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ORIGINAL ARTICLE

Cold-induced stress increases the intensity of *Chlamydia* genital infection in mice

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KEYWORDS
Catecholamines;
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Background/Purpose(s): Genital infection by *Chlamydia trachomatis* (CT) is the most common bacterial sexually transmitted disease worldwide. The infection can cause serious reproductive health complications including pelvic inflammatory disease and infertility. Stress is implicated as a risk factor for various infections; however, its effect on *Chlamydia* genital infection and complications are unknown.
Methods: We investigated the effect of cold-stress on resistance to *Chlamydia* genital infection, stress hormone production, and the functions of immune cells in a mouse model. Mice were infected intravaginally with CT after a 24-day cold-stress application. The course of infection was monitored by cervicovaginal swabbing for isolation of live *Chlamydia* in tissue culture. The production of stress hormones and cytokines in genital tracts, spleen or blood were assessed.
Results: Exposure of mice to 24-day stress resulted in: (a) increased susceptibility to *Chlamydia* genital infection and greater intensity of infection, (b) increased plasma or tissue noradrenaline and adrenaline levels, and (c) decreased mRNA and protein levels of major cytokines and chemokines in the spleen and genital tract.
Conclusion: These results suggest that cold-induced stress induces the production of catecholamines, which may play a critical role in the modulation of the immune system leading to increased susceptibility and greater intensity of *Chlamydia* genital infection that could promote the development of complications.

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Introduction

Genital *Chlamydia trachomatis* (CT) infection is a leading cause of pelvic inflammatory disease (PID), fallopian tube scarring, ectopic pregnancy, infertility, and neonatal conjunctivitis.[1–3] Epidemiologic data from the U.S. Centers for Disease Control and Prevention indicate that CT genital infection is a serious public-health predicament with more than 90 million new cases occurring annually worldwide and 4 million in the United States alone.[4] *Chlamydia* genital infection in the United States disproportionately affects populations of low socioeconomic status, particularly among African Americans. The reasons are not well known; but lack of timely access to quality health care, low status of socioeconomic conditions, and other unknown factors, including stress, may have a role in the persistent high rate of *Chlamydia* genital infection.[5–7]

It is widely believed that psychological or physical stress, resulting from competition, pressure, and increased hardship of life in society, has major impacts on public health. Stress may regulate the host immune response through the neuroendocrine-immune axis, involving glucocorticoids and catecholamines.[8–10] Glucocorticoids and catecholamines can serve as the major mediators of stress responses by modulating signaling events which result in either immunosuppression or immunostimulation in the host.

Animal models that employ physical stressors, which include, restraint, exercise, hypothermia (cold), mild electric foot shock, and suspension, to elicit stress responses have been developed.[11–16] Application of cold water as a stressor in animal models, including mice, has resulted in changes in immune responses, such as levels of immunologic parameters, which correlated with the activity of the neuroendocrine system of corticosteroids and catecholamines.[12]

Differential population susceptibility and incidence of disease have been reported for genital *Chlamydial* infection; and, in the continuing effort to develop prevention and control measures, numerous biological, epidemiologic, and clinical studies of *Chlamydia* genital infection have been undertaken; however, the role of stress in the pathogenesis of *Chlamydial* genital disease and the influence on immune response against *Chlamydia* remain unknown. The focus of this study was to use a cold-stress mouse model to investigate the role of stress in the susceptibility and intensity of genital *Chlamydial* infection and to determine the immune parameters affected. A better understanding of how stress may contribute to the immunopathogenesis of *Chlamydia* infection in a mouse model may allow us to develop more effective prevention and treatment options in humans.

Materials and methods

Chlamydia stock culture and McCoy cells

CT agent of mouse pneumonitis (MoPn) biovar (strain Nigg) and McCoy mouse fibroblast cell line were kindly provided by Dr. Joseph Igietseme, Morehouse School of Medicine, Atlanta, GA, USA.

