Published in final edited form as:

Brain Behav Immun. 2008 May ; 22(4): 552–564. doi:10.1016/j.bbi.2007.11.005.

Stress-induced differences in primary and secondary resistance against bacterial sepsis corresponds with diverse corticotropin releasing hormone receptor expression by pulmonary CD11c⁺ MHC II⁺ and CD11c⁻ MHC II⁺ APCs

Xavier F. Gonzales¹, Aniket Desmutkh¹, Mark Pulse¹, Khaisha Johnson², and Harlan P. Jones^{1,*}

¹ Department of Molecular Biology and Immunology, University of North Texas Health Science Center in Fort Worth, Fort Worth, Texas 76107

² Department of Biology, Fisk University, Chicago, IL

Abstract

Stress responses have been associated with altered immunity and depending upon the type of stressor, can have diverse effects on disease outcomes. As the first line of defense against potential pathogens, alterations in cellular immune responses along the respiratory tract can have a significant impact on the manifestation of local and systemic disease. Utilizing a murine model of respiratory pneumonia, the current study investigated the effects of restraint stress on the induction of primary and secondary immunity along the respiratory tract, influencing host susceptibility. Female CD-1 mice were subjected to three hours of restraint stress over a period of four days followed by primary and secondary *Streptococcus pneumoniae* infection via intranasal route. Stress exposure led to increased retention of bacterial carriage in the lungs, enhanced polymorphonuclear cells and a preferential decrease in pulmonary CD11c⁺ MHC II⁺ cells resulting in delayed lethality during primary infection but significant impairment of acquired immune protection after secondary infection. We also provide evidence to support a role for lung-associated corticotrophin releasing hormone regulation through peripheral CRH and diverse CRH receptor expression by MHC II⁺ antigen presenting cells (APCs). We conclude that repeated restraint stress has distinct influences on immune cell populations that appear to be important in the generation of innate and adaptive immune responses along the respiratory tract with the potential to influence local and systemic protection against disease pathogenesis.

Keywords

restraint stress; respiratory immunity; antigen presentation; corticotrophin releasing hormone

Correspondence: Harlan P. Jones, 3500 Camp Bowie Boulevard, Department of Molecular Biology and Immunology, Phone: (817) 735-2448, Email: hajones@hsc.unt.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Exposure to stressful situations has been associated with health status (Fava et al., 2007; McClellan and Cohen, 2007; Schindler, 1985; Yang and Glaser, 2002), lending credence to the belief that communication between the neuroendocrine and immune systems play a major role in disease susceptibility. Evidence to support this notion originate from previous *in vitro* and *in vivo* studies demonstrating that neuroendocrine response elements of the central and peripheral nervous systems affect immune function including: cellular proliferation, differentiation, cytolytic function and cytokine secretion (Bryndina and Danilov, 2002; Calcagni and Elenkov, 2006; Dhabhar and McEwen, 1996; Dhabhar et al., 1996; Emeny et al., 2007; Engler et al., 2005b; Leu and Singh, 1993; Saha et al., 2001; Sternberg, 2006; Weiss et al., 1989). In this regard, stress-induced immune modulation has been found to be diverse. Experimentally, studies have documented stress susceptibility to predict immune competence based upon strain and gender differences (Li et al., 2000; Sieve et al., 2006). For example, C Kiank et al. demonstrated disease susceptibility to be greater in male mice, highlighting differences in stress responsiveness and inherent hormonal and behavioral dispositions (Kiank et al., 2006). In addition, numerous studies have characterized stress-immune relationships to be dependent upon gender, neuroendocrine response factors, the type and quality of the stress event, antigenic stimuli and host genetic factors (Butcher and Lord, 2004; Engler et al., 2005b; Gross and Siegel, 1981; Ishihara et al., 1999; Ising and Holsboer, 2006; Matalaka, 2003; Okuyama et al., 2007). Furthermore, studies have shown that stress effects to be associated with regional differences in the generation of immune responses (Engler et al., 2005b). Thus, it has been predicted that defining the relationships between neuroendocrine and immune function during disease to be very complex.

As a primary site of pathogenic exposure, immune regulation along the respiratory tract plays a major protective role against potential local and systemic disease. Importantly, because the respiratory tract is constantly exposed to foreign stimuli, the respiratory immune defense system must be able to temper its' activation between innocuous antigen and potential pathogens. Stress exposures have been implicated in the pathogenesis of numerous respiratory disease states: including asthma, bacterial pneumonia, viral infection and tumor development (Boyce et al., 1995; Bryndina and Danilov, 2002; Datti et al., 2002; Engler et al., 2005a; Joachim et al., 2003; Konstantinos and Sheridan, 2001; MacQueen and Bienenstock, 2006; Okuyama et al., 2007). Yet, the mechanisms involved in the activation of neuroendocrine responses that influence the regulation of respiratory immunity remain uncertain. Our mission in understanding the relationships of stress and respiratory immunity is to define how stress mediates cell-specific responses that predict protective verses immunopathogenesis, influencing disease susceptibility.

Utilizing various *in vivo* experimental models of stress, researchers have made progress in this area. For example, previous studies have documented the role that stress has in mediation of viral infection and tumor defenses along the respiratory tract by demonstrating the influence of stress on cell-mediated immune responses and NK cell activity (Engler et al., 2005a; Hunzeker et al., 2004; Konstantinos and Sheridan, 2001; Sheridan et al., 2000; Strange et al., 2000). In particular, Tseng et. al. demonstrated that susceptibility to experimental viral influenza infection was associated with glucocorticoid and opiod-induced suppression of NK activity in response to restraint stress (Tseng et al., 2005). Likewise, studies have shown that in conditions such as asthma, neuroendocrine activation can promote polarization of cell-mediated immune responses, facilitating a hyperactive immune reaction toward innocuous antigens (Datti et al., 2002; Okuyama et al., 2007; Portela Cde et al., 2001). A study by Joachim et. al. showed that stress-induced exacerbation of asthma was associated with a biased activation of Th2 cellular immune responses including: elevations in IL-4 and IL-5 cytokine activity and eosinophillia (Joachim et al., 2003). These and other published studies reveal that

proper orchestration of different cellular components of the immune system in response to various infectious or non-pathogenic insults can be influenced by neuroendocrine activation.

As a leading cause of community acquired respiratory pneumonia, *Streptococcus pneumoniae* (*S. pneumoniae*) infection poses a serious problem for the health care system. Despite therapeutic advances, effective vaccines against recurrent infections are unavailable and therefore resistance against disease hinges greatly on host natural immunity. Protection against *S. pneumoniae* infection involves the activation of the innate and adaptive compartments of the immune defense system. In that proper immune activation can determine the outcome of disease, it is conceivable that stress-associated alterations in immune responsiveness can have a significant impact on host susceptibility. Support for this idea is found in studies demonstrating that children, elderly, hospitalized patients and the immunocompromised, experiencing stressful conditions correlated with increased risk of respiratory-related disease (Butcher and Lord, 2004; Chiang et al., 2007; Ginesu and Pirina, 1995; Graham et al., 2006; Hortal et al., 2007; McClellan and Cohen, 2007). Few experimental studies have investigated the relationships between stress and *S. pneumoniae* infection. In on such study, Murray et. al. demonstrated compensatory effects of cellular host immune defenses was associated with susceptibility to *S. pneumoniae* infection in a model of CRH expression (Murray et al., 2001). Using an experimental model of stress and pneumococcal respiratory infection in mice, the purpose of the current study was to determine the influence of stress on cellular immunity along the respiratory tract and how potential alterations would predict disease susceptibility. In this study, we revealed that stress-induced differences in protective responses during primary and secondary pneumococcal infection are associated with preferences in cellular immune responsiveness along the respiratory tract. We also provide evidence to support a role for lung-associated diversity of corticotrophin releasing hormone (CRH) and CRH receptor expression by antigen presenting cells (APCs), as a potential link in the induction of acquired immune protection in during exposure to stressful events.

