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The protective effects of resveratrol on social stress-induced cytokine release and depressive-like behavior

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Abstract

Social stress is a risk factor for psychiatric disorders, however only a subset of the population is susceptible while others remain resilient. Inflammation has been linked to the pathogenesis of psychosocial disorders in humans and may underlie these individual differences. Using a resident-intruder paradigm capable of revealing individual differences in coping behavior and inflammatory responses, the present study determined if resveratrol (RSV; 0, 10, 30mg/kg/day) protected against persistent stress-induced inflammation in socially defeated rats. Furthermore, the antidepressant efficacy of RSV was evaluated using the sucrose preference test. Active coping rats were characterized by more time spent in upright postures and increased defeat latencies versus passive coping rats. Five days after defeat, flow cytometry revealed enhanced stimulation of inflammatory proteins (IL- β , TNF- α) in spleen cells of passive rats as compared to active coping and controls, an effect that was blocked by both doses of RSV. Furthermore, only passive coping rats exhibited increased proinflammatory proteins (IL-1 β , TNF- α , GM-CSF) in the locus coeruleus (LC), a noradrenergic brain region implicated in depression. Notably, only 30mg/kg RSV blocked LC neuroinflammation and importantly, was the only dose that blocked anhedonia. Alternatively, while stress had minimal impact on resting cytokines in the dorsal raphe (DR), RSV dose-dependently reduced DR cytokine expression. However, this did not result in changes in indoleamine 2,3-dioxygenase activity or serotonin levels. Taken together, these data suggest that social stress-induced depressive-like behavior evident in passive coping rats may be driven by stress-induced neuroinflammation and highlight natural anti-inflammatory agents to protect against social stress-related consequences.

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Keywords

Social Stress; Inflammation; Depression; Resveratrol; Coping

1. Introduction

It has long been recognized that repeated exposure to social stress can result in the development of depression (Almeida, 2005). Affecting approximately 7 percent of adults and 11 percent of adolescents, depression is considered to be one of the most common debilitating diseases in the United States (National institute of mental health 2013a, b), yet there is significant individual variability in susceptibility, which has been attributed to differences in coping style (Veenema et al., 2003). In humans, passive coping, such as avoidance or substance abuse, is associated with increased stress susceptibility, while active coping, such as problem solving, is associated with stress resiliency (Cairns et al., 2014). This phenomenon has also been observed in rodents where passive coping results in the development of physiological and behavioral endpoints comparable to a depressive-like state, which is not evident in animals which adopt active coping strategies (Ahmed et al., 2014; De Miguel et al., 2011; Gomez-Lazaro et al., 2011; Korte et al., 1992; Perez-Tejada et al., 2013; Wood et al., 2010; Wood et al., 2015; Wood et al., 2013).

Interestingly, coping in both humans and rodents has been shown to play a large role in inflammatory responses to an immune challenge. For example, lipopolysaccharide stimulation of cytokine release in whole plasma is greater in passive versus active coping individuals (Bouhuys et al., 2004). Furthermore, unstimulated resting levels of peripheral cytokines, such as interleukin (IL-6) and tumor necrosis factor (TNF)- α are increased in subpopulations of depressed patients (Alesci et al., 2005; Bouhuys et al., 2004; Maes et al., 1997; Miller et al., 2002; Motivala et al., 2005; Musselman et al., 2001; Raison et al., 2013). Although much of what is known about depression and inflammation is obtained from plasma measurements, recent studies detected evidence of increased neuroinflammation during major depressive episodes regardless of peripheral inflammatory status (Setiawan et al., 2015). Data from our lab and others have revealed that social stress can produce increased inflammation both peripherally and centrally and that this increased inflammatory response occurs only in rats that are susceptible to social defeat-induced depressive-like behaviors (Hodes et al., 2014; Li et al., 2015; Wood et al., 2015). As a result, social defeat stress represents a useful tool for identifying how neuroinflammation contributes to the pathophysiology of these stress-related disorders.

Historically depression has been associated with dysfunctions of both the noradrenergic (NE) and serotonergic (5-HT) systems. This is believed to occur through dysregulation of the locus coeruleus (LC), the major source of NE to the brain (Aston-Jones et al., 1995; Ressler and Nemeroff, 2000; Swanson and Hartman, 1976), and the dorsal raphe (DR), a major source of 5-HT (Lechin et al., 2006; Roche et al., 2003). While it is well recognized that these systems may play a significant role in the pathophysiology of depression, the mechanistic basis by which this occurs is still unclear. Interestingly, inflammation has been shown to affect both of these systems; inflammatory cytokines increase the spontaneous

firing rate of the LC (Borsody and Weiss, 2002, 2004) and within the DR they promote serotonergic cell death and a shift away from 5-HT synthesis by activation of the indoleamine 2,3-dioxygenase (IDO) enzymatic pathway (Guillemin et al., 2003; Hochstrasser et al., 2011; Lestage et al., 2002). Likely due, in part, to increased inflammatory responses, enhanced NE tone throughout the brain (Page and Abercrombie, 1999), reduced 5-HT, and elevated kynurenine (Kyn), a neurotoxic byproduct of the IDO pathway, have been associated with depression (Guillemin et al., 2003; Hochstrasser et al., 2011; Lestage et al., 2002). Therefore, this study focused on the contribution of stress-induced neuroinflammation within the LC and DR in the development of depressive-like behaviors.

Given the role that inflammation may play in depressive disorders, anti-inflammatory agents may prove useful as an antidepressant therapy or adjuvant to traditional therapies. Resveratrol (RSV) is a natural, commercially available polyphenol found in the skin of red grapes and red wine (Langcake and Pryce, 1976). RSV has demonstrated anti-inflammatory properties (Donnelly et al., 2004; Falchetti et al., 2001; Fordham et al., 2014) through inhibition of mast cell, macrophage, neutrophil, and microglial production of histamines, cytokines, proteases, nuclear factor-kappa B, and oxidants (de la Lastra and Villegas, 2005; Zhang et al., 2010). These cellular effects are thought to be responsible for RSV's anxiolytic properties (Patki et al., 2013a) as well as its demonstrated anti-depressant efficacy in the forced swim test (Ahmed et al., 2014; Xu et al., 2010), single prolonged stress (Solanki et al., 2015), and Wistar-Kyoto depressive-like rat model (Hurley et al., 2014). The aim of the present study was to test the ability of RSV to suppress stress-induced inflammation resulting from exposure to an ethologically relevant model of social stress in rodents. Furthermore, these studies differentiated between the contribution of central (LC/DR) and peripheral inflammation in the development of a depressive-like phenotype. In addition to measuring neuroinflammation in the LC, these studies quantified the effects of stress and RSV on inflammation and IDO activity within the serotonergic DR.