Animals

Five to 7-week-old female BALB/c mice purchased from Harlem-Sprague Daley (Indianapolis, IN, USA) were given food, water, and libitum and allowed to acclimate to Bluefield State College animal housing conditions for 7 days prior to experimentation. Experimental procedures on mice were approved by the Bluefield State College Institutional Animal Care and Use Committee.

Stress model

Stress was applied by placing mice in a packet filled with 2 cm of cold water (1–4°C) for 5 minutes daily for 24 days. The water level was deep enough to cover their backs while swimming in the cold-water. At the end of each stress period, mice were dried with towels to avoid hypothermia. Non-stressed mice were at room temperature without the treatment of cold-water. All mice received 2.5 mg of progesterone subcutaneously 7 days before infection.

Course of genital *C. trachomatis* infection

Groups of five or six stressed or non-stressed mice were infected intravaginally with 10^3 or 10^7 IFU of CT in a volume of 30 μl of phosphate buffered saline (PBS) while under isoflurane-induced anesthesia. The course of infection was monitored by cervico-vaginal swabbing at 3-day intervals for the first 40 days of the primary course of infection. During secondary infection, stressed mice that resolved a primary infection were subjected to cold-stress again for 10 days and reinfected with a 10^7 IFU/mouse dose of CT at Day 70 of primary infection, then followed by swabbing at 3-day intervals for 21 days. *Chlamydia* shedding in the cervicovaginal vault was measured by the isolation of CT from the swabs in tissue culture followed by immunofluorescence staining and enumeration of inclusions, according to standard procedures.

Determination of plasma and tissue levels of catecholamines

Groups of at least five stressed or non-stressed mice were sacrificed by CO_2 inhalation after 48 hours of CT infection. Blood samples were obtained by cardiac puncture vacutainer with 15% ethylene diamine tetraacetic acid (EDTA). The plasma was separated by centrifugation and the levels of adrenaline (epinephrine) (EP) or noradrenaline (norepinephrine; NE) were determined using the 3-cat enzyme immunoassay (EIA) kit and following the manufactures instructions (Labor Diagnostika Nord GmbH and Co. KG, Germany). Absorbance was read using a microreader plate set to 450 nm. Concentrations of EP or NE were determined by extrapolation from standard values. For levels of EP or NE in the spleens and genital tracts of mice, the tissues

were placed in PBS (7.2 pH) and homogenized using a Tissue Ruptor from QIAGEN (Valencia, CA, USA). Tissue debris was removed by centrifugation at 1000*g*. The levels of EP or NE in the homogenates were determined using the 3-cat EIA kit as above.

Assessment of effect of cold-stress on immune system functions

Luminex assay

After 48 hours of infection, spleens and genital tracts were harvested from stressed or non-stressed mice, pooled in groups, and homogenized using a Qiagen Tissue Raptor. Samples of genital tract or spleen homogenates were tested for the presence of cytokines and chemokines with the milliplex-map mouse 32 cytoikine/chemokine kit from Millipore (Billerica, MA, USA) using the Luminex-100 system according to the manufacturer's instruction. Data were analyzed using the spline curve-fitting method for calculating cytokine/chemokine concentrations in samples.

RNA isolation, cDNA synthesis and QPCR analysis of gene expression

After 48 hours of infection, the genital tracts from stressed or non-stressed mice were harvested and pooled in QIAGEN Allprotect Tissue Reagent to stabilize RNA in the tissue until homogenization. Tissues were disrupted and homogenized in QIAzol Lysis Reagent using a QIAGEN Tissue Ruptor. Total RNA was extracted using a QIAGEN RNeasy Lipid Tissue Mini Kit, by following the manufacturer's instructions. cDNA was synthesized using a RT^2 First Strand cDNA Synthesis Kit from SABiosciences (Valencia, CA, USA). DNA amplification by PCR was performed in a Stratagene Mx3000P PCR system using SABiosciences RT^2 Profiler PCR Array following the manufacturer's instructions.

