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Abstract: Chronic psychological stress impairs antibody synthesis following influenza vaccination. Chronic stress also increases circulating levels of proinflammatory cytokines and glucocorticoids in elders and caregivers, which can impair antibody synthesis. The purpose of this study was to determine whether psychological stress increases *ex vivo* cytokine production or decreases glucocorticoid sensitivity (GCS) of peripheral blood leukocytes from healthy college students. A convenience sample of Reserve Officer Training Corps (ROTC) students completed the Perceived Stress Scale (PSS). Whole

blood was incubated in the presence of influenza vaccine and dexamethasone to evaluate production of interleukin-6 (IL-6), interleukin-1-beta (IL-1 β), tumor necrosis factor-alpha (TNF- α), and interferon-gamma (IFN- γ). Multiple regression models controlling for age, gender, and grade point average revealed a negative relationship between PSS and GCS for vaccine-stimulated production of IL-1 β , IL-6, and TNF- α . These data increase our understanding of the complex relationship between chronic stress and immune function.

Keywords: stress, cytokines, influenza, glucocorticoid sensitivity

The antibody response to vaccination involves a complex, integrated network of immune cells and soluble mediators. Vaccination stimulates a transient increase in proinflammatory cytokine production, which is necessary for antigen presentation and activation of T and B cells. T-helper type 1 (Th1) cells, which drive development of cell-mediated immune responses, also produce proinflammatory cytokines, particularly interferon-gamma (IFN- γ), which suppresses the activity of T-helper type 2 (Th2) cells. Th2 cells produce anti-inflammatory cytokines, particularly interleukin-4, which suppress Th1 activity and drive development of humoral immune responses. Proinflammatory cytokines also stimulate cortisol secretion through direct effects on the adrenal cortex and indirect effects on the hypothalamus and the anterior pituitary. Thus, the production of proinflammatory cytokines by Th1 cells is reduced by the anti-inflammatory effects of increased cortisol secretion and concurrent increased production of anti-inflammatory cytokines by Th2 cells. This cross talk of neuroendocrine and inflammatory mediators is necessary for β -cell differentiation, antibody synthesis, and seroprotection (Pedersen, Zachariae, & Bovbjerg, 2009; Phillips, 2012).

Cortisol secretion is also increased in stressful situations, and stress can have significant effects on the immune response (Bauer, Moriguchi Jeckel, & Luz, 2009; Plotnikoff, Faith, Murgu, & Good, 2007). Acute stress, causing a burst of cortisol secretion prior to vaccination, enhances antibody production in healthy individuals (Edwards et al., 2006). In contrast, chronic stress leads to prolonged activation of the hypothalamic-pituitary-adrenal (HPA) axis and poorer antibody response to vaccination (Pedersen et al., 2009; Phillips, 2012). The negative effects of chronic psychological stress on antibody production following vaccination have been demonstrated to be independent of age of subjects or type of psychological stress

(Pedersen et al., 2009; Phillips, 2012). Chronically stressed individuals also exhibit persistent elevations in serum levels of proinflammatory cytokines such as interleukin-6 (IL-6; Rohleder, 2012). Given the concurrent increase in circulating levels of IL-6 and cortisol, the traditional concept of stress-induced immunosuppression does not offer a clear explanation as to why and how chronic stress blunts the immune response to vaccination.

An alternative explanation is that chronic psychological stress leads to a diminished sensitivity of cells to the anti-inflammatory effects of glucocorticoids (Cohen et al., 2012; Miller, Cohen, & Ritchey, 2002; Rohleder, 2012; Silverman & Sternberg, 2012). The basic paradigm used to assess glucocorticoid sensitivity (GCS) of peripheral blood leukocytes (PBL) is to stimulate cytokine production *ex vivo* using bacterial endotoxin or a mitogen and coincubating the stimulated cells with dexamethasone (DEX), a synthetic form of cortisol, to inhibit cytokine production (Agarwal & Marshall, 2001).

Much of the research demonstrating the effects of chronic stress on cytokine production, immune cell function, and GCS of immune cells has involved PBL from elders or caregivers of persons with cancer or Alzheimer's disease. Little is known about the effects of chronic stress on the production of inflammatory cytokines and GCS of PBL from young, healthy adult populations. The purpose of the present study was to examine associations between perceived stress, cytokine production, and GCS of PBL exposed *ex vivo* to influenza vaccine in a healthy, physically fit population of Reserve Officer Training Corps (ROTC) college students. The central hypothesis is that higher perceived stress will be associated with increased production of proinflammatory cytokines and decreased GCS of PBL stimulated *ex vivo* with influenza vaccine.

