF, and chemokines including CCL2 (MCP-1), CXCL1 (GROα) and CXCL8 (IL-8 mouse homologue) by several cell types (e.g. macrophages, fibroblast, epithelial cells and endothelial cells) (103, 108). Several investigations demonstrated that IL-17 plays a pivotal role in protection against extracellular pathogen infections (145). In contrast, IL-17 is also associated with autoimmune immunopathologies such as allergic asthma, rheumatoid arthritis and Crohn's disease (15, 111, 113, 146). In both immunoprotective and immunopathologic consequences, the role of IL-17 is associated with neutrophil recruitment (147, 148). Thus, neutrophils may be an important cell population responding to the IL-23/IL-17 axis.

NEUTROPHILS AND PATHOGENESIS

Neutrophils are the most abundant cell type among blood leukocytes and are important participants of the innate immune defense system, particularly against bacterial and fungal infections (147, 149-151). During the initiation stages of inflammation, neutrophils are

translocated to inflammatory sites and eliminate pathogens through phagocytosis accompanied with the release of granular lytic enzymes/antimicrobial peptides into the phagosome (149). They also generate reactive oxygen and nitrogen species, which are crucial products for the regulation of microbicidal activity (147, 149). Based on these functional aspects, Alves-Filho et al., demonstrated that neutrophils play crucial roles against acute infectious pathogenesis such as severe sepsis (149). However, several mediators including cytokines and cellular events such as phagocytosis limit destructive damage by neutrophils, which are tightly orchestrated by controlling their life span (152). In the midst of performing bactericidal reactions, apoptotic pathways of neutrophils are also triggered and are eliminated by macrophages (152). By contrast, an uncontrolled life span or activation status of neutrophils facilitates tissue damage by facilitating persistent hyper-inflammatory responses (152). In fact, excessive infiltration and activation of neutrophils contributes to tissue damage in pathologic states such as rheumatoid arthritis (RA), inflammatory bowel disease (IBD) and chronic obstructive pulmonary disease (COPD) (153-159). In support, Aldridge demonstrated that neutrophil recruited by bacterial septic shock potentially mediates adult respiratory distress syndrome (ARDS) (160). Warren et al., also suggested that neutrophils may play central role in pathogenesis of acute lung injury (ALI) (161). Thus, a tight regulation of neutrophil functions is needed to maintain balance between and host protection and pathogenesis.

PART IV

CONCLUDING REMARKS

The relationship between nervous and immune systems is frequently documented to evaluate influences of stress on number of chronic diseases (11-15). However, no clear evidence has been shown to explain direct effects of stress-induced neuroendocrine components in terms of biochemical and cell biological mechanisms on innate and adaptive immunity. We believe that CRH and catecholamines are important factors induced by stress that influence innate and adaptive immunity. We hypothesize that (). To address this hypothesis, we developed the following studies to answer the following questions: 1) What is the impact of CRH and catecholamines on dendritic cell function? 2). What role does IL-23 have in protection and pathogenesis of *S. pneumoniae* infection? 3). Does receptor-mediated stimulation of stress-derived CRH or catecholamines alter immune responses against acute respiratory *S. pneumoniae* infection?

Collectively, we anticipate that this study will provide an overall understanding of possible mechanisms through which stress can affect immune responses, and consequently modulate disease pathogenesis. Furthermore, this study will also suggest pharmacological prevention of stress-induced alteration of immune system through examination of antagonist treatment.

CHAPTER II

EPINEPHRINE AND CRH-PRIMED MURINE BONE MARROW-DERIVED DENDRITIC CELLS FACILITATE PRODUCTION OF IL-17A AND IL-4 BUT NOT IFN-γ BY $CD4^+$ T CELLS

Neuroendocrine stimulation induces a potential regulatory circuit which is related to immune-mediated diseases. However, questions still remain on the behavior of antigen presenting cells (APC) dictated by catecholamines and CRH. The purpose of this study was to examine potential roles of epinephrine and CRH on the fate of bone marrow derived dendritic cell (BMDC)-associated influences on resting $CD4^+$ T cell activation. We hypothesize that pre-exposure of dendritic cells (DCs) to epinephrine or CRH can modify the intensity of cytokine production, leading to preference in resting $CD4^+$ T cell activation. BMDCs were pretreated with epinephrine or CRH for 2 hr followed by subsequent treatment of lipopolysaccharide (LPS). Subsequently, BMDCs were cocultured with purified $CD4^+$ T cells from mouse spleen in the absence or presence of anti-CD3 stimulation in epinephrine-free media. Epinephrine pre-treatment enhanced surface expression of MHCII, CD80 and CD86. Quantitative RT-PCR showed that epinephrine pretreatment induced a significant transcriptional decrease of IL-12p40 and a significant increase of IL-12p35 and IL-23p19. In addition, β2-adrenergic-blockade was

shown to reverse these effects. Epinephrine pretreatment also induced a significant decrease of IL-12p70 and a significant increase of IL-23 and IL-10 cytokine production. Importantly, these changes corresponded with increased IL-4 and IL-17A, but not IFN-γ cytokine production by $CD4^+$ T cells in a β2-adrenergic receptor-dependent manner. Similarly, CRH induced a significant decrease of IL-12p40 and a significant increase of IL-12p35 and IL-23p19 at the transcriptional level. Accordingly, the expression of IL-12p70 protein was significantly decreased whereas IL-23 and IL-10 were significantly increased. CRH-mediated changes in cytokine production lead to increased IL-4 and IL-17A, but not IFN-γ cytokine production by $CD4^+$ T cells in the absence of anti-CD3 stimulation. These results suggest that exposure to stress-derived epinephrine/CRH dictates dendritic cells to generate a dominant Th2/Th17 phenotype in the context of subsequent exposure to a pathogenic stimulus.

INTRODUCTION

Mechanisms which foster imbalanced $CD4^+$ T cellular responses play an important role in the initiation and progression of most chronic diseases. For example, polarized Th2 cellular activation is known to exacerbate allergic asthma (4, 110, 114). Individuals who suffer from irritable bowel disease and rheumatoid arthritis have documented elevations in Th1-mediated inflammatory responses (163-169). Moreover, recent clinical and experimental studies have highlighted the significant role of Th17 as well as T regulatory cell activation in modulation of acute (170, 171) and chronic inflammatory (111-113, 115, 146, 172-179).

Antigen presenting cells (APCs), which present antigen on major histocompatibility complex (MHC) II molecules and produce cytokines/chemokines are principal determinants of $CD4^+$ T cell-mediated inflammatory responses (94). Dendritic cells (DC), which consist of heterogenous phenotypes (180, 181), are capable of presenting antigen, providing co-stimulatory recognition signals, and secreting cytokine, which are required for $CD4^+$ T cell differntiation and function (94, 182). For example, the divergence of $CD4^+$ T cell phenotypes driven by preferences in IL-10, IL-12, IL-23 or TGF-β cytokine production by DC is believed to play a pivotal role in regulation of inflammatory disease (183-189). In particular, there is emerging interest in investigating the relationships between the IL-12/IL-23 balance by DC in controlling the fate of $CD4^+$ T cell responses. IL-12 represents a heterodimeric cytokine comprising of

covalently linked p40 and p35 subunits (122), and is widely known as an important regulator of Th1 cellular responses (190). Through the co-expression of both subunits, IL-12 is biologically active in its secreted form. More recently, a novel subunit p19 that associates with the IL-12p40 subunit was discovered (122). Similar to IL-12, IL-23 cytokine production requires both p19 and shared p40 subunit expression (122). In contrast to IL-12, the production of IL-23 by DC is found to regulate IL-17 production by $CD4^+$ T cells (124). IL-17 plays a pivotal role in the regulation of chemokines and adhesion molecules from epithelial cells and endothelial cells. These regulations are associated with the infiltration of neutrophils and monocytes to inflammatory sites, through binding of its receptor on epithelial cells and endothelial cells (191-193). Thus, given the plasticity of DC to direct the fate of $CD4^+$ T cells, defining factors, which alter their function can be a preemptive target in the prevention and treatment of chronic inflammatory disease.

Perceived stress has emerged as a key causative factor in the initiation and progression of many chronic diseases including irritable bowel disease (194-196), rheumatoid arthritis (197, 198), atherosclerosis (199, 200), and asthma (7, 201, 202). The underlying mechanism of action is thought to be derived from stress-induced neuroendocrine regulation of cellular immune function. In response to stressful experiences (e.g. psychological, physical), activation of the central nervous system leads to neuronal activation and neuropeptide release of stress factors such as catecholamines (norepinephrine and epinephrine), corticosteroids (cortisol), via CRH and ACTH pathway in CNS, parasympathetic-mediated acetylcholine activation, and others (203, 204). Furthermore, through nervous innervations of lymphoid tissue and/or by neuroendocrine receptor expression, immune cells receive nervous system stimuli resulting in altered function (68, 205). Such neuroendocrine-mediated influences on immune function have

since been shown to impact disease susceptibility by facilitating either hyperactivity or suppression of immune responses (59, 68, 205).

The adrenergic (catecholaminergic) stress response pathway is highly integrated within the immune defense system. The sympathetic nervous system innervates almost all major lymphoid tissues such as thymus, spleen, bone marrow, and regional lymph nodes (206). Adrenergic receptors are also expressed on immune cells including NK cells, T cells, and macrophages (59, 63, 207, 208), which exhibit paracrine/autocrine function, through release of catecholamines and the expression of adrenergic receptors (54). In studies by Maestroni et al., DCs were found to express $\alpha 2_A$-, $\alpha 2_C$- β1-, and β2-adrenergic receptors. Importantly, *in vivo* administration of β2-adrenergic antagonists was found to regulate the production of Th1 cytokines IL-2 and IFN-γ in lymph node after adoptive transfer of DC (55, 209). Panina-Bordignon et al., also have shown that β-adrenergic agonists can preferentially prevent IL-12 production and promote Th2 development (56). More recent findings also highlight the potential impact of adrenergic stimulation on Th17 responsiveness associated with IL-23 expression in effector immune cells such as macrophages (210). Thus, the ability of DC to facilitate the activation and effector function of $CD4^+$ T cells in response to adrenergic stimulation could be a determinant in disease pathogenesis under conditions of stress.

In contrast to β2 adrenergic pathway, the direct impact of CRH is still not clearly identified in stress-induced alteration of immune response. CRH has been originally identified in CNS as stress-induced neuroendocrine factor (33) to trigger production of stress-induced hormone, cortisol or corticosterone (33, 35). Two receptor isotypes, CRHR1 and CRHR2 have been identified in several immune cells (44, 121, 211, 212). Furthermore, several findings have

shown potential immunomodulatory effect of CRH in NK cells, macrophages and mast cells (44, 46, 48). Importantly, Gonzales et al., demonstrated that restraint stress induced differential regulation of CRHR1 and CRHR2 transcriptional level in pulmonary $CD11c^{+}MHCII^{+}$ cells (28). This finding implicates that dendritic cells may be a potential target of CRH, mediating their functional modulation by stress through differential CRHR expression. In support, local CRH expression in cellular level has been reported, supporting potential autocrine/paracrine stimulation in cellular level (38, 213, 214) .

Utilizing an experimental system of epinephrine or CRH-mediated regulation of DC activation through MHCII and co-stimulatory signaling molecule (CD80 and CD86) as well as cytokine production (IL-10, IL-12p70 and IL-23, the current study determined the fate of $CD4^{+}$ T cell activation under the control of DC previously influenced by adrenergic and CRH stimulation. The results from the current study provide evidence that adrenergic stimulation can enhance surface expression of MHCII, CD80 and CD86 and also preferentially augment p40, p35, and p19 heterodimeric subunit expression by DC, resulting in a preferential IL-23/IL-17 phenotype in a β2-adrenergic manner. Our studies also provide that direct CRH stimulation on DC also potential initiate similar effect with β2-adrenergic stimulation in DC. These data provide evidence that neuroedocrine effects on APC is important in understanding stress-induced augmentation of $CD4^{+}$ T cell responses that may be important in defining the hidden mechanisms of stress and chronic inflammatory disease.

MATERIALS AND METHODS

Animals

Adult (6–8 weeks of age) female CD-1 mice (Harlan Sprague–Dawley, Indianapolis, Indiana) were used in all studies. Mice were maintained under specific pathogen-free conditions on a 12:12 light/dark cycle (7:00 PM to 7:00 AM). Mice were kept under optimal temperature and humidity controlled conditions and provided proper care as directed by the institutional animal care and use committee. Before bone marrow cell isolation, mice were acclimated at housing facility for 7 days to eliminate shipping stress.

Generation of bone marrow-derived dendritic cell (BMDC)

Bone marrow cells were flushed from the *femur* and *tibia* with wash media (RPMI 1640 with 1% FBS and 1% penicillin / streptomycin) using a 25-gauge needle. After removing red blood cells, using ACK (ammonium-chloride-potassium) lysis method (215), total mononucleated cells were purified by gradient centrifugation using lympholyte M solution (Cedarlane laboratories Ltd. Hornby, ON, Canada). Cells were maintained in RPMI 1640 media containing 10% FBS and 1% penicillin / streptomycin supplemented with recombinant murine GM-CSF (10 ng/ml) (Biosource, invitrogen cytokines & signaling, Camarillo, CA) and IL-4 (10 ng/ml) (R&D systems, Inc. Minneapolis, MN). All floating cells and loosely adherent cells were removed by gentle swirling and fresh media was replaced on day 3. On day 6, half amount of fresh media was gently added to cell culture. On day 7, cells were transferred to either 6 well

plates with $1X10^6$ cells per well or 48 well plates with $1X10^5$ cells per well for experiments. Purity of $CD11c^+$ cells was confirmed by flow cytometry (~90%).

Cell treatment and harvest

BMDC plated on 6 well- or 48 well-plates were exposed to 10^{-6} M of epinephrine in the presence or absence of the selective β2-adrenergic antagonist, butoxamine (Sigma) or 2 x 10^{-6}M of CRH (Sigma, St. Louis, MO) for 2 hr. After 2hr epinephrine exposure, cells were stimulated by lipopolysaccharde (LPS) (Sigma) (1 μg/ml) for an additional 3 hr. For gene expression analysis, 1 ml of TriZol reagent (Invitrogen.Co. Carlsbad, CA) was added to each well of a 6 well-plate, and cell lysates were collected for mRNA expression analysis. Similarly, BMDC cultured in 48-well plates were pre-exposed to epinephrine in the presence or absence of butoxamine or pre-exposed to CRH for 2hr. BMDC culture supernatants were collected 24hr after LPS stimulation and stored at -80° C until analysis. For ELISA analysis, culture supernatant was collected from 48-well plates after 24 hr of stimulation.

Flow cytometry

Collected BMDC were incubated with anti-CD16/CD32 FcRII/III blocker at 4°C for 10 min. Two-color immunofluorescence staining was performed using PE-labeled anti-mouse CD11c with either FITC-labeled anti-mouse CD80, FITC-labeled anti-mouse CD86 or FITC-labeled anti-mouse MHCII in 4°C for 30 min. After two time of cell washing to eliminate non-binding antibodies, positive cells for immunostaining were identified using cytomic FC500 flow

cytometry analyzer (Beckman-Coulter). Further analysis of mean fluorescence intensity and generation of histograms were performed using FlowJo analysis program (version 8.8.7, Ashland, OR). Fc blocker and antibodies were purchased from BD biosciences (BD biosciences, San Jose, CA)

Magnetic cell sorting for $CD4^+$ T cell and BMDC-$CD4^+$ T cell coculture

$CD4^+$ T cells were purified and enriched from total splenocytes using IMagnet $CD4^+$ T Lymphocyte Enrichment Set (BD PharMingen Inc., San Jose, CA) as described in procedures from the manufacturer. Briefly, after elimination of red blood cell using ACK lysis method (215), total splenocytes were incubated with an optimal concentration of biotin-conjugated antibody cocktail (BD PharMingen Inc.) containing anti-mouse CD8a, anti-mouse CD11b, anti-mouse CD45R/B220, anti-mouse CD49b and anti-mouse TER-119/Erythroid for 20 min on ice, followed by incubation with streptavidin-conjugated magnetic nanoparticles. Non-$CD4^+$ T cells were selected by magnetic force using BD IMagnet (BD PharMingen Inc.). Purity of enriched $CD4^+$ T cells was analyzed using flow cytometry technique (~ 93%). BMDC were plated (1 x 10^5 cells/well) in 48 well plates and pre-primed for 2 hr by epinephrine (10^{-6} M) in the absence or presence of butoxamine (10^{-5} M) followed by subsequent LPS stimulation (1 μg/ml) for 24 hr. 4 x 10^5 purified $CD4^+$ T cells were added to each well with fresh new media after removing old media from BMDC culture to prevent direct epinephrine stimulation on T cells. To mimic T cell receptor (TCR)-independent or dependent CD4+ T cell activation, BMDC-CD4+ T cell coculture was performed in the absence [(-)CD3] or presence [(+)CD3] of anti-CD3

monoclonal antibody (2.5 μg/ml) (BD PharMingen Inc.). Culture media was collected after 96 hr incubation period and used for cytokine expression analysis.

Quantitative real-time RT-PCR

Total RNA was extracted from BMDC culture. After adjustment to a concentration of 1 μg per reaction, reverse transcription was performed using MLV (Molony murine leukemia virus) reverse transcriptase (Promega, Madison, WI) as previously described (216). After cDNA synthesis, real-time PCR was performed using SYBR green techniques to evaluate mRNA expression levels of cytokine genes. Selected target and house keeping gene primer sets; IL-12p40, IL-12p35, IL-23p19, IL-10 and GAPDH (glyceraldehydes-3-phosphate dehydrogenase) was purchased from SAbioscience Inc. (SAbiosicence, Frederick, MD). Real-time SYBR master mix was purchased from Applied Biosystem (Applied Biosystems, Foster City, CA). Thermal cycling procedure was performed in a 20 μl reaction volume using a StepOne system (Applied Biosystems). Differences in gene expression were determined by relative quantification between cDNA templates from un-stimulated control cells and cells from each experimental group including LPS-treated positive controls. The expression of the housekeeping gene GAPDH was used as an internal control 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) - ΔCT (GAPDH). The $\Delta\Delta CT$ value of cDNA amplification from the control group was considered the calibrator for baseline levels of mRNA expression. Data were expressed as the ratio of target gene expression of each group of various treatment subjects to the target gene expression of the control group, resulting in fold difference in target gene mRNA levels.

Enzyme-linked immunosorbent assay (ELISA)

IFN-γ, IL-4, IL-10, IL-17A, IL-12p70 and IL-23 were determined by sandwich ELISA method. All procedures were performed as described by the manufacturer. Briefly, flat-bottomed 96-well plates were coated with an optimal concentration of capture antibody and followed by blocking (10% FBS in PBS). After incubation of samples at 4°C for 16 hr, plates were incubated with biotin-conjugated detection antibody and streptavidin-HRP (horseradish peroxidase). After adding tetramethylbenzidine (TMB) peroxidase substrate solution (Rockland Immunochemicals, Inc. Gilbertsville, PA), the concentration of each cytokine was determined according to the standard curve generated by reference concentration of cytokine at wavelength of 450 nm detected by colorimetric plate reader (Biotek Instruments Inc. Winooski, VT). ELISA antibody set and recombinant cytokine for standard were purchased from eBiosicence (eBiosciences, San Diego, CA) for IL-12p70 and IL-23 ELISA sets, BD PharMingen Inc. for IFN-γ, IL-4, IL-10 ELISA sets and IL-17A antibody pairs and R&D Systems (R&D Systems Inc. Minneapolis, MN) for recombinant IL-17A.

Statistical analysis

Statistical analysis was performed using GraphPad Prism Version 4.0 (GraphPad Software, San Diego, USA). For multi-experimental group analysis, data were subjected to one-way and two-way ANOVA (analysis of variance) followed by post hoc tests (Newman-Keuls and Bonfferoni) for group differences. All data are expressed as means ± standard error of mean (SEM). The two-tailed level of significance was set at $p \leq 0.05$ for group differences.

RESULTS

Pre-exposure to epinephrine enhances surface expression of MHCII and co-stimulatory molecules (CD80 and CD86) on LPS-stimulated BMDC.

We examined the expression of MHCII and co-stimulatory (CD80 and CD86) molecules from epinephrine pre-treated BMDC, which are stimulated by LPS. As expected, LPS stimulation significantly increased the intensity of MHCII, CD80 and CD86 as compared to un-stimulated BMDC. Epinephrine exposure prior to LPS-exposure induced a further significant enhancement of intensity of these molecules (figure 1). Interestingly, introduction of the β2 adrenergic antagonist, butoxamine during epinephrine pre-exposure attenuated MHCII and CD86 but not CD80 (supplemental figure 1).

Exposure to epinephrine downregulates gene expression of IL-12p40, but upregulates IL-12p35 and IL-23p19 by BMDC in response to LPS stimulation.

We analyzed BMDC-associated cytokine gene expression that typically dictates Th1 and Th17 differentiation. IL-12p40 is heterodimerized with IL-12p35 or IL-23p19 and constitutes each IL-12p70 or IL-23. Epinephrine-pretreated BMDC showed a significant ($p \leq 0.05$) decrease in the transcription level of IL-12 p40 (1160 fold) as compared to LPS-stimulated BMDC. In contrast, transcription of the IL-12 p35 and IL-23 p19 was significantly ($p \leq 0.05$) increased by 18 and 25-fold by epinephrine pre-treatment, respectively (figure 2A). β2-adrenergic receptor is known as a major receptor isotype involved in catecholamine-mediated responses in immune cells. We examined whether epinephrine-mediated effects of IL-12p40, IL-

12p35 and IL-23p19 heterodimer expression was impacted by β2-adrenergic receptor activation. Using a selective β2 adrenergic receptor antagonist, butoxamine, a transcriptional down-regulation of IL-12 p40 by epinephrine challenge was significantly ($p \leq 0.05$) recovered in a dose-dependent manner ($10^{-4} \sim 10^{-7}$ M). Conversely, increased transcriptional levels of IL-12p35 and IL-23p19 were significantly ($p \leq 0.05$) reduced by butoxamine (10^{-4} M) (figure 2B).

IL-12p70, IL-23 and IL-10 cytokine production by BMDC exposed to epinephrine corresponds with preferences in p35 and p19 subunit expression.

We determined protein expression level of IL-12p70, IL-23 and IL-10 within culture supernatants of BMDC after 2hr pre-exposure of epinephrine at 10^{-6} followed by additional LPS stimulation for 24 hr. IL-12p70 was significantly ($p \leq 0.05$) decreased by epinephrine pretreatment in response to LPS stimulation as compared to LPS treatment alone. In contrast, exposure to epinephrine prior to LPS stimulation induced a significant ($p \leq 0.05$) increase in IL-23 as compared to LPS stimulation alone. In addition, IL-10, a Th2 promoting-cytokine was significantly ($p \leq 0.05$) increased in cultures of LPS stimulated BMDC pre-exposed to epinephrine as compared to LPS stimulation alone (figure 3).