2. Materials and Methods

2.1 Animals

Male Sprague Dawley rats (225–250g, intruder or controls) and Long-Evans retired breeders (650–850g, residents) (Charles River, Wilmington MA) were individually housed in standard cages with ad libitum access to food and water while maintaining a 12-hour light/dark cycle. Care and use of the animals was approved by the University of South Carolina's IACUC and was in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

2.2 Resveratrol Treatment

Trans-resveratrol (Cayman Chemical Company, Ann Arbor, MI) was dissolved in a solution consisting of 10% ethanol (Ultra Pure, Darien, CT), 15% Cremophor (Fisher Scientific, Waltham, MA), and 75% saline (Abbott Laboratories, Chicago, IL) to a concentration of 10 or 30 mg/ml. These doses were chosen as 10 and 30 mg/kg RSV treatment selectively produces peripheral anti-inflammatory effects (Hong et al., 2008; Singleton et al., 2010), yet

only the higher dose of 30 mg/kg accesses the brain as identified by high performance liquid chromatography studies (Wang et al., 2002). Therefore, vehicle (0), 10, or 30 mg/kg RSV (ip) was administered to rats beginning 7 days before control/defeat exposure and ending on the last day of defeat. All tissue was collected 5 days after the final treatment with RSV (see Figure 1 for a brief study timeline). Since the half-life of RSV following repeated dosing is reported to be 2.5 hours, RSV is not likely to persist in plasma or brain tissue at time of collection (Almeida et al., 2009).

2.3 Social Stress (Resident-Intruder paradigm)

This animal model is modified from the version developed by Miczek (Miczek 1979) and identical to our previous publications (Wood et al., 2010; Wood et al., 2015; Wood et al., 2013). Long-Evans retired breeders were screened for their level of aggression prior to being included in the study. Inclusion criteria consisted of 1) exhibiting an attack latency of less than 60 seconds, 2) total number of attacks ≤ 4 within the first 5 minutes, and 3) effective attacks that did not result in injury to the intruder. Sprague-Dawley rats were randomly assigned into the “intruder” or “control” group. Intruders were exposed to a different Long-Evans retired breeder for 30 minutes for five consecutive days and behavioral responses of the resident and intruder were noted in addition to defeat latency. After exhibiting a supine posture, or after 15 minutes, whichever came first, intruders were placed behind a Plexiglas partition within the resident’s cage for the remainder of the 30-minute defeat period. Each day following social defeat intruders were returned to their home cage. Control animals were not present in the room during social defeat exposures and control manipulation consisted of being placed into a novel cage behind a partition for 30 minutes/day. Defeat exposures were video recorded for a subset of rats from each treatment group. An experimenter blinded to the treatment conditions quantified the duration of time each intruder spent in an upright posture upon the 5th day of social defeat as previously reported (Wood et al., 2010).

2.4 Sucrose Preference

All experimental animals were subject to the sucrose preference test 2–3 days prior to stress/control exposure and 4 days after the final stress/control exposure as previously published (Wood et al., 2015). Sucrose preference ($[\text{volume } 1\% \text{ sucrose}/\text{total volume consumed}] \times 100$) was calculated for the first hour of the dark period.

2.5 Tissue Collection

Trunk blood, brains, and spleens were collected upon time of sacrifice 5 days after the final defeat or control exposure. Brains were flash frozen in isopentane, and stored at -80°C . The posterior brain was sliced coronally using a cryostat up to the most caudal level of the LC (Bregma -10.08 mm, Paxinos and Watson) at which time bilateral 1 mm deep punches were taken using a 1 mm wide tissue biopsy punch (Harvard apparatus, Holliston, MA). Coronal slices were continued after LC recovery to the most caudal level of the DR (Bregma -8.40 mm, Paxinos and Watson) at which time a 2 mm deep punch was taken. Tissue punches were stored at -80°C .

2.6 Tissue Homogenization

LC and DR tissue punches were homogenized in 200 μ L of tissue lysis buffer (500 mM NaCl, 50 mM Tris HCl pH 7.6, 10% NP-40, 70% glycerol) with 0.02% protease phosphatase inhibitor cocktail (Thermo Scientific, Waltham, MA) in conjunction with mechanical disruption with a bullet blender (Next Advance, Averill Park, NY) at 4°C. Protein concentrations of the supernatant were assessed using a BCA assay (Thermo Scientific, Waltham, MA) according to the manufacturer's protocol and read using a Synergy 2 Multi-Mode plate reader (Bio Tek, Winooski, VT) with Gen5 software (Bio Tek, Winooski, VT). The remaining tissue homogenate was stored at -80°C.

2.7 Bio-Plex Cytokine Analysis

In order to determine the inflammatory levels in brain and plasma of experimental animals, a 10-plex Bio-Plex Assay (Bio-Rad Laboratories, Hercules CA) was used to quantify expression of IL-1 β , IL-2, IL-4, IL-6, IL-10, IL-13, granulocyte macrophage colony stimulating factor (GM-CSF), monocyte chemoattractant protein (MCP)-1, interferon (INF)- γ , and TNF- α . Homogenized LC samples were diluted to 275 μ g/mL and plasma samples were diluted 1:4 as per Bio-Plex Assay Quick Guide 4 protocol. All samples (LC, DR, and plasma) and standards were run in duplicate according to manufacturer protocol. Plates were read using a Bio-Rad Bio-Plex system (Hercules, CA). A range of absolute plasma, LC, and DR Bio-Plex values for vehicle treated rats is available in the supplementary materials (Supplementary Table 1). However, in order to account for inter-kit variability within the Bio-Plex Assays the same vehicle treated control samples were run on each plate and data were normalized to percent of vehicle controls run on each respective plate.