Materials and methods

Mice

Adult (7–8 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 housed 5 to a cage under optimal temperature and humidity controlled conditions and provided proper care as directed by the institutional animal care and use committee. All mice were allowed to acclimate to the colony until of age prior to experiment.

Stress paradigm

Restraint stress (RS) was induced in mice by placing them in a sterile 50 ml conical tube supplied with air holes for sufficient ventilation. All mice were stressed daily for 4 days performed for 3 hrs exactly between the hours of 1:00 PM and 4:00 PM. No food or water was supplied to subjects during the stress period, including non-restraint stress (NRS) animals, which remained in their home cage environment.

Bacteria and infection

Streptococcus pneumoniae strain #6301 (ATCC, Manassas, VA) was grown overnight to achieve mid-log phase cultures on Blood Agar plates. Mice were infected with *S. pneumoniae* (10^6 cells) in a volume of 40 μ l of Todd Hewitt broth (Sigma, St. Louis, MO) by intranasal inoculation. Similar groups of mice were inoculated with Todd Hewitt broth (Sigma Aldrich) to serve as vehicle control. All inoculations were performed during the same time in which the stress paradigm was performed. Prior to infection and sacrifice, mice were

anesthetized by intramuscular injection of ketamine-xylazine. In selected experiments survival analysis was performed.

Colony forming assay

Bacterial loads in lung, spleen and blood were evaluated at different time points after *S. pneumoniae* infection. After sacrifice, organs were harvested and homogenized in PBS. Ten-fold serial dilutions of organ homogenates were plated in triplicate onto blood agar plates and incubated at 37° C overnight. Colonies on plates were enumerated, and the results were expressed as log₁₀ CFU per µl.

Cell Isolation

Bronchiolar lavage cells were isolated from bronchiolar lavage fluid (BALF). Specifically, lungs were perfused twice via tracheal instillation of 1–2 ml of wash media using 25 gauge blunted syringe needle. BALF containing cells was aspirated and the volume of fluid retrieved was recorded. Single cell suspensions from lung tissue were prepared as previously described (Jones et al., 2001). Briefly, lungs were infused with sterile PBS to eliminate contaminating RBCs. Lung tissue was separated into single lobes finely minced and placed into digestion medium containing 500 units/ml collagenase type II (Worthington, Lakewood, NJ.) and 50 units/ml DNase (Sigma Aldrich, St. Louis, MO). Lung tissue was digested for 1.5 hr per lung. After digestion, lung digestions were passed through a nylon mesh filter (LabPak, Depew, NY.) into sterile 15 ml conical tubes and washed 2X in wash media (Hyclone, Logan, UT.). Lymphocytes were prepared by ficoll-hypaque (Lympholyte M, Cedarlane, Laboratories, Ltd., Ontario, CA) centrifugation. Spleen cell suspensions were prepared by pressing spleen tissue through sterile mesh. Contaminating RBCs were removed using ACK lysis buffer as previously described (Kruisbeek, 1999). Whole blood samples were prepared from cardiac puncture and placed into sterile tubes containing heparin-sulfate to impede coagulation.

Flow cytometry

The phenotype of immune cells was determined by characterization of cell surface markers using flow cytometric methods. Lung single cell suspensions were washed and incubated with an optimal concentration of anti-FcBlock Antibody (clone 2.4G2) (BD PharMingen, San Diego, CA) to block non-specific binding to FcRs for 30 min. Two-color immunofluorescent staining was performed by incubating cells with combinations of pre-titrated concentrations of the following antibodies: anti-PE GR1⁺, anti-FITC MHC II⁺, anti-FITC F4/80⁺, anti-FITC CD11b⁺, anti-PE CD3⁺, anti-FITC CD4⁺ or anti-FITC-CD8⁺ and anti-PE-Cy7 CD45R. Detection of CD11c⁺ cells was performed using biotin-conjugate anti-CD11c⁺ antibody followed by secondary labeling using streptavidin Texas Red conjugate. Antibodies were purchased from BD PharMingen, San Diego, CA. with the exception of CD45R conjugated antibody that was purchased from Invitrogen, Corporation (Invitrogen, Corporation, Carlsbad, CA) and biotin-conjugated CD11c⁺ antibody and streptavidin Texas Red that were purchased from eBioscience (eBioscience, San Diego, CA.). Gating of lymphocytes was identified by forward-scatter/side-scatter profile. Percent positive staining was determined by subtracting autofluorescence from cells stained with appropriate anti-fluorescent labeled iso-typed controls (BD PharMingen). Data was collected on an FC500 flow cytometer (Beckman Coulter, Miami, FL.). Further analysis was performed using EPIC software (Beckman Coulter). Absolute cell numbers was determined by multiplying the percent positive cells by the total number of cells isolated from lung tissue.

Cell Sorting

The purification of pulmonary APC populations was performed by cell-sorting techniques. Briefly, total pulmonary lymphocytes were labeled with Texas Red-anti CD11c⁺ (eBioscience)

and FITC anti-MHC II⁺ (BD, PharMingen). After labeling, total lung lymphocytes were sorted using an InFlux cell sorter (Cytospeia, Seattle, WA). CD11c⁺ MHC II⁺ and CD11c⁻ MHC II⁺ cell populations were collected. Selected cell populations were determined to have a purity of at least 99%; analyzed using Spigot software (Cytospeia).

Lung frozen sectioning and histological evaluation

To evaluate lung airway inflammation, mice were sacrificed and lungs were perfused with sterile cold PBS intratracheally, followed by infusion of 2% paraformaldehyde (Sigma Chemical, Co., St. Louis, MO). Fixed tissues were then incubated in a 2% formaldehyde bath for 4 hours and overnight in 30% sucrose solution at 4° C. The following day, tissues were embedded in OCT freezing medium (Sakura Finetechnical, Torrance, CA) and stored at -80° C until analysis. 10 µm sections were prepared using Ultrapro 5000 cryostat (Vibratome, St. Louis, MO) and mounted on FisherBrand SuperFrost/plus microscope slides (Fisher Scientific, Hampton, NH). Slide preparations were stained with hematoxylin and eosin (FisherBrand). Images were taken using an Olympus AX70 fluorescent microscope (Olympus, Centerville, PA) and analyzed using Image Pro X software (Olympus).