BMDC-CD4+ T cell coculture preferentially induce IFN-γ, but not IL-4 and IL-17A

We first determined the level of IFN-γ, IL-4 and IL-17A cytokine production by $CD4^+$ T cell without BMDC coculture in the absence and presence of soluble anti-CD3 stimulation to examine T cell receptor-dependent cytokine production. Anti-CD3 stimulation induced IL-4,

IFN-γ and IL-17A cytokine production with IL-17A being the highest concentration detected in culture supernatants. None of these cytokines were detected in $CD4^+$ T cell culture without anti-CD3 stimulation (figure 4). For comparison, we examined the intrinsic ability of un-stimulated BMDC to induce cytokine production by $CD4^+$ T cells in absence or presence of anti-CD3 stimulation. IFN-γ was the only cytokine detected in $CD4^+$ T cell culture supernatants in the presence of un-stimulated BMDC without anti-CD3 stimulation. A further significant ($p \leq 0.05$) increase in IFN-γ cytokine production was observed in the presence of un-stimulated BMDC given CD3-mediated stimulation of $CD4^+$ T cells as compared to $CD4^+$ T cell cultures without BMDC in the presence of anti-CD3 antibody. In contrast, the presence of un-stimulated BMDC significantly ($p \leq 0.05$) attenuated IL-4 cytokine production as compared to $CD4^+$ T cell only cultures in the presence of anti-CD3 antibody. Interestingly, IL-17A cytokine production by $CD4^+$ T cells alone or in the presence of BMDC was not detected in the absence of anti-CD3 antibody. Furthermore, BMDC did not significantly alter IL-17A cytokine production by $CD4^+$ T cells in the presence of anti-CD3 antibody stimulation as compared to their $CD4^+$ T cell only counterparts ($p \leq 0.05$). Thus, the innate ability of resting BMDC was demonstrated to facilitate a preference for IFN-γ production, but not for IL-4 or IL-17A.

Epinephrine promotes IL-4 and IL-17A, but not IFN-γ under BMDC-mediated preferential cytokine production of $CD4^+$ T cells.

We next determined $CD4^+$ T cell cytokine production mediated by epinephrine-primed BMDC. IFN-γ production by $CD4^+$ T cells in the absence or presence of anti-CD3 antibody was not significantly attenuated by epinephrine-treated BMDC. However, LPS-stimulated and

epinephrine-primed LPS-stimulated BMDC showed significantly ($p \leq 0.05$) decreased IFN-γ production by anti-CD3-stimulated $CD4^+$ T cells as compared to un-stimulated and epinephrine only experimental conditions group (figure 5). This response was modestly reversed in the presence of butoxamine (data not shown). In response to LPS stimulation, pre-treatment of epinephrine supported a significant ($p \leq 0.05$) increase in IL-4 production as compared to BMDC stimulated with LPS alone in the absence or presence of anti-CD3 stimulation (figure 5). In support, BMDC exposed to epinephrine with butoxamine significantly ($p \leq 0.05$) reduced IL-4 production by $CD4^+$ T cells in the absence of anti-CD3 stimulation and modestly diminished IL-4 production in the presence of anti-CD3 stimulation (data not shown). LPS stimulation of BMDC exposed to epinephrine resulted in a significant ($p \leq 0.05$) increase of IL-17A production by $CD4^+$ T cells in the absence of or presence of anti-CD3 stimulation (figure 6A) as compared to LPS only stimulation. Importantly, this increase in IL-17A was significantly ($p \leq 0.05$) attenuated in the presence of butoxamine within both conditions (figure 6B).

CRH stimulation prior to LPS stimulation in BMDC resulted in similar cytokine production pattern with β2 adrenergic stimulation.

We also analyzed IL-12p40, IL-12p35 and IL-23p19 gene expression in CRH-pretreated BMDC. IL-12p40 was significantly ($p \leq 0.05$) decreased in the transcription level (790 fold) as compared to LPS-stimulated BMDC. In contrast, transcription of IL-12 p35 and IL-23 p19 was significantly ($p \leq 0.05$) increased by 17 and 14-fold by CRH pre-treatment, respectively (figure 7). We also determined protein expression level of IL-12p70, IL-23 and IL-10 under CRH pre-treatment followed by LPS challenge. IL-12p70 was significantly ($p \leq 0.05$)

decreased by CRH pretreatment in response to LPS stimulation as compared to LPS treatment alone. In contrast, the same condition induced significant ($p \leq 0.05$) increase in IL-23 and IL-10 as compared to LPS stimulation alone (figure 8).

CRH also promotes IL-4 and IL-17A, but not IFN-γ in the absence of anti-CD3 stimulation.

We also determined $CD4^+$ T cell cytokine production mediated by CRH-primed BMDC. IFN-γ production by $CD4^+$ T cells in the absence or presence of anti-CD3 antibody was not significantly attenuated by CRH-treated BMDC. However, LPS-stimulated and CRH-primed LPS-stimulated BMDC showed significantly ($p \leq 0.05$) decreased IFN-γ production by anti-CD3-stimulated $CD4^+$ T cells as compared to un-stimulated and CRH only experimental conditions group (figure 9). In response to LPS stimulation, pre-treatment of BMDC with CRH supported a significant ($p \leq 0.05$) increase in IL-4 or IL-17A production as compared to LPS stimulation alone only in the absence of anti-CD3 stimulation (figure 9). However, CRH pre-treatment on BMDC did not show controllability on IL-4 or IL-17A production by cocultured $CD4^+$ T cells in the presence of anti-CD3 stimulation

DISCUSSION

Both anecdotal and experimental evidence support a link between stress and disease susceptibility, particularly in the case of chronic inflammatory conditions (7, 194-196, 198, 200-202). For years, researchers have considered this phenomenon to result from biological

interactions between the central nervous and immune systems. Yet, to date the mechanisms of action that define their relationship remain unresolved.

An abundance of knowledge demonstrates the important role that $CD4^+$ T cells have in mediating various inflammatory diseases. Few reports however, distinguishes neuroendocrine-mediated DC responsiveness as a potential pathway influencing innate (217) and adaptive immunity (218), including determination of $CD4^+$ T cell phenotypes. Thus, there is a further need to define the cellular immune mechanisms influenced by stress response factors.

The current study focused on the role of epinephrine and CRH as major stress response factor regulating DC function. First, we demonstrated a potential impact of epinephrine on DC surface markers. As shown in figure 1, we demonstrated that epinephrine exposure contributes to LPS-induced increase in surface CD80, CD86 and MHCII expression. Such findings demonstrate the potential relevance of epinephrine's influence during the initial stages of DC activation and maturation independent of antigen exposure. In that epinephrine exposure enhanced MHCII as well as CD80 and CD86 co-stimulatory molecule expression suggests that epinephrine supports an elevated DC activation status. This is in contrast to the effects previous reported regarding the role of corticosteroids in which, attenuation of activation was observed by DC (219, 220). This finding suggests that specific epinephrine action on DC maturation and activation can be controlled in a β2-adrenergic dependent manner. This is important in light of the reported influence that differential co-stimulatory expression by DC has on priming of T cell responses (221). Thus, further investigation of adrenergic receptor specificity may provide novel insight of epinephrine actions on DC maturation and co-stimulatory signaling pathways important for the cellular interactions between DC and T cells, constituting T cell activation.

Antigen presentation by DC is essential for the initiation and maintenance of adaptive $CD4^+$ T cell responses through antigen-specific recognition between MHC II - T cell receptor complexes and co-stimulatory signaling pathways. Equally important is the paracrine release of cytokines and/or chemokines by DC that direct the differentiation of $CD4^+$ T cells into Th1, Th2, T regulatory and Th17 subsets (95, 222). For the purpose of understanding how neuropeptide stimulation can impact DC cytokine production, our initial studies examined the influence of exposing BMDC to epinephrine or CRH prior to lipopolysaccharide (LPS) exposure, which is known to induce co-stimulatory molecule expression and cytokine responses through toll-like receptor-4 (TLR4) signaling pathways (223-225). Previous studies have documented the specificity of cytokine secretion by DC to direct $CD4^+$ T cell cytokine production (95). Recently, the IL-12/IL-23 from APC has received significant attention regarding protection and disease pathogenesis (125). DC-associated IL-12 and IL-23 cytokine production have a key role in dictating DC ability to instruct $CD4^+$ T cell phenotypes (226). IL-12 cytokine production promotes the differentiation of Th1 effector cells (190). IL-23 production is known to induce the production of antimicrobial peptides via IL-22 (132, 227) and maintain the Th17 cell subset (107, 124), which is an important mediator of adaptive immune responses (228) as well as regulation of innate responses, including neutrophil recruitment (191, 193). In the current study, we demonstrated that BMDC exposed to epinephrine followed by LPS induces a preferential reduction in IL-12p40 mRNA transcripts. In contrast, IL-23p19 and IL-12p35 mRNA expression was significantly increased in BMDC exposed to epinephrine or CRH followed by LPS stimulation (figure 2 and figure 7). As shown in figure 2B, selective β2-antagonist (e.g. butoxamine) demonstrated a dose-dependent impact on BMDCs p40, p35, and p19 subunit transcription, suggesting a bias for IL-23 on the basis of p19 subunit expression. This finding

corresponded with ability of butoxamine to attenuate MHCII and CD80 expression (data not shown). Although further clarification is still required on receptor isotype responsible on this influence, CRH also showed similar impact on those subunits. Thus, providing evidence of a novel mechanism where DC capacity to instruct the induction of cytokine production, particularly IL-17A is dependent on β-adrenergic stimulation.

Importantly, such preferences in gene expression were substantiated by dose- associated epinephrine dependent decreases in IL-12p70 protein and a concomitant increase in IL-23 protein (figure 3 and figure 8). Furthermore, our results demonstrated the preferential increase in IL-10 production in response to epinephrine treatment. IL-10, a key facilitator of Th2 and regulatory $CD4^+$ T cell responses is prevalent in many chronic inflammatory disease states (188, 229, 230). Previous studies have documented preferential Th2 responses driven by adrenergic stimulation (56, 65, 226). Together, these findings suggested that epinephrine might direct DC cytokine phenotypes that potentially predict downstream cytokine production by $CD4^+$ T cell populations.

Based on the above studies, we predicted that preferences in cytokine production (e.g. decreased IL-12p70 and increased IL-23) by DC would subsequently lead to a preferential Th17 phenotype. First, we examined the intrinsic effect of un-stimulated BMDC to regulate $CD4^+$ T cell cytokine production. DC have been shown to promote Th1 differentiation through a preference in IL-12p70-dependent IFN-γ cytokine production by $CD4^+$ T cells (231). Alternately, DC can also support tolerogenic $CD4^+$ T cell responses by diminishing IFN-γ production (232). In comparison to cytokine production by $CD4^+$ T cells alone, BMDC promoted marked IFN-γ cytokine production in both absence and presence of soluble anti-CD3 stimulation (figure 4). Our

data also demonstrated a dichotomy between IFN-γ and IL-4 in the presence of adrenergic or CRH stimulation (figure 5 and Figure 9). Most striking however, was the ability of LPS-stimulated BMDC to facilitate *de novo* IL-17A by $CD4^+$ T cells independent of CD3-stimulation at levels significantly higher than IFN-γ. Moreover, epinephrine was found to significantly increase this response that was reversed by butoxamine treatment (figure 6B). To our knowledge, this is the first study to test IL-17A cytokine production by a heterogenous population of resting $CD4^+$ T cells in the absence of TCR-mediated activation in the context of LPS-associated TLR-4 DC activation. This suggests a potential novel mechanism in which action of epinephrine or CRH on DC in the absence of TLR activation constitutes a default mechanism toward IL-17A production. Presumably, this result could serve as a mode of by-passing antigen-dependent MHC-TCR responses for the purpose of innate immune activation. Further investigation will likely provide important insight into default actions of DC in the face of neuroendocrine stimuli.

We next examined the impact of BMDC in the presence of $CD4^+$ T cells, which were stimulated through TCR by CD3 antibody. As expected, $CD4^+$ T cells responded to CD3 stimulation by producing IFN-γ, IL-4 and IL-17A (figure 5, 6 and 9). In contrast to what was observed in the absence of CD3 stimulation, we expected that BMDC exposed to LPS would accentuate IFN-γ by CD3-stimulated $CD4^+$ T cells. Surprisingly, lower IFN-γ production was detected in the presence of LPS-stimulated BMDC as compared to $CD4^+$ T cells cocultured with unstimulated BMDC, which produced the highest levels of IFN-γ. One explanation, may be the necessity for optimal signaling by DC-$CD4^+$ T cell interaction in the midst of LPS-associated maturation and activation of DC. This would be consistent with previous reports suggest

functional inhibition or tolerance of T cells in response to DC (234, 235). Yet, despite this observation, epinephrine or CRH's effect on BMDC resulted in a similar negative impact on IFN-γ production. IL-4 production by CD3-stimulated $CD4^+$ T cells was decreased in the presence of BMDC regardless of treatment. This is consistent with BMDC preference for robust IL-12p70 cytokine production corresponding with conservative IL-10 production as well as the strong IL-17A production. In support, previous studies have demonstrated DC as potent regulators of Th1 responses (236). In the absence or presence of anti-CD3 stimulation, BMDC exposed to epinephrine in the context of LPS stimulation enhanced IL-4 compared to LPS only condition. Together, these studies demonstrated for the first time that epinephrine or CRH pre-exposure of BMDC can preferentially induce the activation of Th17 phenotype as evidence of robust IL-17A cytokine production by $CD4^+$ T cells. Hence, priming BMDC, in the presence of epinephrine or CRH by the stimulation of LPS, served as a catalyst for induction of IL-17A cytokine production.

Earlier studies support the sympathetic nervous system as an active participant in regulation of proinflammatory conditions (237, 238). However, the specific immune cell-types influenced remain unresolved. $CD4^+$ T cells have become a major focus of chronic inflammatory disease, especially the impact of the recently defined Th17 subset (111, 113, 115, 146, 175, 239). Whether Th17 cells play an instrumental role in neuroendocrine-associated inflammatory disease remains undefined. In the current study, we focused on DC response as a catalyst for cytokine production by a heterogenous population of resting $CD4^+$ T cells. Recently, Seiffert et al., showed that epidermal langerhans cells as well as DC-like cell lines respond to epinephrine through β–adrenergic stimulation, resulting in impaired IFN-γ-associated contact

hypersensitivity responses (240). In a report related to our current study, Maestroni demonstrated that BMDCs exposed to epinephrine influenced IL-12p40 cytokine production and Th1 development (55, 65). Unique to this study however, is the findings that not only does epinephrine exposure decrease IL-12 production (IL-12p40 mRNA and IL-12p70 cytokine production), but also results in a preferential increase in IL-23 response through induction of IL-23p19 and a reduction in IL-12p40 mRNA expression that corresponded with elevations in IL-23 cytokine production. The ability of epinephrine to influence $CD4^+$ T cell cytokine production in this study had both similarities and differences compared with previous *in vitro* studies. Maestroni et al. (55, 65) and others (56) demonstrated that adrenergic stimulation modulates $CD4^+$ T cell responses. Specifically, Maestroni et al. highlighted the ability of norepinephrine exposure to decrease IFN-γ cytokine production in a mixed DC-$CD4^+$ T cell culture system. However, in contrast to other reports, we examined the exclusive impact of epinephrine solely on BMDC cultures and its subsequent ability to elicit $CD4^+$ T cell cytokine production in an epinephrine-free co-culture system. Also, in contrast to our current findings, Goyarts et al., demonstrated a decrease in IL-12p40 and IL-23 cytokine production by human cord blood $CD34^+$ precursor cells (241). Such discrepancies may be explained by differences between human and murine progenitors as well as *in vitro* culture conditions for the generation and maintenance of human verses murine DC (241-244). In contrast to adrenergic pathway, no clear evidence has shown the role of CRH on $CD4^+$ T cell mediated adaptive immunity. Thus, our findings may provide a understanding of the novel peripheral role of CRH on DC functions. In addition, further investigation using primary DC populations such as splenic or lymphatic DC will further validate the function of DC in physiological conditions under catecholaminergic stimulation.

In summary, DC function represented by the type of cytokines and chemokines they secrete is critical for programming the type and quality of $CD4^+$ T cell responses. Importantly, because inflammatory conditions mediated by $CD4^+$ T cells require DC regulation, understanding the mode of action will likely benefit the development of therapies which control chronic disease. In recent years, the IL-17 cytokine family has become the center of attention in regulation of inflammatory disease states including arthritis, colitis, asthma and cardiovascular disease (111, 113, 115, 146, 175, 239). Likewise, the role that stress plays in exacerbated inflammatory conditions is believed to be pivotal in disease management. Here, we examine the relationship between adrenergic nervous system activation on DC as a determinant in the fate of T cell cytokine production representing Th1, Th2 and Th17 phenotypes. Moreover, the consequence of adrenergic influences, DC may also contribute to innate immune responses, particularly through antigen-independent *de novo* Th17 pathway. As shown in our hypothetical model, our findings suggest that adrenergic stimulation can affect inflammatory conditions by favoring Th17 differentiation through augmentation of DC cytokine functioning (figure 10). Importantly, we hypothesize that differential pathways can be driven by epinephrine through selectivity of β2-adrenergic and CRH responses that can result in $CD4^+$ T cell cytokine production by non-classical DC-$CD4^+$ T cell interaction. On the basis of these findings, future understanding of the type of adrenergic receptor expression by DC that alter their function will provide important insight toward understanding their role in orchestration of $CD4^+$ T cell-mediated inflammatory conditions, considering stress as a catalyst.

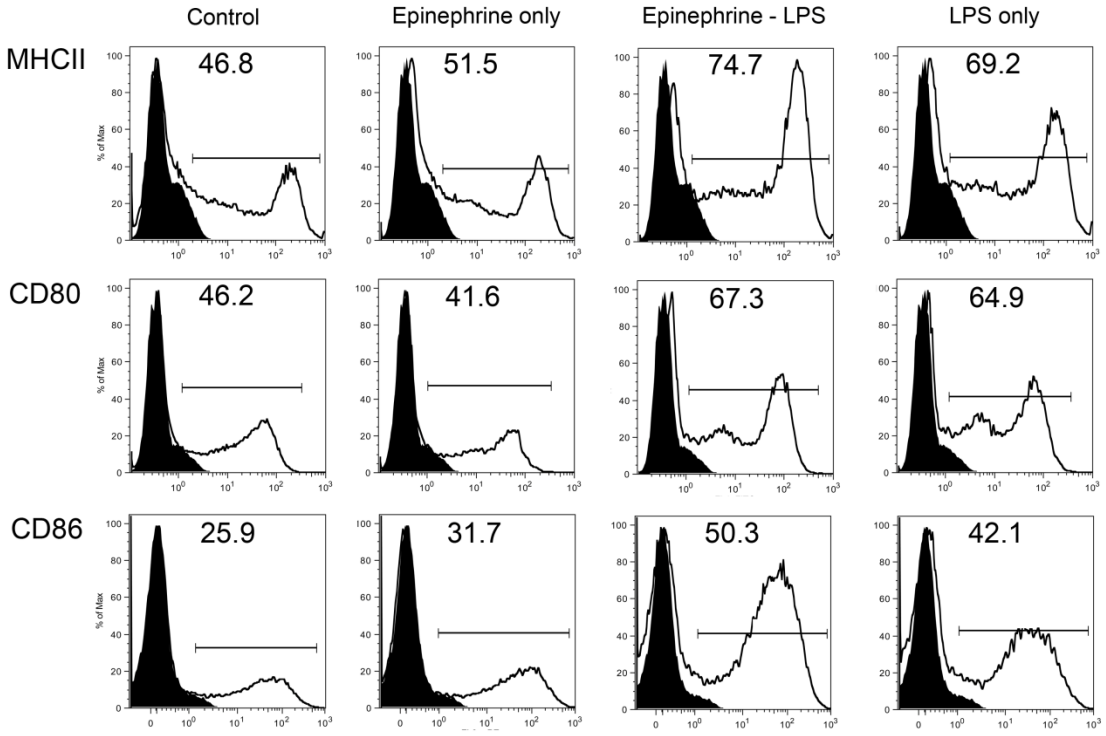

Control
Epinephrine only
Epinephrine - LPS
LPS only
MHCII
CD80
CD86
46.8
51.5
74.7
69.2
46.2
41.6
67.3
64.9
25.9
31.7
50.3
42.1
% of Max

Figure 1. Epinephrine enhances LPS-stimulated BMDC maturation. Percentage of positive cell population for MHCII and co-stimulatory (CD80 and CD86) molecule was determined by flow cytometry after 24 hr of LPS stimulation in either epinephrine-pretreated or non-treated group. Cell population in black area indicates no-staining control. Numbers on each histogram indicate the percentage of positive cell population for each molecule from representative data of experimental groups (n=3).

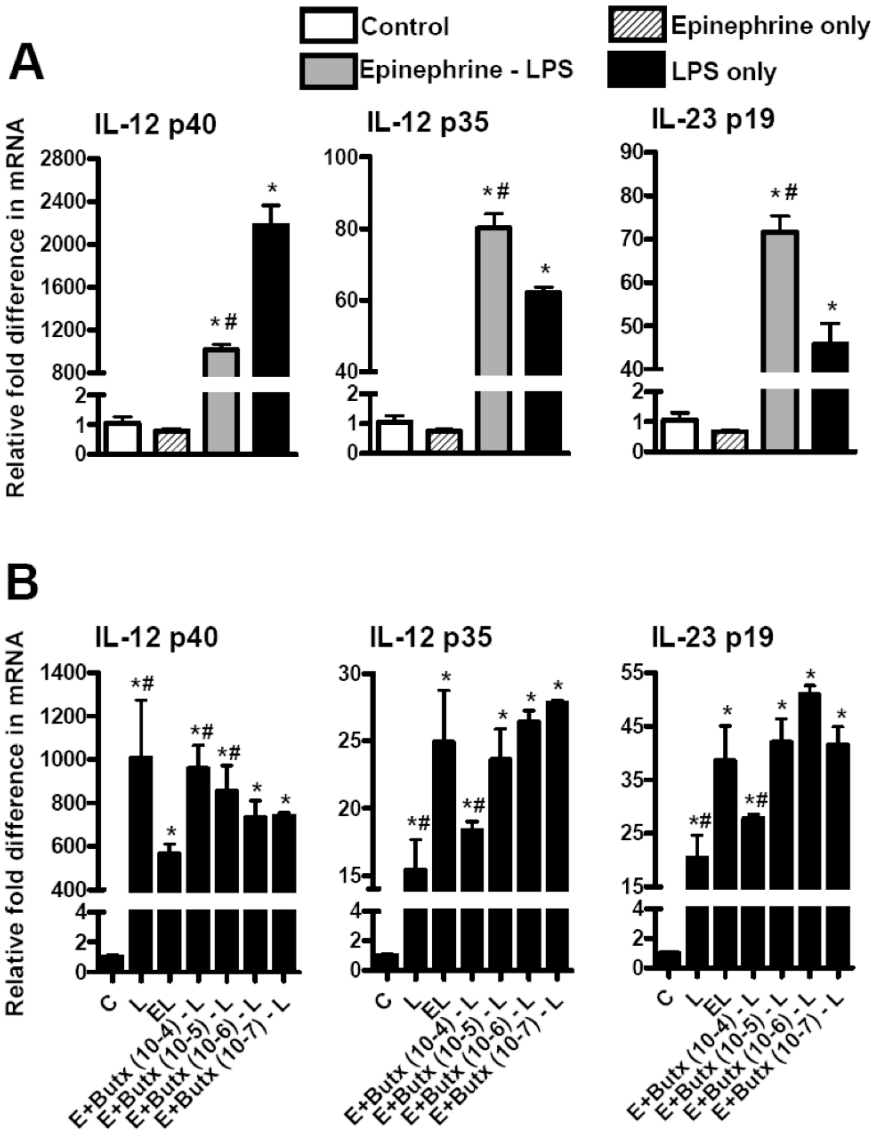

A
Control
Epinephrine only
Epinephrine - LPS
LPS only
IL-12 p40
IL-12 p35
IL-23 p19
Relative fold difference in mRNA
B
IL-12 p40
IL-12 p35
IL-23 p19
Relative fold difference in mRNA
C
L
EL
E+Butx (10-4) - L
E+Butx (10-5) - L
E+Butx (10-6) - L
E+Butx (10-7) - L

Figure 2. $CD4^+$ T cell fate-determining cytokine expression by BMDCs is altered by exposure to epinephrine. Total RNA was extracted from BMDCs. mRNA transcription of IL-12p40, IL-12p35 and IL-23p19 was analyzed by quantitative real-time RT-PCR technique. Relative fold difference of mRNA transcription in epinephrine only group, epinephrine-LPS treated (EL) group and LPS only group was compared to un-stimulated control BMDC group as 1. Asterisk (*) indicates significant group differences in control/epinephrine only vs other groups and sharp (#) indicates significant differences in epinephrine pre-treated (EL) vs LPS only group (A). BMDCs were also treated by epinephrine (10^{-6} M) with or without various concentrations (10^{-4} ~ 10^{-7} M) of butoxamine for 2 hr followed by LPS (1 μg/ml) challenge for additional 3 hr. All data represent mean (N=3) ± standard error. Asterisk (*) indicates significant difference in control vs other groups and sharp (#) indicates significant differences in EL vs butoxamine treated and LPS only group. To present group differences, one-way ANOVA was used followed by Student-Newman posthoc test.