We used the Web-based RT^2 Profiler PCR Array data Analysis of SABiosciences following the instructions of the manufacturer. Briefly, Ct values for cytokines or chemokines were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by subtracting the average Ct value for each treatment group. $\Delta\Delta$Ct values were the normalized gene expression of stressed minus by the normalized gene expression in the non-stressed mice. Internal control of our experiment was the non-stressed chlamydia infected mice. Fold-change values were the normalized gene expression of stressed mice divided by the normalized gene expression in the non-stressed mice. Fold-regulation represented fold change results to describe whether there was or not down-regulation or up-regulation genes of interest in stressed mice. Accordingly, fold-change values greater than 1 indicated a positive or up-regulation of gene expression, whereas fold change values less than 1 indicated a negative or gene down-regulation.

Statistical analysis

Student's *t*-test was used to test statistical significance between any two groups. ANOVA was used to test statistical difference between more than two groups. Level of statistical significance was at $p < 0.05$.

Results

Stress increases the intensity of a primary genital CT infection in mice

Using the cold water-induced stress model, we investigated the effect of stress on the intensity and the course of a primary genital CT infection in female mice. The results from infection with a lower inoculum of 10^3 IFU/mouse showed that stressed mice suffered a greater intensity of infection, measured by the mean IFU/ml, and longer duration of infection than the non-stressed mice ($p < 0.01$); this is shown in Fig. 1A. However, at the higher inoculum of 10^7 IFU, the intensity of the infection was higher in the stressed animals only at the initial stages (Day 9) of the infection (Fig 1B). All mice eventually cleared the shedding by Day 40 after the primary infection. The results indicated

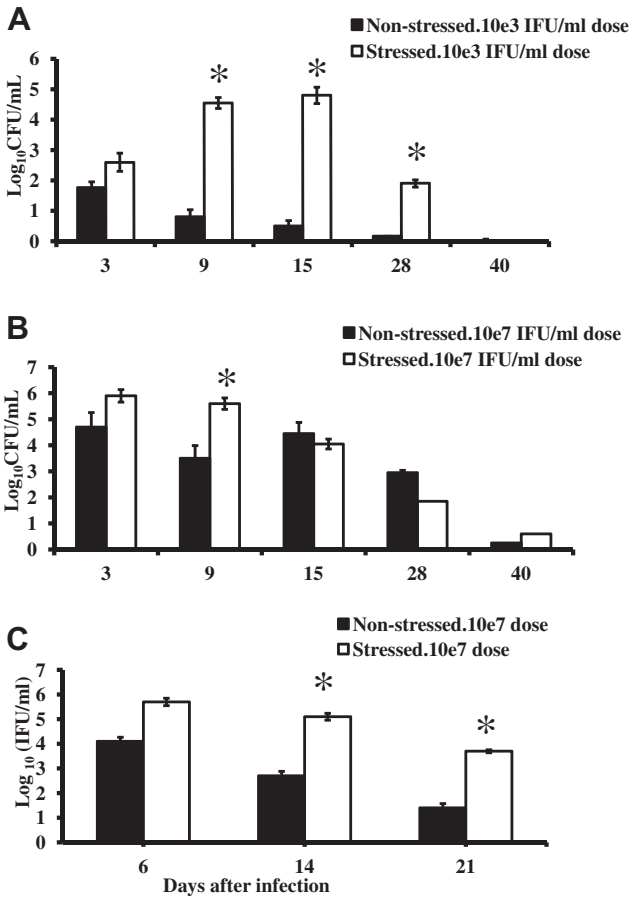

Figure 1. Kinetics of *Chlamydia* shedding in genital tract. (A) Primary *Chlamydia* genital infection in stressed mice infected with of 10^3 IFU/ml as low dose; (B) primary *Chlamydia* genital infection in non-stressed mice infected with of 10^7 IFU/ml as low dose; (C) secondary *Chlamydia* genital infection in stressed and non-stressed mice infected with 10^7 IFU/ml. Each data point is mean $\pm$ standard deviation of log10 inclusion forming unit/ milliliter representing combined results of two separate experiments ($n = 5$ to 6 mice per experiment). *Denotes significant statistical difference between experimental groups at the level of ($p \leq 0.05$).

that cold-induced stress could increase the intensity of a primary genital *Chlamydial* infection.