2.8 Flow Cytometry of Splenic Cells

To determine the effect of RSV on intracellular cytokine expression following exposure to social stress, flow cytometry was conducted. Primary spleen cells were prepared on the day of sacrifice and stimulated with 2 μ g/ml concanavalin-A (Sigma-Aldrich, St. Louis MO) overnight at 37°C/ 5% CO₂ to stimulate immune cell proliferation. The following day, 4 hours before the immunostaining procedure began, intracellular trafficking was halted using Golgi Stop as per the manufacturer's instructions (BD Biosciences, San Jose CA) and aliquoted to facilitate immunostaining of four individual inflammatory cytokines. Rat FITC anti-CD4 cell surface antibody (or isotype control) was added to label the T-helper cells (Biolegend, San Diego CA). The cells were fixed and permeabilized using BD cytofix/cytoperm (BD Biosciences, San Jose CA) as per the manufacturer's protocol. Primary antibodies used to label inflammatory cytokines were PE-anti rat TNF α (Biolegend, San Diego CA), Biotin anti-rat IL-1 β (Abcam, Cambridge MA), mouse anti-rat IL-6 (Invitrogen, Camarillo CA), and PE-anti-rat IL-10 (BD Biosciences, San Jose CA) or appropriate isotype controls. For primary antibodies not fluorescently labeled an appropriate secondary antibody with a conjugated fluorophore was used (PE-anti-Biotin and PE-anti-mouse; Biolegend, San Diego, CA). Following the staining procedure, all samples were stored in BD cytofix/cytoperm staining buffer, and protected from light at 4°C until they were analyzed by flow cytometry the following day (FC 500, Beckman Coulter, Fort Collins Co).

2.9 SIRT-1 ELISA

To determine whether RSV altered expression of the NAD-dependent deacetylase Sirtulin (SIRT) 1, LC homogenates (150 µg/mL) were analyzed using a SIRT 1 ELISA kit (Abcam, Cambridge, MA) following the manufacturer's protocol.

2.10 IDO enzymatic activity assay

To determine the effects of social stress and RSV treatment on IDO in the DR, an IDO activity assay was conducted and modified from Matin and colleagues (Matin et al., 2006). This assay quantified IDO enzymatic activity by measuring the conversion of its substrate tryptophan to KYN; therefore greater IDO activity is indicated by increased synthesis of KYN. Briefly, 20 µL of DR homogenate was added to 420 µL of assay buffer [50 mM potassium phosphate buffer pH 6.5 (Monobasic, Acros Organics, NJ; Dibasic, MP Biomedicals, Solon, OH), 20 mM ascorbic acid pH 7 (BDH Lab Depot, Dawsonville, GA), 10 µM methylene blue, 100 µg/mL catalase (Fisher Scientific, Waltham, MA), 408 µM L-Tryptophan (Acros Organics, NJ)]. L-Kyn (Fisher Scientific, Waltham, MA) standards (0, 1, 5, 7.5, 10, 25, 50, 75, 100 µM) were prepared from 500 µM stock solutions in assay buffer. Standards and samples were incubated at 37°C for 1 hour. After incubation, 80 µL of 1 M NaOH was added to each sample and standard, vortexed, and incubated for 15 minutes at 60°C. Samples were vortexed and 240 µL of each sample and standard were added to a 96 well plate in duplicate. The fluorescence of Kyn was determined using a Synergy 2 Multi-Mode plate reader (Bio Tek, Winooski, VT) with Gen5 software (Bio Tek, Winooski, VT) and excitation wavelength of 360 nm and emission of 460 nm. For assay validation see Supplemental Section 1 and Supplemental Figures 1–4. All IDO activity assay data was normalized to BCA protein concentration.

2.11 High Performance Liquid Chromatography (HPLC) 5-HT measurement

To determine the effects of social stress on 5-HT levels, HPLC analysis of DR tissue was performed (adapted from (Cheng et al., 1994) and (Ueyama et al., 2003)). DR homogenates were spiked with 10 nM dihydroxybenzylamine (DHBA, Sigma-Aldrich, St. Louis, MO) to serve as an internal standard and filtered using Amicon Ultra 0.5 mL Centrifugal filters with a molecular weight cut off of 3kD (Millipore, Billerica, MA) at 16,000xg for 30 minutes at 4°C. HPLC mobile phase consisted of 90 mM sodium phosphate monobasic, 50 mM citric acid, 1.7mM 1-octanesulfonic acid, and 50 µM disodium EDTA pH 3.4 and 10% acetonitrile. 5-HT (Sigma-Aldrich, St. Louis, MO) standards were made at 500 nM, 200 nM, 100 nM, and 10 nM with diluted antioxidant solution (350 µL perchloric acid, 200 µL EDTA, 1.3 g sodium bisulfate, deionized water 300 mLs). All 5-HT concentrations were normalized to BCA protein values.

2.12 Statistical Analysis

Rats were statistically separated into passive and active coping groups using a k-means cluster analysis of average defeat latency using JMP (Version 10, SAS, Cary, NC) as previously reported (Wood et al., 2015). In addition, for each data set, outliers were identified and removed using the Modified Thompson Tau outlier test. All data were subjected to a standard Two-Way ANOVA ($\alpha = 0.05$) to determine effects of treatment and

stress followed by Fisher LSD post-hoc analysis using GraphPad Prism 6 software (La Jolla, CA). Pearson correlations (two-tailed, $\alpha = 0.05$) to determine relationships between cytokines and Kyn levels in the DR were also completed using GraphPad Prism 6 (La Jolla, CA).

3. Results

3.1 Effect of RSV on social defeat behavior and anhedonia

Rats in each treatment group were statistically split into the passive or active coping phenotype (Table 1) using a k-means cluster analysis of average defeat latency as previously reported (Wood et al., 2015). Two-way ANOVA analysis of these data sets indicated an effect of treatment ($F(2,39) = 4.4$; $p < 0.05$) as 30 mg/kg RSV treated active coping rats exhibited higher defeat latencies as compared to active coping rats treated with 0 and 10 mg/kg RSV. However, RSV had no significant effect on behavior in passive coping rats. As anticipated, there was also a significant difference in defeat latency between the two clusters of rats classified as passive and active coping phenotypes ($F(1,39) = 184.5$, $p < 0.0001$). In addition, Two-way ANOVA indicated an effect of coping phenotype on upright postures on both days 1 ($F(1,26) = 12.6$; $p < 0.01$) and 5 ($F(1,28) = 12.3$; $p < 0.01$; Figure 2A) of defeat, such that rats classified as passive coping spent significantly less time in upright postures on both days as compared to their active coping counterparts. Furthermore, RSV treatment did not alter the amount of time spent in upright postures across passive or active coping groups.