Method

A cross-sectional descriptive design was used to evaluate the effects of psychological stress on proinflammatory cytokine production and GCS of PBL stimulated *ex vivo* with influenza vaccine. Subjects were full-time, military college students in the ROTC program of a large Midwestern university. Inclusion criteria included able to read, write, and speak English; general good health with no preexisting

conditions (e.g., asthma, Crohn's disease, Cushing's disease, cardiovascular disease, metabolic syndrome, type 2 diabetes, atherosclerosis, chronic obstructive pulmonary disease [COPD], chronic pain, pregnancy/lactation); no recent acute illness or use of antibiotic, steroidal, or anti-inflammatory (e.g., albuterol inhalers, DEX, nonsteroidal anti-inflammatory drugs [NSAIDs], Humira, tumor necrosis factor-alpha [TNF- α] blockers, methotrexate), or antidepressant medications (e.g., Paxil, Zoloft) in the past 6 weeks (with the exception of oral contraceptives). The criteria were designed to minimize the interference of factors known to affect the production of proinflammatory cytokine production or immune function. Demographic data were collected from consented subjects including age, race, ethnicity, gender, and marital status; rank, years of military service, military service branch, active duty/reserve duty status, and deployment history; and cumulative grade point average (GPA), student class rank, and scholarship status. All study procedures were reviewed and approved by the Institutional Review Board (IRB) for human subjects biomedical research at The Ohio State University. Subjects were provided a gift card in the amount of US\$10 following completion of data collection. All data were collected between the hours of 9 to 11 a.m. during the months of March through May.

Measures of Psychological Stress

Psychological stress was measured using the Perceived Stress Scale (PSS), a 10-item questionnaire used to elicit an individual's evaluation of stressful experiences in the past month. The responses are rated on a Likert-type scale from 0 to 4 (0 = *never*, 1 = *almost never*, 2 = *sometimes*, 3 = *fairly often*, 4 = *very often*) with higher scores indicating more perceived psychological stress. Reliability and internal validity have been reported as high with α from .84 to .86 (Cohen, Kamarck, & Mermelstein, 1983). The PSS has been validated with diverse populations, including military academy students (Glaser et al., 1999).

Physiological Measures

Cytokine production

Production of IFN- γ , interleukin-1-beta (IL-1 β), IL-6, and TNF- α by PBL was induced ex vivo by incubating whole blood with Afluria trivalent inactivated influenza vaccine that contained the following three strains for 2011-2012: A/California/7/09 (H1N1)-like virus (pandemic (H1N1) 2009 influenza virus); A/Perth /16/2009 (H3N2)-like virus; and B/Brisbane/60/2008-like virus (CSL Biotherapies, Parkville, Australia). Whole blood cultures have been shown to produce results equivalent to those obtained with isolated PBLs (De Groot et al., 1992).

GCS

GCS was determined by coincubating vaccine-stimulated PBLs with DEX (Sigma-Aldrich, St. Louis, MO) at a final concentration of 200 nM to determine the inhibition of IFN- γ , IL-1 β , IL-6, and TNF- α production (Agarwal & Marshall, 2001; Miller et al., 2002). Pilot work using PBLs from three healthy non-ROTC volunteers confirmed that this concentration of DEX produced 80% to 90% inhibition of IL-6 production by PBLs stimulated with influenza vaccine. The following formula was used to quantify percent inhibition of cytokine production:

$$\% \text{ Inhibition} = 1 - \frac{\text{stimulated cytokine level with DEX}}{\text{stimulated cytokine level without DEX}} \times 100,$$

$$\text{for example, } \% \text{ Inhibition} = 1 - \frac{20 \text{ pg/ml}}{100 \text{ pg/ml}} \times 100$$

for example = 80% inhibition of cytokine production

A lower percent inhibition of cytokine production indicated greater resistance to anti-inflammatory effects of DEX.