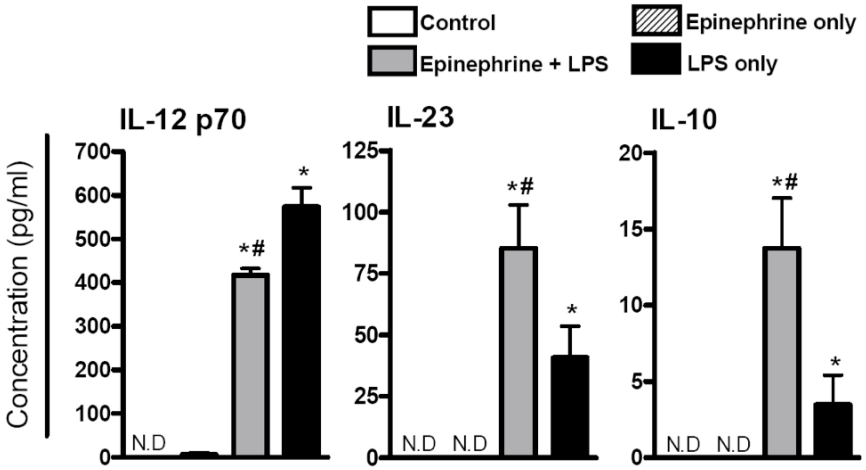

Control
Epinephrine only
Epinephrine + LPS
LPS only
IL-12 p70
IL-23
IL-10
Concentration (pg/ml)
700
600
500
400
300
200
100
0
125
100
75
50
25
0
20
15
10
5
0
N.D
N.D
N.D
N.D
N.D
*#
*
*#
*
*#
*

Figure 3. IL-12p70, IL-23 and IL-10 cytokine production by BMDCs exposed to epinephrine. BMDCs were treated by epinephrine (10^{-6} M) for 2 hr followed by LPS (1 μg/ml) challenge for additional 24 hr. Representative protein expression of IL-12p70, IL-23 and IL-10 from 3 independent experiments was determined from culture media using ELISA technique. All data represent mean (N=3) ± standard error. One-way ANOVA (analysis of variance) was used to determine group differences followed by Student-Newman posthoc test. Asterisk (*) indicates significant difference between control/epinephrine-only group and either EL or LPS-only group. Sharp (#) indicates significant difference between EL and LPS-only group. N.D: not detected.

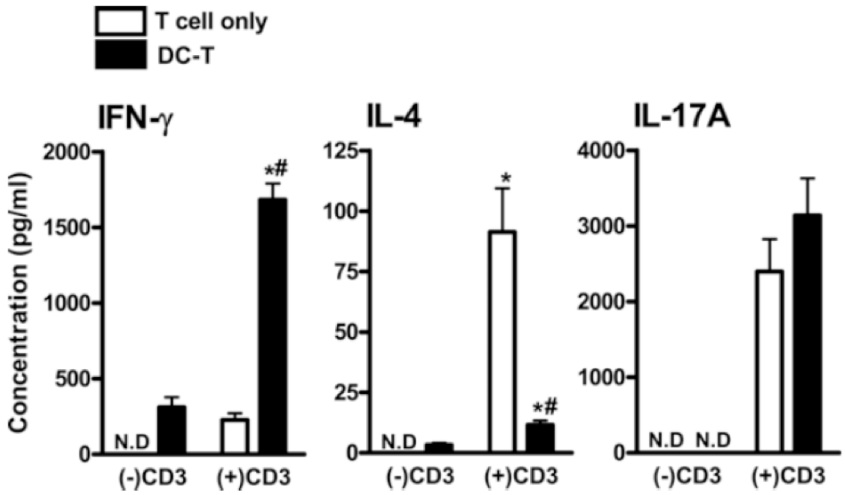

T cell only
DC-T
IFN-γ
IL-4
IL-17A
Concentration (pg/ml)
2000
1500
1000
500
0
125
100
75
50
25
4000
3000
2000
1000
N.D
N.D
N.D
N.D
*
*#
*#
(-)CD3
(+)CD3
(-)CD3
(+)CD3
(-)CD3
(+)CD3

Figure 4. BMDCs support preferential cytokine production of CD3-activated $CD4^+$ T cells. Purified $CD4^+$ T cells were cocultured with BMDCs for 96 hr with or without anti-CD3 monoclonal antibody (2.5 μg/ml). Protein expression of IFN-γ, IL-4, and IL-17A was determined from culture media collected from coculture. All data represent mean (from 3 independent experiments with n=3~5) ± standard error. Two-way ANOVA followed by Bonferroni posthoc test was used to determine group differences. Asterisk (*) indicates statistical difference between (-)CD3 and (+)CD3 group and sharp (#) indicates statistical difference of BMDC-$CD4^+$ T cell coculture group from T cell only control group in each (-)CD3 and (+)CD3 group. N.D: not detected

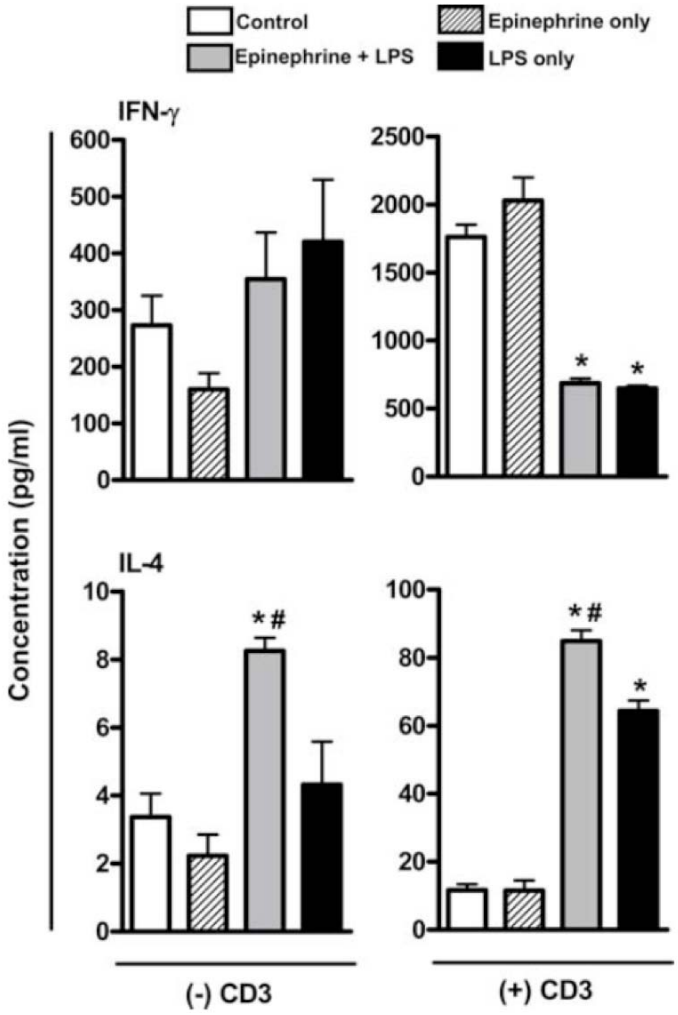

Control
Epinephrine only
Epinephrine + LPS
LPS only
IFN-γ
IL-4
Concentration (pg/ml)
(-) CD3
(+) CD3
*
*#

Figure 5. Epinephrine supports IL-4 production, but not IFN-γ by $CD4^+$ T cells. Purified $CD4^+$ T cells were cocultured with epinephrine-pretreated BMDCs (as shown in figure 2) in epinephrine-free new culture media for 96 hr with or without anti-CD3 monoclonal antibody (2.5 μg/ml). Protein expression of IFN-γ and IL-4 was determined from collected culture media. All data represent mean (from 3 independent experiments with n=3~5) ± standard error. One-way ANOVA (analysis of variance) was used to determine group differences followed by Student-Newman posthoc test. Asterisk (*) indicates significant difference ($p \leq 0.05$) between control/epinephrine-only group and either EL or LPS-only group. Sharp (#) indicates significant difference ($p \leq 0.05$) between EL and LPS-only group.

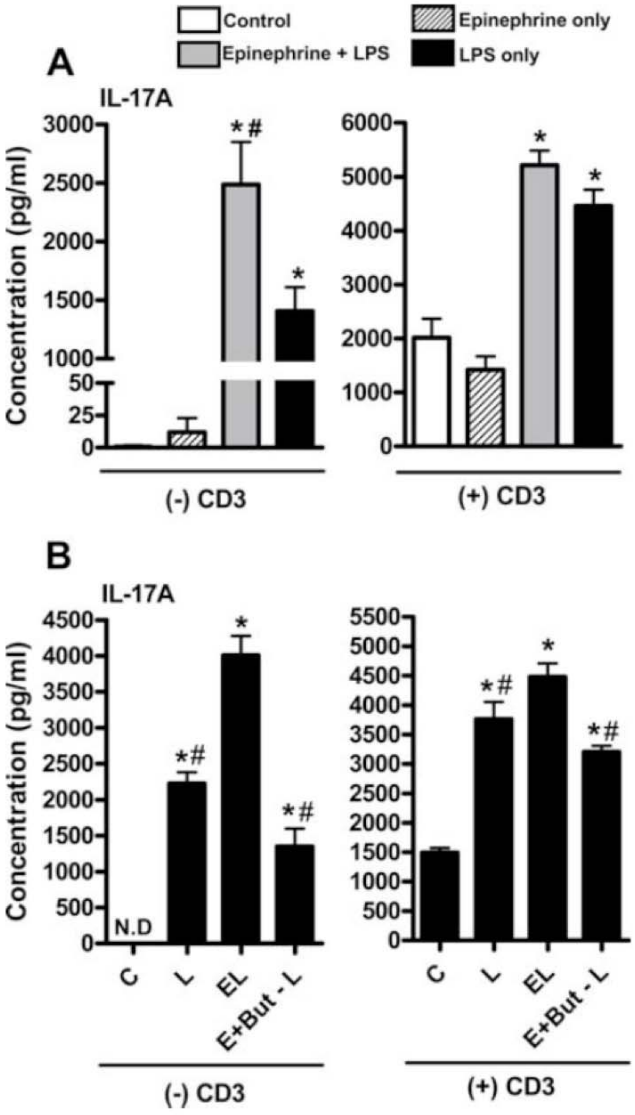

Control
Epinephrine only
Epinephrine + LPS
LPS only
A
IL-17A
Concentration (pg/ml)
3000
2500
2000
1500
1000
50
25
0
6000
5000
4000
3000
2000
1000
0
*#
*
(-) CD3
(+) CD3
B
IL-17A
Concentration (pg/ml)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
N.D
C
L
EL
E+But - L
(-) CD3
(+) CD3

Figure 6. Epinephrine supports IL-17A production through β2-adrenergic receptor by $CD4^+$ T cells. Purified $CD4^+$ T cells were cocultured with epinephrine-pretreated BMDCs in epinephrine-free culture media for 96 hr with or without anti-CD3 monoclonal antibody (2.5 mg/ml). Protein expression of IL-17A was determined from collected culture media. All data represent mean (from 3 independent experiments with n=3~5) ± standard error. Asterisk (*) indicates significant difference in control/epinephrine-only group vs other groups and sharp (#) indicates significant difference between EL and LPS-only group (A). BMDCs were also cocultured with purified $CD4^+$ T cells after stimulation by either epinephrine only or epinephrine with butoxamine (10^{-6} M). Concentration of IL-17A was determined from culture media after coculture after 96 hrs. All data represent mean (n=3) ± standard error. Asterisk (*) indicates significant difference in control vs other groups and sharp (#) indicates significant difference of EL group from butoxamine-treated or LPS-only positive control (B). One-way ANOVA (analysis of variance) was used to determine group differences followed by Student-Newman posthoc test. N.D: no detective cytokine concentration.

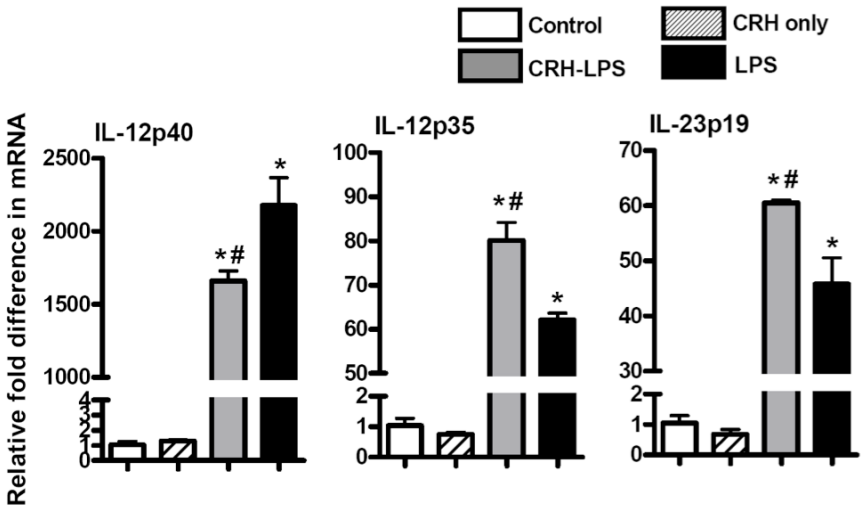

Control
CRH only
CRH-LPS
LPS
Relative fold difference in mRNA
IL-12p40
IL-12p35
IL-23p19
2500
2000
1500
1000
4
3
2
1
0
100
90
80
70
60
50
2
1
0
70
60
50
40
30
2
1
0
*#
*
*#
*
*#
*

Figure 7. $CD4^+$ T cell fate-determining cytokine expression by BMDCs is predicted by exposure to CRH. Total RNA was extracted from BMDCs. BMDCs were treated by CRH (2×10^{-6} M) for 2 hr followed by LPS (1 μg/ml) challenge for additional 24 hr (CRH-LPS). Relative fold difference of mRNA transcription was determined using real-time RT-PCR technique as compared to other groups, no treatment (control), CRH only and LPS only as positive control. Asterisk (*) indicates significant group differences in control/CRH only vs other groups and sharp (#) indicates significant differences in CRH pre-treated (EL) vs LPS only group.

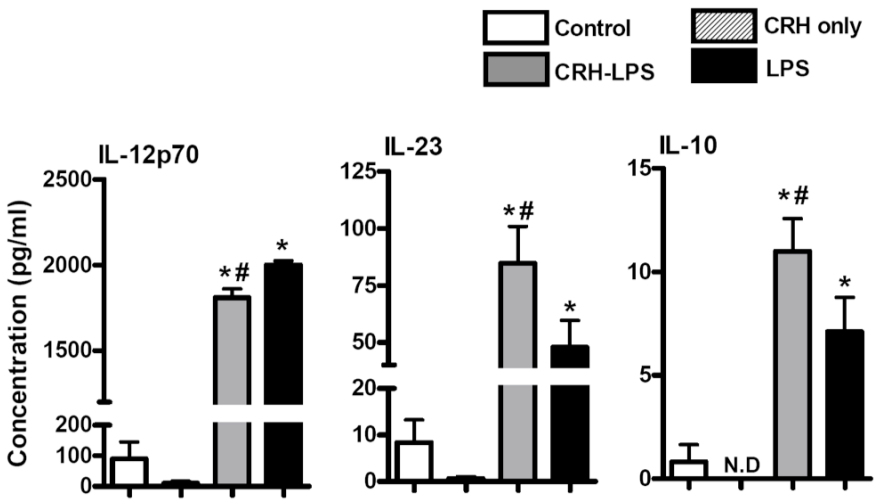

Control
CRH only
CRH-LPS
LPS
IL-12p70
IL-23
IL-10
Concentration (pg/ml)
2500
2000
1500
200
100
0
125
100
75
50
20
10
0
15
10
5
0
*#
*
N.D

Figure 8. IL-12p70, IL-23 and IL-10 cytokine production by BMDCs exposed to CRH. BMDCs were treated by CRH (2 x 10^{-6} M) for 2 hr followed by LPS (1 μg/ml) challenge for additional 24 hr (CRH-LPS). Representative protein expression of IL-12p70, IL-23 and IL-10 from 3 independent experiments was determined from culture media using ELISA technique as compared to other groups, no treatment (control), CRH only and LPS only as positive control. All data represent mean (N=3) ± standard error. One-way ANOVA (analysis of variance) was used to determine group differences followed by Student-Newman posthoc test. Asterisk (*) indicates significant difference between control/CRH-only group and either CRH-LPS or LPS-only group. Sharp (#) indicates significant difference between CRH-LPS and LPS-only group.

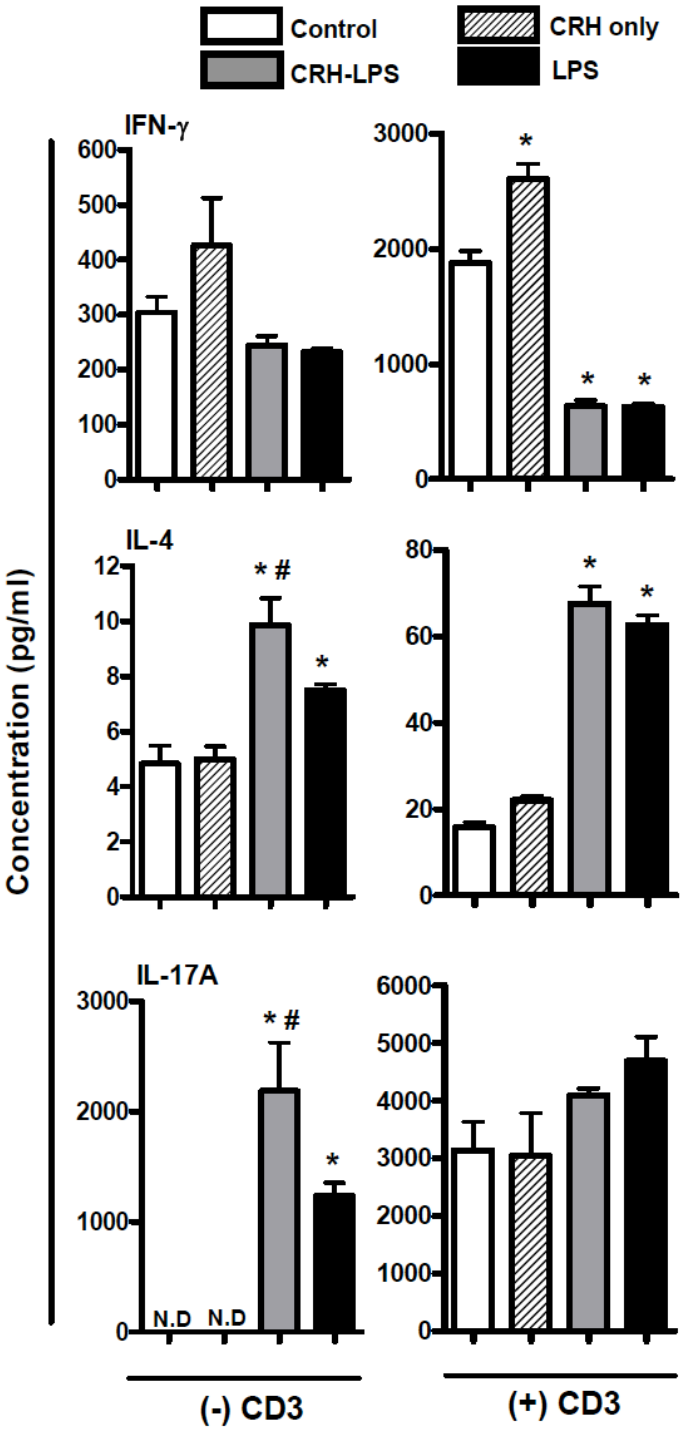

Control
CRH only
CRH-LPS
LPS
IFN-γ
IL-4
IL-17A
Concentration (pg/ml)
N.D
N.D
(-) CD3
(+) CD3

Figure 9. CRH supports IL-4 and IL-17A production, but not IFN-γ by $CD4^+$ T cells in the absence of CD3 stimulation. Purified $CD4^+$ T cells were cocultured with CRH-pretreated BMDCs in CRH-free new culture media for 96 hr with or without anti-CD3 monoclonal antibody (2.5 μg/ml). Protein expression of IFN-γ and IL-4 was determined from collected culture media. All data represent mean (from 3 independent experiments with n=3~5) ± standard error. One-way ANOVA (analysis of variance) was used to determine group differences followed by Student-Newman posthoc test. Asterisk (*) indicates significant difference ($p \leq 0.05$) between control/CRH-only group and either CRH-LPS or LPS-only group. Sharp (#) indicates significant difference ($p \leq 0.05$) between EL and LPS-only group.