To assess the development of a depressive-like phenotype, a sucrose preference test was administered four days after the final social defeat or control manipulation as previously published (Wood et al., 2015). This particular method of assessing depressive-like behavior was chosen as it is a non-stressful means of measuring depressive-like behavior and will not result in stimulated cytokine release as compared to other tests like the forced swim test, which has been shown to result in cytokine release in the brain (Llorens-Martin et al., 2016). Two-way ANOVA of sucrose preference indicated a stress x treatment interaction ($F(4,40) = 2.8$; $p < 0.05$; Figure 2B). Importantly, vehicle-treated passive coping rats exhibited significantly less sucrose preference than vehicle-treated control rats (Figure 2B). Furthermore, RSV effectively blocked the development of anhedonia as passive coping rats treated with the highest dose of RSV exhibited increased hedonic behavior (increased sucrose preference) compared with passive coping rats treated with vehicle. It should be noted that although the 10 mg/kg RSV treatment did function to modestly increase sucrose preference in passive coping rats, post hoc analysis did not indicate a significant difference between 0 and 10 mg/kg RSV groups, and it was not until the highest dose that a full blockade of anhedonic behavior was observed. No significant differences were detected across doses for active coping rats or rats with a history of control. Importantly, based on the sucrose preference test conducted prior to defeat/control exposure, there were no pre-existing differences between stress coping conditions ($F(2,30) = 1.79$, $p = 0.18$) or RSV treatment ($F(2,30) = 1.17$, $p = 0.32$) groups nor were there significant variations in total volume consumed between stress ($F(2,61) = 0.67$, $p = 0.51$) and treatment groups ($F(2,61) = 1.83$, $p = 0.17$).

3.2 Effect of RSV on peripheral inflammation

Five days after the final social defeat exposure Bio-Plex analysis detected no significant differences in resting cytokine expression in the plasma as a result of stress or treatment (Supplementary Table 2). Since the spleen is a major site of initiation of the peripheral immune response, splenic cells were harvested and flow cytometry confirmed that social defeat significantly increased concanavalin-A stimulated TNF- α and IL-1 β levels selectively in the passive coping phenotype (Figures 3 and 4). Post hoc analysis revealed increased TNF- α in vehicle-treated passive coping rats compared to both vehicle-treated control and active coping animals ($p < 0.0001$). Furthermore, the effect of stress on TNF- α in passive coping rats was blocked by treatment with both 10 and 30mg/kg RSV (Figure 3; stress x treatment interaction: $F(4,49) = 7.0$; $p = 0.0001$). Similarly, increases in the percentage of CD4 $^{+}$ cells expressing IL-1 β were evident in vehicle-treated, passive coping rats and blocked by both doses of RSV (Figure 4; stress x treatment interaction: $F(4,50) = 3.9$; $p = 0.0073$). IL-6 was not elevated in rats with a history of stress, however there was an effect of treatment ($F(2,50) = 31.8$; $p < 0.0001$), with a reduction in IL-6 levels in all rats treated with 30mg/kg RSV vs. vehicle, regardless of stress history (Supplemental Figure 5). There were no effects of stress or treatment on anti-inflammatory IL-10 levels.

3.3 Effect of RSV on inflammation in the LC

Consistent with our previous findings (Wood et al., 2015), cytokine levels were elevated in the LC of passive coping rats (Figure 5). The present study determined that RSV treatment prior to and during social stress exposure significantly blocked the development of increased cytokine expression within the LC measured 5 days after stress. Specifically, IL-1 β (Figure 5A), GM-CSF (Figure 5B), and TNF- α (Figure 5C) exhibited stress-induced increases that were dependent on coping style (effect of stress group: IL-1 β : $F(2,58) = 10.6$, $p < 0.0001$; GM-CSF: $F(2,60) = 8.2$, $p < 0.001$; TNF- α : $F(2,55) = 5.2$, $p < 0.01$). Passive coping rats treated with vehicle and 10mg/kg RSV exhibited significantly elevated levels of IL-1 β and GM-CSF protein in the LC as compared to controls and active coping rats. A similar trend was observed with TNF- α , where passive coping vehicle-treated rats exhibited significantly elevated levels compared with their active coping counterparts (Figure 5C). Treatment with RSV reduced cytokine expression in a dose dependent manner for all three proinflammatory cytokines (effect of treatment: IL-1 β : $F(2,58) = 11.0$, $p < 0.0001$; GM-CSF: $F(2,60) = 12.0$, $p < 0.0001$; TNF- α : $F(2,55) = 13.9$, $p < 0.0001$). Treatment effects were also detected for IL-2 ($F(2,45) = 11.3$; $p < 0.001$), IL-6 ($F(2,56) = 5.0$, $p < 0.05$), IL-10 ($F(2,41) = 12.6$, $p < 0.0001$), and MCP-1 ($F(2,53) = 20.3$, $p < 0.0001$), such that 30 mg/kg RSV significantly reduced cytokine expression (See supplemental Figure 5). A significant stress x treatment interaction ($F(4, 58) = 2.5$, $p < 0.05$) was evident in IL-4 within the LC, such that treatment with 30mg/kg RSV produced increased IL-4 expression in all 3 stress conditions compared with vehicle-treated controls (Figure 5D). Interestingly, active coping rats appeared more sensitive to the IL-4-inducing effects of RSV as the 10mg/kg dose significantly and selectively increased IL-4 in active coping rats.

It has been suggested that RSV may increase SIRT-1 (Borra et al., 2005), a member of the histone deacetylase family that is involved in inflammatory processes, while stress generally decreases histone deacetylase function (Renthall et al., 2007). However in the present study,

there were no differences in SIRT-1 expression within the LC between control (335.4 ± 12.3 pg/mL, $n=5$) and passive coping (311.6 ± 10.0 pg/mL, $n=6$) rats. Furthermore, prior treatment with 30 mg/kg RSV did not alter SIRT-1 expression (control: 302.5 ± 10.0 , $n=6$; passive coping: 308.1 ± 11.1 , $n=5$).