Real-time RT-PCR

Total RNA (1 µg per 10 µl reaction) from lung cell preparations was reverse transcribed using Molony murine leukemia virus reverse transcriptase (Promega, Madison, WI) as previously described (Sun et al., 2006). After cDNA synthesis, real-time RT-PCR was performed using SYBR green techniques. Selected target gene primer sets; CRHR1, CRHR2, were purchased from SuperArray, Bioscience Inc. (SuperArray, Frederick, MD). Real-time SYBR master mix was also purchased from SuperArray. PCRs were performed in 25 µl reactions using a Smart Cycler system (Cepheid, Sunnyvale, CA). PCR reactions were performed as previously described (Sun et al., 2006). The threshold of the growth curve (*CT*) was set at a value of 35. Differences in gene expression were determined by relative quantification between cDNA templates from *S. pneumoniae*-infected RS mice and *S. pneumoniae*-infected NRS mice. The expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to normalize target gene expression between samples. Differences in target gene expression was calculated using the following formula: $\Delta\Delta^{CT} = \Delta^{CT}(\text{target gene}) - \Delta^{CT}(\text{GAPDH})$. The $\Delta\Delta^{CT}$ value of cDNA amplification from the NRS group was considered the calibrator for baseline levels of mRNA expression. Data was expressed as the ratio of target gene expression of RS-treated subjects to the target gene expression of the NRS-treated group, resulting in fold difference in target gene mRNA levels.

Statistical analysis

Statistical analysis was performed using GraphPad Prism Version 4.0 for MacIntosh (GraphPad Software, San Diego, USA). For multi-experimental group analysis, data were subjected to analysis of variance (univariate ANOVA) followed Post Hoc tests (Tukey) for group differences. For analysis of two-group differences, student's *t* test was employed, followed by Post Hoc analysis. Differences in survival between experimental groups were determined using survival chi square analysis. All data are expressed as the mean ± SEMs. The two-tailed level of significance was set at $p \leq 0.05$.

Results

RS does not increase susceptibility to primary *S. pneumoniae* infection, but does impair acquired resistance to re-challenge

We examined the effect of RS treatment on susceptibility to *S. pneumoniae* infection. Mice exposed to RS experienced significant weight loss as compared to control NRS mice prior to

infection ($t(38) = 4.986$, $p \leq 0.0001$) and among non-survivors ($t(38) = 3.059$, $p \leq 0.0041$) (Fig. 1). RS exposure resulted in a delayed onset of endotoxemia, resulting in significantly ($\chi^2 = 98.67$, $p \leq 0.001$) fewer deaths within the first 96 hrs of infection (Fig. 2A). However, no significant ($\chi^2 = 1.095$, $p \leq 0.5178$) difference in overall percent survival was found between RS and NRS mice exposed to *S. pneumoniae*. We also determined the effect of RS on natural resistance among survivors of primary *S. pneumoniae* infection. RS and NRS survivors were re-challenged 7 days following initial infection. As shown in fig. 2B, a significant ($\chi^2 = 4.598$, $p \leq 0.0320$) decrease in percent survival was found among RS-treated mice.

RS survivors from re-challenge demonstrate greater chronic lung injury

We compared the histopathological changes in the lungs between NRS and RS treated mice exposed to primary infection and of NRS and RS survivors from re-exposure to *S. pneumoniae*. Despite minimal differences in cellular consolidation within the alveolar spaces between NRS and RS survivors from primary infection (Fig. 3., 3 and 4), NRS mice demonstrated a preferential monocytic infiltrate as compared to a polymorphonuclear infiltrate among RS mice (Fig. 3., 5 and 6). In contrast, RS-treated mice subjected to secondary infection, demonstrated greater gross immunopathological changes as defined by a preferential polymorphonuclear infiltrate within the alveolar spaces as compared to NRS challenged mice (Fig. 3., 9 and 10).

RS decreases pulmonary bacterial clearance

We determined the bacterial loads within the lung, blood and spleen of RS and NRS mice 18 hr after *S. pneumoniae* infection. No differences in the number of organisms were found within the spleen and blood between RS and NRS mice 18 hr after infection. In contrast, a significantly ($t(12) = 2.577$, $p \leq 0.0242$) higher number of organisms were apparent in the lungs of RS-treated mice than NRS mice (Fig. 4).

RS influences leukocyte distribution along the respiratory airways in response to *S. pneumoniae* infection

We assessed whether differences in the distribution of immune cells along the respiratory tissues would be observed in response to RS. Differential cell characterization within the bronchiolar lavage fluid (BALF), examination of immune cell numbers in respiratory draining lymphnodes and phenotypic examination of lymphocytes isolated from the lung interstitial tissues was performed. The absolute number of polymorphonuclear cells (PMNs), monocytes and lymphocytes from BALF and cellular phenotypes resident in interstitial lung tissue of NRS and RS infected mice were determined. Total BALF cell numbers were significantly higher ($F = 5.047$; $p \leq 0.0172$) in RS stressed mice exposed to *S. pneumoniae* than in respective controls (Fig. 5A). PMNs were significantly ($F = 11.46$; $p \leq 0.0029$) increased among RS infected mice as compared to respective controls (Fig. 5A). By contrast, monocyte numbers among RS-infected were significantly ($F = 8.433$; $p \leq 0.0043$) diminished as compared to NRS-infected mice (Fig. 5A). Figure 5B confirms morphologically the distribution of PMNs and monocytes distributed in BALF of each experimental group.

We also determined the changes in immune cell populations in the draining respiratory nodes (LRN) and total lung tissue of mice exposed to RS. No significant differences in total LRN were found (NRS-broth; $5.79 \times 10^6 \pm 2.3 \times 10^6$, RS-broth; $5.5 \times 10^6 \pm 1.5 \times 10^6$, NRS-infected; $1.0 \times 10^7 \pm 8.9 \times 10^6$, RS-infected; $6.2 \times 10^6 \pm 2.1 \times 10^6$; $n=4$). Whereas RS led to a moderate increase in the total number of BALF cells, RS exposure was found to elicit a decrease in lymphoid infiltrate within the interstitial lung tissue (Fig. 6). Despite the observed decrease in total cells, Gr1⁺CD11b⁺ cells; markers typically used to designate neutrophils were the major cell-type found to significantly increase ($F=505.9$; $p \leq .0001$) in response to *S. pneumoniae* infection (Fig. 6). On the contrary, RS-treatment corresponded with a significant ($F=18.58$; p

≤ 0.0082) decrease in a subset of APCs; CD11c⁺ MHC II⁺ (DCs) and B220⁺ (B Cells) ($F=10.44$; $p \leq 0.0231$) 18 hrs after infection (Fig. 6). No significant changes in F4/80⁺ MHC II⁺ APCs (Macrophages) subset or CD3⁺ CD4⁺ and CD3⁺ CD8⁺ T cells were found (data not shown).

Diverse CRH receptor expression by lung APCs in response to restraint stress and infection

Utilizing real-time RT-PCR methods, we examined total CRH expression in lung tissue as well as CRHR1 and CRHR2 receptor expression by pulmonary CD11c⁺ MHC II⁺ and CD11c⁻ MHC II⁺ cells of infected mice exposed to RS. CRH mRNA levels were significantly ($t = (3) 10.71$; $p \leq .0086$) increased in the lungs of RS-infected mice (Fig. 7A). CRHR1 mRNA levels by CD11c⁺ MHC II⁺ was significantly ($F= 48.41$; $p \leq 0.0013$) increased in the lungs of RS-infected mice as compared to non-stressed mice. However, CRHR2 expression by CD11c⁺ MHC II⁺ cells among RS-infected mice was not significantly different as compared to NRS-infected mice (Fig 7B). The converse was shown by lung CD11c⁻ MHC II⁺ cells, CRHR2 expression was preferentially increased (4-fold) and CRHR1 mRNA levels were only moderately increased (Fig. 7B).