Hypothetical model of epinephrine/CRH mediated regulation in CD4+ T cell activation

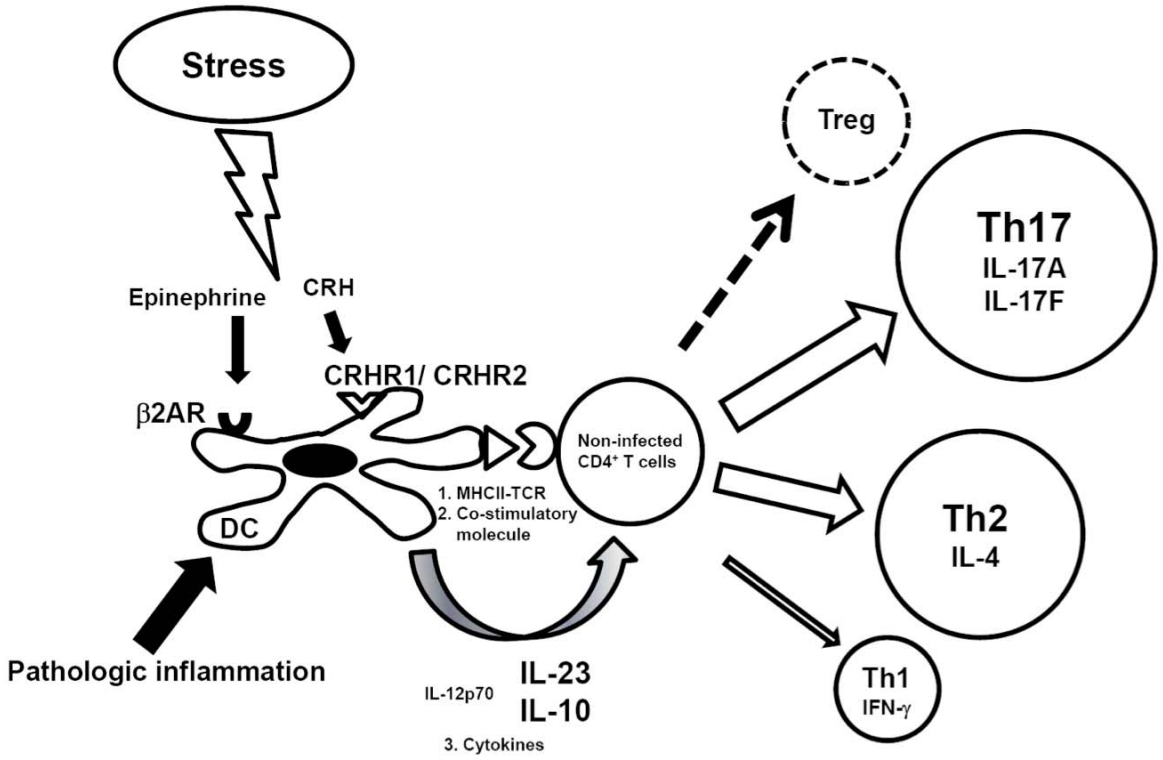
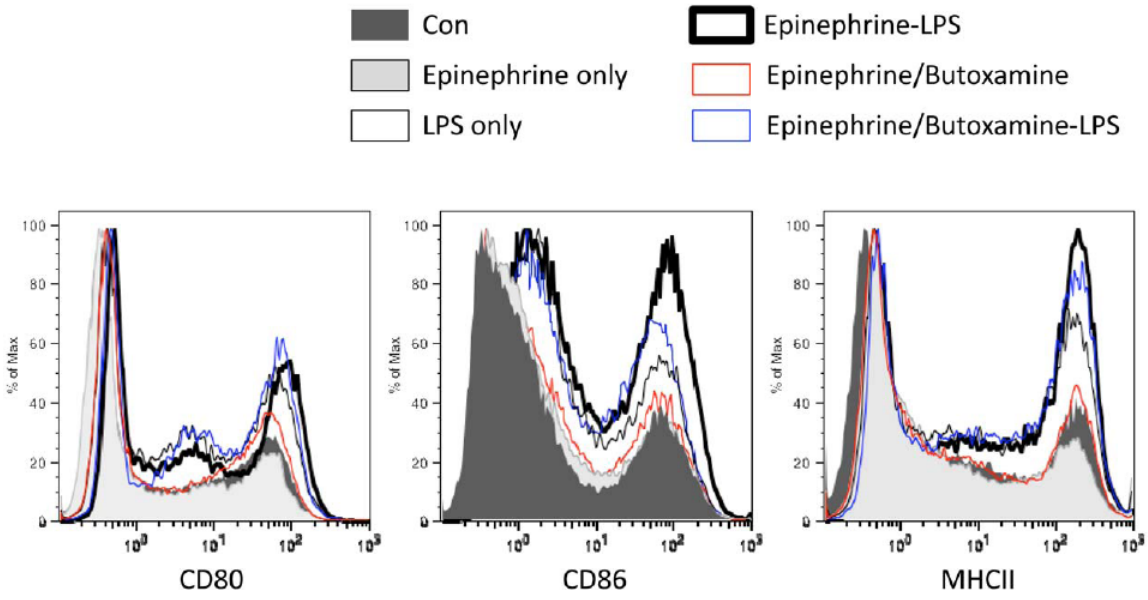


Figure 10. Hypothetical model of epinephrine/CRH-mediated regulation of dendritic cell function to generate adaptive immunity. Diagram represents our hypothetical model that stress-induced epinephrine impacts dendritic cells through β2-adrenergic receptor and CRH receptor 1 and 2 to generate dominant IL-23 and IL-10 production in response to pathogenic activation, and as a consequence, drive $CD4^+$ T cell-mediated adaptive immunity into Th2/Th17 type. β2AR: β2-adrenergic receptor, CRHR1: CRH receptor 1. CRHR2: CRH receptor 2.

Supplemental figure 1.



Supplemental figure 1. Flow cytometry for MHCII, CD80 and CD86 surface expression on BMDC. Surface expression of MHCII and co-stimulatory (CD80 and CD86) molecule was determined by flow cytometry after 24 hr of LPS stimulation. Experimental groups were non-stimulated control (con), epinephrine pretreated with no LPS stimulation (epinephrine only), epinephrine/butoxamine pretreated with no LPS stimulation (epinephrine/butoxamine only), epinephrine pretreated with LPS stimulation (epinephrine-LPS), epinephrine/butoxamine pretreated with LPS stimulation (epinephrine/butoxamine-LPS) and stimulated by only LPS (LPS) groups.

CHAPTER III

IL-23 MEDIATES NEUTROPHIL INDEPENDENT PROTECTION ON AGAINST PULMONARY *STEPTOCOCCUES PNEUMONIAE*–INDUCED SEPSIS IN MICE

IL-23 plays a crucial role in establishing host defenses against extracellular pathogens. Further investigation is still required to define the impact of IL-23 on acute pulmonary *S. pneumoniae* infection. We hypothesize that IL-23 plays an important role in protection against *S. pneumoniae*-induced sepsis. Utilizing IL-23p19 deficient mice, we determined bacterial load, cytokine production and the contribution of neutrophils against *S. pneumoniae* infection using monoclonal antibody-mediated systemic neutrophil depletion. The absence of IL-23 induced a significantly higher bacterial load in lung 2 days after *S. pneumoniae* infection. The bacterial load in the blood was also significantly higher in IL-23 knockout mice 18 hr and 2 days after infection. IL-6 and IL-12p70 cytokine production was significantly reduced in bronchiolar lavage fluid (BALF) of IL-23p19 knockout mice. IL-23 deficiency also resulted in a significant reduction in IL-17A and IFN-γ cytokine production by lung lymphocytes as compared to wild type mice. In addition, the absence of IL-23 corresponded with lower neutrophils in lung at 18 hr and higher neutrophils in blood 2 days after infection. These finding suggested impaired neutrophil recruitment into lung. Interestingly, neutrophil depletion did not impact bacterial load in lung and blood in both IL-23 competent and deficient mice. These findings demonstrated that

IL-23 plays a yet undefined protective role independent of neutrophilic host defenses against pulmonary *S. pneumoniae* infection. Future investigations are needed to clarify novel defense mechanisms generated by IL-23 against *S. pneumoniae* infection.

INTRODUCTION

Community acquired pneumonia (CAP) is a leading cause of morbidity and mortality attributed by infectious disease (245, 246). *Streptococcus pneumoniae* (*S. pneumoniae*) is a gram-positive bacterium and the most frequent cause of acute lower respiratory infection and death due to sepsis (247). Immune defense against pulmonary *S. pneumoniae* infection represent a double-edge sword, in which hypo or hyper-inflammatory responses can lead to insufficient bactericidal effects or promote acute and chronic immunopathogenesis, respectively (248, 249). Thus, clinical outcomes of *S. pneumoniae*-induced sepsis rely heavily on maintaining a balanced immune response.

Neutrophils are the most abundant leukocytes in blood circulation that largely participate in the defense against bacterial and fungal pathogens (250, 251). During the initial stages of *S. pneumoniae* infection, neutrophils are recruited locally by chemotactic factors (e.g. chemokines) produced by lung epithelial and endothelial cells in response to bacterial invasion and breach of the lung epithelial barrier (252). Through adhesion molecule expression, neutrophils leave the blood and cross into the tissue site where they are capable of phagocytosis and oxidative-mediated intracellular killing of bacterium (253). By contrast, neutrophils can promote immunopathogenic responses through destructive tissue damage in an attempt to clear the infection. Previous studies have documented that hyperactive oxidative responses by neutrophils contribute to excessive inflammation and tissue damage, prompting escape of *S. pneumoniae* from the pulmonary compartment, resulting in systemic spread and septic conditions (254, 255).

Thus, neutrophils are an immune constituent representing the dichotomy between protection and pathogenesis against *S. pneumoniae* infection.

Cytokine balance is believed to have a critical influence in achieving optimal immune defenses against respiratory pathogens, including *S. pneumoniae* (256). IL-23 is a heterodimeric cytokine composed of IL-12p40 shared with IL-12p70 and a unique IL-23p19 subunit (122) preferentially produced by macrophages and dendritic cells (122). IL-23 plays pivotal roles in maintaining interleukin-17 (IL-17) production by $CD4^+$ T helper (107) and $CD8^+$ cytotoxic T cells (257), which are important for the recruitment of immune cells, particularly neutrophils to sites of infection by the induction of various chemotactic factors such as CXCL1 and CXCL8 (191). IL-23 is also important for the induction of IL-22, a known mediator of antimicrobial peptide production (130-132). Increasing evidence suggest IL-23 may be a focal mediator in balancing inflammatory responses against pathogens. For example, IL-23 is known to mediate protection against immunopathology by *Toxoplasma gondii* (132). Kleinschek et al., also showed that pathogenesis against fungal infection of *Cryptococcus neoformans* was significantly decreased by systemic administration of IL-23 through (130, 131). These findings support a central role of IL-23 in regulating effective cytokine communication against bacterial pathogenesis. Few studies have investigated the role of IL-23 in regulation of *S. pneumoniae* infection. Ma et al., showed that morphine-induced severe pathogenesis in *S.pneumoniae* infection was closely related with decreased IL-17 production associated with lower IL-23 (162). However, no direct evidence has shown IL-23-dependency related to subsequent cytokine responses and its impact on neutrophil cell recruitment against pulmonary *S. pneumoniae* infection. The current study examined the relationship between the recruitment of neutrophils and the activation of inflammatory cytokines, controlled by IL-23 expression. IL-23 deficiency

resulted in higher bacterial burdens, particularly in blood at early time points. Impaired IL-17A production corresponded with lower neutrophil percentages in lung in the absence of IL-23. However, neutrophil depletion did not impact bacterial clearance. Our results indicate a potential novel role of IL-23 independent of neutrophils function.

MATERIALS AND METHODS

Mice

C57BL/6 (B6) mice were purchased from the Charles River (Charles River Laboratories International Inc. Wilmington, MA). IL-23p19 KO mice were kindly provided by Dr. Nico Ghilardi at Genentech, Inc. Female mice (6~8 weeks old) were used for all experiments. Mice were maintained under specific pathogen-free conditions on a 12:12 light/dark cycle (7:00 PM to 7:00 AM) with food and water *ad libitum*. All animal studies were performed under the approval of the Institutional Animal Care and Use Committee at the University of North Texas Health Science Center.

Streptococcus pneumoniae and intranasal infection

Streptococcus pneumoniae (*S. pneumoniae*) strain #6301 (ATCC, Manassas, VA) was grown for 16 hr to obtain mid-log phases cultures on blood agar plates. Mice were intranasally infected with *S. pneumoniae* (2 x10^6 cells) in a volume of 40 μl of Brain-Heart Infusion Broth (EMD, EMD Chemicals Inc. Gibbwtown, NJ) after anesthesia.

Colony forming assay

Bacterial load in lung, spleen and blood were evaluated at two different time points (18 and 48 hr) after infection. Upon sacrifice, tissues were harvested and homogenized in PBS. Tenfold serial dilution of organ homogenates were plated onto blood agar plates and incubated overnight at 37°C with 5% CO_2. Colonies on plates were enumerated, and the results were expressed as $\log_{10}$CFU per ml of wet volume of organ.

BALF isolation, single cell preparation of total lung cells and in vitro culture.

Bronchoalveolar lavage fluid (BALF) was prepared by intratracheal perfusion with 1ml of 1x PBS using 25G blunt-end needle. After removing cells by centrifugation, collected BALF was used for ELISA analysis. Single cell suspensions of mononuclear cell from lung tissue were prepared as previously described (258, 259). Briefly, lung tissues were finely minced after separation into single lobe and incubate in collagenase digestion media containing 300 unit/ml collagenase type II (Worthington, Lakewood, NJ) and 50 unit/ml DNase (Sigma–Aldrich, St. Louis, MO) in RPMI 1640 culture media for 1 hr 30 min. After digestion, lungs were passed through a nylon mesh filter (LabPak, Depew, NY) into sterile 50 ml conical tubes and washed twice in wash media (Hyclone, Logan, UT). Lung mononuclear cells were prepared by ficoll-hypaque (Lympholyte M, Cedarlane, Laboratories, Ltd., Ontario, CA) centrifugation. Spleen cell suspensions were prepared by mashing spleen tissue through sterile mesh. Contaminating RBCs were removed using ACK lysis buffer as previously described (215). Isolated cells were further characterized by flow cytometry or used for *in vitro* culture. 4 x 10^5 cells were placed in each

well of flat-bottom 96 well plates with or without heat-killed *S. pneumoniae* (HKSP) under at a ratio of 1:500 (cells: HKSP). After 96 hr of culture, supernatant was collected and used for ELISA to evaluate cytokine concentrations.

Peripheral blood mononuclear cell (PBMC) preparation for flow cytometry

Whole mouse blood was collected by retro-orbital bleeding method using heparinized capillary glass tube after anesthesia. After collection, whole blood was placed in heparinized tube. Red blood cells were lyzed by 20 volume of ACK lysis buffer at room temperature for 10 min. After centrifugation, cells were washed twice with 1xPBS containing 1% FBS to eliminate lyzed blood cell debris.

Flow cytometry

Mouse PBMC or isolated total lung cells were characterized by fluorescence immunostaining and flow cytometryic analysis. Single cell suspension of PBMC or lung cells in flow staining buffer were incubated for 10 min at 4 °C with anti-Fc receptor antibody (Fc blocker, clone 2.4G2) (BD Pharmingen, San Diego, CA) to prevent non-specific binding of antibody Fc region to Fc receptor. Two color immunostaining to identify neutrophils was performed for 30 min in 4 °C using PE labeled anti-mouse Ly6G (1A8) and PEcy7 labeled anti-mouse CD11b. After washing, cells were fixed using 2 % paraformaldehyde. Gating of live cells was identified by forward-scatter/side-scatter profile. Percent positive staining was determined by subtracting autofluorescenced cells from non-stained negative control. Data were collected on

Cytomic FC500 (Beckman–Coulter, Miami, FL). Further analysis was performed using CXP software (Beckman–Coulter). Absolute cell numbers was determined by multiplying the percent positive cells by the total number of cells isolated from lung tissue.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of IFN-γ, IL-4, IL-6, IL-10, IL-17A and IL-12p70 in BALF or total lung cell culture were determined by sandwich ELISA method. All procedures were performed as described by the manufacturer. Briefly, flat-bottom 96-well plates were coated with optimal concentration of capture antibody for 16 hr at 4°C and followed by blocking (10% FBS in PBS) for 16 hr at 4°C. After incubation of samples at 4°C for 16 hr, plates were incubated with biotin-conjugated detection antibody and streptavidin-HRP (horseradish peroxidase. After adding tetramethylbenzidine (TMB) peroxidase substrate solution (Rockland Immunochemicals, Inc. Gilbertsville, PA), the concentration of each cytokine was determined according to standard curved generated by reference concentration of cytokine at wavelength of 450 nm detected by colorimetric plate reader (Biotek Instruments Inc. Winooski, VT). ELISA antibody set and recombinant cytokine for standard were purchased from eBiosicence (eBiosciences, San Diego, CA) for IL-12p70, BD PharMingen Inc. for IFN-γ, IL-4, IL-6, IL-10 ELISA sets and IL-17A antibody pairs and R&D Systems (R&D Systems Inc. Minneapolis, MN) for recombinant IL-17A.

Neutrophil depletion

Neutrophils were depleted by intraperitoneal injection of purified Ly6G (1A8) monoclonal antibody (BioXcell, West Lebanon, NH) with concentration of 0.5 mg/mouse in 500 µl of sterile 1X PBS. Injection was performed every 24 hr for two days. Depletion was confirmed by differential staining of whole blood (~98 %).

Statistical analysis

Statistical analysis was performed using GraphPad Prism Version 5.0 (GraphPad Software, San Diego, USA). For multi-experimental group analysis, data were subjected to one-way and two-way ANOVA (analysis of variance) followed by post hoc tests (Newman-Keuls and Bonfferoni) for group differences. All data are expressed as means ± standard error of mean (SEM). The level of significance was set at $p \leq 0.05$ for group differences.

RESULTS

IL-23 deficiency promotes bacterial dissemination from the lung.

To investigate the role of IL-23 on resistance against pulmonary *S. pneumoniae* infection, bacterial colonization between IL-23p19 gene deficient (KO) and wild-type (WT) C57/BL6 mice was monitored in the lung, spleen and blood. No significant difference in bacterial numbers in the lung was found 18 hr after infection. Yet, a significantly ($p \leq 0.05$)

higher number of bacteria were present in blood of IL-23 deficient mice. By 48 hrs, IL-23 KO mice revealed significantly ($p \leq 0.05$) higher bacterial counts in both lung and blood. No significant differences between IL-23p19 KO and WT mice were found in spleen at both time points after infection (figure 1).

Reduced production of IL-6, IL-12p70, IL-17A and IFN-γ is associated with IL-23 deficiency against pulmonary S. pneumoniae infection.

Preferences in cytokines produced during acute bacterial infection can impact disease outcome. We determined whether the lack of IL-23 cytokine production could impact cytokine responses during acute *S. pneumoniae* infection. Protein concentration of IL-4, IL-6, IL-10, IL-12p70, IL-17A and IFN-γ was determined from BALF and total lung immune cell cultures stimulated with heat-killed *S. pneumoniae* antigen. First, we examined protein concentration of primary pro-and anti-inflammatory cytokines, IL-6, IL-10 and IL-12p70. BALF from IL-23p19KO mice demonstrated a significantly ($p \leq 0.05$) lower IL-6 concentration after 18 hr of infection as compared to WT mice. IL-12p70 was also significantly lower ($p \leq 0.05$) in IL-23p19KO mice as compared to WT mice. No significant difference was shown in IL-10 or IL-17A between WT and IL-23p19KO mice (figure 2).

We next examined protein concentration of T cell-derived cytokines IL-17A, IFN-γ and IL-4 influenced by IL-6, IL-12p70 and IL-10 from lung tissue. Total lung immune cells were isolated 18 hr after infection from WT and IL-23p19KO mice and cultured with presence or absence of HSKP for 96 hr. We determined the concentration of IFN-γ, IL-4 and IL-17A

cytokine concentration from culture supernatant. Interestingly, as compared to WT, total lung cells from IL-23p19KO mice produced significantly ($p \leq 0.05$) lower concentration of IFN-γ by HKSP stimulation. HKSP stimulation on total lung cells also induced further production of IL-17A. However, concentration of IL-17A in total lung cells isolated from IL-23p19KO cells was significantly ($p \leq 0.05$) lower as compared to WT cells. In addition, concentration of IL-4 was not influenced by HKSP stimulation and no significant difference was shown between WT and IL-23p19KO cells with or without HKSP stimulation (figure 3).

IL-23 deficiency abrogates neutrophilic response to pulmonary S. pneumoniae infection.

Based on the findings above, we predicted that an attenuation in IL-17 production due to the lack of IL-23 would regulate neutrophillic responses. Thus, we determined the percentage of neutrophils in response to nasal *S. pneumoniae* infection in lung and blood. The percentage of neutrophil in the lung of IL-23p19 KO mice was significantly ($p \leq 0.05$) lower at 18 hr after *S. pneumoniae* infection as compared to WT counterparts. No significant difference in blood was found at 18 hr after *S. pneumoniae* infection (figure 4A). By contrast, the percentage of neutrophils in blood was significantly ($p \leq 0.05$) higher in IL-23p19KO mice at 2-days after *S. pneumoniae* infection as compared to WT. The significantly ($p \leq 0.05$) higher neutrophil in blood of IL-23p19KO mice was shown with no difference in lung neutrophil between WT and IL-23p19KO (figure 4B). These findings indicate that a correlation between IL-23-mediated IL-17 cytokine activation and nuetrophillic responses was associated with disease progression.

Neutrophil depletion does not impact bacterial load in the absence of IL-23 against S. pneumoniae infection.

To confirm the functional impact of IL-23 deficiency on neutrophil responses, we examined impact of IL-23 deficiency in the presence or absence of neutrophils, and how this would impact S. *pneumoniae* infection. Mice were administered anti-mouse Ly6G monoclonal antibody to deplete neutrophils. Mice were intranasally infected with *S. pneumoniae* and bacterial burden in lung, spleen and blood was examined after 18 hrs. No significant difference of lung bacterial load between isotype control and neutrophil depleted group was shown in both WT and IL-23p19KO mice. No detectable CFU was shown from spleen (data now shown). Bacterial load in IL-23p19KO was significantly higher in blood as compared to their counterpart. However, neutrophil depletion did not alter blood bacterial burden as compared to isotype control in both WT and IL-23p19KO mice (figure 5). Thus, our results suggested a neutrophil-independent mechanism associated with *S. pneumoniae*-induced pathogenesis.

DISCUSSION

The IL-23/IL-17 cytokine axis is an important regulator of various bacterial infections (260). In addition to its impact on IL-17 activity, IL-23 also induces production of IL-22, which is important for anti-microbial peptide production in response to bacterial infection (131, 132). Ma et al., also suggested that downregulation of IL-23 associated with IL-17 is accompanied with severe pathogenesis by *S. pneumoniae* infection (162). However, to date, no study has directly demonstrated the role of IL-23 in regulation of immune responses against *S.*

pneumoniae. Based on these findings, we determined role of IL-23 in a *S. pneumoniae* infection model using genetic deficient animal model for IL-23p19 subunit

IL-17A is known as a key cytokine produced by Th17 T cells which binds to target cells including epithelial and endothelial cells. Activation of epithelial and endothelial cells by IL-17A results in chemokine production and upregulation of adhesion molecules such as CXCL1, ICAM-1 (191, 193, 261, 262). These events ultimately contribute to the recruitment of immune cells such as neutrophils and monocytes to the site of inflammation (263). It is known that IL-23 is essential in maintaining Th17 phenotype (107), as evidence in studies, which demonstrated that a lack of IL-23 resulted in decreased IL-17A production (126, 127). In support, our data showed that the absence of IL-23 resulted in a significant decrease of IL-17A production by HKSP-stimulated total lung tissue cells (figure 3). Importantly, a higher percentage of neutrophils were detected in blood whereas significantly lower percentage was detected in lung at 18 hrs (figure 4). This result demonstrated that impaired neutrophil recruitment into lung was induced by a lower production of IL-17A is due to the absence of IL-23. In support, Meeks et al., reported that the IL-23/IL-17 axis is important for optimal neutrophil recruitment into the liver, which is essential for clearance of *Listeria monocytogenes* (264). Therefore, our findings suggest that an absence of IL-23 also significantly influences expression of IL-17A in a pulmonary *S. pneumoniae* infection.

Epithelial and endothelial cell activation by IL-17A binding results in neutrophil recruitment (191). Neutrophils are recruited in response to chemotatic factors produced from sites of inflammation. Through their phagocytic activity and production of reactive oxygen species (ROS) production to kill pathogens, neutrophils plays a pivotal role in innate immune

response against infectious diseases by extracelluar and intracellular pathogens such as *Listeria monocytogenes*, *Salmonella typhimurium*, and *S. pneumoniae* (264-270). In support, Aujla et al., suggested that the IL-23/IL-17 axis provides a critical barrier system against extracellular bacteria by regulating chemokine gradients for neutrophil emigration (271, 272). In this report, neutrophils are proposed to prevent dissemination and increase clearance of invading bacteria. Furthermore, Noriega et al., also showed that neutrophils are protective against dissemination of *Salmonella typhimurium* in intestine (273). By comparison, our data demonstrated higher bacterial load in lung and blood, particularly at an earlier time point in blood (18hr), representing early dissemination. Furthermore, the lack of IL-23 potentially induced less neutrophil infiltration into lung as compared to WT mice (figure 4). However, surprisingly, neutrophil depletion (figure 5) showed no differences in lung and blood bacterial load compared with the presence of neutrophils at 18 hr after *S. pneumoniae* infection. This suggests that neutrophils may not be a major cell population involved in bacterial clearance against acute *S. pneumoniae* infection. Interestingly, several studies have shown that neutrophil-mediated inflammation resulted in destructive tissue damage and increase pathogenesis (153, 157-160). To date, the role of neutrophils during *S. pneumoniae* infection remains contradictory with respect to their role in pathogenesis and protection. Garvy et al., reported that depletion of neutrophil resulted in greater severity of pneumococcal pneumonia by *S. pneumoniae* infection in adult and neonatal mice (274). In contrast, Marks et al., showed depletion of neutrophil improved survival rate and induced less bacteremia (275).