3.4 Effect of RSV on inflammation in the DR

We previously reported that social stress down regulated IL-1 β in the DR of active coping rats when measured 24 hours after stress (Wood et al., 2015). Consistent with this, in the present study, expression of select cytokines were decreased in the DR of socially defeated rats (Table 2). Specifically, stress and treatment produced a significant interaction resulting in reduced IL-2 ($F(4,38)=7.5$, $p<0.001$) and IL-4 ($F(4,44)=4.6$, $p<0.01$) expression regardless of coping style. There was also a general effect of treatment for the remaining 7 analytes (Table 4: IL-1 β : $F(2,42)=12.4$, $p<0.0001$; IL-6: $F(2,45)=17.7$, $p<0.0001$; IL-10: $F(2,40)=16.86$, $p<0.0001$; IL-13: $F(2,43)=13.0$, $p<0.0001$; GM-CSF: $F(2,40)=4.5$, $p<0.05$; TNF- α : $F(2,43)=7.1$, $p<0.01$; MCP-1: $F(2,42)=8.3$, $p<0.001$) such that RSV treatment functioned to lower cytokine levels regardless of stress history with exception of IL-6, which demonstrated a dose dependent increase. Treatment effects for IL-6, TNF- α , and MCP-1, although statistically significant, likely are not biologically relevant due to the modest changes exhibited by these cytokines. Importantly, while no general effects of stress were detected for IL-1 β and IL-10, vehicle treated passive coping rats did exhibit significant reductions of these cytokines within the DR as compared to their control and active coping counterparts (Table 2).

Stress and inflammation are well known to result in a shift in 5-HT metabolism towards the production of Kyn through increased IDO activity (Guillemin et al., 2003; Hochstrasser et al., 2011; Lestage et al., 2002). Therefore, the present study conducted an IDO enzymatic activity assay and measured 5-HT levels by HPLC within the same DR samples that were used to quantify cytokine levels. There were no significant effects of treatment or stress history on IDO activity levels (Supplemental Table 3). Importantly, no differences were determined between vehicle treated control, passive, and active coping groups ($F(2,3)=0.2574$, $p=0.79$). Furthermore, HPLC analysis of 5-HT concentrations within DR homogenates indicated that neither stress nor RSV treatment produced significant alterations in 5-HT levels at rest 5 days after the completion of social defeat (Supplemental Table 3).

4. Discussion

The findings in this study are the first to explore the effects of the natural anti-inflammatory agent RSV on susceptibility to social stress-induced inflammation and depressive-like behavior. Socially defeated rats characterized as passive coping exhibited sensitized release of IL-1 β and TNF- α by CD4 $^{+}$ T-cells following concanavalin-A stimulation, which was blocked by both doses of RSV. Unlike findings in the periphery, which required stimulation with concanavalin-A, long lasting neuroinflammation was detected under resting conditions selectively in the LC of passive coping rats. Only the highest dose of RSV inhibited the depressive-like anhedonia and remarkably, this was also the only dose that inhibited LC neuroinflammation in passive coping rats. These data suggest that LC neuroinflammation

plays a key role in susceptibility to social stress-induced depressive-like responses, specifically in passive coping individuals. These data also highlight the importance of differentiating between peripheral and central inflammation. Furthermore, the contrast between enhanced pro-inflammatory cytokines in the LC and suppression within the DR illustrates the brain region specificity of stress-induced changes in inflammation, and underscores the concept that changes in the plasma may not represent the state of inflammation in the brain.

One key feature of the resident-intruder paradigm is that it can produce depressive-like behaviors in rodents (Gomez-Lazaro et al., 2011; Hammels et al., 2015; Perez-Tejada et al., 2013; Wood et al., 2010; Wood et al., 2015). In agreement with our previous studies, active coping during social defeat was characterized by greater time in upright postures, increased defeat latencies and resiliency to the negative behavioral consequences of social stress as indicated by intact hedonic behavior in the sucrose preference test following stress (Wood et al., 2010; Wood et al., 2015). RSV treatment did not shift the behavioral response during social defeat from a passive to an active response; however, it did attenuate the anhedonic behavior exhibited by passive coping rats in a dose dependent manner. Importantly, other studies have demonstrated the anti-depressant efficacy of RSV using pharmacological (Ahmed et al., 2014), single prolonged stress (Solanki et al., 2015), and genetic models of depressive-like responses (Hurley et al., 2014), as well as in the forced swim test (Xu et al., 2010). Notably, our study is the first to use RSV in the context of social stress and provides evidence that a natural anti-inflammatory agent can produce an anti-depressant-like effect in an ethologically relevant model.

Much of the clinical evidence regarding inflammation in depressive disorders reflects changes in plasma, where increases in IL-6, TNF- α , and C-reactive protein have been correlated with symptom severity (Alesci et al., 2005; Maes et al., 1997; Miller et al., 2002; Motivala et al., 2005; Musselman et al., 2001). Recently we demonstrated that passive coping in response to repeated social stress in rodents can result in robust increases in plasma MCP-1 when analyzed 24 hours after stress exposure (Wood et al., 2015). The present study expanded upon these findings by evaluating resting cytokine levels 5 days after the final defeat exposure. During this extended time following stress, resting circulating cytokine levels were no longer elevated. It has been determined that cytokines such as TNF- α , GM-CSF, and IL-2 reach complete peripheral clearance between 25 and 58 hours respectively (Foxwell et al., 1998; Gillies et al., 1993). Therefore, it is not surprising that the present study did not determine measurable differences in rats at rest 5 days (120 hours) after the final social defeat/control exposure.

Although resting cytokine levels were normalized 5 days post stress in our study, there is a vast body of literature indicating that inflammatory priming can also occur following stress exposure (Fonken et al., 2016; Frank et al., 2012; Frank et al., 2011; Weber et al., 2013; Wohleb et al., 2014a). Priming can result in a sensitized inflammatory response both in the periphery and the brain to a future stress exposure or endotoxin challenge (Wohleb et al., 2014a). Spleen cells play an important role in the peripheral immune response in part because highly morphologically flexible splenic CD4⁺ T-cells can rapidly secrete cytokines upon stimulation by circulating cytokines (DuPage and Bluestone, 2016). Although it has