Discussion

The studies presented here demonstrate that repeated regiments of acute restraint stress elicits selective cellular immune responsiveness along the respiratory tract, leading to divergent outcomes between primary and secondary *S. pneumoniae* infection. Such findings suggests that stress-induced preferences in the induction of pulmonary immune responses are likely to play a critical role in determining the course of disease. To date, the exact regulatory pathways which stress responses influence cellular immune function, particularly along the respiratory tract are not fully understood. Here, we demonstrate stress-induced preferences in the distribution of pulmonary cellular populations as a potential mechanism responsible for the contrasting outcomes of primary and secondary pneumococcal infection. We also implicate peripheral CRH and CRH receptor expression in the lung and pulmonary APCs respectively as a potential link between neuroendocrine-immune interactions in the generation of acquired immune protection.

Many studies have reported the generation of immune responses to be dependent on a broad range of physiological, environmental and genetic variables. Likewise, exposure to various types of stress has been characterized for the type of immune response that is affected, depending on its duration (e.g. short periods verses longer exposure times) (Ishihara et al., 1999; Okuyama et al., 2007; Persoons et al., 1997). By applying 4 consecutive days of repeated durations of stress (3 hrs) and subsequent delivery of *S. pneumoniae* along the respiratory airways 18 hrs later, our findings demonstrated that subjecting female mice to this form of stress did not diminish overall lethality compared to NRS mice. In fact, RS provided temporary protection by delaying the onset of death within the first 0–96 hrs that gradually declined throughout the course of primary infection which was consistent with the indistinguishable differences in lung histopathology found between NRS and RS mice. This finding was in contrast to our prediction considering the significant decrease in weight among mice subjected to RS prior to infection. Support for such findings has been documented previously. For example, C. Kiank et al. demonstrated that female BALB/c mice were more resistant toward bacterial infections given RS exposure despite increased weight loss (Kiank et al., 2006). Thus, based upon these results, we suspected that this form of stress was consistent with previous studies demonstrating the temporal immuno-enhancing effects of acute RS (Fleshner et al., 1998; Roozen and Magnusson, 1996) at least to the extent that did not result in increased susceptibility in female mice.

Lethality from bacterial dissemination and sepsis due to *S. pneumoniae* infection is often dependent upon the degree of bacterial outgrowth from the respiratory tract into systemic

compartments (e.g. blood). Given that no significant difference in disease outcome was found between mice exposed to RS as compared to NRS mice, suggested that little if any differences in bacterial colonization along the respiratory tract and subsequently within the peripheral tissues would be observed. Alternatively, one might consider that the observed delay in onset of death among RS-treated mice could be a result of a preferential inhibition of initial bacterial colonization within the lungs. On the contrary, the results from this study demonstrated a preferential retention of bacterial growth within the lung and no significant differences in outgrowth in the blood or spleen 18 hrs after infection. One potential explanation for these findings could be that the preference for bacterial colonization in the lung under stress conditions may be preventing dissemination allowing for delayed sepsis, but ultimately without providing a selective advantage of survival. We considered whether the initial bacterial load masked resistance among RS mice. The fact that reducing the infection dose to the LD⁵⁰ demonstrated a similar survival pattern (data not shown), suggest that initial pulmonary carriage was not influencing the effects of stress. Furthermore, we found that after 36 hr, the extent of bacterial outgrowth in the lungs, spleen and blood were similar between RS and NRS mice and would therefore correspond with little differences in overall survival (data not shown). Based upon these findings, we concluded that restraint stress demonstrated transient protective influences, presumably by elevating the status of innate immune responses during primary infection.

Natural acquired resistance to *S. pneumoniae* infection is believed to be an important factor in preventing recurrent disease. In that no differences in host susceptibility and at least temporal protection upon stress exposure was observed, one might expect that the induction of natural resistance would reflect a similar pattern. However, in contrast to primary infection, resistance against re-challenge was shown to be significantly altered by demonstrating the following: 1) re-challenging surviving subjects previously exposed to RS led to a significant decrease in overall survival as compared to NRS survivors and 2) greater histopathological changes were found in the lungs of RS survivors, indicating chronic lung injury. Based upon these findings, we concluded that although initial exposure did not adversely affect primary resistance to sepsis, RS exposure was substantial enough to diminish acquired adaptive immune responses leading to decreased survival and chronic pulmonary disease.

The respiratory airways serve as an initial site of defense against local and potentially systemic disease. Thus, it is likely that stress-induced alterations in local respiratory immune responses could facilitate host susceptibility, warranting an investigation of the phenotypes governing respiratory immune defenses against *S. pneumoniae* infection. The preferential increase in PMNs/neutrophils within BALF prior to and after infection is consistent with its documented role in early host defense against bacterial infection (Edwards et al., 1982; Gordon et al., 1986; Hart et al., 1986; Mold et al., 1982) and may explain the observed delayed onset of death given the enhanced BAL infiltrate of PMNs in response to RS. Utilizing this model of stress, we have documented early chemokine activity prior to and 3hr post-infection (unpublished data), suggesting that RS maybe enhancing cellular recruitment of innate immune cell populations (e.g. neutrophils) that could potentially influence protection. In support Speyer et. al. demonstrated that during sepsis, alterations in chemokine activity resulted in trafficking of PMNs to the lung (Speyer et al., 2004). Interestingly, pulmonary monocytes (comprising of macrophages), which also have an important role in immune surveillance and constitute important liaisons and activators of acquired immunity, were not responsive toward RS within the BALF. Alveolar macrophages, a major constituent of the monocytic population, are known to play a major regulatory role as phagocytes in defense against bacterial infections (Marriott et al., 2006). Thus, the findings presented here are seemingly contradictory with respect to the lack of bacterial clearance and delayed lethality. In support of our observations, other studies have demonstrated that neuroendocrine activation can promote disease pathogenesis along the respiratory tract that is mediated in part by altered neutrophil function (Chang et al., 2004;

Meagher et al., 1996; Nishida et al., 1998). Thus, one might consider that although neutrophilic recruitment under RS conditions may be associated with the observed delayed onset of death, the lack of bacterial clearance may be a result of a dysregulation in their functional response or other cellular components that are instrumental in neutrophil function (e.g. complement activation) (Gordon et al., 1986; Liles and Klebanoff, 1995; Noya et al., 1993). Also, the lack of the activation by alveolar macrophages in response to infection may have been a contributing factor for increased bacterial load within the lungs of RS mice. In support, Flesher et. al. have previously demonstrated that nitric oxide activation by peritoneal macrophages required pre-activation via antigenic recognition and its effect was dependent upon time between stress and antigenic exposure (Fleshner et al., 1998). Thus, at least given our current stress regimen prior to antigenic challenge, impairment of bactericidal activity along the respiratory tract contradictory to the delayed lethality may be facilitated by a lack of continuity between neutrophilic and macrophages or other innate cell populations.