Stress increases the intensity of secondary genital CT infection in mice

Since reinfections are a significant cause of pathologies associated with genital chlamydial infection, we investigated the effect of cold-induced stress on the intensity of a secondary infection in mice. Preliminary studies showed that previously stressed animals that cleared a primary infection and recovered from stress cleared the secondary infection at the same rate as non-stressed immune mice (data not shown); so mice were stressed again before the secondary infection. Results presented in Fig. 1C show that the intensity of the secondary genital infection was significantly greater in mice that were stressed after resolving a primary infection compared to non-stressed mice ($p < 0.05$). The results indicated that stress could also increase the intensity of secondary genital chlamydial infection; however, sustained stress was required for the effect.

Stress alters the levels of key immune parameters in response to genital chlamydial infection

We measured the levels of key cytokines and chemokines that are part of host innate and adaptive immune responses during c*hlamydial* infection in stressed animals. Results showed that proteins of key immunostimulatory cytokines, including interferon-gamma (IFN-g), inteleukin-1beta (IL-1b, IL-4, IL-12p40, and 12p70 (data not shown)), were suppressed in the genital tract of stressed mice compared to the non-stressed; however, tumor necrosis factor alpha (TNF-αα) level was slightly higher in stressed compared to non-stressed mice (Fig. 2). Moreover, level of the immunosuppressive cytokine IL-10 was elevated in the stressed mice (Fig. 2E). As shown in Fig. 3A,B,C, the immunostimulatory chemokines such as Interferon gamma-induced Protein- 10 (IP-10), regulated upon activation normal T-cell expressed, presumably secreted (RANTES), macrophage inflammatory protein 1 alpha (MIP-1a), were suppressed in the spleens of stressed compared to the non-stressed mice. Thus, the results suggested that cold-stress could suppress the local and systemic induction of a majority of key immunostimulatory cytokines and chemokines and stimulate the expression of key immunosuppressive cytokines, especially IL-10.

As shown in Tables 1 and 2, the mRNA levels of IFN-g, IP-10, and IL-18, key Th-1 cytokines were suppressed by 47%, 82%, and 97%, respectively, while IL-10 was up-regulated by 90% suggesting the correlation of Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Luminex results. The overall results suggested that the increased intensity of genital *Chlamydial* infection in cold-stressed animals may be is caused by the alteration in host immune response in stressed animals. However, the mechanism of immune modulation by stress is unknown and further investigation is needed.

Stress increases the plasma levels of stress hormones in mice

We hypothesized that the stress hormones EP and NE, known to modulate the host immune function, may play a role in the immune alteration against *Chlamydial* infection following a cold-induced stress. Figs. 4A and 4B show that the plasma levels of EP and NE were significantly