been demonstrated that depression is associated with a reduction in peripheral CD4⁺ T cells due to greater apoptosis (Eilat et al., 1999; Ivanova et al., 2007; Szuster-Ciesielska et al., 2008), these cells exhibit exaggerated release of proinflammatory TNF- α and IL-6 (Duggal et al., 2014) and can readily traffic to the brain to promote neuroinflammation and neurodegeneration (Stuart et al., 2015; Toben and Baune, 2015). In congruence with findings by Gomez-Lazaro and colleagues (Gomez-Lazaro et al., 2011) indicating greater splenic immune responses in passive coping mice, the present study identified enhanced concanavalin-A induced expression of TNF- α and IL-1 β in CD4⁺ T cells selectively in passive coping rats compared with controls and active coping defeated rats. Therefore, our findings in spleen cells supports the idea that inflammatory cell priming has occurred and therefore further identifying the role of priming in this susceptible phenotype will be an important advance in understanding the stress-related consequences that promote increased stress susceptibility. Importantly, further exploring alterations in other immune cell types including CD3⁺, CD8⁺, and CD11b⁺ cells would provide additional information on the status of the inflammatory phenotype of splenic cells. There is limited evidence to suggest that social stress can alter the expression of CD3⁺ (Dominguez-Gerpe and Rey-Mendez, 2001) and CD8⁺ (Stefanski and Engler, 1998) T cells. However, social defeat stress has been shown to increase the number and reactivity of CD11b⁺ cells (Heidt et al., 2014; Wohleb et al., 2014b; Wohleb et al., 2013), resulting in enhanced CD11b⁺ cell trafficking to the brain (Wohleb et al., 2012; Wohleb et al., 2014a; Wohleb et al., 2014b; Wohleb et al., 2013), and may serve as a predictor of social stress susceptibility (Hodes et al., 2014). Therefore, further evaluation of social defeat-induced changes of these cell types, specifically with regard to CD11b⁺ cells, will provide valuable insight into the inflammatory cross-talk between the periphery and the brain and represents an important future direction in this model.

Limited information exists on inflammation in the brains of depressed patients; however, cytokine expression has been found to be increased in the cerebrospinal fluid of a subset of depressed patients compared with non depressed controls (Devorak et al., 2015; Kern et al., 2014; Sasayama et al., 2013). Interestingly, a recent advance in this field revealed greater microglial activation in the brains of depressed patients, demonstrating a potential mechanism for this neuroinflammation (Setiawan et al., 2015). Many parallels have been found between these studies in humans and neuroinflammatory changes that occur as a consequence of social stress in rodents. For example, social defeat has been shown to increase the number of immune competent cells such as microglia and macrophages in whole brain homogenates (Audet et al., 2011; De Miguel et al., 2011; Wohleb et al., 2012; Wohleb et al., 2011). Our previous work demonstrates that stress can produce distinctively different effects on neuroinflammation 24 hours after stress that is contingent upon the specific brain region and upon the coping style used during stress (Wood et al., 2015). The present study extended these findings by demonstrating that social defeat stress produces long lasting increases in pro-inflammatory cytokines in the LC of passive coping rats, which also exhibit increased susceptibility to depressive-like behaviors. It should also be noted, however, that elevated pro-inflammatory cytokines are not necessarily selective for vulnerable phenotypes, but rather are dependent upon the brain region. As a result, increased pro-inflammatory cytokines may also be associated with resilience as demonstrated by De

Miguel and colleagues who identified greater IL-1 β expression within the hypothalamus of active coping rats (De Miguel et al., 2011).

While this and other studies have demonstrated an anti-depressant effect of RSV, the molecular mechanism by which this occurs is unclear, yet there are several possibilities. One possibility is through RSV's potent anti-neuroinflammatory capabilities in stress sensitive brain regions. For example, cytokines have an excitatory effect directly on LC-NE neurons (Borsody and Weiss, 2002, 2004) likely mediated by activation of the glutamatergic system (Kamikawa et al., 1998). Exaggerated activation of the LC-NE system is associated with the pathophysiology of depression (Wong et al., 2000). Therefore, RSV's potent inhibitory effects on neuroinflammation within the LC would serve to inhibit exaggerated activation of this depressogenic brain region. Alternatively, as identified in the brains of RSV-treated rats in the present study, RSV has also been shown to increase the release of the anti-inflammatory cytokine IL-4 in isolated peripheral mononuclear cells (Falchetti et al., 2001). These data suggest that RSV may also function through the recruitment of endogenous anti-inflammatory pathways.

In addition to RSV's potent anti-inflammatory effects, it has also been suggested to inhibit nuclear factor kappa B through histone deacetylation by SIRT-1 (Bagul et al., 2015; Borra et al., 2005), a factor which has been linked to the development of depressive-like states (Haslund-Vinding et al., 2016; Patki et al., 2013b; Vilhardt et al., 2016). In the present study there were no differences in SIRT-1 protein levels between stress or treatment groups. However, we cannot rule out the possibility that stress and RSV may have altered SIRT-1 activity rather than absolute expression levels or that measuring SIRT-1 levels 5 days after stress is not optimal to detect these changes. Finally, RSV and RSV-rich grape powder also exert strong inhibition of oxidative stress (Olas and Wachowicz, 2004; Patki et al., 2013a). Importantly, reactive oxygen species are increased in the brain following social defeat (Kanarik et al., 2011; Patki et al., 2013b), and specifically within the LC oxidative stress has been shown to desensitize mu opioid receptors (Pablos et al., 2015), a major endogenous mechanism that serves to reduce LC activity following stress (Chaijale et al., 2013; Reyes et al., 2015). Therefore, by reducing the production of reactive oxygen species in the LC, RSV would serve to protect the endogenous inhibitory enkephalinergic input following social defeat (Reyes et al., 2015). Taken together, there are likely several mechanisms by which RSV is exerting its protective effects and determining which are recruited to protect against the repercussions of social defeat stress will be an important advance in understanding the protective effects of RSV.

Distinct from stress effects on neuroinflammation in the LC, we previously reported that social stress downregulates IL-1 β and MCP-1 protein in active coping rats 24 hours after stress exposure (Wood et al., 2015). In the present study, decreased cytokine expression was not only evident in active coping rats, but by 5 days post stress, this potentially protective adaptation was recruited in all stressed animals regardless of coping strategy. Furthermore, RSV functioned to further reduce cytokine expression in the DR, regardless of stress history. Importantly, these opposing adaptations in neuroinflammation within the DR versus the LC indicate the intricate nature of stress-induced neuroinflammation and highlight the region specificity and temporal regulation of stress-induced neuroinflammatory phenotypes.