Subsequently to collecting lavage fluid, we further assessed the phenotypic distribution of residual immune cells within the lung parenchyma and draining respiratory nodes. While no significant changes in cellular responses were observed in the respiratory draining lymphnodes, we noted a preferential reduction in the total lymphoid infiltrate within the lungs in response to RS, suggesting the mobilization of immune cells within the alveolar spaces in response to infection. This would be consistent with the observed BALF-associated increase in cellular infiltrate demonstrated in RS-infected subjects. The depression of total cells within the lungs involved a selective distribution of cellular populations among RS-infected subjects. Namely, while GR1⁺ CD11b⁺ cells (typically markers for neutrophils) were increased in the lungs, F4/80⁺ MHC II⁺ specific staining of macrophages demonstrated no increase in their response given RS toward *S. pneumoniae* infection. Interestingly, however, we found a significant decrease in CD11c⁺ MHC II⁺ and B220⁺ (B cells) cells among RS infected mice. Therefore, while similar trends in neutrophil and macrophage responses were observed along the respiratory tract, the finding that other major cell types were dramatically decreased was interesting and provided a potential explanation for the differential effect on primary and acquired immune protection given RS. Specifically, the diminished number of CD11c⁺ MHC II⁺ cells along the respiratory tract in response to stress during infection, suggest a potential mechanism responsible for the observed decrease in host protection. CD11c⁺ MHC II⁺ cells comprise mainly of dendritic cells (DCs) and can elicit protection against *S. pneumoniae* (Colino and Snapper, 2007). However, more studies are needed to define the mechanisms influencing DC functionality under stress conditions. Our results demonstrating a concomitant decrease in CD11c⁺ MHC II⁺ cells with decreased acquired protection provides a potential explanation for the observed difference in primary and secondary responses to *S. pneumoniae* infection. Moreover, decreases in B cell populations would be consistent with altered priming of adaptive immune protection via impaired humoral and cell-mediated immune responses (van Rossum et al., 2005). In support, alterations in adaptive immunity have been previously described to influence disease susceptibility under stress conditions (Cao and Lawrence, 2002; Mediratta and Sharma, 2002). Future studies are planned to examine the relationships between stress and priming of humoral and T cell-mediated immune responses to primary and secondary infection.

Stress-associated activation of the CNS results in the neuroendocrine response through the hypothalamic-pituitary axis (HPA) resulting in corticosterone release and the autonomic response via stimulation of catecholamines such as norepinephrine. Indeed, the findings presented here suggest the activation of both stress response pathways in modulation of the cellular immune responses generated during primary and secondary infection. For example, stress exposure has been documented to be a trigger of both the hypothalamic and adrenergic nervous systems with the capacity to amplify local innate and inflammatory responses as a first line of defense against acute infection (Ader and Cohen, 1993). In particular, numerous studies

have highlighted the significance of adrenergic activation as primary mediators during early stages of disease (Cao et al., 2003), while prolonged stress exposure may be responsible in regulation of adaptive immune responses through HPA-mediate corticosterone activity (Truckenmiller et al., 2006). Therefore, although the contribution of catecholamines and/or corticosteroids were not demonstrated in the current study, the findings here suggest that preferences in their activity will be important in defining the underlying differences in disease outcome.

Once believed to be primarily derived from the HPA, CRH can also be expressed peripherally at sites of inflammation and believed to impact immune responses (Jain et al., 1991; Murray et al., 2004). CRH activation has been shown to modulate several disease states including: colitis (Anton et al., 2004), asthma (Silverman et al., 2004; Tantisira et al., 2004) and rheumatoid arthritis (Crofford et al., 1992; Jessop et al., 2001). To date, numerous cellular immune targets have been documented including: mast cells, T cells, B cells, neutrophils and macrophages (Agelaki et al., 2002; Baker et al., 2003; Cao et al., 2006; Cao et al., 2005; Iavicoli et al., 1998; Radulovic et al., 1999). The functional activity of CRH is regulated by two major receptors, CRHR1 and CRHR2 having diverse affinities for CRH and the CRH homologues, Urocortin (UCN1–3) (Jain et al., 1991; Jessop et al., 2001). A number of studies have implicated a role for CRH and its receptors as inflammatory mediators (Moffatt et al., 2006; Papadopoulou et al., 2005; Radulovic et al., 1999). To our best knowledge, we report for the first time the expression of CRH receptors by pulmonary CD11c⁺ MHC II⁺ cells; a subset of DCs. Specifically, we provide *in vivo* evidence that CD11c⁺ MHC II⁺ cells and CD11c[−] MHC II⁺ cells (presumably macrophages and B cells) exhibit diverse CRH receptor expression in the lungs of mice exposed to a stress event. Moreover, our finding that CRH was expressed in the lungs and significantly increased given RS exposure provides the potential for direct ligand-receptor interaction. In that CD11c⁺ MHC II⁺ cells revealed a preferential increase in CRHR1 expression by DCs whereas CD11c[−] MHC II⁺ cells expressed higher levels of CRHR2 is intriguing. Previous studies have described a function role of CRHR1 expression on macrophages and regulation of anti-inflammatory activity by macrophages in the presence of CRH1 and CRH agonist (Tsatsanis et al., 2007). Thus, based upon these findings one may speculate that differential receptor expression could have influences on APC function and therefore impact downstream APC-T cell interactions, leading to altered adaptive immune responses (e.g. cytokine production by T cells). Although not examined in the current study, we cannot discount the potential influence of CRH regulation of neutrophils that were observed to have opposing responsiveness within the airways as compared to immune cells populations examined. Previous studies have demonstrated the role of CRH on neutrophil function (Iavicoli et al., 1998). It would be interesting to determine whether diverse cellular effects correspond with CRH/CRH receptor regulation. Thus, given these results, we believe that further investigation into the role of CRH on antigen presenting cell function as well as on other target cell populations will reveal potential mechanisms responsible for stress-induced alterations in immune function along the respiratory tract.

In conclusion, our findings restate the complexities of stress responses on immune regulation. The studies presented increase our knowledge of the effects that a given stress event can impact pulmonary immune responses and how such changes influence both innate and acquired immunity. Importantly, the studies begin to address how peripheral CRH activation on APC function as a mechanism influencing stress-effects on immune modulation.

Acknowledgments

We would like to acknowledge Dr. Jerry Simecka, Byung-Jin Kim and Xiangli Sun for guidance in the execution of studies and preparation of this manuscript. The project described was supported by Grant Number P20-MD001633 from NCMHD and its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NCMHD.