The type of cytokine responses generated during acute and chronic lung disease is important in determining disease outcome. Interestingly, IL-6 and IL-12p70 (figure 4) cytokine production was significantly impaired in the absence of IL-23 against *S. pneumoniae* infection,

which corresponded with an impaired production of IFN-γ by total lung cell cultures in the presence of HKSP stimulation (figure 5). IL-6 plays pivotal role in proinflammatory responses in several animal bacterial infection models (276-278). With TGF-β, IL-6 is also key cytokine to induce IL-17A producing T cells (103, 123). Dubin et al., previously showed that deficiency of IL-23 resulted in significantly lower IL-6 production against mucoid *Pseudomonas aeruginosa* lung infection in mice (279).

IL-12p70, mainly produced by antigen presenting cells such as dendritic cells and macrophages, is also an important cytokine associated with the induction of Th1 adaptive immunity, including the regulation of IFN-γ production, (105). Shan et al., also reported that IL-23 promoted the production of Th1 cytokines such as IFN-γ, IL-12 and TNF-α (280). Furthermore, Wetering et al., suggested positive feedback loop in which Salmonella-induced IL-23 can enhance IL-12 production with IL-1β (281). In addition, Lin et al., showed that IL-17 is required for IL-12 production against intracellular pathogen *Francisella tularensis* (282). In this report, they suggested a potential regulatory role of IL-23/IL-17 cell pathway to promote IL-12-associated Th1 cell immunity for host responses to an intracellular pathogen. In fact, as shown figure 1, lung *S. pneumoniae* burden was significantly higher in IL-23p19 KO mice which correspond with impaired level of IL-6, IL-12p70, IL-17A and IFN-γ (figure 3 and 4). Therefore, considering previous findings, our data suggests that IL-23 may play a novel role in protection against pulmonary *S. pneumoniae* infection through supporting proinflammatory immune responses conducted by early proinflammatory cytokines and Th1& Th17 cytokines. On the basis of these findings, we propose that IL-23 may play a novel role in preventing early escape of bacteria from site of infection. In human clinical studies, O′Dwyer et al., showed that

severe sepsis in human intensive care unit (ICU) patients is closely related with IL-23 deficiency (283). Neutrophils have been suggested as an effector cell type important in the prevention of systemic dissemination of pathogens. Interestingly however, our data demonstrated that neutrophils did not contribute early dissemination of *S. pneumoniae* induced by IL-23 deficiency (figure 5). These findings suggest that IL-23 may constitutes effective defense system to prevent systemic septicemia against *S. pneumoniae* infection independent of neutrophilic-associated mechanisms. A future understanding of IL-23 cytokine network that connect other cytokines and cell types will provide important insight toward understanding their role in acute pulmonary infection of extracellular bacterial pathogen such as *S. pneumoniae*.

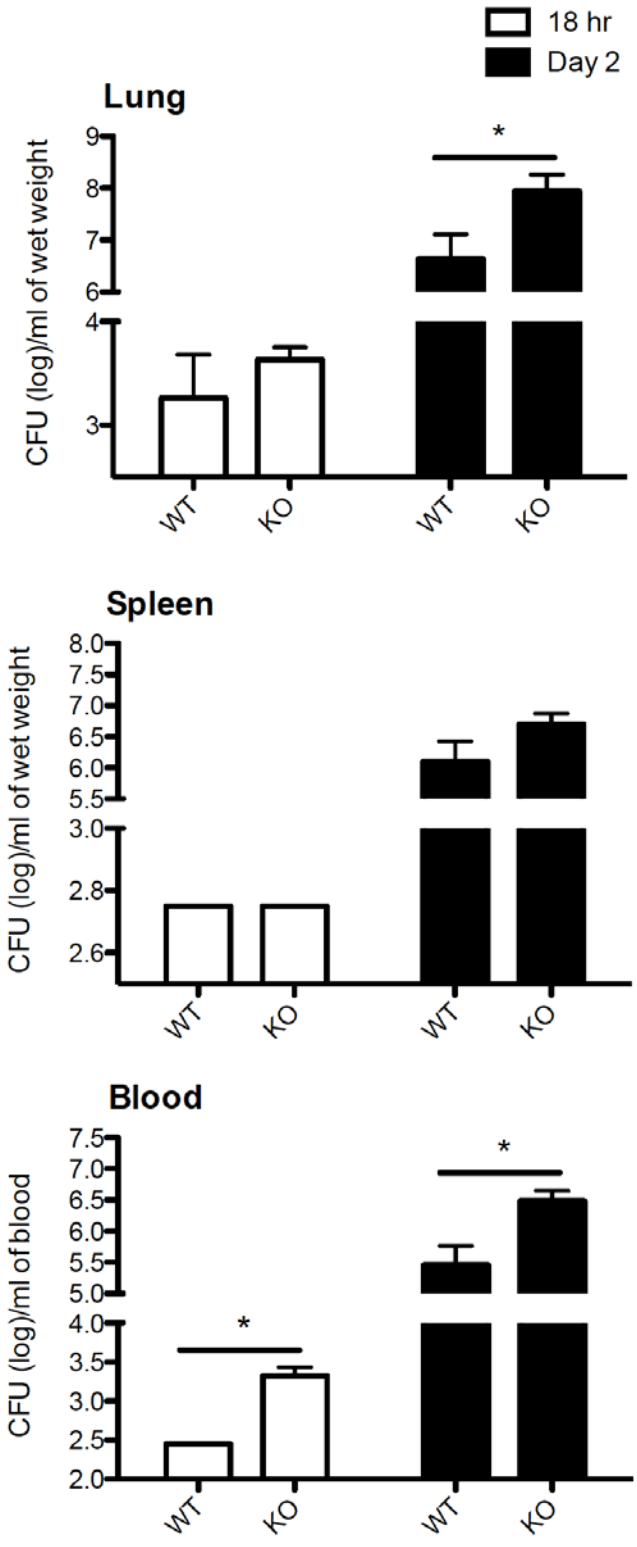

18 hr
Day 2
Lung
CFU (log)/ml of wet weight
9
8
7
6
4
3
*
WT
KO
WT
KO
Spleen
CFU (log)/ml of wet weight
8.0
7.5
7.0
6.5
6.0
5.5
3.0
2.8
2.6
WT
KO
WT
KO
Blood
CFU (log)/ml of blood
7.5
7.0
6.5
6.0
5.5
5.0
4.0
3.5
3.0
2.5
2.0
*
*
WT
KO
WT
KO

Figure 1. Absence of IL-23 induced impaired bacterial clearance in lung and blood. Bacterial CFU was determined from lung, spleen and blood after 18 hr and 2 days of *S. pneumoniae* infection. Data represent mean ± SEM of mice (n=10). Two-way ANOVA was used for testing group variance. Asterisk indicates difference between strain in same time line.

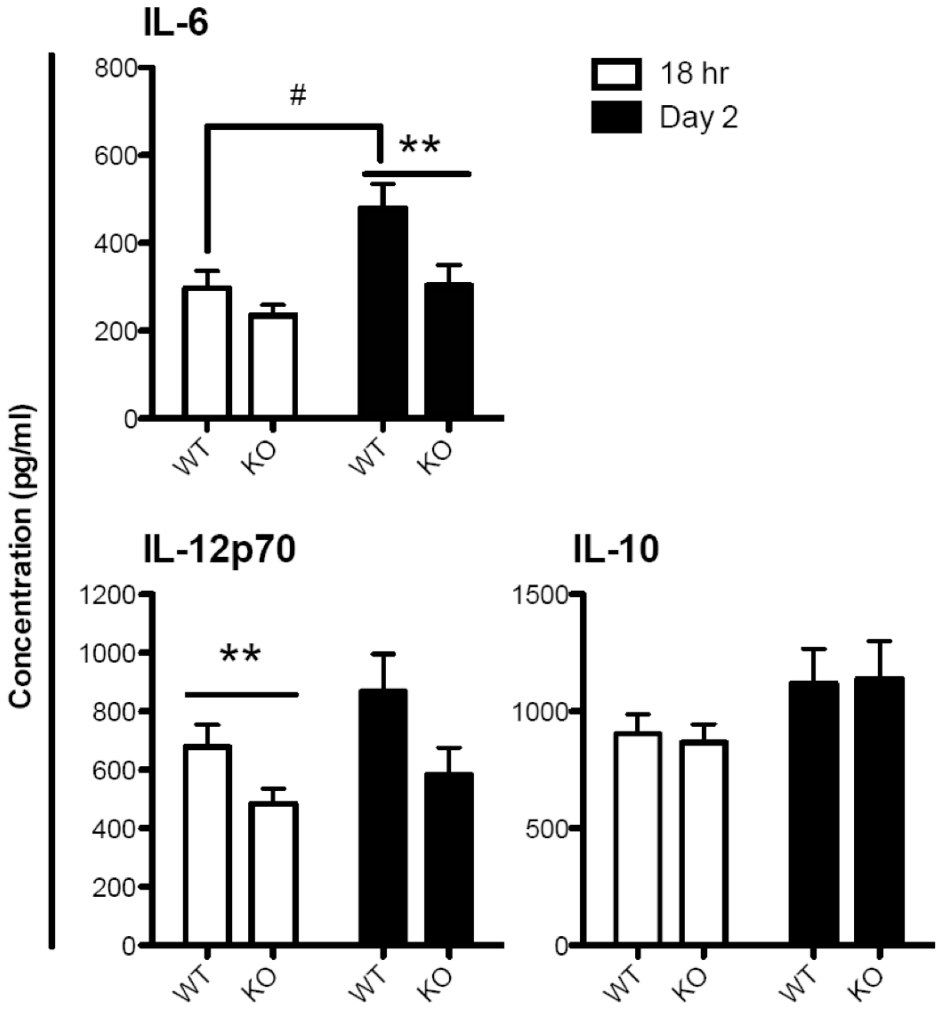

IL-6
18 hr
Day 2
Concentration (pg/ml)
800
600
400
200
0
#
**
WT
KO
WT
KO
IL-12p70
1200
1000
800
600
400
200
0
**
WT
KO
WT
KO
IL-10
1500
1000
500
0
WT
KO
WT
KO

Figure 2. IL-6 and IL-12p70 cytokine production was impaired in BALF by absence of IL-23 against *S. pneumoniae* infection. IL-6, IL-10, IL-12p70 and IL-17A cytokine production was determined in BALF after 18 hr of *S. pneumoniae* infection by ELISA. Data represent mean ± SEM of mice (n=10). Two-way ANOVA was used for testing group variance. Asterisk indicates difference between strain in same time line and sharp indicates difference between different time points.

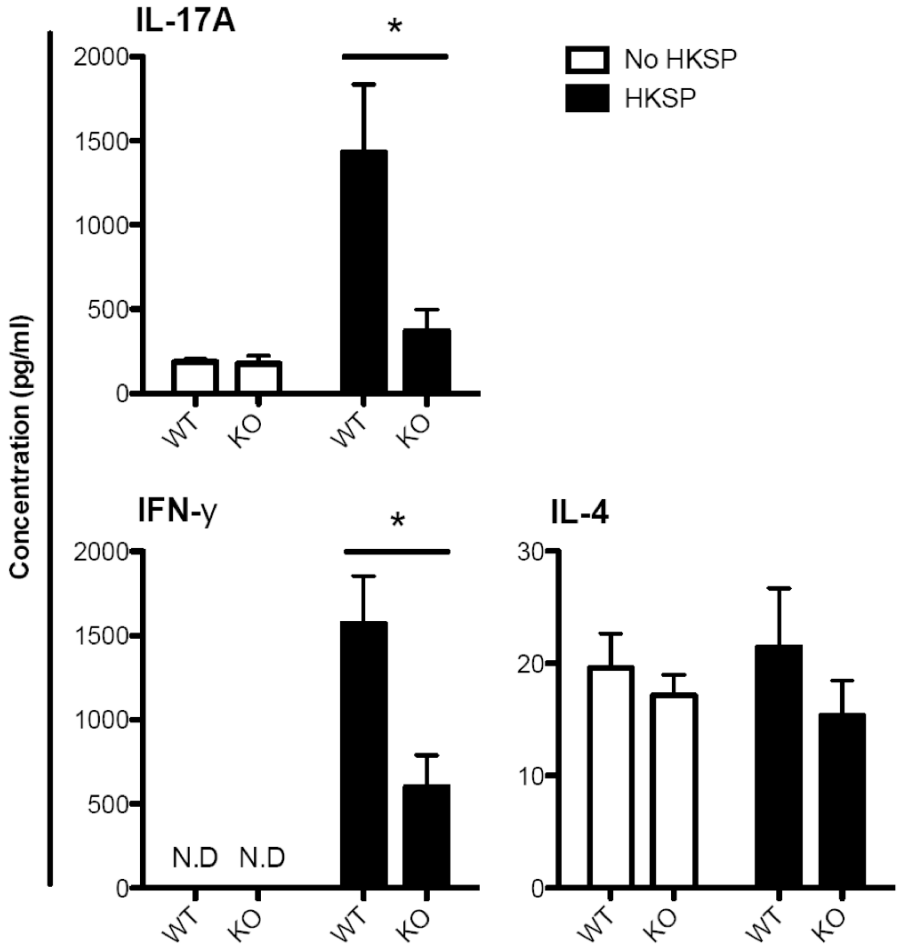

IL-17A
*
No HKSP
HKSP
Concentration (pg/ml)
2000
1500
1000
500
0
WT
KO
WT
KO
IFN-γ
*
N.D
N.D
IL-4
30
20
10

Figure 3. IL-23 deficient total lung cells from *S. pneumoniae*-infected mice produced impaired IFN-γ and IL-17A in response to HKSP stimulation. IFN-γ, IL-4 and IL-17A cytokine production was determined from culture supernatant of total lung cell isolated after 18 hr of *S. pneumoniae* infection with or without HKSP stimulation by ELISA. Data represent mean ± SEM of cytokine concentrations from mice (n=10). Two-way ANOVA was used for testing group variance. Asterisk indicates difference between strain in same time line and sharp indicates difference between different time points.

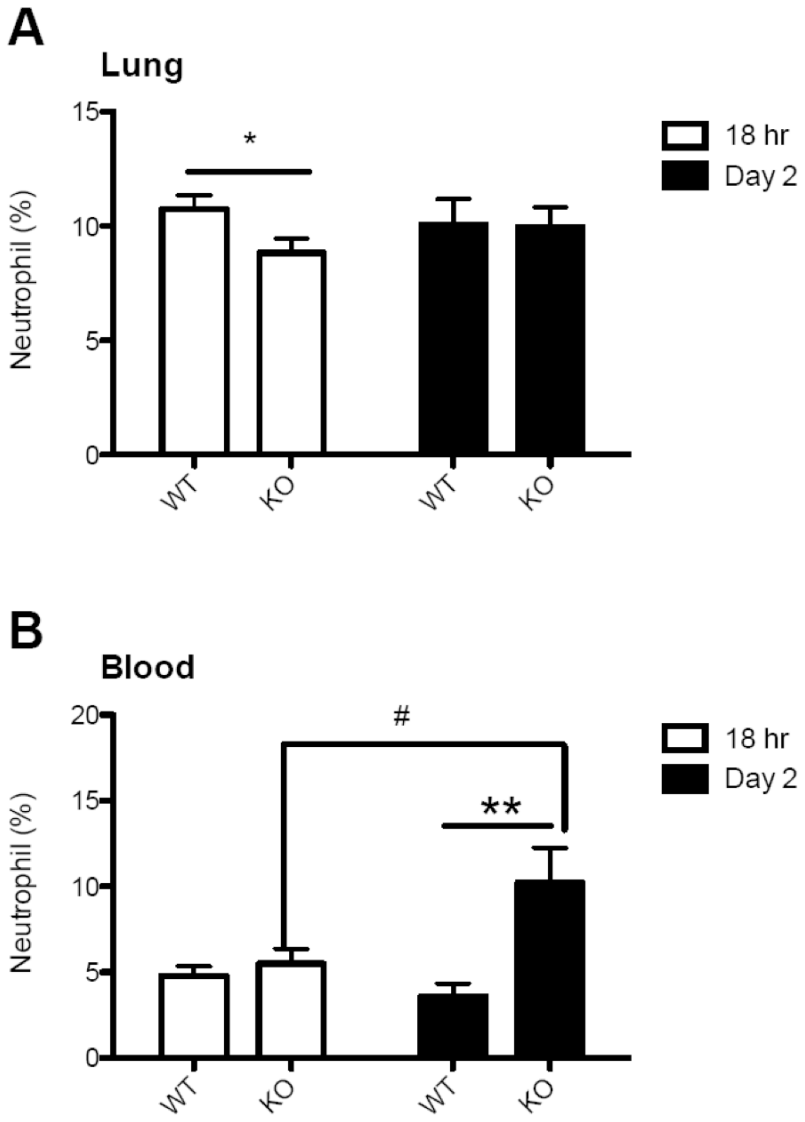

A
Lung
Neutrophil (%)
15
10
5
0
*
WT
KO
WT
KO
18 hr
Day 2
B
Blood
Neutrophil (%)
20
15
10
5
0
#
**
WT
KO
WT
KO
18 hr
Day 2

Figure 4. IL-23 deficiency abrogates neutrophilic response to pulmonary S. pneumoniae infection. Population of neutrophils ($Ly6G^+$ $CD11b^+$) was determined from lung and whole blood by flow cytomtey after 18 hr and 2 days after *S. pnumoniae* infection. Data represent mean ± SEM of mice (n=15). Two-way ANOVA was used for testing group variance. Asterisk indicates difference between strain in same time line and sharp indicates difference between different time points.

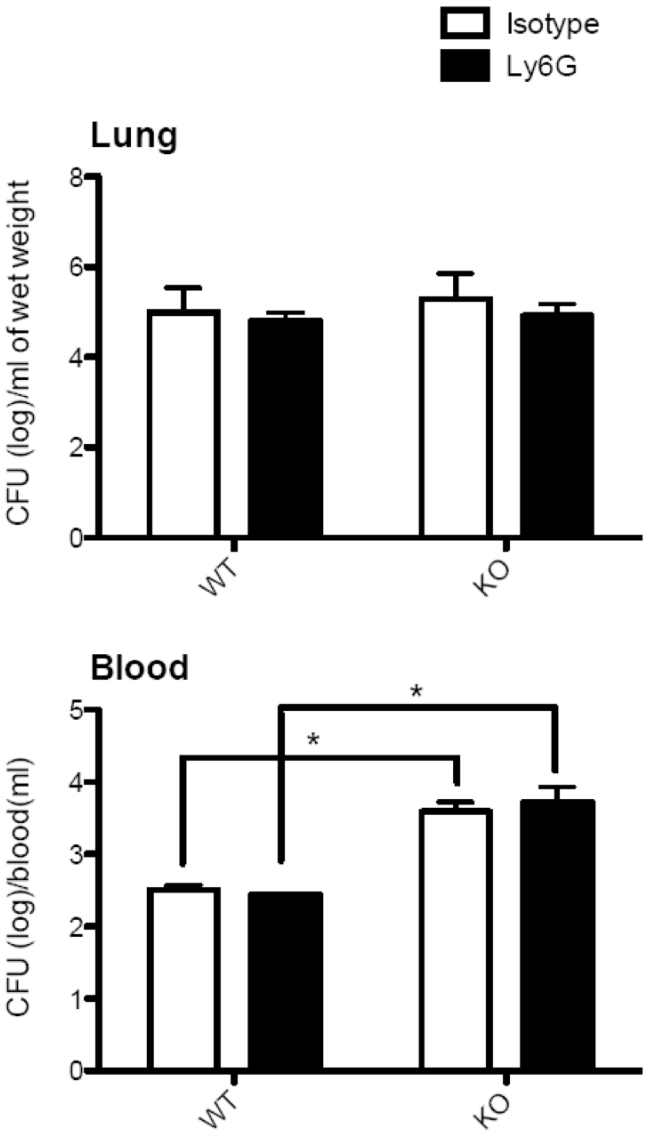

Isotype
Ly6G
Lung
CFU (log)/ml of wet weight
8
6
4
2
0
WT
KO
Blood
CFU (log)/blood(ml)
5
4
3
2
1
0
*
*
WT
KO

Figure 5. Neutrophil depletion did not impact bacterial load in the absence of IL-23 against *S. pneumoniae* infection. Mice were intraperitoneally administered with Ly6G (1A8) monoclonal antibody (0.5 mg/mouse/day) or isotype antibody for two days prior to infection. Bacterial CFU was determined from lung and blood after 18 hr of *S. pneumoniae* infection. Data represent mean ± SEM of mice (n=5). Two-way ANOVA was used for testing group variance. Asterisk indicates difference between strain in same time line and sharp indicates difference between different time points. D.L: detection limit.

CHAPTER IV

ADMINISTRATION OF NEUROENDOCRINE FACTOR ANTAGONISTS ALTER IMMUNE RESPONSES AGAINST PULMONARY *STREPTOCOCCUS PNEUMONIAE* INFECTION

Stress-derived corticotrophin-releasing hormone (CRH) and catecholamines impact immune cells indirectly through nervous system innervation of lymphoid organs and peripheral tissues, but also directly in an autocrine/paracrine receptor-mediated manner. However, questions still remain regarding the impact that CRH and catecholaminergic regulation of immune function has in modifying disease pathogenesis, particularly under conditions of stress. Utilizing a murine model of acute pulmonary *S. pneumoniae* infection and restraint stress, we hypothesized that functional blockade of CRH receptor isotypes or the β2 adrenergic receptor during restraint stress would exhibit receptor-specific disease pathogenesis. Selective receptor antagonists for CRH receptor (CRHR) 1 (antalarmin), CRHR2 (astressin2B) and β2 adrenergic receptor (butoxamine) were administered intraperitoneally prior to restraint stress followed by intranasal pulmonary *S. pneumoniae* infection. Antagonist administration did not impact restraint stress-induced loss of weight or hypothermia as compared to non-stressed mice. However, *S. pneumoniae* infection following stress mediated different alterations in body weight and temperature in non-stressed and antalarmin-administered mice compared to their restraint-

stressed counterparts administered astress2B or butoxamine. Antalarmin administration induced significantly higher lethality in *S. pneumoniae* infection whereas astressn2B exhibit significantly higher resistance against *S. pneumoniae* infection. Antalarmin administration induced significantly higher increase in bacterial load in lung and blood between 18 hr to 36 hr after infection. Furthermore, antalarmin administration resulted in a significantly higher IL-17A production in bronchioalveolar lavage (BALF) which was associated with a significantly higher percentage of neutrophils in BALF, lung, spleen and blood. In contrast, astressin2B administration induced significantly higher percentage of dendritic cells and eosinophils in lung. Lung $CD4^+$ and $CD8^+$ T cells were significantly increased by butoxamine administration as compared to other experimental conditions. Data indicate that activation of each CRHR1, CRHR2 and β2 adrenergic receptors resulted in differential effects on immune responses against *S. pneumoniae* infection, correlating with disease outcome. Our findings provide a further understanding of how stress-derived neuroendocrine factors directly impact immune responses related to immunopathology and immunoprotection. These findings suggest the potential therapeutic use of CRH antagonist and/or agonists as adjunctive therapy against acute septicemia by pulmonary *S. pneumoniae* infection.

INTRODUCTION

Complications due to bacterial respiratory infection are a leading cause of death among immunocompromised hosts (e.g. infants, elderly and critical care patients), (284) of which stress is considered a predictor of risk (285). The events leading to the onset and progression of severe disease during acute respiratory infection is due to and imbalance in immune responses. In an attempt to locally eradicate pathogens, suppressed immune responses due to corticosteroid treatment can blunt the induction of innate immune responses (286). Alternatively, a robust activation of inflammatory mediators most commonly referred as “cytokine storm” (287), is known to facilitate the recruitment of neutrophils and other cellular constituents to the inflamed site of infection and promote immune-mediated pathologies (288). Subsequently, acute tissue injury compromises capillary structure, vascular endothelial integrity resulting in functional deficits. Ultimately, bacteremic conditions lead to multiple organ failure and death (287).