Like cytokine effects within the LC, the serotonergic DR is also sensitive to pro-inflammatory cytokines. Stress and cytokines have both been shown to shift catabolism of 5-HT to Kyn through increasing activity of IDO, resulting in increased levels of Kyn and decreased levels of 5-HT (Guillemin et al., 2003; Hochstrasser et al., 2011; Lestage et al., 2002). We hypothesized that the reduction in cytokines in the DR of socially stressed rats, and the further reduction in cytokines from RSV treatment, may function as a protective mechanism to maintain or increase 5-HT levels in the face of stress. However, in the present study resting levels of IDO enzymatic activity and 5-HT concentration within the DR were unchanged. This may be explained by findings that elevated levels of proinflammatory cytokines such as TNF- α and INF- γ increase IDO enzymatic activity (Robinson et al., 2005), yet reductions in proinflammatory cytokines below non-stressed/resting levels may not necessarily exhibit regulatory control over IDO activity. Therefore, since social defeat produces decreases in select cytokines within the DR it follows that IDO activity would not be altered. In support of this, evaluation of the IDO-Kyn pathway at earlier time points after stress revealed significant social stress-induced increases in Kyn production in the DR when measured 24 hours after the completion of social defeat (Fuertig et al., 2016). Additionally, in the present study 5-HT cells were not isolated from the DR, therefore we can not rule out the possibility that the heterogeneous cellular population that made up the DR tissue homogenates may have masked stress induced effects on 5-HT and IDO activity. Therefore, IDO and 5-HT measurements at earlier timepoints in a population of isolated 5-HT neurons will be important in confirming the potential effect of stress or RSV on the DR-5-HT system in passive and active coping socially defeated rats.

5. Conclusion

There is evidence to suggest that traditional antidepressant therapies only moderately reduce depressive scores, achieving only 30% remission in patients (Kessler et al., 2005; Krishnan and Nestler, 2008; Pfau and Russo, 2015) and in many cases do not function to treat the underlying inflammation in the subsets of patients exhibiting elevated inflammatory markers (Maes et al., 1997; Uher et al., 2014). While it has been suggested that the use of anti-inflammatories as monotherapy for depression or as adjuvants to anti-depressants is largely ineffective (Eyre et al., 2015), most clinical trials to date did not measure inflammation in the depressed patients. On the other hand, a clinical trial using Infliximab, a TNF antagonist, was shown to be efficacious in reducing both inflammation and depression in patients which presented with the highest levels of plasma C-reactive protein (Raison et al., 2013). Taken together, these data suggest that treatment with anti-inflammatory agents could be a useful therapeutic or adjuvant for treating depression selectively in patients who present with high levels of inflammation. Natural compounds, such as RSV, which have demonstrated high tolerability by patients (la Porte et al., 2010) could therefore become a useful tool to enhance the efficacy of current antidepressant therapy for these subsets of patients. However, future studies designed to better understand the mechanism by which natural anti-inflammatory agents such as RSV attenuates depressive-like behavior will be key to enhancing the clinical value of RSV. This will include examining the role of inflammation on various neurotransmitters and neuropeptides implicated in depression. RSV been shown to alter both 5-HT and NE levels (Sarubbo et al., 2015; Xu et al., 2010), inhibits 5-HT and NE re-uptake

(Yanez et al., 2006), and inhibits monoamine oxidase activity (Xu et al., 2010; Yanez et al., 2006). Therefore, understanding how RSV intervenes the stress induced adaptations within the brain will be critical in furthering our understanding of RSV's therapeutic potential.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Passive coping during social stress results in depressive-like behavior in rats.
- This behavior is associated with greater inflammation in the spleen and locus coeruleus.
- Pretreatment with 10 mg/kg Resveratrol blocks stress-induced splenic inflammation.
- 30 mg/kg Resveratrol pretreatment blocks depressive behavior and neuroinflammation.

Figure 1. Study design and time line

Rats were treated with 0 (Vehicle, Veh), 10, or 30 mg/kg RSV for 7 days prior to the start of social stress and throughout the social stress period (D-7 – D5). Two days prior to the onset of social defeat (SD) or control (Con) exposure (D-2) rats were exposed to a sucrose preference test. Sucrose preference was conducted 4 days after SD or Con (D9, 7–8pm) and approximately 14 (± 2) hours later (D10), all rats were sacrificed.

Figure 2. Effects of stress and RSV treatment on behavior
(A) Upright postures during social defeat on day 5 of defeat and (B) sucrose preference.
 Active and passive coping rats exhibited distinctly different time spent in upright posture regardless of treatment (* $p < 0.05$ vs. within treatment active coping group). Decreased sucrose preference in passive coping rats was dose dependently blocked by RSV treatment (** $p < 0.01$ vs. 0 mg/kg control and active coping, α $p < 0.001$ vs. 30 mg/kg passive coping; β $p < 0.01$ vs. 30 mg/kg passive coping).

Figure 3. Effects of stress and resveratrol treatment on splenic TNFα levels 5 days post social defeat or control exposure

RSV decreased expression of the pro-inflammatory cytokine TNFα following Con-A stimulation in splenic cells of passive coping rats. Data are expressed as the percentage of total CD4⁺ cells expressing TNFα. (A, B, C) Data shown are flow cytometry dot plots from one representative animal in each group shown. (D) Graph of combined data from all experiments (n=8 [control + vehicle]; n=7 [passive + vehicle]; n=8 [active + vehicle]; n=5 [control + 10mg/kg RSV]; n=8 [passive + 10mg/kg RSV]; n=4 [active + 10mg/kg RSV]; n=5 [control + 30mg/kg RSV]; n=9 [passive + 30mg/kg RSV]; n=4 [active + 30mg/kg RSV]). Fisher's LSD post-hoc analysis revealed statistical significance (*f* $p < 0.0001$ vs. control + vehicle; * $p < 0.0001$ vs. active + vehicle; ** $p < 0.0001$ vs. passive + vehicle).

Figure 4. Resveratrol decreases splenic IL-1 β levels 5 days post social defeat in passive coping rats. Effects of stress and resveratrol treatment on splenic IL-1 β levels 5 days post social defeat or control exposure

RSV decreased Con-A stimulated splenic IL-1 β expression in passive coping rats. Data are expressed as the percentage of total CD4⁺ cells expressing IL-1 β . (A, B, C) Data shown are flow cytometry dot plots from one representative animal. (D) Graph of combined data from all experiments (n=8 [control + vehicle]; n=8 [passive + vehicle]; n=8 [active + vehicle]; n=5 [control + 10mg/kg RSV]; n=8 [passive + 10mg/kg RSV]; n=4 [active + 10mg/kg RSV]; n=5 [control + 30mg/kg RSV]; n=9 [passive + 30mg/kg RSV]; n=4 [active + 30mg/kg RSV]). Fisher's LSD post-hoc analysis revealed statistical significance (f $p < 0.0001$ vs. control + vehicle; * $p < 0.01$ vs. active + vehicle; ** $p < 0.05$ vs. passive + vehicle; *** $p < 0.01$ vs. passive + vehicle).