References

- Ader R, Cohen N. Psychoneuroimmunology: conditioning and stress. *Annu Rev Psychol* 1993;44:53–85. [PubMed: 8434895]
- Agelaki S, Tsatsanis C, Gravanis A, Margioris AN. Corticotropin-releasing hormone augments proinflammatory cytokine production from macrophages in vitro and in lipopolysaccharide-induced endotoxin shock in mice. *Infect Immun* 2002;70:6068–74. [PubMed: 12379683]
- Anton PM, Gay J, Mykoniatis A, Pan A, O'Brien M, Brown D, et al. Corticotropin-releasing hormone (CRH) requirement in *Clostridium difficile* toxin A-mediated intestinal inflammation. *Proc Natl Acad Sci U S A* 2004;101:8503–8. [PubMed: 15159534]
- Baker C, Richards LJ, Dayan CM, Jessop DS. Corticotropin-releasing hormone immunoreactivity in human T and B cells and macrophages: colocalization with arginine vasopressin. *J Neuroendocrinol* 2003;15:1070–4. [PubMed: 14622437]
- Boyce WT, Chesney M, Alkon A, Tschann JM, Adams S, Chesterman B, et al. Psychobiologic reactivity to stress and childhood respiratory illnesses: results of two prospective studies. *Psychosom Med* 1995;57:411–22. [PubMed: 8552730]
- Bryndina IG, Danilov GE. [Substance P as a factor enhancing resistance of the surfactant lung system to chronic immobilization stress]. *Russ Fiziol Zh Im IM Sechenova* 2002;88:84–9. [PubMed: 11868267]
- Butcher SK, Lord JM. Stress responses and innate immunity: aging as a contributory factor. *Aging Cell* 2004;3:151–60. [PubMed: 15268748]
- Calcagni E, Elenkov I. Stress system activity, innate and T helper cytokines, and susceptibility to immune-related diseases. *Ann N Y Acad Sci* 2006;1069:62–76. [PubMed: 16855135]
- Cao J, Cetrulo CL, Theoharides TC. Corticotropin-releasing hormone induces vascular endothelial growth factor release from human mast cells via the cAMP/protein kinase A/p38 mitogen-activated protein kinase pathway. *Mol Pharmacol* 2006;69:998–1006. [PubMed: 16332989]
- Cao J, Papadopoulou N, Kempuraj D, Boucher WS, Sugimoto K, Cetrulo CL, et al. Human mast cells express corticotropin-releasing hormone (CRH) receptors and CRH leads to selective secretion of vascular endothelial growth factor. *J Immunol* 2005;174:7665–75. [PubMed: 15944267]
- Cao L, Hudson CA, Lawrence DA. Acute cold/restraint stress inhibits host resistance to *Listeria monocytogenes* via beta1-adrenergic receptors. *Brain Behav Immun* 2003;17:121–33. [PubMed: 12676574]
- Cao L, Lawrence DA. Suppression of host resistance to *Listeria monocytogenes* by acute cold/restraint stress: lack of direct IL-6 involvement. *J Neuroimmunol* 2002;133:132–43. [PubMed: 12446016]
- Chang LC, Madsen SA, Toelboell T, Weber PS, Burton JL. Effects of glucocorticoids on Fas gene expression in bovine blood neutrophils. *J Endocrinol* 2004;183:569–83. [PubMed: 15590983]
- Chiang WC, Teoh OH, Chong CY, Goh A, Tang JP, Chay OM. Epidemiology, clinical characteristics and antimicrobial resistance patterns of community-acquired pneumonia in 1702 hospitalized children in Singapore. *Respirology* 2007;12:254–61. [PubMed: 17298459]
- Colino J, Snapper CM. Dendritic cell-derived exosomes express a *Streptococcus pneumoniae* capsular polysaccharide type 14 cross-reactive antigen that induces protective immunoglobulin responses against pneumococcal infection in mice. *Infect Immun* 2007;75:220–30. [PubMed: 17043104]
- Crofford LJ, Sano H, Karalis K, Webster EL, Goldmuntz EA, Chrousos GP, et al. Local secretion of corticotropin-releasing hormone in the joints of Lewis rats with inflammatory arthritis. *J Clin Invest* 1992;90:2555–64. [PubMed: 1281840]
- Datti F, Datti M, Antunes E, Teixeira NA. Influence of chronic unpredictable stress on the allergic responses in rats. *Physiol Behav* 2002;77:79–83. [PubMed: 12213504]
- Dhabhar FS, McEwen BS. Stress-induced enhancement of antigen-specific cell-mediated immunity. *J Immunol* 1996;156:2608–15. [PubMed: 8786326]
- Dhabhar FS, Miller AH, McEwen BS, Spencer RL. Stress-induced changes in blood leukocyte distribution. Role of adrenal steroid hormones. *J Immunol* 1996;157:1638–44. [PubMed: 8759750]
- Edwards KM, Gewurz H, Lint TF, Mold C. A role for C-reactive protein in the complement-mediated stimulation of human neutrophils by type 27 *Streptococcus pneumoniae*. *J Immunol* 1982;128:2493–6. [PubMed: 7077077]

- Emeny RT, Gao D, Lawrence DA. Beta1-adrenergic receptors on immune cells impair innate defenses against *Listeria*. *J Immunol* 2007;178:4876–84. [PubMed: 17404268]
- Engler A, Roy S, Sen CK, Padgett DA, Sheridan JF. Restraint stress alters lung gene expression in an experimental influenza A viral infection. *J Neuroimmunol* 2005a;162:103–11. [PubMed: 15833365]
- Engler H, Engler A, Bailey MT, Sheridan JF. Tissue-specific alterations in the glucocorticoid sensitivity of immune cells following repeated social defeat in mice. *J Neuroimmunol* 2005b;163:110–9. [PubMed: 15885313]
- Fava GA, Fabbri S, Sirri L, Wise TN. Psychological factors affecting medical condition: a new proposal for DSM-V. *Psychosomatics* 2007;48:103–11. [PubMed: 17329602]
- Fleshner M, Nguyen KT, Cotter CS, Watkins LR, Maier SF. Acute stressor exposure both suppresses acquired immunity and potentiates innate immunity. *Am J Physiol* 1998;275:R870–8. [PubMed: 9728086]
- Ginesu F, Pirina P. Etiology and risk factors of adult pneumonia. *J Chemother* 1995;7:277–85. [PubMed: 8568539]
- Gordon DL, Johnson GM, Hostetter MK. Ligand-receptor interactions in the phagocytosis of virulent *Streptococcus pneumoniae* by polymorphonuclear leukocytes. *J Infect Dis* 1986;154:619–26. [PubMed: 2943825]
- Graham JE, Christian LM, Kiecolt-Glaser JK. Stress, age, and immune function: toward a lifespan approach. *J Behav Med* 2006;29:389–400. [PubMed: 16715331]
- Gross WB, Siegel PB. Long-term exposure of chickens to three levels of social stress. *Avian Dis* 1981;25:312–25. [PubMed: 7020680]
- Hart PH, Spencer LK, Nikoloutsopoulos A, Lopez AF, Vadas MA, McDonald PJ, et al. Role of cell surface receptors in the regulation of intracellular killing of bacteria by murine peritoneal exudate neutrophils. *Infect Immun* 1986;52:245–51. [PubMed: 3514456]
- Hortal M, Estevan M, Iraola I, De Mucio B. A population-based assessment of the disease burden of consolidated pneumonia in hospitalized children under five years of age. *Int J Infect Dis* 2007;11:273–7. [PubMed: 16997592]
- Hunziker J, Padgett DA, Sheridan PA, Dhabhar FS, Sheridan JF. Modulation of natural killer cell activity by restraint stress during an influenza A/PR8 infection in mice. *Brain Behav Immun* 2004;18:526–35. [PubMed: 15331123]
- Iavicoli S, Lopez-Perez E, Buehring GC, Thomas HA, Wei ET, Kishimoto T. Bipolar-shape response of human neutrophils to corticotropin-releasing factor. *Eur J Pharmacol* 1998;349:301–6. [PubMed: 9671111]
- Ishihara Y, Matsunaga K, Iijima H, Fujii T, Oguchi Y, Kagawa J. Time-dependent effects of stressor application on metastasis of tumor cells in the lung and its regulation by an immunomodulator in mice. *Psychoneuroendocrinology* 1999;24:713–26. [PubMed: 10451907]
- Ising M, Holsboer F. Genetics of stress response and stress-related disorders. *Dialogues Clin Neurosci* 2006;8:433–44. [PubMed: 17290801]
- Jain R, Zwickler D, Hollander CS, Brand H, Saperstein A, Hutchinson B, et al. Corticotropin-releasing factor modulates the immune response to stress in the rat. *Endocrinology* 1991;128:1329–36. [PubMed: 1999154]
- Jessop DS, Harbuz MS, Lightman SL. CRH in chronic inflammatory stress. *Peptides* 2001;22:803–7. [PubMed: 11337094]
- Joachim RA, Quarcoo D, Arck PC, Herz U, Renz H, Klapp BF. Stress enhances airway reactivity and airway inflammation in an animal model of allergic bronchial asthma. *Psychosom Med* 2003;65:811–5. [PubMed: 14508025]
- Jones HP, Hodge LM, Fujihashi K, Kiyono H, McGhee JR, Simecka JW. The pulmonary environment promotes Th2 cell responses after nasal-pulmonary immunization with antigen alone, but Th1 responses are induced during instances of intense immune stimulation. *J Immunol* 2001;167:4518–26. [PubMed: 11591779]
- Kiank C, Holtfrete B, Starke A, Mundt A, Wilke C, Schutt C. Stress susceptibility predicts the severity of immune depression and the failure to combat bacterial infections in chronically stressed mice. *Brain Behav Immun* 2006;20:359–68. [PubMed: 16330179]