Acute and chronic stress is known to disrupt immune function, increasing the risk of infection, cancer and chronic inflammatory disease (195, 197, 204, 285, 289). The central nervous system (CNS) elicits neuroendocrine and neurotransmitter activity that can directly or indirectly influence immune function in a receptor-mediated mechanism or though nervous system innervations of major lymphoid tissues, respectively (3, 31, 31, 32). The most common mechanisms are stress-induced corticosteroid and adrenergic CNS activation, which are found to impart both hypo- and hyper-responsiveness of innate and adaptive compartments of the immune system (34, 35).

Corticotropin releasing hormone (CRH) is a 41-amino acid peptide primarily produced in the hypothalamus and brain regions (33), where it plays an important role in behavior and autonomic responses to stress (34, 35). The functional activity of CRH is regulated by two major receptors, CRHR1 and CRHR2, having diverse affinities for CRH and CRH homologues, Urocortin (UCN1-3). CRH receptors are further diversified into CRHR1 and CRHR2 alpha and beta subtypes (36, 37). CRH and URN1 have highest affinity for CRHR1, whereas UCN2 and UCN3 share high affinity for CRHR2. Previous studies also demonstrate that CRH is present in peripheral tissues including lung, intestine and placenta (28, 38, 40, 214, 290, 291). CRH receptor regulation of peripheral physiological functioning is documented in cardiac (292) and mucosal tissues (293-295) and on immune cell populations (44, 213). Furthermore, CRH responses have been linked to the regulation of inflammatory conditions including colitis, tumor development and asthma (296-298). Importantly, previous studies have documented that selective CRH receptor 1 and 2 antagonists can alter inflammatory responses and dampen disease severity (299, 300). However, whether CRH-CRH receptor interactions can impact disease outcome due to respiratory infection under conditions of central nervous system activation is unknown. In recent studies, Gonzales et al., have shown that during *S. pneumoniae* infection, CRH expression is increased in total lung tissue (28). Furthermore, ongoing studies in our laboratory demonstrate that diversity of CRH receptor expression on immune cells can dictate cytokine responses *in vitro* (unpublished data). Together, these findings support the hypothesis that CRH can be a key regulator of targeting inflammatory responses revealed by stress an impact severity of bacterial infection.

In response to perceived stress, activation of SNS results in production of several neurotransmitters including epinephrine and norepinephrine at the nerve end, innervate immune

organs(51, 53, 206). Catecholaminergic neurotransmitters, epinephrine and norepinephrine are derived from common precursor amino acid molecule, tyrosine, and elicit their immunomodulatory functions. Although adrenergic receptor subtypes (α1, α2, β1, β2 and β3) are known to be expressed from some immune cells such as macrophages and dendritic cells, little evidence has found to clearly define their immunological influences except β2 adrenergic receptor. β2 adrenergic receptors are abundantly expressed on several immune cells including Th1 CD4+ T cells, NK cells, macrophages and dendritic cells (55-59). Several findings have shown β2 adrenergic receptor-mediated suppression of Th1 and enhancement of Th2 type of adaptive immunity *in vitro* and *in vivo* (55, 56). However, no clear evidence has shown impact of stress-mediated β2 adrenergic receptor activation on respiratory infectious disease.

The current study demonstrates that CRH and catecholamines under restraint stress alters immune response against *S. pneumoniae* with receptor-mediated manner. These data provide an understanding of the potential mechanisms, in which stress-derived neuroendocrine factors can improve or exacerbate pathogenesis against infectious disease. Furthermore, these finding suggest potential biomarker for stress-induced disease enhancement.

MATERALS AND METHODS

Animals

Adult (6–8 weeks of age) female CD-1 mice (Harlan Sprague–Dawley, Indianapolis, Indiana) were used in all studies. Mice were maintained under specific pathogen-free conditions on a 12:12 light/dark cycle (7:00 PM to 7:00 AM). Mice were kept under optimal temperature

and humidity controlled conditions and provided proper care as directed by the institutional animal care and use committee.

Stress paradigm and pharmacologic agents

Restraint stress (RS) was induced as described previously (28). Briefly, mice were placed in a sterile 50 ml conical tube supplied with air holes for sufficient ventilation. RS was performed for 3 hr (exactly from 1:00 PM to 4:00 PM) and repeated for 4 days. CRHR1 and CRHR2 antagonist, antalarmin (1 mg/kg) and astressin2B (100 μg/kg), and β2 adrenergic receptor antagonist, butoxamine (2 mg/kg) (Sigma-Aldrich, St. Louis, MO) were administered intraperitoneally before 3hr stress period for all stress paradigm. Sham administration with 1 x PBS was performed in non-stressed (home) and RS mice as a vehicle control (supplemental figure 5). Food and water were deprived during each RS session, including all home with sham administration (home-sham), RS with sham administration (RS-sham), RS with antalarmin administration (RS-ANT), RS with astressin2B administration (RS-AST), and RS with butoxamine administration (RS-Butx) groups (table 1). Body weight change was daily monitored at the beginning of each stress procedure. Body temperature was also measured at the first day of RS and at 24 hr after the last RS procedure.

Bacteria and infection

Streptococcus pneumoniae (*S. pneumoniae*) strain #6301 (ATCC, Manassas, VA) was grown for 16 hr to obtain mid-log phases cultures on blood agar plates. Mice were intranasally

infected with *S. pneumoniae* (5 x10^5 cells) in a volume of 40 μl of Brain-Heart Infusion Broth (EMD, EMD Chemicals Inc. Gibbwtown, NJ) after anesthesia (supplemental figure 5).

Mice survival study

Survival of mice was monitored after infection. Body weight corresponding infection-induced sickness was monitored prior to infection and at 24 hr after infection. Bacterial load in lung spleen and blood were evaluated at two different time points (18 and 36 hr) after infection.

Colony forming assay

To access bacterial burden in lung, spleen and blood after infection, organs were harvested and homogenized in PBS. Tenfold serial dilutions of organ homogenates were plated in triplicate onto blood agar plates and incubated at 37°C with 5% CO_2 overnight. Colonies on plates were enumerated, and the results were expressed as $\log_{10}$CFU per μl of wet volume of organ.

BALF isolation, single cell preparation of total lung cells for flow cytometry.

Bronchioalveolar lavage fluid (BALF) was prepared by intratracheal perfusion with 1ml of 1x PBS using 25G blunt-end needle. After removing cells by centrifuge, collected BALF was used for ELISA analysis. Single cell suspensions of mononuclear cell from lung tissue were prepared as previously described (258, 259). Briefly, lung tissues were finely minced after

separation into single lobe and incubate in collagenase digestion media containing 300 unit/ml collagenase type II (Worthington, Lakewood, NJ) and 50 unit/ml DNase (Sigma–Aldrich, St. Louis, MO) in RPMI 1640 culture media for 1 hr 30 min. After digestion, lungs were passed through a nylon mesh filter (LabPak, Depew, NY) into sterile 50 ml conical tubes and washed twice in wash media (Hyclone, Logan, UT). Lung mononuclear cells were prepared by ficoll-hypaque (Lympholyte M, Cedarlane, Laboratories, Ltd., Ontario, CA) centrifugation. Contaminating RBCs were removed using ACK lysis buffer as previously described (215). Isolated cells were characterized by flow cytometry.

Flow cytometry

Isolated total lung cells were characterized by fluorescence immunostaining and flow cytometry analysis. Single cell suspension of lung cells in flow staining buffer was incubated with anti-Fc receptor antibody (Fc blocker, clone 2.4G2) (BD Pharmingen, San Diego, CA) to prevent non-specific binding of antibody Fc region to Fc receptor on cells for at least 10 min on ice. Two or three color immunostaining for identifying cell phenotype was performed for 30 min in 4 °C using following antibodies: PE labeled anti-mouse CD11c, Alexaflour 488 (AF488) labeled anti-mouse F4/80, PE labeled anti-mouse Ly6G (1A8), PEcy7 labeled anti-mouse CD11b, PE labeled anti-mouse SiglecF, PE labeled anti-mouse CD3, FITC labeled anti-mouse CD8a, PEcy7 labeled anti-mouse CD4, FITC labeled anti-mouse B220 and APC labeled CD19. Single color staining was also performed for voltage compensation. After washing step, cells were fixed using 2 % paraformaldehyde. Gating of cells was identified by forward-scatter/side-scatter profile. Percent positive staining was determined by subtracting

autofluorescenced cells from non-stained negative control. Data were collected on Cytomic FC500 flow cytometry analyzer (Beckman–Coulter, Miami, FL). Further analysis was performed using CXP software (Beckman–Coulter). Absolute cell numbers was determined by multiplying the percent positive cells by the total number of cells isolated from lung tissue. Antibodies were purchased from BD Pharmingen (BD Bioscience, San Jose, CA) except anti-mouse APC-labeled CD19 and AF488-labeled F4/80 (Caltag, Invitrogen Co, Carlsbad,CA).

Enzyme-linked immunosorbent assay (ELISA)

Interleukin-5 (IL-5), interleukin -10 (IL-10), interleukin-12p70 (IL-12p70) and interleukin-17A (IL-17A) cytokine production was determined by sandwich ELISA method from bronchioalveolar lavage fluid (BALF). All procedures were performed as described by the manufacturer. Briefly, flat-bottomed 96-well plate was coated with optimal titration of capture antibody and followed by blocking (10% FBS in PBS). After incubation of samples at 4°C for 16 hr, plates were incubated with biotin-conjugated detection antibody and streptavidin-HRP (horseradish peroxidase. After adding tetramethylbenzidine (TMB) peroxidase substrate solution (Rockland Immunochemicals, Inc. Gilbertsville, PA), the concentration of each cytokine was determined according to standard curved generated by reference concentration of cytokine at wavelength of 450 nm detected by colorimetric plate reader (Biotek Instruments Inc. Winooski, VT). ELISA antibody set and recombinant cytokine for standard were purchased from eBiosicence (eBiosciences, San Diego, CA) for IL-12p70 ELISA sets, BD PharMingen Inc. for IL-5, IL-10 ELISA sets and IL-17A antibody pairs and R&D Systems (R&D Systems Inc. Minneapolis, MN) for recombinant IL-17A.

Statistical analysis

Statistical analysis was performed using GraphPad Prism Version 5.0 (GraphPad Software, San Diego, USA). For multi-experimental group analysis, data were subjected to one-way ANOVA (analysis of variance) followed by post hoc tests (Newman-Keuls and Bonfferoni) for group differences. All data are expressed as means ± standard error of mean (SEM). The two-tailed level of significance was set at $p \leq 0.05$ for group differences.

RESULTS

Administration of pharmacologic antagonists does not impact mal-adaptive clinical signs as a consequence of restraint stress.

Stress exposure has previously been documented to elicit mal-adaptive responses to external stimuli resulting in altered physiological homeostasis (1-3). Such stress-induced effects can be reversed or accentuated by targeting neuroendocrine-associated receptors through the use of pharmacological antagonists (299, 300). We determined the influence of antagonist administration on changes in body mass and temperature during the course of the 4-day stress protocol prior to and following *S. pneumoniae* infection. Home-sham mice demonstrated a significant ($p \leq 0.05$) weight gain over the 4-day stress protocol. In contrast, during the 4 days of stress exposure, no difference in body weight loss between mice subjected to restraint stress and systemic injection of the selective CRH receptor and β2 adrenergic receptor antagonists was demonstrated as compared to their restraint stress and sham injection counterparts. Body temperature in RS-sham and RS with antagonist-administered mice was significantly ($p \leq 0.05$)

diminished as compared to home-sham group (figure 1A). Following infection, home-sham mice demonstrated a significantly ($p \leq 0.05$) greater loss in weight as compared to all mice exposed to restraint stress with or without prior administration of all selective antagonists. Interestingly, RS-AST mice demonstrated a significant ($p \leq 0.05$) gain in weight after infection compared to its sham injected and antagonist administered stressed counterparts. In addition, body temperature was significantly ($p \leq 0.05$) diminished among home-sham mice as compared to RS-sham, RS-AST and RS-Butx mice, but not RS-ANT mice. No statistical difference was found between restraint stressed and all antagonist-administered mice (figure 1B).

Selective CRH receptor antagonists, astressin2B and antalarmin result in divergent survival among RS mice after S. pneumoniae infection.

The impact of CRH and β2 adrenergic receptors under restraint stress on survivorship in response to nasal-pulmonary *S. pneumoniae* infection was determined by the administration of selective CRH and β2 adrenergic receptor antagonists. As previously reported (28), delayed lethality, albeit insignificant, was induced in mice exposed to restraint stress without administration of antagonists as compared to their non-stressed-non antagonists-administered counterparts, resulting in no significant differences in survival rate after 96 hr (figure 2A and 2B). At 56 hr after infection, RS with astressin2B administration started to show enhanced survival rate as compared to all experimental groups. However, no significant difference was shown in lethality by *S. pneumoniae*. Interestingly, administration of antalarmin (RS-ANT) resulted in a significant ($p \leq 0.05$) increase in lethality as compared to astressin2B administration at 76 hr (figure 2B). Notably, sham administration in non-stressed and restraint stressed group

resulted in similar but not identical pattern of survival curve in comparison with same groups without sham administration (supplemental figure 1), suggesting additional stress by intraperitoneal administration procedure.

Antagonists-effects on bacterial outgrowth in lungs and peripheral tissues directly correlate with survival.

The propensity of bacterial growth in lung, spleen and blood were determined by bacterial load increased from 18 to 36 hr following infection, controlling for restraint stress exposure and administration of antagonists (figure 3). In the lung, stress alone (RS sham) did not influence the bacterial colonization as compared to home-sham mice. Antalarmin administration during stress procedure resulted in a significantly ($p \leq 0.05$) higher increase of bacteria in lung than Home sham and RS sham-treated mice. Administration of astressin2B resulted in a modest yet insignificant increase in bacterial growth. Butoxamine administration however, resulted in a significant attenuation of bacterial growth among stressed mice compared to both RS-ANT and RS-AST mice. A similar effect was observed in blood. A significantly ($p \leq 0.05$) higher number of bacterium was present in the blood of RS-ANT mice as compared to RS-sham and RS-AST mice. Comparable numbers of bacterium were present in the blood of home-sham and RS-Butx mice as compared to RS-ANT mice. No significant differences in the number of bacterium were apparent in the spleen regardless of experimental treatment (figure 3).

CRH receptor-1 antagonist, antalarmin preferentially accentuates stress-induced IL-17A cytokine production in bronchiolar lavage fluid (BALF) of S. pneumoniae-infected mice.

IL-5, IL-10, IL-12p70 and IL-17A were measured in BALF at 18 hr following *S. pneumonaie* infection among non-stressed and stressed mice, controlling for antagonists administration (figure 4). With the exception of IL-17A, mice exposed to stress alone (RS sham) or administered selective CRH receptor and β2-adrenergic receptor antagonist demonstrated as significant ($p \leq 0.05$) increase in IL-5, IL-10 and IL-12p70 as compared to their non-stressed (Home sham) counterparts. RS mice, which were administered antalarmin during, demonstrated a significantly ($p \leq 0.05$) higher concentration of IL-17A compared to home sham and RS sham-treated mice as well as mice administered astressin2B and butoxamine (figure 4).

Antalarmin-associated increase in BALF IL-17A corresponds with increases in BALF, lung and blood neutrophils.

IL-17A induces chemotactic activity in epithelial and endothelial cells to recruit neutrophils into site of inflammation (191). In BALF and blood, antalarmin-administration with RS induced the most significantly ($p \leq 0.05$) higher increase of neutrophil percentage as compared to home-sham and RS-sham mice as well as astressin2B and butoxamine administered RS mice. In similar pattern, lung neutrophil percentage in antalarmin-administered group was significantly ($p \leq 0.05$) higher as compared to home-sham and RS-AST mice (figure 5).

Antagonist administration resulted in changes in lung immune cell population with different preference in response to S. pneumoniae infection.

Expression of CRHRs or β-adrenergic receptor has been detected in several immune cells (28, 44, 55, 57, 58, 211, 213, 301). As shown in figure 6A, we examined influences of restraint stress and antagonist administration on antigen presenting cell populations (APCs), eosinophils (figure 6) and lymphocytes ($CD4^+$ and $CD8^+$ T cells, B cells) (figure 7) in lung against *S. pneumoniae* infection. Dendritic cells from RS-AST group exhibited significantly higher percentage in lung as compared to other groups and similar result was shown in macrophages with no statistical significance (figure 6). Although neutrophils are important cell types for bacterial clearance and host defense, other leukocytes such as eosinophils are also involved in host immune system with various functions such as enzyme and cytokine productions (302, 303). Therefore, we examine population changes of lung eosinophils in the absence and presence of antagonist administration under RS condition. RS-sham, RS-ANT and RS-AST mice showed significantly ($p \leq 0.05$) higher lung eosinophils as compared to home sham and RS-Butx mice. Further significantly ($p \leq 0.05$) higher eosinophils percentage was shown in restraint stressed and RS-AST mice as compared to RS-ANT (figure 6). We next examined effects of restraint stress and antagonist administration on lymphocytes, $CD4^+$/$CD8^+$ T cells and B cells. $CD8^+$ T cells isolated from restraint stressed showed significantly ($p \leq 0.05$) higher population as compared to home-sham and RS-AST mice. Butoxamine administration in RS mice induced similar population changes in restraint stressed group with significant ($p \leq 0.05$) difference from home-sham, RS-ANT and RS-AST mice. Similar pattern was shown in $CD4^+$ T cell population with no significant difference except between RS-AST and RS-Butx ($p \leq$

0.05). In addition, B cell population was not significantly changed by RS or antagonist administration (figure 7).

DISCUSSION

Perceived stress is believed to be a link between psychosocial input and a number of factors that influence health outcomes. Although numerous investigations have shown possible roles of stress as a factor regulating several immunopathologic responses, further investigation is required to identify the underlying mechanisms of neuroendocrine factors' influence on immunological pathways. This study determined the potential role of stress-induced CRH and catecholamines in mediating disease outcome. We pursued our investigation using a pharmacological antagonist approach, which targeted their effects by selective blockade of CRH and β2 adrenergic receptors. Specifically, prior to restraint stress, mice were administered antalarmin (selective CRHR1 antagonist), astressin$_2$B (selective CRHR2 antagonist) or butoxamine (selective β2-adrenergic receptor antagonist) by intraperitoneal administration prior to daily stress procedure. Following the last stress session, mice were infected with *S. pneumoniae* and pathogenesis was monitored.

As shown in figure 3, bacterial burden in lung and blood was dramatically increased from 18 hr to 36 hrs and these changes directly corresponded with the survival rate of mice (figure 2). Interestingly, in comparison of two receptor isotypes for same ligand, our data clearly demonstrated that blocking CRHR2 via astressin2B resulted in enhanced protection among other groups against death caused by pneumococcal sepsis whereas administration of the CRHR1

antagonist, antalarmin showed the least resistance as compared to all experimental groups, including non-stressed mice. Thus, suggesting a dichotomous relationship between CRH receptor isotypes. The role of these two receptors on inflammatory responses is still contraversial. Wlk et al., showed that blockade of CRHR1 provided benefits to improve disease pathogenesis in Toxin A-induced intestinal inflammation (304). In contrast, CRHR2 is also known as an important inflammatory mediator in intestine (299). Overall previous findings suggest that both CRHR1 and CRHR2 are involved in inflammatory responses with the potential to mediate opposing outcomes of disease under conditions of stress-induced activation of CRH.

The events that mediate sepsis caused by infectious disease are known to depend on the type and quality of inflammatory responses determined by immune reactivity to the inciting pathogen. In the current study, our data demonstrated that blockade of CRHR1 with antalarmin resulted in a higher percentage of neutrophils in BALF and lung corresponding with a preferential BALF IL-17A cytokine production as compared to blockade of CRHR2 against *S. pneumoniae* infection (figure 4). This may be due to skewed priming on CRHRs by systemic blockade of the other receptor isotype, leading to a unique inflammatory response by one receptor. By this concept, our results suggested that CRHR2 induced intense IL-17 dependent inflammatory responses represented by significantly higher propensity of neutrophil recruitment associated with tissue damage and systemic septic conditions. By contrast, CRHR1 ligation with its natural ligand CRH or its homologues UCN 1-3 (36, 37), results in different immune responses from CRHR2, more effective for protection against *S. pneumoniae* infection.

Interestingly, systemic blockade of CRHRs resulted in different preferences of immune cell populations in lung. As mentioned earlier, antalarmin administration induced higher

neutrophil percentages in lung after infection (figure 5). In general, neutrophils constitute the first defense line against external pathogen infection to protect host (91, 149, 150, 152, 274, 305). Interestingly, neutrophils also showed functional discrepancies on pathogenesis by destructive tissue damage. Our neutrophil depletion data showed that neutrophil depletion did not impact bacterial clearance in lung, but significantly impacted systemic compartments including spleen and blood at early time point (18 hr) after *S. pneumoniae* infection (supplemental figure 4). Data suggested that antalarmin administration-mediated excessive neutrophil may result in higher lung damage rather than bacterial clearance.

In contrast, astressin2B administration supported higher population of dendritic cells, macrophages and eosinophils in lung after infection (figure 6). In support, several evidences have demonstrated that CRH may be a direct modulator on their immunological functions through their receptors. It is still unclear whether CRH impact dendritic cell function. However, Gonzales et al provided evidence that dendritic cells express transcriptional level of CRHR1 and CRHR2 and their expression is altered in response to restraint stress (28). Macrophages are known to express both CRHR1 and CRHR2 (46). Tsatsanis et al reported that CRHR2 mediates macrophage apoptosis in LPS-mediated inflammatory responses (306). Likewise neutrophil, eosinophils is known to express CRHR1 (212). Furthermore, eonisophil also express endogenous CRH at intestine in response to stress (213), suggesting autocrine stimulation on their own function.

As compared to CRHR, blocking β2 adrenergic receptor by butoxamine administration show enhanced but not significant protection in survival rate of acute pneumococcal septic model at the end of survival kinetics as compared to non-stressed or antalarmin-administered RS

mice. Butoxamine administration also showed less protective effects as compared to astressin2B administration. Furthermore, systemic blockade of β2 adrenergic receptor by butoxamine administration induced significantly higher percentage of $CD4^+$ and $CD8^+$ T cells in lung as compared to CRHR antagonist administration after *S. pneumoniae* infection (figure 7). This data suggests that stress-induced catecholamines may prevent proliferation or recruitment of T cells necessary for bacterial clearance or initiation of adaptive immunity at the site of inflammation. This different propensity of β2 adrenergic receptor activity may be due to different distribution of β2 adrenergic receptor expression on particular cell types such as T cells. In support, Th1 $CD4^+$ and NK cells express β2 adrenergic receptor and their IFN-γ production and proliferation are suppressed through β2 adrenergic receptor (59). In contrast, by the absence of β2 adrenergic receptor expression, Th2 CD4+ cells are negative from β2 adrenergic receptor-mediated influences (63, 64). However, no clear evidence has shown CRHR expression or functional role of CRH on T cells or B cells. One more implication is that the effect of β-adrenergic blocking might be important for generating adaptive immune system important on re-exposure to same organism such as secondary infection as suggested in chapter 3 for $CD4^+$ T cell activation. Therefore, our finding suggested that distribution and expression of CRHRs or β2 adrenergic receptor is distinct from each other and perform differential role on immune responses.