Procedure

Flyers were posted on university bulletin boards in the ROTC unit building. If a potential subject agreed to participate, IRB-approved consent forms were completed. Following enrollment, subjects were seated in a private room for 30 min and completed the demographic data form and the 10-item PSS. Blood (10 ml) was then drawn from the antecubital vein into heparin-coated vacutainer tubes (Becton-Dickinson, Franklin Lakes, NJ). The blood tubes from each individual were placed in an individually labeled, biologic specimen bag on an ice-pack and transported to the laboratory within 2 hr of collection. All blood samples were collected between the hours of 9 to 11 a.m. over a 3-month period (March through May).

Three milliliters of whole blood were diluted 1:1 with complete 1640 RPMI medium containing no fetal calf serum (Gibco by Life Technologies) and 1.8 ml of the diluted blood was placed into each of three wells of a six-well culture plate. Well 1 received an additional 0.2 ml medium to determine spontaneous, unstimulated cytokine production, well 2 received 0.1 ml of the influenza vaccine and 0.1 ml of medium to determine vaccine-induced cytokine production, and well 3 received 0.1 ml of influenza vaccine and 0.1 ml of DEX to determine GCS. The final volume in each well was 2.0 ml. The samples were incubated for 72 hr at 37°C with 5% CO₂, after which the culture fluid was aspirated from each well and centrifuged at 500g (1,250 rpm) for 10 min. The cell-free supernatant fluids were collected and stored in 500 µl aliquots in a -80°C freezer until assayed for cytokine levels.

The concentrations of IFN-γ, IL-1β, IL-6, and TNF-α in the supernatant fluids were determined using 4-Plex I Ultra-Sensitive Kits for IFN-γ, IL-1β, IL-6, and TNF-α (Meso Scale Discovery [MSD]) according to the manufacturer's instructions. Intra-assay and inter-assay coefficients of variation were <5% and <10%, respectively. Sensitivity of detection varied by cytokine: IFN-γ = 0.55 pg/ml, IL-1β = 2.4 pg/ml, IL-6 = 0.7 pg/ml, and TNF-α = 0.2 pg/ml. All samples were run at the same time according to the manufacturer's instructions. Samples were batched by subject and assayed in duplicate.

Analysis

Descriptive statistics were calculated to ensure the quality of the data (check distributions, examine outliers) and to describe the socio-demographic characteristics of the sample. Untransformed concentrations of each cytokine are reported as mean and standard deviation. Following univariate analyses, bivariate analyses were conducted using Pearson correlation coefficients for relationships between continuous variables. One-way ANOVA with post hoc procedures was used to evaluate differences in cytokine levels in supernatant fluids from the three culture conditions. Multiple regression models were fit to examine whether PSS predicts GCS (percent inhibition of influenza vaccine-stimulated cytokine production) of IFN- γ , IL-1 β , IL-6, and TNF- α , controlling for age, gender, race, and student cumulative GPA. We first fit our models with the PSS alone. We then added each control variable individually and then as a set of control variables. A sample size of 55 achieved 90% power at an a priori significance level set at .05 to detect an R^2 of .15 (medium effect) attributable to 1 predictor in a multiple regression model, adjusting for three control variables with an R^2 of .10 (Cohen, 1988). Data were analyzed using the Statistical Package for Social Sciences (SPSS) software (version 19.0).

Results

A convenience sample of 61 healthy male and female military (active duty, reservist, and veteran) full-time college students with an average age of 22 years (ranging from 18 to 37 years) were enrolled over a period of 3 months. Demographic, educational, and military characteristics of the sample are displayed in Table 1.

Table I. Demographic, Education, and Military Service Characteristics of the Sample ($n = 61$).

	<i>n</i> (%)
Gender	
Male	46 (75)
Female	15 (25)
Race/ethnicity	
White	53 (86)
African American	1 (2)
Asian	5 (8)
Native American Indian	1 (2)
Unknown	1 (2)
Student cumulative GPA	
4.0 GPA	4 (6)
≥ 3.8 to < 4.0 GPA	9 (15)
3.5-3.79 GPA	16 (26)
3.0-3.49 GPA	20 (33)
≥ 2.0 to < 3.0 GPA	12 (20)
Military Service Branch	
Army	12 (20)
Navy and Marine Corps	46 (75)
Air Force	3 (5)
Military Duty status	
Active duty	13 (21)
Active reserves	7 (12)
Inactive reserves	41 (67)
Years of military service	
1-4 years	50 (82)
5-8 years	8 (13)
9-16 years	3 (5)

Note. GPA = grade point average.