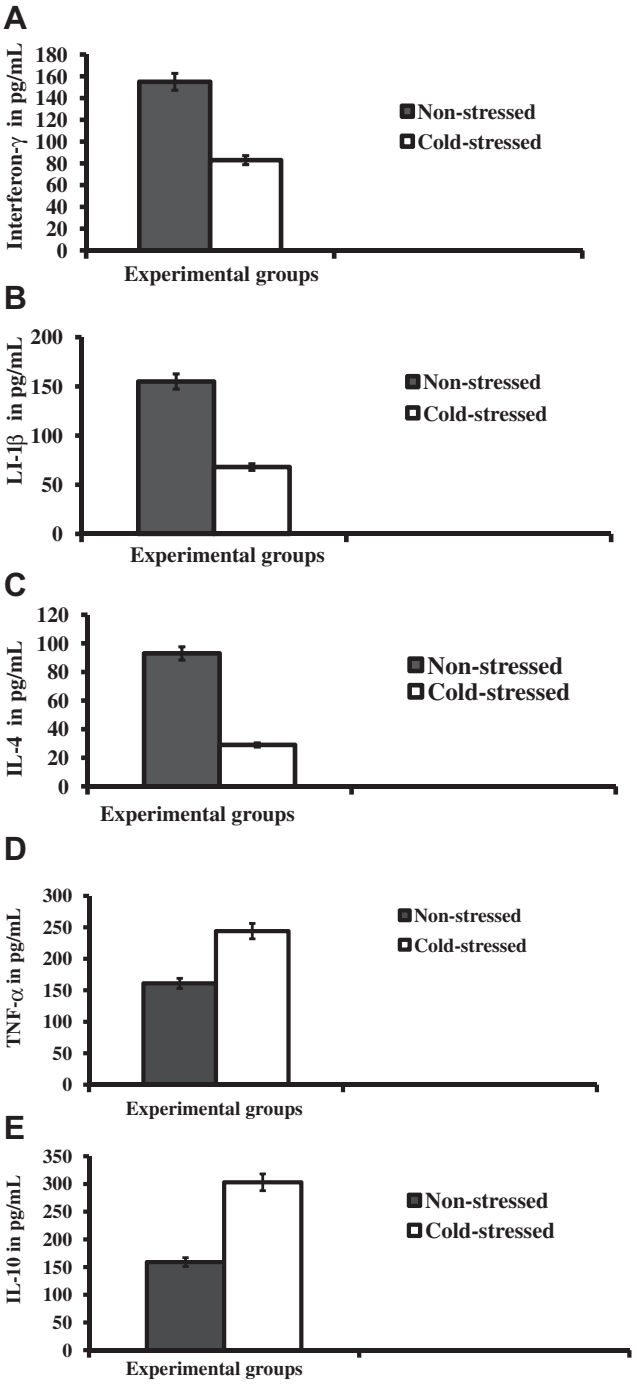

Figure 2. Relative production of immune stimulatory or suppressor cytokines in genital tract of *Chlamydia trachomatis* infected mice subjected to cold-water stress. (A) The levels of interferon-gamma; (B) IL-1b; (C) IL-4; (D) TNF-alpha; (E) interleukin-10 were measured by using a Millipore Mouse Cytokine/Chemokine Millipex map kit, following the manufacturer's instructions. Each point value represents the means ± standard deviation cytokines of two or three wells ($n = 5$ or 6 mice for each group of experiments). IL = interleukin; TNF = tumor necrosis factor.

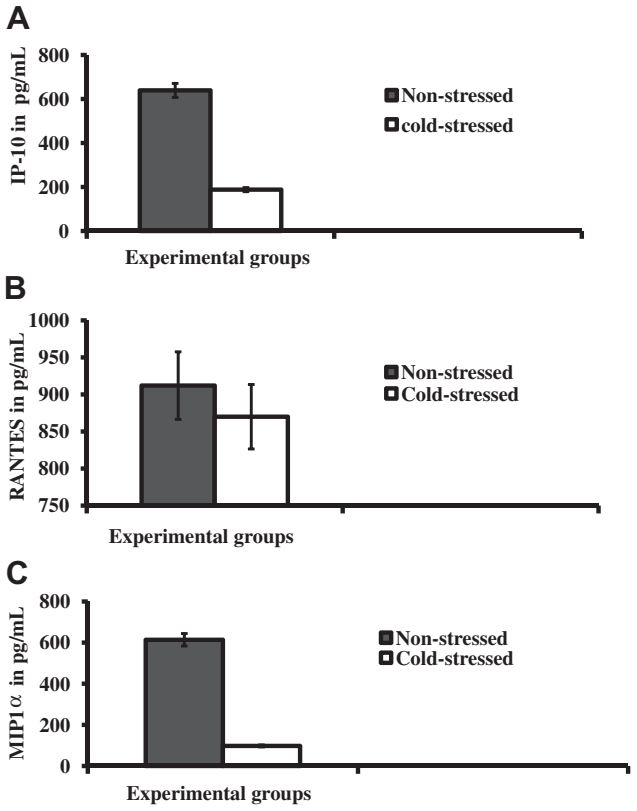

Figure 3. Relative production of chemokines in spleens of *Chlamydia trachomatis* infected mice subjected to cold-water stress. The levels of chemokines: (A) IP-10; (B) RANTES; (C) MIP-1a were measured by using a Millipore Mouse Chemokine Millipex map kit, following the manufacturer's instructions. Each point value represents the means ± standard deviation of two or three wells ($n = 5$ or 6 mice for each group of experiments).