Figure 5. Resveratrol decreases neuroinflammation in the LC of passive coping rats in a dose dependent manner

Social defeat resulted in elevated levels of inflammatory cytokines in vehicle and 10 mg/kg RSV treated passive coping rats as evidenced by increased IL-1 β (A), GM-CSF (B), and TNF- α (C) in LC homogenates (within treatment comparisons: α $p < 0.05$ passive vs. control and active; β $p < 0.05$ passive vs. control; γ $p < 0.05$ passive vs. active). Importantly, only treatment with 30 mg/kg RSV completely blocked social stress induced inflammation in passive coping rats. Surprisingly, 30 mg/kg RSV also resulted in increased concentrations of anti-inflammatory IL-4 (D). Active coping rats were more sensitive to this effect as they exhibited a significant increase from controls and passive coping rats when treated with 10 mg/kg RSV (within treatment: f $p < 0.05$ active vs. control and passive).

Table 1
Quantification of passive and active coping phenotypes

K-means cluster designations of defeat latency are reported as average (s) $\pm$ SEM. Post-hoc analysis indicated 30 mg/kg treated active coping rats had significantly higher defeat latencies compared to 0 and 10 mg/kg treated active coping rats (* $p < 0.05$).

Dose of RSV	Cluster 1 (Passive)	Cluster 2 (Active)
0	208.7 $\pm$ 47.3	651.3 $\pm$ 50.4
10	230.3 $\pm$ 21.8	702.1 $\pm$ 27.2
30	279.9 $\pm$ 8.4	853.9 $\pm$ 46.2 *

Table 2
The effect of stress and resveratrol treatment on cytokine expression in the DR

RSV treatment reduced cytokine expression in a dose dependent manner. Furthermore, a history of social defeat stress reduced cytokine expression of IL-1 β , IL-2, IL-4, and IL-10 (f $p < 0.05$ vs. 0 mg/kg Con + AC; ** $p < 0.01$, *** $p < 0.0001$ vs 0 mg/kg Con). Data are expressed as percent of vehicle (0 mg/kg) control (average $\pm$ SEM) and sample size (n) for control (Con), passive coping (PC), and active coping (AC) groups.

Cytokine Analyte	0 mg/kg RSV			10 mg/kg RSV			30 mg/kg RSV			Tx Effect
	Con	PC	AC	Con	PC	AC	Con	PC	AC	
IL-1 β	100.0 $\pm$ 1.7 (4)	87.6 $\pm$ 2.5 (7) f	96.1 $\pm$ 3.3 (8)	91.8 $\pm$ 4.1 (5)	95.6 $\pm$ 1.3 (6)	101.1 $\pm$ 5.0 (4)	86.4 $\pm$ 4.4 (4)	83.6 $\pm$ 2.4 (9)	81.6 $\pm$ 1.1 (4)	$p < 0.0001$
IL-2	96.3 $\pm$ 4.8 (4)	70.4 $\pm$ 2.7 (7) ***	74.8 $\pm$ 3.7 (8) ***	77.0 $\pm$ 4.1 (5)	81.9 $\pm$ 0.9 (5)	87.7 $\pm$ 2.3 (3)	66.8 $\pm$ 4.6 (4)	64.9 $\pm$ 1.3 (7)	62.0 $\pm$ 2.1 (4)	$p < 0.0001$
IL-4	100.0 $\pm$ 1.0 (4)	88.6 $\pm$ 2.1 (8) **	90.7 $\pm$ 2.3 (8) **	90.8 $\pm$ 1.5 (5)	95.4 $\pm$ 2.3 (7)	95.5 $\pm$ 2.1 (4)	81.5 $\pm$ 1.7 (4)	83.2 $\pm$ 1.4 (9)	80.0 $\pm$ 1.6 (4)	$p < 0.0001$
IL-6	100.0 $\pm$ 0.4 (5)	102.3 $\pm$ 0.6 (9)	102.0 $\pm$ 0.7 (8)	101.8 $\pm$ 0.7 (5)	101.0 $\pm$ 0.5 (7)	100.9 $\pm$ 0.8 (4)	104.0 $\pm$ 0.5 (4)	103.8 $\pm$ 0.4 (9)	104.4 $\pm$ 0.5 (4)	$p < 0.0001$
IL-10	100.0 $\pm$ 0.6 (4)	91.0 $\pm$ 1.2 (7) **	94.6 $\pm$ 2.6 (8)	95.8 $\pm$ 1.6 (5)	96.6 $\pm$ 1.5 (6)	95.7 $\pm$ 2.4 (4)	86.3 $\pm$ 1.2 (4)	88.2 $\pm$ 1.0 (8)	88.1 $\pm$ 2.0 (4)	$p < 0.0001$
IL-13	100.0 $\pm$ 2.7 (5)	97.4 $\pm$ 1.2 (6)	101.6 $\pm$ 2.0 (8)	98.7 $\pm$ 2.2 (5)	102.4 $\pm$ 2.2 (7)	105.3 $\pm$ 2.1 (4)	94.5 $\pm$ 2.8 (4)	94.4 $\pm$ 1.2 (9)	91.3 $\pm$ 1.1 (4)	$p < 0.0001$
GM-CSF	100.0 $\pm$ 1.6 (4)	82.7 $\pm$ 6.4 (7)	96.6 $\pm$ 5.8 (7)	104.2 $\pm$ 10.3 (4)	93.7 $\pm$ 5.9 (7)	114.5 $\pm$ 17.3 (4)	85.3 $\pm$ 5.2 (4)	83.1 $\pm$ 7.1 (8)	83.7 $\pm$ 4.1 (4)	$p < 0.05$
TNF- α	100.0 $\pm$ 0.9 (4)	98.6 $\pm$ 0.4 (8)	98.3 $\pm$ 1.2 (7)	98.6 $\pm$ 0.5 (5)	98.7 $\pm$ 1.1 (7)	99.9 $\pm$ 1.4 (7)	95.9 $\pm$ 0.3 (4)	96.4 $\pm$ 0.8 (9)	96.7 $\pm$ 0.5 (4)	$p < 0.01$
MCP-1	100.0 $\pm$ 0.9 (4)	98.7 $\pm$ 0.9 (8)	98.3 $\pm$ 1.2 (7)	98.6 $\pm$ 0.5 (5)	98.7 $\pm$ 1.1 (6)	99.9 $\pm$ 1.4 (4)	94.9 $\pm$ 1.0 (4)	96.4 $\pm$ 0.8 (8)	96.7 $\pm$ 0.5 (4)	$p < 0.001$