Demographics and Psychological Stress

Scores on the 10-item PSS ranged from 0 to 36, with a mean score (with standard deviation) of 12.82 ($SD = 6.32$). No demographic characteristics were significantly correlated with PSS scores.

Cytokine Production

The mean levels of IFN- γ , IL-1 β , IL-6, and TNF- α in whole blood culture fluid supernatants from the following conditions: (a) nonstimulated cells, (b) influenza vaccine-stimulated cells, and (c)

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vaccine and DEX-stimulated cells, are represented in Figure 1. Based on significant ANOVA, a multiple comparison post hoc procedure using the Dunnett's T3 method was conducted to evaluate pairwise contrasts for each cytokine. All of the pairwise comparisons of culture conditions (A-B, A-C, B-C) were significantly different ($p < .001$) for IFN- γ , IL-6, and TNF- α . Only condition groups (b) and (c) were found to be significantly different ($p < .001$) for IL-1 β (Figure 1b).

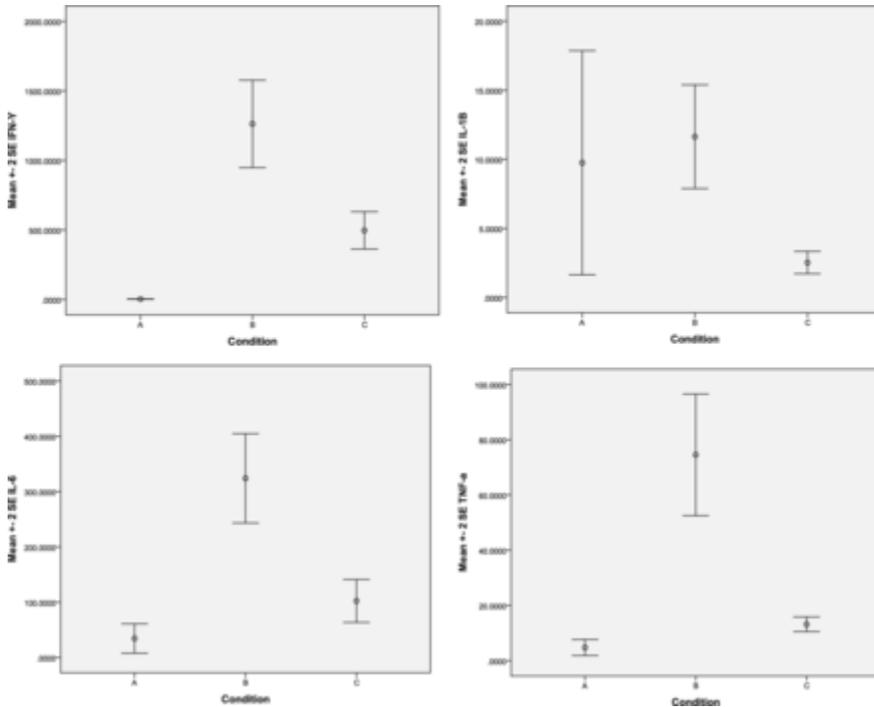


Figure 1. Differences in cytokine production by peripheral blood leukocytes based on culture conditions.

Note. Mean levels of (a) IFN- γ , (b) IL-1 β , (c) IL-6, and (d) TNF- α . Whole blood was diluted 1:1 with medium and incubated for 72 hr. Cytokine production was measured in culture fluid using methods of ELISA. A = spontaneous, nonstimulated production; B = stimulated by influenza vaccine; C = inhibited by dexamethasone. Results are shown as pg/ml with means \pm SEM. IFN- γ = interferon-gamma; IL-1 β = interleukin-1-beta; IL-6 = interleukin-6; TNF- α = tumor necrosis factor-alpha; ELISA = enzyme-linked immunosorbent assay; SEM = standard error of the mean.

Psychological Stress and Stimulated Cytokine Production

The relationship between perceived stress (PSS) and production of each cytokine was estimated with Pearson correlations. Levels of IFN- γ , IL-1 β , IL-6, and TNF- α in supernatants from the nonstimulated

(control) cultures were not significantly correlated with scores on the PSS. Similarly, cytokine levels in influenza-stimulated cultures were not significantly correlated with scores on the PSS.