higher in stressed compared to non-stressed animals ($p < 0.02$). Tissue atrophy assessment revealed that the average body weight of stressed and non-stressed mice was statistically unchanged at 18.42 ± 0.77 versus 17 ± 0.91 g, respectively ($p < 0.15$). However, the average weight of the spleens changed slightly from 90.55 mg (non-stressed) to 72.61 mg (stressed) ($p < 0.045$).

Stress elevates the levels of catecholamines in spleen and genital tract during *C. trachomatis* infection

To assess the effect of persistent or chronic cold-induced stress on stress hormone production in lymphoid organs and genital tract during infection, we measured the levels of NE and EP in the spleens and genital tracts of stressed and non-stressed mice. We hypothesized that NE or EP levels is greater in the spleens and genital tracts of stressed mice. Mice exposed to cold-stress had a mean value of 114.5 ± 4 pg/ml of NE in the spleen compared to 75 ± 5 in non-stressed mice. Similarly, the genital tracts of stressed mice had a mean value of 90 ± 7 pg/ml NE compared to 70 ± 5 of non-stressed mice. Similarly, the spleen homogenates had a mean of 13 ± 1 ng/ml and 3 ± 1 ng/ml of EP for stressed and non-stressed mice, respectively. The EP level in the genital tract of stressed mice was 90 ng/ml compared with 32 ng/ml in non-stressed mice. This elevated level of catecholamines, particularly NE production locally in the spleen and the genital tract as a result of cold stress may suggest that these stress hormones could contribute to impairment of the immune system during chlamydia infection.

Table 1 Stress decreases gene expression of cytokines in the genital tract during *Chlamydia* infection

Gene	PCR data analysis of selected cytokines				
	Average[a]	ST DEV	ΔΔCt[b]	Fold Regulation[c]	*p* value (<0.05)
IL-1α	26.13	3.77	0.98	−1.02	0.04
IL-1β	26.68	2.63	0.95	1.05	0.04
IL-4	31.14	1.38	0.58	1.58	0.05
IL-10	28.73	0.09	0.50	1.50	0.05
IL-13	32.11	3.33	0.74	−1.35	0.07
IL-18	26.54	3.61	0.35	−2.84	0.01
IL-20	32.22	3.21	0.57	−1.76	0.05
TNF-α	30.95	0.29	0.78	1.38	0.02
IFN-γ	29.45	0.40	1.09	−1.99	0.05

[a] The average threshold cycle (Ct) for each molecule across all repetitions of RT-PCR.
[b] Values were the normalized gene expression of stressed minus by the normalized gene expression in the non-stressed mice.
[c] Gene up regulation or down regulation as compared to the control. Values greater than 1 indicated the up regulation, whereas values less than 1 indicated gene down regulation of genes of interest.
IFN = interferon; IL = interleukin; RT-PCR = reverse transcriptase polymerase chain reaction; ST DEV = standard deviation; TNF = tumor necrosis factor.

Discussion

Stress is caused by different situations, including socio-economic conditions, health status, and environmental and community pressures. The differential population susceptibility and incidence of disease often reported for *Chlamydia* genital infection would suggest a role for stress in the pathogenesis of C*hlamydia* genital disease. We predicted that stressing mice for a period of 24 days would result in suppression of the activities of immune cells. We investigated the possible role of cold-induced stress in the differential susceptibility and incidence of genital *Chlamydia* genital infection and complications in a mouse to mimic our understanding of the differential chlamydia incidence and disease. We hypothesized that stress would increase the severity of genital *C. trachomatis* infection by modulating the immune response against the infection. To test this hypothesis we used a murine cold-stress model to investigate the role of stress in the susceptibility and intensity of genital chlamydial infection and analyzed the immune parameters affected. A significant increase in *Chlamydia* shedding in stressed compared to non-stressed