Notably, all experimental groups exposed to RS showed similar patterns of physiological parameters including body weight and temperature, regardless of antagonist administration after stress period (figure 1A). It is well known that CNS induced significant metabolic disorders in the processing of stress and resulted in alteration of body mass and temperature (307). Therefore, changes in body weight and temperature may indicate CNS

controllability on stress. Our data clearly demonstrated that antagonist administration during stress paradigm may not influence CNS controllability against stress. In contrast, body weight and temperature changes after *S. pneumoniae* infection (figure 1B) was significantly altered and corresponded with survival rate and bacterial load (figure 2 and figure 3) modified by antagonist administration. As clinical indicator, severe loss of weight and hypothermia are important physiological parameters in systemic septicemia (308) and this may be due to severe inflammatory response against infection (309). Therefore, our data provided implication that antagonist administration influences on local immune responses separated from direct CNS controllability against stress. In support, serum corticosterone concentration was not significantly influenced by restraint stress or antagonist administration after *S. pneumoniae* infection (supplemental data figure 2). Corticosterone is glucocorticoid hormone produced in adrenal gland in response to stress process in CNS via CRH and ACTH (34, 35). Thus, our data demonstrated that stress-induced neuroendocrine factors, particularly CRH and catecholamines, directly impact local immune system against *S. pneumoniae* infection independent from CNS controllability. Several findings support direct influences of neuroendocrine factors on local tissues. Peripheral nervous systems (PNS) are innervated in several organs including lung and immune organs such as thymus, lymph node and spleen. In particular, sympathetic nervous system is known as a major source of catecholamines in response to stress. Not only CNS-derived CRH, ganglion cells in PNS also produced local CRH by stress (310). Gonzales et al also demonstrated transcriptional regulation of CRH gene by RS in lung (28). Furthermore, we also demonstrated CRH protein expression in lung (supplemental figure 3). In addition, several investigations have shown expression of CRH, catecholamines and their receptors in cellular level from immune cells, suggesting autocrine and paracrine stimulation (44, 54, 213, 290).

In summary, our results provide evidence of the differential and diverse influences that neuroendocrine factors portray in controlling bacterial pathogenesis under conditions of stress.

Experimental groups	Description
Home-sham	Non-stressed mice group with sham administration
RS-sham	Restraint stressed mice group with sham administration
RS-ANT	Restraint stressed mice group with antalarmin administration
RS-AST	Restraint stressed mice group with astressin2B administration
RS-Butx	Restraint stressed mice group with butoxamine administration

Table 1. Abbreviations describing experimental groups for restraint stress with antagonist administration. Selective receptor antagonists were administered before restraint stress (RS) paradigm. Non-stressed groups were described as “home” group and restraint stressed groups were described as “RS” group. Vehicle control with 1x PBS administration were described as “sham”. Each antalarmin, astressin2B and butoxamine administration were described as each “ANT”, “AST” and “Butx”.

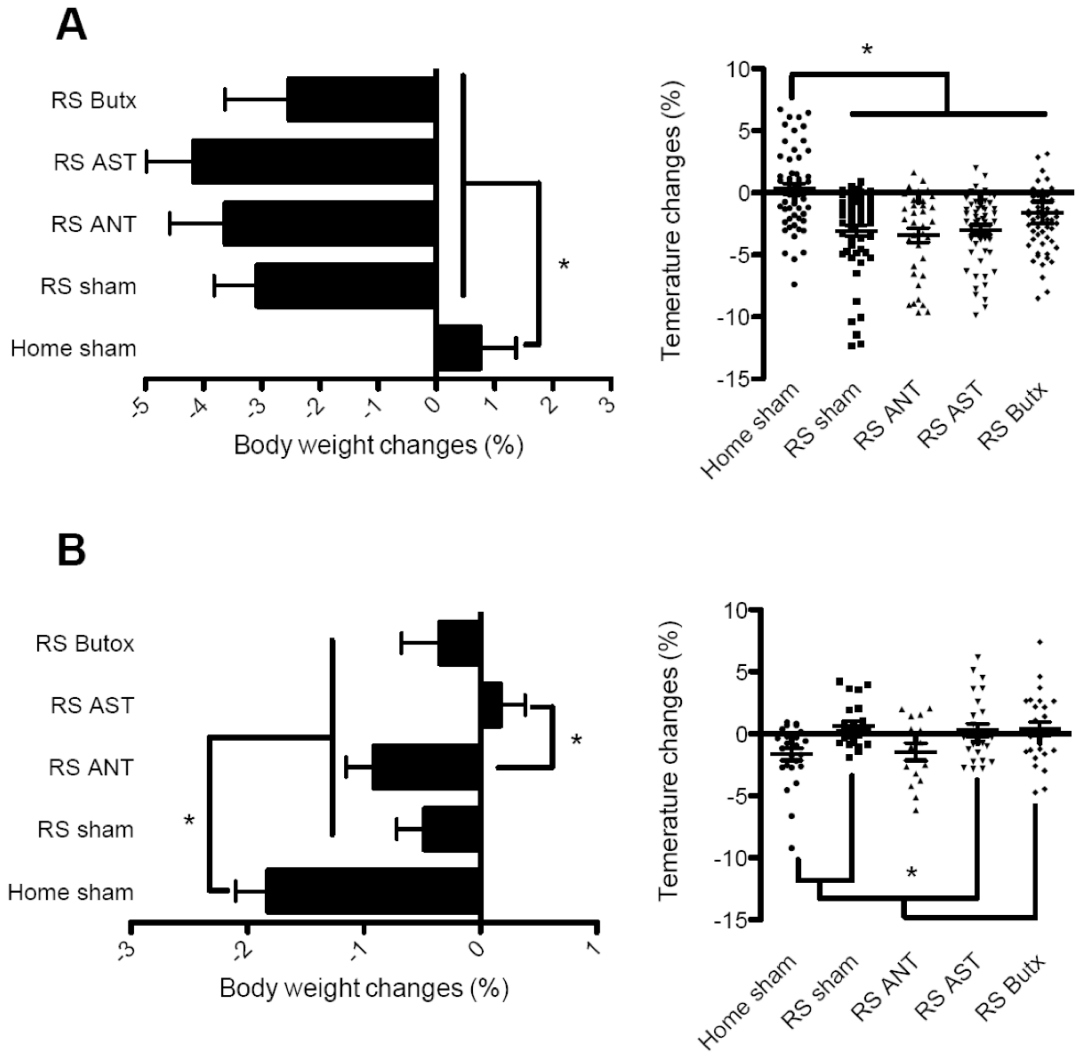

A
RS Butx
RS AST
RS ANT
RS sham
Home sham
-5
-4
-3
-2
-1
0
1
2
3
Body weight changes (%)
*
Temerature changes (%)
10
5
0
-5
-10
-15
*
Home sham
RS sham
RS ANT
RS AST
RS Butx
B
RS Butox
RS AST
RS ANT
RS sham
Home sham
-3
-2
-1
0
1
Body weight changes (%)
*
*
Temerature changes (%)
10
5
0
-5
-10
-15
*
Home sham
RS sham
RS ANT
RS AST
RS Butx

Figure 1. Administration of antagonist showed no effect on body weight and temperature changes by restraint stress but alters body weight and temperature by *S. pneumoniae* infection. Changes of body weight and temperature were determined by comparison between pre- and post-restraint stress paradigm (A) or before and after 24 hr of *S. pneumoniae* infection (B). Percentage of weight and temperature changes was represented as mean ± SEM from n=35 per group. Group differences were analyzed using one-was ANOVA. Asterisk indicates significant ($p \leq 0.05$) difference between groups.

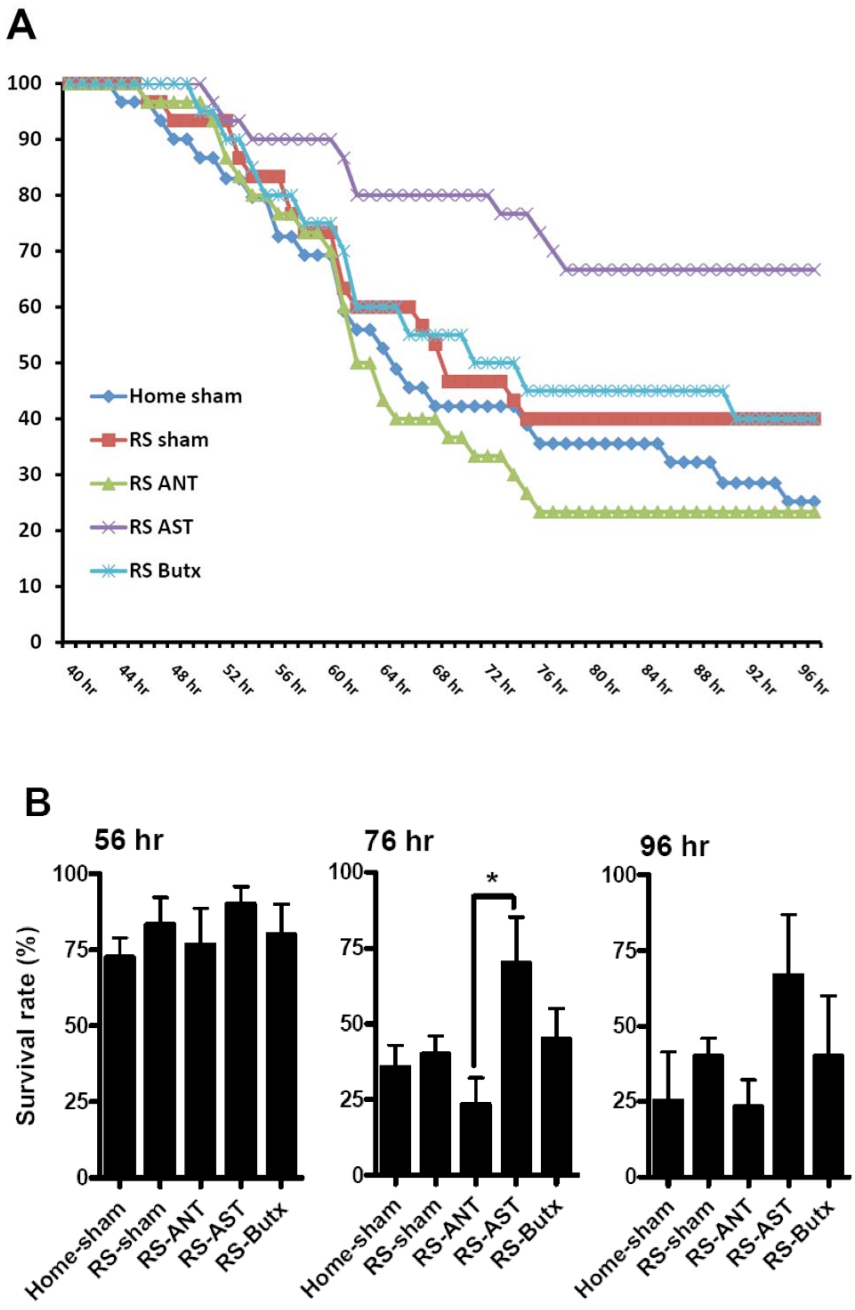

A
100
90
80
70
60
50
40
30
20
10
0
Home sham
RS sham
RS ANT
RS AST
RS Butx
40 hr
44 hr
48 hr
52 hr
56 hr
60 hr
64 hr
68 hr
72 hr
76 hr
80 hr
84 hr
88 hr
92 hr
96 hr
B
56 hr
76 hr
96 hr
Survival rate (%)
100
75
50
25
0
*
Home-sham
RS-sham
RS-ANT
RS-AST
RS-Butx

Figure 2. Astressin2B administration in restraint stress enhanced survival rate against *S. pneumoniae* infection. After 4 days of restraint stress procedure with antagonist administration, mice were infected with *S. pneumoniae* (5×10^5 cells/mouse) intranasally. Survival rate was monitored and recorded as kinetics (A) or 3 representative time points (B). Each kinetics or bar graphs represent percentage of survival rate at each times points from 3 independent experiments with total mice (n=30). Asterisk in figure (B) indicate significant differences between each group.

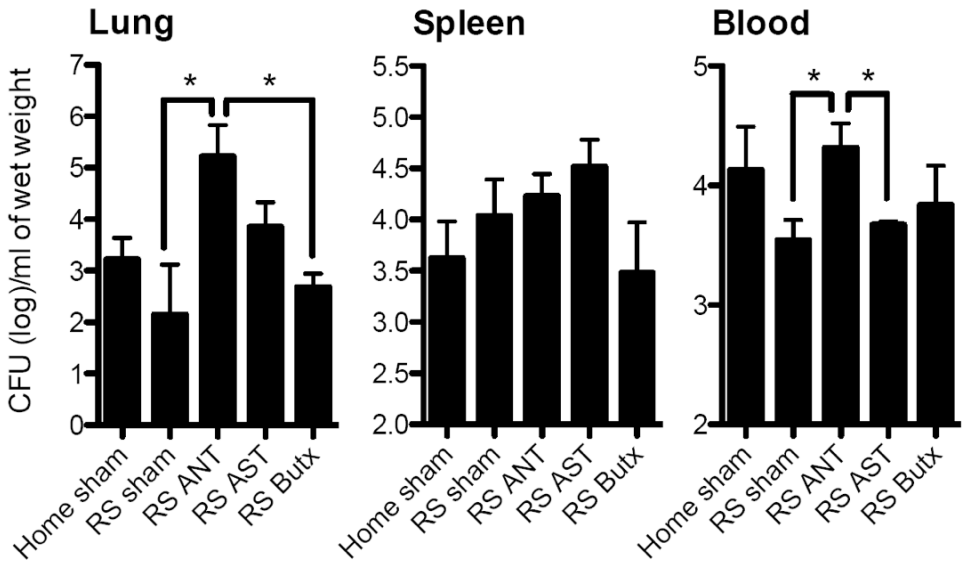

Lung
Spleen
Blood
CFU (log)/ml of wet weight
Home sham
RS sham
RS ANT
RS AST
RS Butx
*
*

Figure 3. Administration of antalarmin with restraint stress significantly increased bacterial CFU in lung blood from 18 hr to 36 hr after pulmonary *S. pneumoniae* infection. Mice were administered receptor antagonists, antalarmin, astressin2B and butoxamine prior to receive restraint stress. After 4 days of stress, mice were intranasally infected with *S. pneumonie* (5×10^5 CFU/mouse). After 18 and 36 hr of infection, bacterial CFU was determined from lung, spleen and blood. Data represent increase of CFU during 12 hrs by subtracting 18 hr data from 36 hr as mean±SEM of n=10 per group. Group differences were analyzed using one-was ANOVA. Asterisk indicates significant ($p \leq 0.05$) difference between groups.

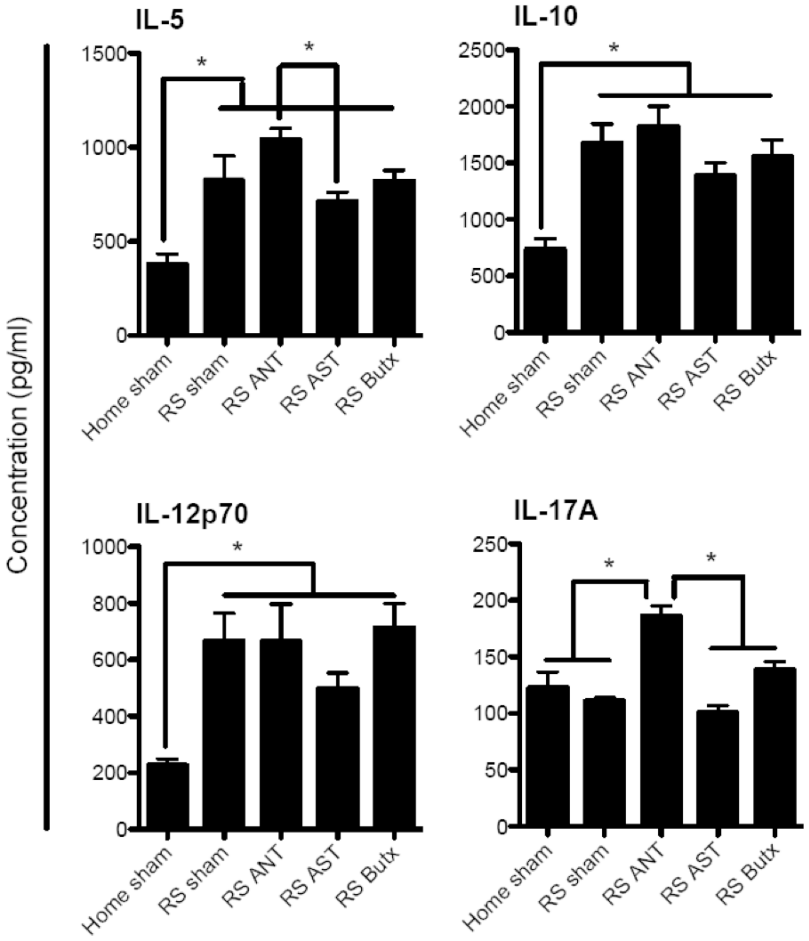

IL-5
IL-10
IL-12p70
IL-17A
Concentration (pg/ml)
Home sham
RS sham
RS ANT
RS AST
RS Butx
*

Figure 4. Administration of antagonist resulted in changes of BALF cytokine production in response to pulmonary *S. pneumoniae* infection. IL-5, IL-10, IL-17A and IL-12p70 cytokine production was determined from BALF of mice (n=5) per group using ELISA. Group differences were analyzed by one-way ANOVA. Asterisk indicates significant ($p \leq 0.05$) difference between all experimental groups.

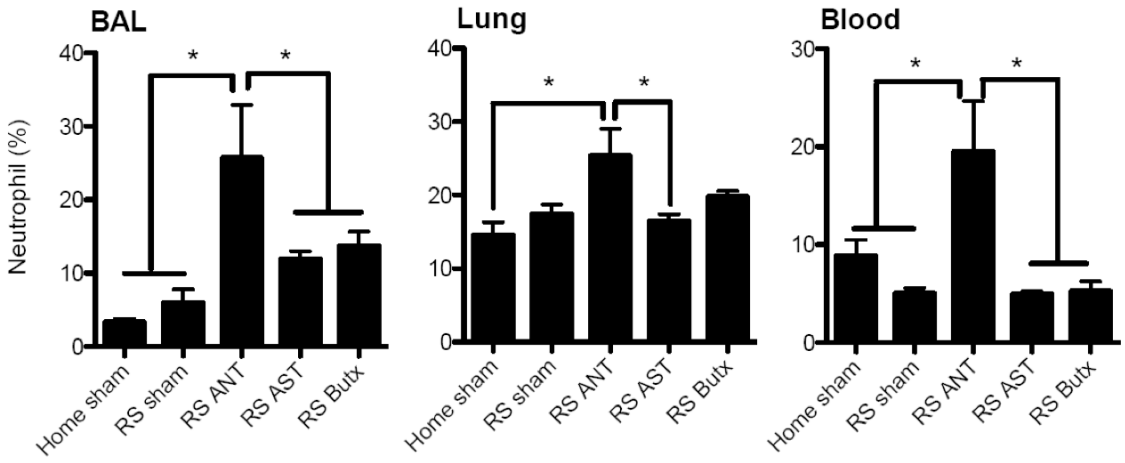

BAL
Lung
Blood
Neutrophil (%)
40
30
20
10
0
*
*
Home sham
RS sham
RS ANT
RS AST
RS Butx

Figure 5. Administration of antalarmin increased neutrophil in BAL, lung and blood against *S. pneumoniae* infection. Neutrophils ($Ly6G^+$ $CD11b^+$) were characterized from BAL, lung and blood of mice (n=5) after 18 hr of infection by flow cytometry. Group differences were analyzed by one-way ANOVA. Asterisk indicates significant ($p \leq 0.05$) difference between all experimental groups.

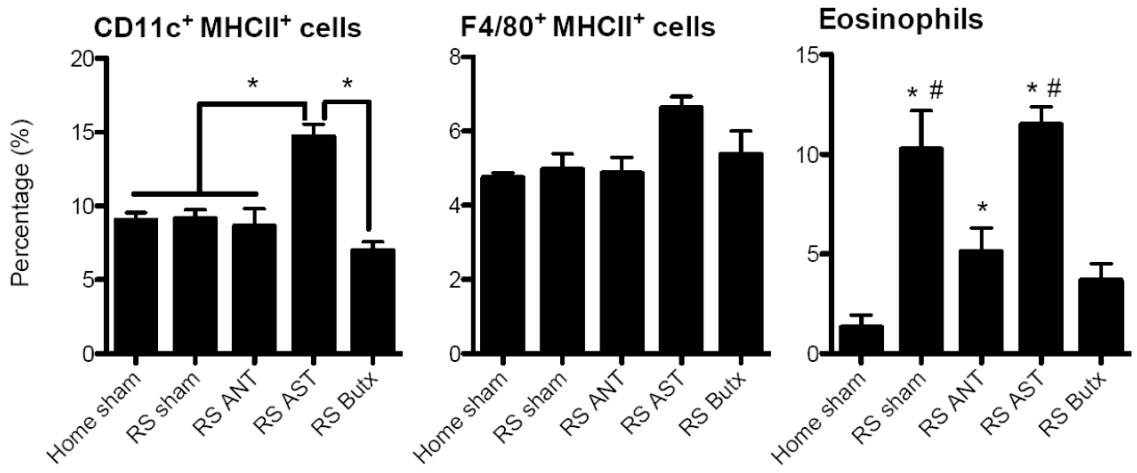

CD11c+ MHCII+ cells
F4/80+ MHCII+ cells
Eosinophils
Percentage (%)
20
15
10
5
0
8
6
4
2
0
15
10
5
0
* *
* #
* #
*
Home sham
RS sham
RS ANT
RS AST
RS Butx

Figure 6. Antagonist administration under restraint stress alters dendritic cell, macrophage and eosinophil population against *S. pneumoniae* infection. Dendritic cells ($CD11c^{+}MHCII^{+}$), macrophages ($F4/80^{+}MHCII^{+}$) and eosinophils ($SiglecF^{+}CD11b^{+}$) were characterized from total lung cells isolated from mice (n=5) after 18 hr of infection by flow cytometry. Data represent mean ± SEM of target cell percentage. Group differences were analyzed by one-way ANOVA. Asterisk indicates significant ($p \leq 0.05$) difference between indicated experimental groups. In eosinophil data, asterisk indicates significant ($p \leq 0.05$) difference from home-sham group, and sharp (#) indicates significant ($p \leq 0.05$) difference from RS-ANT group

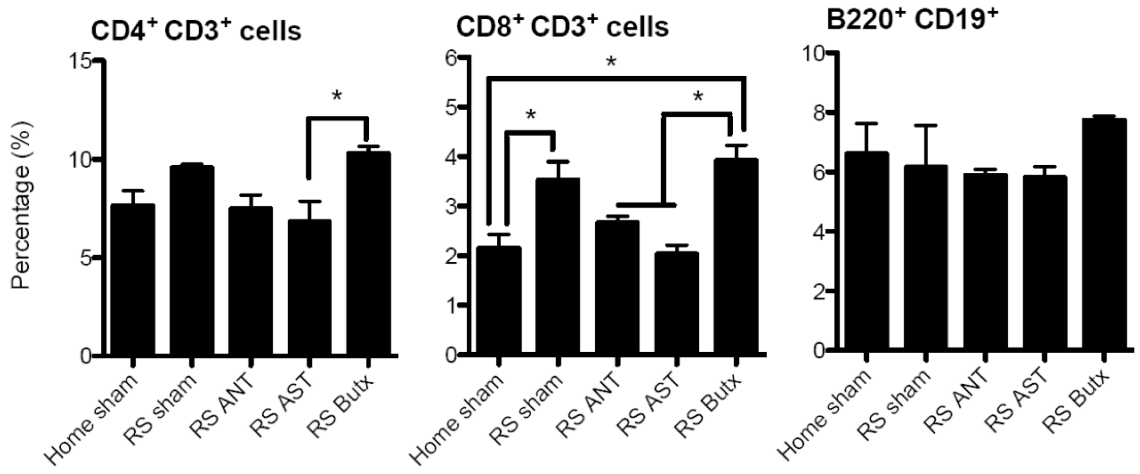

CD4+ CD3+ cells
CD8+ CD3+ cells
B220+ CD19+
Percentage (%)
Home sham
RS sham
RS ANT
RS AST
RS Butx

Figure 7. Antagonist administration under restraint stress alters lymphocyte population against *S. pneumoniae* infection. $CD4^+$, $CD8^+$ T cells and B cells were characterized from total lung cells isolated from mice (n=5) after 18 hr of infection by flow cytometry. Data represent mean ± SEM of target cell percentage. Group differences were analyzed by one-way ANOVA. Asterisk indicates significant ($p \leq 0.05$) difference between all experimental groups.