Psychological Stress and GCS

The PSS scores and percent inhibition of cytokine production for IFN- γ , IL-1 β , IL-6, and TNF- α are represented in Figure 2. The relationship between stress (PSS) and GCS for production of each cytokine was estimated with Pearson correlations. PSS scores were significantly negatively correlated with levels of IL-1 β ($r = -.420, p < .01$), IL-6 ($r = -.296, p < .05$), and TNF- α ($r = -.259, p < .01$) in the supernatant fluids from DEX-treated cultures. There was no significant relationship between PSS and GCS for IFN- γ .

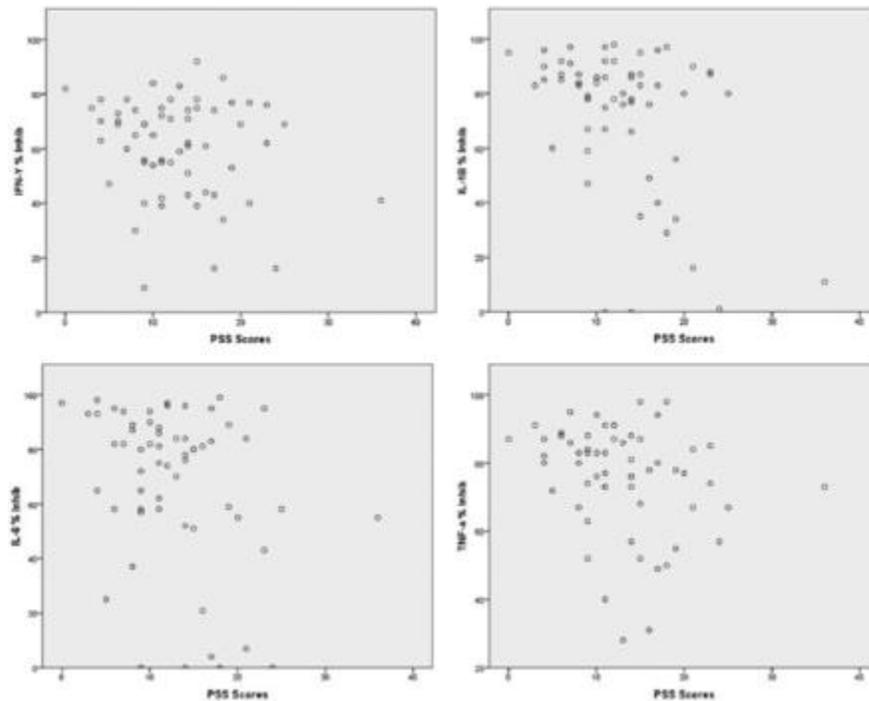


Figure 2. PSS scores and percent inhibition of cytokine production.

Note. PSS scores and percent inhibition of (a) IFN- γ , (b) IL-1 β , (c) IL-6, and (d) TNF- α . PSS scores = 0-40. Percent inhibition of cytokine production = 0%-100%. PSS = Perceived Stress Scale; IFN- γ = interferon-gamma; IL-1 β = interleukin-1-beta; IL-6 = interleukin-6; TNF- α = tumor necrosis factor-alpha.

The relationship between stress (PSS) and GCS (percent inhibition) of vaccine-stimulated production of IL-1 β , IL-6, and TNF- α

was then examined using multiple regression models controlling for factors that could affect perceived stress or cytokine production: age, gender, race, and student cumulative GPA. Based on the nonsignificant relationship between the predictor, PSS, and the outcome GCS for IFN- γ production, further analysis for this cytokine was not warranted.

For GCS of IL-1 β production, the regression model fit the data, $F(df = 1, 59) = 12.637, p < .01$. There was a strong negative relationship between PSS and GCS for IL-1 β production ($\beta = -.420, t = -3.55, p < .01$) when controlling for age, gender, race, and GPA. Similarly, there was a strong negative relationship between PSS and GCS (percent inhibition) of IL-6 production ($\beta = -.296, t = -2.36, p < .05$), and between PSS and GCS of TNF- α production ($\beta = -.259, t = -2.060, p < .05$) when controlling for age, gender, race, and GPA.