- Konstantinos AP, Sheridan JF. Stress and influenza viral infection: modulation of proinflammatory cytokine responses in the lung. *Respir Physiol* 2001;128:71–7. [PubMed: 11535264]
- Kruisbeek, A., editor. Isolation and fractionation of mononuclear cell populations. Vol. 1. 1999.
- Leu SJ, Singh VK. Suppression of in vitro antibody production by corticotropin-releasing factor neurohormone. *J Neuroimmunol* 1993;45:23–9. [PubMed: 8331162]
- Li KS, Liege S, Moze E, Neveu PJ. Plasma corticosterone and immune reactivity in restrained female C3H mice. *Stress* 2000;3:285–98. [PubMed: 11342394]
- Liles WC, Klebanoff SJ. Regulation of apoptosis in neutrophils--Fas track to death? *J Immunol* 1995;155:3289–91. [PubMed: 7561020]
- MacQueen GM, Bienenstock J. Stress and immune regulation. *Clin Exp Allergy* 2006;36:969–71. [PubMed: 16911352]
- Marriott HM, Hellewell PG, Cross SS, Ince PG, Whyte MK, Dockrell DH. Decreased alveolar macrophage apoptosis is associated with increased pulmonary inflammation in a murine model of pneumococcal pneumonia. *J Immunol* 2006;177:6480–8. [PubMed: 17056580]
- Matalka KZ. Neuroendocrine and cytokines-induced responses to minutes, hours, and days of mental stress. *Neuro Endocrinol Lett* 2003;24:283–92. [PubMed: 14646999]
- McClellan CB, Cohen LL. Family functioning in children with chronic illness compared with healthy controls: a critical review. *J Pediatr* 2007;150:221–3. 223 e1–2. [PubMed: 17307532]
- Meagher LC, Cousin JM, Seckl JR, Haslett C. Opposing effects of glucocorticoids on the rate of apoptosis in neutrophilic and eosinophilic granulocytes. *J Immunol* 1996;156:4422–8. [PubMed: 8666816]
- Mediratta PK, Sharma KK. Differential effects of benzodiazepines on immune responses in non-stressed and stressed animals. *Indian J Med Sci* 2002;56:9–15. [PubMed: 12508625]
- Moffatt JD, Lever R, Page CP. Activation of corticotropin-releasing factor receptor-2 causes bronchorelaxation and inhibits pulmonary inflammation in mice. *Faseb J* 2006;20:1877–9. [PubMed: 16855006]
- Mold C, Edwards KM, Gewurz H. Effect of C-reactive protein on the complement-mediated stimulated of human neutrophils by *Streptococcus pneumoniae* serotypes 3 and 6. *Infect Immun* 1982;37:987–92. [PubMed: 7129640]
- Murray SE, Lallman HR, Heard AD, Rittenberg MB, Stenzel-Poore MP. A genetic model of stress displays decreased lymphocytes and impaired antibody responses without altered susceptibility to *Streptococcus pneumoniae*. *J Immunol* 2001;167:691–8. [PubMed: 11441072]
- Murray SE, Rosenzweig HL, Johnson M, Huising MO, Sawicki K, Stenzel-Poore MP. Overproduction of corticotropin-releasing hormone blocks germinal center formation: role of corticosterone and impaired follicular dendritic cell networks. *J Neuroimmunol* 2004;156:31–41. [PubMed: 15465594]
- Nishida K, Ohta Y, Ishiguro I. Contribution of NO synthases to neutrophil infiltration in the gastric mucosal lesions in rats with water immersion restraint stress. *FEBS Lett* 1998;425:243–8. [PubMed: 9559657]
- Noya FJ, Baker CJ, Edwards MS. Neutrophil Fc receptor participation in phagocytosis of type III group B streptococci. *Infect Immun* 1993;61:1415–20. [PubMed: 8454344]
- Okuyama K, Ohwada K, Sakurada S, Sato N, Sora I, Tamura G, et al. The distinctive effects of acute and chronic psychological stress on airway inflammation in a murine model of allergic asthma. *Allergol Int* 2007;56:29–35. [PubMed: 17259807]
- Papadopoulou NG, Oleson L, Kempuraj D, Donelan J, Cetrulo CL, Theoharides TC. Regulation of corticotropin-releasing hormone receptor-2 expression in human cord blood-derived cultured mast cells. *J Mol Endocrinol* 2005;35:R1–8. [PubMed: 16326828]
- Persoons JH, Moes NM, Broug-Holub E, Schornagel K, Tilders FJ, Kraal G. Acute and long-term effects of stressors on pulmonary immune functions. *Am J Respir Cell Mol Biol* 1997;17:203–8. [PubMed: 9271308]
- Portela Cde P, Massoco Cde O, de Lima WT, Palermo-Neto J. Stress-induced increment on total bronchoalveolar cell count in OVA-sensitized rats. *Physiol Behav* 2001;72:415–20. [PubMed: 11274686]
- Radulovic M, Dautzenberg FM, Sydow S, Radulovic J, Spiess J. Corticotropin-releasing factor receptor 1 in mouse spleen: expression after immune stimulation and identification of receptor-bearing cells. *J Immunol* 1999;162:3013–21. [PubMed: 10072553]