Table 2 Stress decreases gene expression of chemokines in the genital tract during *Chlamydia* infection

Gene	Q PCR data analysis of selected cytokines				
	Average[a]	ST DEV	ΔΔCt[b]	Fold Regulation[c]	*p* value (<0.05)
MCP1	30.30	1.44	0.31	−3.23	0.03
MIP1α	31.03	2.95	0.81	−1.23	0.04
MIP1β	31.37	1.23	1.07	1.17	0.03
RANTES	29.34	2.05	0.32	−3.17	0.02
IP10	31.41	0.93	0.80	−1.25	0.05
GPR9	31.86	3.33	0.14	−7.34	0.02

[a] The average threshold cycle (Ct) for each molecule across all repetitions of RT-PCR.
[b] Values were the normalized gene expression of stressed minus by the normalized gene expression in the non-stressed mice.
[c] Gene up- or down-regulation as compared to the control. Values greater than 1 indicated the up regulation, whereas values less than 1 indicated gene down regulation of genes of interest.

mice early in the primary and secondary infections was observed indicating that stress application may cause increased susceptibility to infection. It was noteworthy that stressed mice, which received a low dose infection, provided an opportunity to decipher the higher intensity and longer duration of genital *Chlamydial* infection than infected non-stressed mice. This observation suggested that stress treatment might alter the kinetics of the course of genital infection and evolution into disease onset. These results are corroborated by our previous findings in a murine model system that stress applied in the form of hind-limb suspension or restraint decreased resistance to *Klebsiella pneumoniae*.[13,14] Other research groups have also demonstrated that cold-stress applications to mice increased susceptibility to *Toxoplasma gondii* infection.[15,16] Thus, stress may have a suppressive effect on host resistance to infection in general.

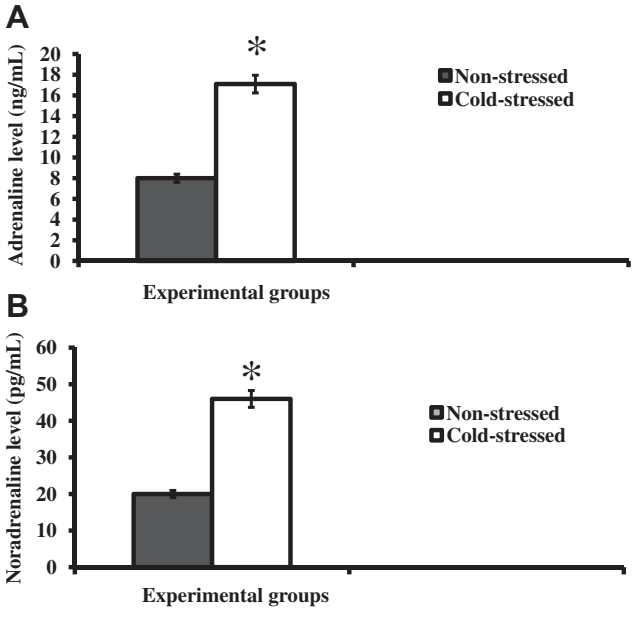

Figure 4. Effect of stress on production of (A) plasma adrenaline; (B) and noradrenaline measured by enzyme linked immunosorbent assay (ELISA). Each point represents the mean ± standard deviation of two experiments ($n = 5$ to 7 mice for each experiment). Statistical differences between treatments were evaluated by Student's t test. *Denotes significant statistical differences between the experimental groups at the level of ($p \leq 0.05$).