Discussion

Prior research has demonstrated that perceived stress is associated with impaired antibody responses to influenza vaccination in healthy young adults, similar to that often seen in older, chronically stressed adults (Pedersen et al., 2009). Increased production of proinflammatory cytokines or reduced sensitivity to the inhibitory effects of cortisol likely contributed to the impaired antibody response to vaccination in chronically stressed or older populations. The present study examined whether psychological stress experienced by young, healthy ROTC college students might affect the inflammatory response of PBL to influenza vaccine or the suppressive effects of DEX on vaccine-stimulated cytokine production. It was hypothesized that subjects with greater psychological stress would have greater cytokine production and lower GCS in an ex vivo laboratory model of influenza vaccine challenge.

Psychological Stress

The average score on the 10-item PSS was 12.82 ($SD = 6.32$), similar to U.S. norms but lower than the average score of 16.78 ($SD = 6.86$) reported by young adults 18 to 25 years of age (Cohen & Janicki-Deverts, 2012). No demographic variable was correlated with the PSS scores.

Psychological Stress and Vaccine-Stimulated Cytokine Production

It was hypothesized that PBL from subjects with higher psychological stress would have increased production of IFN- γ , IL-1 β , IL-6, and TNF- α in response to stimulation by influenza vaccine. The results demonstrate that PBL of military students with higher PSS scores tended to produce more IFN- γ , IL-1 β , IL-6, and TNF- α in response to influenza vaccine challenge ex vivo, but this relationship was not statistically significant as hypothesized. This may be a result of small sample size, the young age of the subjects, or the relatively low scores on the PSS.

DEX-Induced Suppression of Vaccine-Stimulated Cytokine Production

The concentration of IFN- γ , IL-1 β , IL-6, and TNF- α in culture fluids from the influenza vaccine-stimulated and the influenza and DEX condition were significantly different, demonstrating that DEX had a significant inhibitory effect on vaccine-stimulated cytokine production. This finding is consistent with previous studies reporting the effects of DEX-induced suppression of ex vivo inflammatory cytokine production (Agarwal & Marshall, 2001; Cohen et al., 2012; Miller et al., 2002). In contrast, vaccine-stimulated production of IFN- γ was not suppressed by DEX. Given the importance of IFN- γ in the immune response to viral infection, this observation requires further study.

In the present study, 75% of the subjects were male, consistent with the general military population as reported by the Department of Defense (2013). Gender was not associated with PSS, GCS, or production of proinflammatory cytokines. However, male subjects tended to exhibit higher cytokine levels in fluids from all three ex vivo culture conditions. This finding is aligned with previous findings by Wirtz, von Känel, Rohleder, and Fischer (2004) who reported that IL-6 and TNF- α production were higher in men, and glucocorticoid-induced inhibition of lipopolysaccharide (LPS) -stimulated IL-6 and TNF- α production, was less pronounced in men than in women.

Psychological Stress and GCS

It was hypothesized that higher perceived stress would decrease GCS of PBL stimulated with influenza vaccine. We found that the PSS score was inversely related to DEX inhibition of vaccine-stimulated production of IL-1 β , IL-6, and TNF- α . These data are consistent with previous research demonstrating that chronic stress induces glucocorticoid resistance in PBL of caregivers of children with developmental disabilities and patients with cancer and Alzheimer's disease (Cohen et al., 2012). Our findings suggest that perceived stress will reduce GCS in healthy young ROTC college students, which may explain in part the previously reported effect of perceived stress on the antibody response to influenza vaccination in healthy college undergraduate students (Burns, Carroll, Drayson, Whitham, & Ring, 2003).

This study had a number of limitations. Participants may have received the Afluria influenza vaccine in the past, or been exposed to influenza strains related to those in the vaccine. This could affect ex vivo PBL responses to the vaccine. The FDA has recommended this vaccine for several years, and ROTC students receive annual influenza vaccinations, which may affect generalizability of findings to non-ROTC or older populations. The cross-sectional design prevents making any causal inferences regarding the relationship between psychological stress and GCS of PBL stimulated with influenza vaccine. The small sample size and unique, predominately male population of ROTC college students limits generalizability of findings. Although gender was not associated with PSS, cytokine production, or GCS, the small number of female subjects did not allow us to rule out potential effects of menstrual hormones or contraceptives on cytokine production or GCS. Because the study population lacked diversity, effects of race or ethnicity on cytokine production and GCS of PBL also could not be determined.

Article Notes

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Notes

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