- Roozen AW, Magnusson U. Effects of short-term restraint stress on leukocyte counts, lymphocyte proliferation and lysis of erythrocytes in gilts. *Zentralbl Veterinarmed B* 1996;43:505–11. [PubMed: 8921740]
- Saha B, Mondal AC, Basu S, Dasgupta PS. Circulating dopamine level, in lung carcinoma patients, inhibits proliferation and cytotoxicity of CD4+ and CD8+ T cells by D1 dopamine receptors: an in vitro analysis. *Int Immunopharmacol* 2001;1:1363–74. [PubMed: 11460316]
- Schindler BA. Stress, affective disorders, and immune function. *Med Clin North Am* 1985;69:585–97. [PubMed: 3892192]
- Sheridan JF, Stark JL, Avitsur R, Padgett DA. Social disruption, immunity, and susceptibility to viral infection. Role of glucocorticoid insensitivity and NGF. *Ann N Y Acad Sci* 2000;917:894–905. [PubMed: 11270350]
- Sieve AN, Steelman AJ, Young CR, Storts R, Welsh TH, Welsh CJ, et al. Sex-dependent effects of chronic restraint stress during early Theiler's virus infection on the subsequent demyelinating disease in CBA mice. *J Neuroimmunol* 2006;177:46–62. [PubMed: 16762424]
- Silverman ES, Breault DT, Vallone J, Subramanian S, Yilmaz AD, Mathew S, et al. Corticotropin-releasing hormone deficiency increases allergen-induced airway inflammation in a mouse model of asthma. *J Allergy Clin Immunol* 2004;114:747–54. [PubMed: 15480311]
- Speyer CL, Gao H, Rancilio NJ, Neff TA, Huffnagle GB, Sarma JV, et al. Novel chemokine responsiveness and mobilization of neutrophils during sepsis. *Am J Pathol* 2004;165:2187–96. [PubMed: 15579460]
- Sternberg EM. Neural regulation of innate immunity: a coordinated nonspecific host response to pathogens. *Nat Rev Immunol* 2006;6:318–28. [PubMed: 16557263]
- Strange KS, Kerr LR, Andrews HN, Emerman JT, Weinberg J. Psychosocial stressors and mammary tumor growth: an animal model. *Neurotoxicol Teratol* 2000;22:89–102. [PubMed: 10642118]
- Sun X, Jones HP, Hodge LM, Simecka JW. Cytokine and chemokine transcription profile during *Mycoplasma pulmonis* infection in susceptible and resistant strains of mice: macrophage inflammatory protein 1beta (CCL4) and monocyte chemoattractant protein 2 (CCL8) and accumulation of CCR5+ Th cells. *Infect Immun* 2006;74:5943–54. [PubMed: 16988274]
- Tantisira KG, Lake S, Silverman ES, Palmer LJ, Lazarus R, Silverman EK, et al. Corticosteroid pharmacogenetics: association of sequence variants in CRHR1 with improved lung function in asthmatics treated with inhaled corticosteroids. *Hum Mol Genet* 2004;13:1353–9. [PubMed: 15128701]
- Truckenmiller ME, Bonneau RH, Norbury CC. Stress presents a problem for dendritic cells: corticosterone and the fate of MHC class I antigen processing and presentation. *Brain Behav Immun* 2006;20:210–8. [PubMed: 16504465]
- Tsatsanis C, Androulidaki A, Dermitzaki E, Gravanis A, Margioris AN. Corticotropin releasing factor receptor 1 (CRF1) and CRF2 agonists exert an anti-inflammatory effect during the early phase of inflammation suppressing LPS-induced TNF-alpha release from macrophages via induction of COX-2 and PGE2. *J Cell Physiol* 2007;210:774–83. [PubMed: 17117478]
- Tseng RJ, Padgett DA, Dhabhar FS, Engler H, Sheridan JF. Stress-induced modulation of NK activity during influenza viral infection: role of glucocorticoids and opioids. *Brain Behav Immun* 2005;19:153–64. [PubMed: 15664788]
- van Rossum AM, Lysenko ES, Weiser JN. Host and bacterial factors contributing to the clearance of colonization by *Streptococcus pneumoniae* in a murine model. *Infect Immun* 2005;73:7718–26. [PubMed: 16239576]
- Weiss JM, Sundar SK, Becker KJ, Cierpial MA. Behavioral and neural influences on cellular immune responses: effects of stress and interleukin-1. *J Clin Psychiatry* 1989;50(Suppl):43–53. discussion 54–5. [PubMed: 2654130]
- Yang EV, Glaser R. Stress-induced immunomodulation and the implications for health. *Int Immunopharmacol* 2002;2:315–24. [PubMed: 11811934]

Fig. 1. Percent weight loss among control (no stress) and stressed (Restraint stress) subjects before *S. pneumoniae* (10^6 cells) infection and at time of death. Significant differences are shown (*t* test, * $p \leq 0.05$). Vertical bars represent mean percent loss in weight $\pm$ standard error ($n = 38$ /experimental condition). N.D. indicates no weight loss detected.

Fig. 2.

Stress effects bacterial sepsis after primary and secondary pneumococcal infection. Survival among subjects exposed to stress (Restraint) and primary infection (A) and secondary challenge (B). Stepwise survival analysis indicated significant differences at selected time intervals indicated by asterisk (*). Results are the mean $\pm$ SE of $n=20$ mice per group; Chi Square (χ^2) survival analysis.

Primary Infection

B **Fig. 3.**

Histological changes in the lungs of stressed mice exposed to primary and secondary *S. pneumoniae* infection. 1–10, Hematoxylin and eosin-stained lung sections. 1 and 2, Unstressed and restraint stressed broth exposed lungs respectively, 10x magnification. 3 and 4, Unstressed and restraint stressed *S. pneumoniae* infected lungs, 10x magnification. 5 and 6 represents 20x magnification of Unstressed and restraint stressed infected lungs. Black arrows highlight preferential alveoli monocyte and polymorphonuclear infiltrates, 5 and 6 respectively. 7 and 8, Unstressed and restraint stressed lungs of survivors exposed to secondary *S. pneumoniae* infection (10x magnification). 9–10, 20x magnification of 7 and 8. Black arrows highlight preferential alveoli monocyte (9) and extensive polymorphonuclear infiltrates (10). Lung sections are representative of (n=4) mice per experimental condition.

Fig. 4. Impaired bacterial clearance from the lungs of mice exposed to restraint stress. The number of *S. pneumoniae* colony forming units (CFUs) in the lungs, spleen and blood was enumerated 18 hrs after infection. Vertical bar and error bars represent mean $\pm$ SE (n=5). *, Denotes statistical difference ($p \leq 0.05$) between RS and NRS groups.

B.

Fig. 5.

A, Distribution of polymorphonuclear cells (PMNs) and monocytes within the bronchiolar lavage fluid (BALF) among unstressed and RS mice infected with *S. pneumoniae*. Vertical bar and error bars represent mean $\pm$ SE (n=10) of total BALF cells, PMNs, and Monocytes/ml. Asterisks (*) denote statistical difference ($p \leq 0.05$) between experimental groups. B, 1–4 represents cytopspin preparations of BALF cells stained with hematoxylin and eosin. 1 and 2, Unstressed (NRS) and stressed (RS) broth inoculated mice. 3 and 4, NRS and RS infected mice.

Fig. 6.

Numbers of immune cell subpopulations in the lungs of mice in the unstressed and stress condition. Data represents the numbers of the total cells (A) and specified cell-types (B) among unstressed (NRS) and stressed (RS) mice 18 hrs after infection or sham inoculation. The vertical bar and error bars represent the mean $\pm$ SE (n=4) per experimental group. Asterisks (*) Denotes significant ($p \leq 0.05$) differences in the number specific cells from NRS between groups.

Fig. 7.

Lung-associated corticotropin releasing hormone (CRH) gene expression corresponds with CRH receptor-1 and receptor-2 (CRHR1 and CRHR2) gene expression by lung CD11c⁺ MHC II⁺ in response to *S. pneumoniae*. The levels of CRH mRNA gene expression in total lung tissue of stressed mice (RS) as compared to non-stressed (NRS) after infection (A); The levels of CRHR1 and CRHR2 mRNA gene expression by CD11c⁺ MHC II⁺ and CD11c⁻ MHC II⁺ cells (B). Data expressed as the fold difference in mRNA gene levels between NRS and RS experimental groups. The vertical bar and error bars represent the mean $\pm$ SE (n=2) independent experiments. Asterisks (*) Denotes significant ($p \leq 0.05$) differences between experimental conditions.