MATERIAL AND METHOD FOR SUPPLEMENTAL FIGURES

Corticosterone immunoassay

Concentration of blood serum corticosterone was determined using Correlate-EIA Corticosterone kit (Assay designs, Inc. Ann Arbor, MI) and all procedures for competitive immunoassay were performed as described by manufacturer. Briefly, 100 μl of serum samples were placed in pre-coated well with serial-diluted standard and various blanks for 2 hr at room temperature. After 3 times of washing, 200 μl of substrate solution was added in each well and incubated for 1 hr. After adding 50 μl of stop solution, samples were read at optical density 405 nm. Corticosterone concentration was calculated using standard curve obtained from percent bound (Net OD/Net Bo; 0 pg/ml standard OD × 100).

CRH determination by western blotting

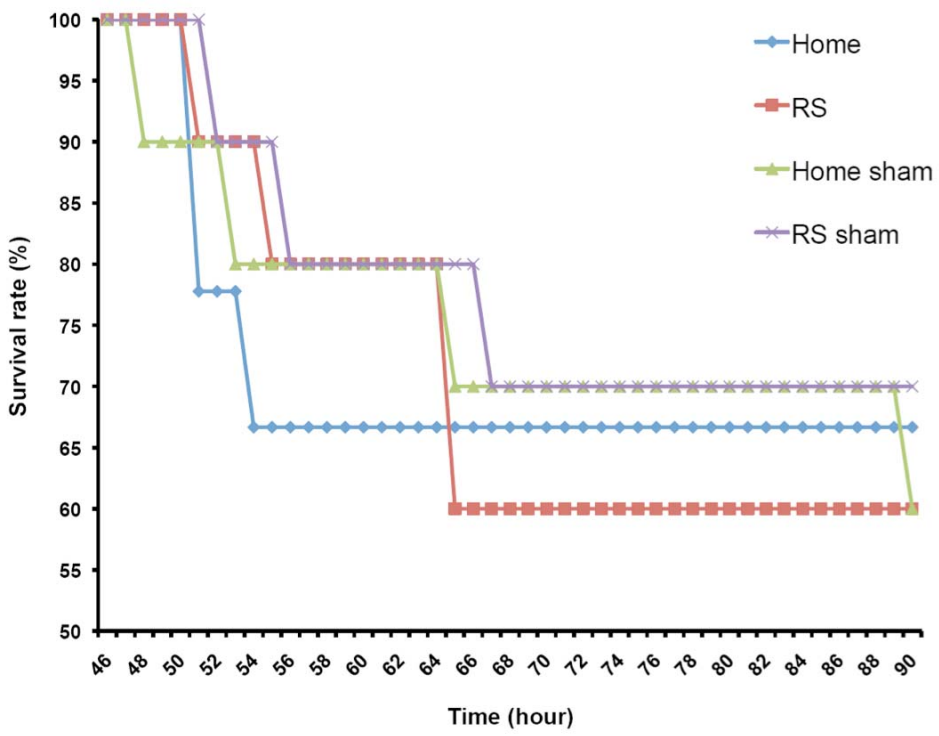
Lung tissues were ground to a fine powder in liquid nitrogen and homogenized with 50 mM potassium phosphate buffer containing 0.3 M KBr and 3 mM EDTA, ph 7.4, and 1:1000 dilution of a protease inhibitor cocktail (100 μM 3-(2-aminoethyl) benzenesulfonyl fluoride, 10 μM leupeptin, 10 μM E-64, 1 μM bestatin, 15 nM aprotinin, 1.0 μM pepstatin A) (Sigma). The protein concentration of tissue homogenates was determined by Lowry assay. Protein aliquots equivalent to 100 μg of total lung protein were dissolved and boiled in Laermmili buffer containing 5 % β-mercaptoethanol for 5 min, separated 12 % SDS-PAGE, and transferred to PVDF (polyvinylidene difluoride) membranes (Bio-Rad). After being blocked in 5 % skim milk,

membrane was incubated with primary antibodies for rabbit anti-CRH (Santa Cruz) and β-actin in 4°C overnight. Secondary antibody (Goat anti-rabbit IgG) conjugated to horseradish peroxidase (Jackson immunoresearch) was added for 2 hr at room temperature. CRH and b-actin band were visulalized by the ECL system (Alpha Innotech) using a FluorChem imaging system (Alpha Innotech). Total brain extract was used as a positive control for CRH protein expression.

Neutrophil depletion

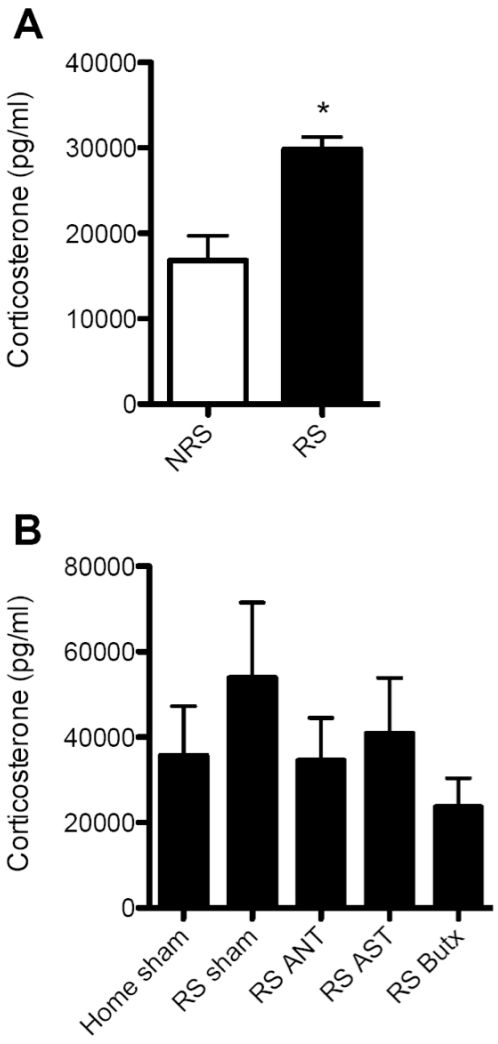
Neutrophils were depleted by intraperitoneal injection of purified Ly6G (1A8) monoclonal antibody (BioXcell, West Lebanon, NH) with concentration of 0.5 mg/mouse in 500 µl of sterile 1X PBS. Injection was performed every 24 hr for two days and depletion was confirmed by differential staining of whole blood (~98 %).

Supplemental figure 1



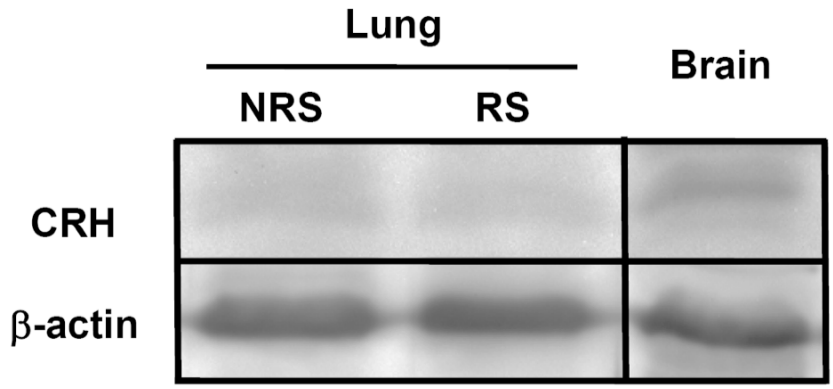
Supplemental figure 1. Sham injection may provide additional signals affecting stress-induced modification in survival rate against *S. pneumoniae* infection. After 4 days of restraint stress procedure with sham administration, mice were infected with *S. pneumoniae* (5×10^5 cells/mouse) intranasally. Survival rate was monitored and recorded as kinetics. Each kinetics or bar graphs represent percentage of survival rate with total mice (n=10).

Supplemental figure 2.



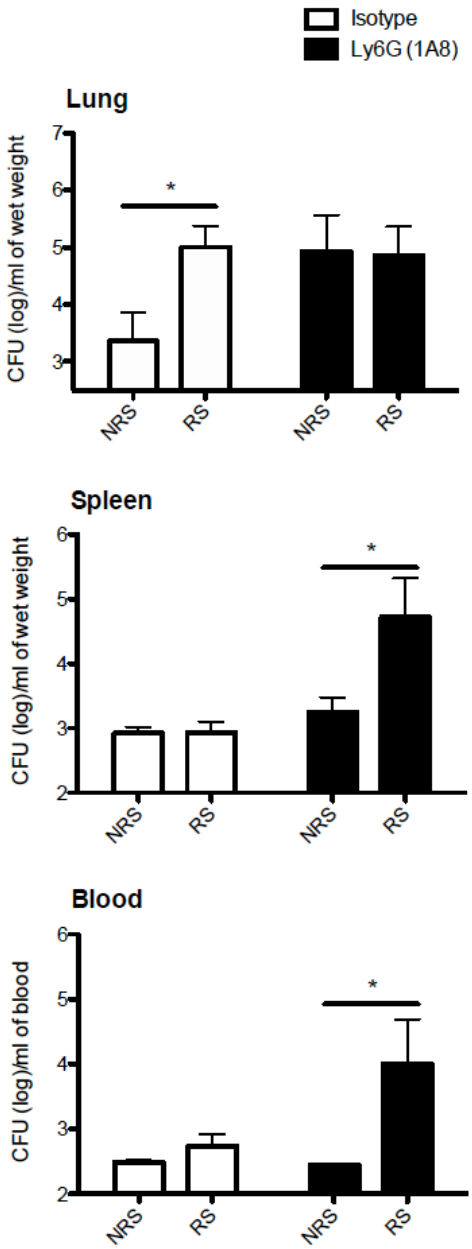
Supplemental figure 2. Serum corticosterone level was increase by restraint stress but, not changes by *S. pneumoniae infection* following restraint stress regardless antagonist administration. Mouse serum was collected from whole blood. Conecentration of corticosterone was determined after stress paradigm (A) or after stress paradigm with antagonist administration followed by *S. pneumoniae* infection (B) using competitive ELISA as described in method. Vertical bar graph represents mean ± SEM. To determine statistical differences between group, non-parametric t-test (A) and one-way ANOVA (B) was used.

Supplemental figure 3



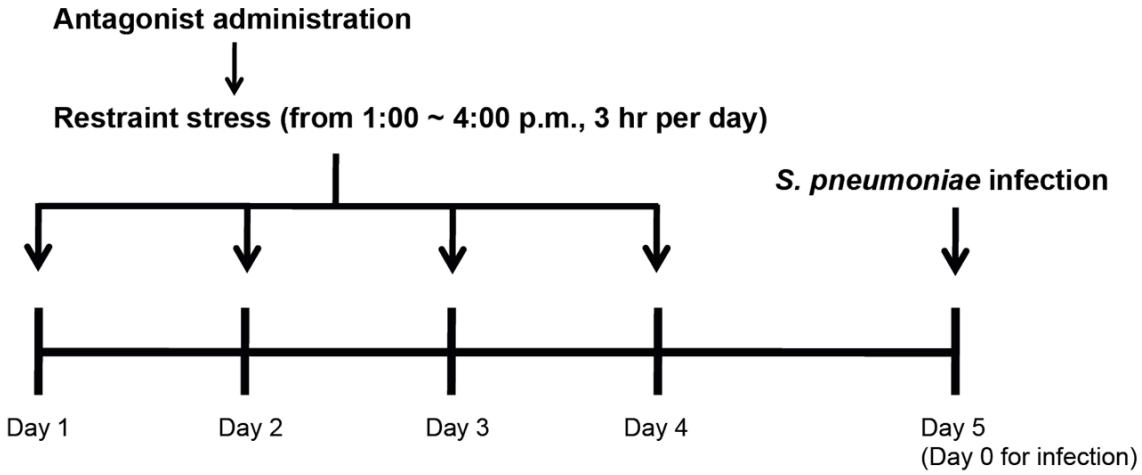
Supplemental figure 3. CRH is detected in protein level in lung. Protein expression level of CRH was determined by immunoblotting from total lung protein extract. Total protein extract from brain was used for positive control.

Supplemental figure 4



Supplemental figure 4. Restraint stress influenced neutrophil function for bacterial clearance in lung but not in spleen and blood. Mice were received restraint stress for 4 day as described. At day 3 and 4 of stress, neutrophil depletion antibody was infected to mice. After 4 days of stress, mice were intranasally infected with *S. pneumonie* (5×10^5 CFU/mouse). Bacterial CFU was determined in lung, spleen and blood from neutrophil-depleted mice after 18 hr of infection.

Supplemental figure 5



Supplemental figure 5. Disgram for restraint stress paradigm with antagonist administration followed by *S. pneumoniae* infection. Prior to restraint stress (RS), mice were administered with 1x PBS as vehicle control or antagonists. RS was induced by placing mice in a sterile 50 ml conical tube supplied with air holes for sufficient ventilation. RS was performed for 3 hr (exactly from 1:00 PM to 4:00 PM) and repeated for 4 days. *S. pneumoniae* were intranasally inoculated to mice after 24 hr of last stress paradigm.

CHAPTER V

DISCUSSION

These studies highlight the impact neuroendocrine factors have in modulating immune responses against pulmonary bacterial infectious disease. In addressing the hypothesis that stress-derived catecholamines and CRH have predominant roles in modulation of immune responses, several important findings are clarified; 1) Epinephrine and CRH directly promote IL-23 and IL-10 cytokine production by DC in response to a bacterial-associated constituent, LPS to direct $CD4^+$ T cell activation into Th17 and Th2 cells. 2) IL-23 plays an important role on bacterial clearance and preventing sepsis in a neutrophil-independent manner against pulmonary *S. pneumoniae* infection, suggesting a novel mode of action. 3) Systemic blockade of stress-related neuroendocrine factors in the midst of stress results in divergent disease outcomes against *S. pneumoniae* infection. The overall findings are discussed in this chapter to draw conclusions and relationships between each study with the anticipation of formulating new hypotheses for future research.

DC perform crucial roles to process invading pathogens through molecular pattern recognition molecules, uptake of antigens by phagocytosis/pinocytosis processes and presentation of antigenic epitopes on major histochemical complex (MHC) molecules. Through these processes, DC also produce cytokines influencing pro-inflammatory and anti-inflammatory responses of other immune cells as a primary means of inducing adaptive T cell differentiation

and activation. Previous research has demononstrated that DCs express both adrenergic and CRH receptors. Our data directly demonstrated the potential immunomodulatory effect of epinephrine and CRH to direct cytokine production in response to bacterial pattern molecule LPS, a potent ligand for TLR4. Prior to these studies, β2 adrenergic regulation on Th1 and Th2 adaptive immunity had been well-established (55, 56). Particularly, previous studies have demonstrated that by suppressing Th1 proliferation and effector functions, β2 adrenergic stimulation supports Th2 dominant adaptive immunity pathways (55, 56). In our studies, the results shown in chapter 2 demonstrate new findings. First, pre-exposure to epinephrine dictates non-activated DCs to produce IL-23 and IL-10 dominant cytokine production. Second, epinephrine-primed DCs support IL-17A and IL-4 production by activated $CD4^+$ T cells. Importantly, these results are the first evidence that epinephrine-primed DCs can generate $CD4^+$ T cell activation without further exposure of exogenous epinephrine to $CD4^+$ T cells. Similarly, CRH stimulation also supported IL-23 and IL-10 cytokine production from DCs and IL-17A and IL-4 in $CD4^+$ T cells. Although these findings demonstrate the impact APC-CRH interactions, further studies are needed. For example, we and others demonstrated that DC express both CRH receptors (e.g. detection of transcriptional expression of both CRHR1 and CRHR2) [published and unpublished data] However, studies are required to define the receptor isotypes responsible on CRH's impact dictated by preferential CRH receptor activity. Such studies utilizing pharmacological-specific receptor agonists and antagonists could be used to address this question as well as the use of CRH receptor deficient animal models.

Based on our *in vitro* finding that IL-23/IL-17 axis are possibly under control of stress-derived neuroendocrine factors, our next question aimed to examine the role on IL-23

related to pulmonary *S. pneumoniae* infection using an IL-23 genetic deficient animal model. IL-23 plays an important role in immune defenses against bacterial infection. As expected, the absence of IL-23 resulted in less bacterial clearance in lung and a higher rate of bacterial dissemination. These results were accompanied with impaired production of IL-17A, predicting a dysregulation on neutrophil recruitment, which is reported to be the first defense line against bacterial infection including *S. pneumoniae* (147, 149, 265, 274). Accordingly, an attenuated neutrophil recruitment into lung was shown in IL-23 deficient mice corresponding with elevations in bacterial growth as well as diminished pro-inflammatory cytokine production. Surprisingly however, neutrophil depletion did not show a significant impact on *S. pneumoniae* induced pathogenesis. Rather, our data showed that neutrophil depletion did not exacerbate bacterial burden in both IL-23 competent and deficient mice. These results implicate that IL-23 plays novel role in a neutrophil-independent manner in protection against *S. pneumoniae* infection. One possible explanation supporting the independence of IL-23 and neutrophil function could be directly or indirectly related to the regulation of cytokine responses in the absence of IL-23. In support, we found that the proinflammatory cytokines IL-12p70 and IL-6 were significantly impaired in the lung brochoalveolar lavage space in the absence of IL-23. IL-12p70 is a major APC-derived cytokine known to trigger IFN-γ-mediated Th1 cellular immunity, which supports protection against bacterial infection. Importantly, IFN-γ cytokine production by isolated lung lymphocytes was significantly impaired. IL-6 is also an important cytokine activating several immune cells supportive of proinflammatory immune responses against bacterial invasion including chemokine and complement activity. Thus, we anticipate that future studies aimed at dissecting these and other possible influences by IL-23 could provide valuable

insight regarding the immunological responses involved in protection and disease pathogenesis against *S. pneumoniae* infection.

The intent of our research is to understand the role that stress plays in disease susceptibility through defining the pathways linking stress response factors and immune function. We have previously reported that stress exposure impacts disease susceptibility against *S. pneumoniae* infection. However, the identification of stress factors mediating immune responses in this model remained unknown. Utilizing an *in vitro* model system, our initial investigation defined epinephrine and CRH as putative stress factors involved in regulation of immune function in response to LPS, a bacterial-associated component. In particular, epinephrine and CRH was found to be significant stress factors regulating IL-23/IL-17A responses. Such findings provided interest in defining the role of IL-23 in regulation of immune defenses against *S. pneumoniae* infection. In this regard, our studies utilizing a genetic model of IL-23 deficiency revealed the importance of IL-23 and immune defenses. Together, these findings provided impetus to investigate whether CRH and epinephrine in our model of restraint stress against *S. pneumoniae* infection was linked to disease susceptibility.

Using pharmacological antagonists, we determined whether blockade of CRHR1, CRHR2 or β2-adrenergic receptors would impact the disease outcome of mice subjected to restraint stress and infection. Indeed, selective receptor-mediated blockade resulted in significant alterations in the quality of immune responses generated under conditions of stress and infection. Most impressive was the dichotomy of immunological responses influenced by CRHR1 and CRHR2 antagonists and their concomitant difference in disease outcome. Whereas CRHR1 antagonist administration accentuated inflammatory responses depicted by increased

neutrophils and elevations in IL-17A, CRHR2 blockade resulted in an opposite effect (e.g. lower neutrophils and lower IL-17A production along the respiratory airways). This finding suggests a strong regulatory role for CRHR1 and CRHR2 activity on inflammatory responses that is related to the severity of *S. pneumoniae* infection under conditions of stress. As shown in chapter 3, neutrophils may not be a major cell population required for protection from *S. pneumoniae*. However, in the context of stress, our results suggest that control of neutrophil function could be regulated through neuroendocrine responses. In this regard, a skewed CRHR1 or CRHR2 response may be a determinant of the functional role of neutrophils and whether they provide benefit to the resolution of *S. pneumoniae* infection. In total, these findings implicate the different actions of these two receptor isotypes for the same ligand CRH involved in neutrophillic responses.

Protection against *S. pneumoniae* infection does not solely depend on neutrophils, but also requires the surveillance by other immune system constituents including DCs, macrophages, eosinophils, B cells and T cells. These cells are known to express CRHRs and also play a pivotal role on clearance as components of innate and adaptive immune responses. For instance, DCs process extracellular pathogens through phagocytosis/pinocytosis to generate effective T cell-mediated immune systems. Macrophages are also known to be effective phagocytes for bacterial clearance including *S. pneumoniae* infection (313). In addition, eosinophils are also an important source of cytokines required for effective protection against pathogen invasion (303). Our results suggest that these cell types may have a different CRHR isotype distribution from neutrophils such as CRHR1-dominant expression. This is evidence by a preference in CRHR1 activation by pharmacological blockade of CRHR2 correlating with altered cellular distribution in pulmonary compartments. Further investigation is required to define distribution of CRHR isotypes in

various immune cells in response to *S. pneumoniae* infection and how CRH-CRHR activity impacts disease outcome.

Catecholamine-driven responses in the context of stress exposure are major regulators of immune responses. Our studies investigated the role of adrenergic stimulation in response to stress and the resultant impact on *S. pneumoniae* infection. The results from our study demonstrate that blocking β2 adrenergic receptor did not show a great influence in bacterial susceptibility or protection. However, interestingly, β2 adrenergic blockade altered different lung immune cell populations in comparison with CRH receptor antagonists. Both $CD4^+$ and $CD8^+$ T cells were increased in lung among mice administered a β2 adrenergic antagonist in response to *S. pneumoniae* infection. Th1 CD4+ T cells are known to express β2 adrenergic receptors, which result in an inhibition of their proliferation as well as function (63). In contrast, Th2 type $CD4^+$ T cells do not express β2 adrenergic receptors (63) and no clear evidence has yet been shown of their receptor expression on Th17 $CD4^+$ T cells or IL-17 producing $CD8^+$ T cells. Furthermore, β2 adrenergic stimulation also inhibits production of IL-12p70 by APC as well as suppresses IL-2 production required for Th1 cell proliferation (55). Considering previous studies and our current findings, we propose that β2 adrenergic blocking may directly or indirectly facilitate T cell associated cell-mediated immune responses and impact disease outcome. Thus, future studies will be to define T cell subpopulations influenced by blocking β2 adrenergic signaling and determine their contribution to protection or immune-mediated disease pathogenesis.

Overall, this study demonstrates the potential immunomodulatory role of stress-derived catecholamines and CRH on DC to generate $CD4^+$ T cell activation against bacterial pathogenesis using an *in vitro* system. Moreover, we identified a potential novel role of IL-23,

which is mainly controlled by CRH and catecholamines, against acute pulmonary *S. pneumoniae* infection. We also identified the impact of CRHRs and β2 adrenergic receptors on disease susceptibility, cytokine production and immune cell populations against restraint stress followed by pulmonary *S. pneumoniae* infection. Further investigations are still required to define biochemical and cell physiological networks in response to stress. However, we believe our finding highlighted several critical questions how stress can directly impact on immune system. Specifically, we clearly showed; 1) the impact of neuroendocrine factor on novel Th17 immunity, 2) potential novel role of IL-23 independent from neutrophil network and 3) the diverse susceptibly against septic lethality by different neuroendocrine factor receptors. Based these findings, future investigations will be further advanced to define influences of stress on human health through understanding immunoprotective or immunolopathologic impact of stress.

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