The highly intense shedding of *Chlamydia* by stressed mice during early stage of infection indicated an inability to control the early stages of the infection, suggesting that stress may compromise the innate and/or adaptive immune resistance to genital infection. Several published studies have reported that long-term stress suppressed the ability of the immune system to fight viral, bacterial, fungal and parasitic infections.[8–11] Thus, various stressors appear to be able to modulate the function of the immune system, which subsequently leads to decreased resistance to various forms of microbial infections. Our results revealed decreased production of immune stimulatory cytokines and chemokines after cold-stress, which are consistent with previous reports that exposure of mice to cold water led to a decrease in the secretion of cytokines.[15–17] It has been well recognized that the host immunity developed after genital infection is crucial for clearance of *Chlamydia*.[18,19] in this study, stressed mice that suffered greater intensity and prolonged *Chlamydia* infection than non-stressed mice had decreased levels of key proinflammatory cytokines (IFN-g, RANTES, MIP-1a, and IP-10) that are crucial for *Chlamydial* control and increased level of at least the principal suppressive cytokine, IL10. Furthermore, we investigated the likely molecular mechanism of cold-stress enhancement of susceptibility of mice to *Chlamydia* genital infection and its impact on the immune system. We hypothesized that the stress hormones, such as noradrenaline, may mediate the down-regulation of the immune response by lessening its ability to suppress microbial growth or directly enhancing *Chlamydia* growth in the infected cells. Our results revealed that the catecholamines, EP and NE, were significantly more expressed in the systemic and local lymphoid and genital tract tissues of stressed compared to non-stressed mice. Thus, these hormones could mediate the enhanced growth of *Chlamydia* in the stressed mice by suppressing the immune response or by their direct action on the microbe, since they have been shown to directly enhance the growth and expression of virulence factors in a variety of gram-negative bacteria.[20,21] The catecholamines could also promote the greater intensity of *Chlamydial* growth in the stressed mice by a combination of suppression of immune response, enhancement of *Chlamydia* propagation, and expression of virulence factors. In addition, stress-mediated suppression of host immune response against *Chlamydia* and the increased susceptibility of the animals may be due to a lack of recruitment and activation of inflammatory cells by chemotactic factors.[19,22]

In summary, our results indicate that stress leads to increased susceptibility of mice to *Chlamydia trachomatis* genital infection, causing significantly higher titers of *Chlamydia* shedding at early times after a primary or

secondary infection. While both stressed and non-stressed mice were susceptible to chlamydial infection, stressed mice had much more intense and longer infection duration. Key immune parameters that drive Th1 response known to be crucial for clearance including IFN-g and MIP-1a were down regulated in stressed mice while a key immunosuppressive cytokine, IL-10 was up regulated. Collectively, our findings demonstrated that cold-induced stress promotes greater susceptibility and intense infectivity of *Chlamydia* in mice by mechanisms that target the suppression of immune stimulatory cytokines. We speculate that the catecholamines may play a role in the immunosuppression. Norepinephrine receptors expressed on immune cells may bind norepinephrine and interfere with the function of NF-kB, which regulates the expression of cytokines and chemokines.[22–24] Our results appear to be corroborated by documented reports that sympathetic nerves in the lymphoid organs release norepinephrine which stimulates the b2 adrenergic receptor (b2ADR) expressed on $CD4^+$ and B cells, leading to modulation of immunity.[22–24]

In conclusion, the initial observations in this study could serve as the basis for designing studies to assess in greater detail how stressful conditions may promote susceptibility to chlamydial infection and disease onset, the associated immunological alterations, and immunomodulatory mechanisms. To our knowledge, this is the first report to examine the effect of stress on *Chlamydia* genital infection.

Application of cold water as a physical or psychological stressor in animal models including mice had resulted in changes such as levels of immunological parameters and the neuroendocrine system of corticosteroids and catecholamines.[11] Surprisingly, level of stress cold-treated or restraint mice were almost identical (data not shown). Studies have shown that, mice and rats exposed to cold-water stress displayed decreased numbers of immune cells and a decreased capacity to secrete certain cytokines. We feel that this study is important to investigate because psychological or physical stressors may lead to increased stress hormone production and have profound effects on health including prevalence of chlamydia genital infection. Evidence shows that these stressors generally are greater in populations of lower socioeconomic status, in which there are increased health concerns. However, the present study is an initial stage and does not fully define the underlying link between animal model and human participants. We believe however that this study may offer important direction for further investigation of the mechanisms of stress on *Chlamydia* genital infection and immunity in humans.

Acknowledgments

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