Negative Affectivity and Inflammation: Moderation by the Glucocorticoid Receptor-9beta Polymorphism

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NEGATIVE AFFECTIVITY AND INFLAMMATION: MODERATION BY THE GLUCOCORTICOID RECEPTOR-9BETA POLYMORPHISM

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Growing evidence suggests that negative emotions may play a role in the upregulation of innate inflammatory processes and associated disease risk. Although the hypothalamic-adrenal-pituitary (HPA) axis is believed to play a role in these associations, empirical evidence is mixed. Inconsistencies may reflect individual differences in glucocorticoid receptor (GR) sensitivity, the capacity of immune cells to respond to anti-inflammatory effects of GC-mediated signaling. We examined whether genotypes of a single nucleotide polymorphism (SNP) in the GR gene—GR-9beta (rs6198), the minor (G) allele of which confers reduced GR sensitivity—moderated associations of negative affectivity (NA) and inflammatory activity. Subjects were middle-aged men and women of European ancestry participating in two phases of the Adult Health and Behavior Project (51% F; 30-54yr). NA was assessed by the neuroticism subscale of the NEO Personality Inventory and by the Positive and Negative Affect Schedule. In a subset of participants in the second phase of the AHAB project (AHAB-2) (51% F; 30-54yr), peripheral

inflammation was additionally assessed by in vitro stimulated production of IL-6, interleukin-1beta (IL-1β), tumor necrosis factor-alpha (TNF-α), and interleukin-8 (IL-8), while NA was also assessed by ecological momentary assessment. In bivariate and hierarchical regression analyses adjusted for potential covariates, none of the NA measures predicted any of the inflammatory cytokine levels. However, subjects homozygous for the GR-9β G allele had higher IL-6 levels than those of alternate genotypes after adjustment for age, gender, and years of school (β = .057, p = .045, R^2 = .047). Although GR-9β genotype was not associated with any of the stimulated cytokines, the interaction of GR-9β was significant, and showed that greater PANAS NA scores associated with greater IL-1β levels, but only among G allele homozygotes (β = .127, p = .027, ΔR^2 = .015). While these results raise the possibility that GR-9β G allele homozygotes show increased inflammatory susceptibility to negative emotionality, this pattern was not retained in analyses of circulating IL-6, or stimulated production of IL-6, TNF-α, and IL-8. We found limited evidence that genotypes of the GR-9β polymorphism moderated associations of NA and measures of peripheral inflammation.

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1.0 INTRODUCTION

1.1 PSYCHOLOGICAL FACTORS AND CHRONIC DISEASE

Accumulating evidence shows that psychological factors play a role in determining overall health and susceptibility to disease. For example, it is now widely accepted that psychosocial factors including depressive symptoms and hostile dispositions predict cardiovascular disease morbidity and mortality (reviewed by Celano & Huffman, 2011; Suls, 2013), and that psychological stress predicts susceptibility to and course of infectious disease (Pedersen, Zachariae, & Bovbjerg, 2010). Recent research attention has focused on identifying psychobiological pathways that contribute to these relationships. In this regard, growing evidence shows that the immune system is one pathway by which psychosocial factors "get under the skin" to influence disease susceptibility. Inflammation is an innate immune process that promotes recovery from acute infection and injury and is thought to pay a key role in the association of psychosocial factors with disease risk. Activation of inflammatory processes are adaptive in the short-term; however, chronic inflammation is known to contribute to the pathogenesis and course of many chronic diseases of aging, including cardiovascular disease, diabetes, autoimmune and neurodegenerative diseases (e.g. Alzheimer's disease), and certain cancers (Gorelick, 2010; Grivennikov, Greten, & Karin, 2010; Libby, 2008; Zeyda & Stulnig, 2009). Interestingly, these same diseases are associated with psychosocial risk, raising the

possibility that psychological processes may modulate disease risk by increasing inflammatory activity.

1.2 THE ROLE OF INFLAMMATION

To date, the majority of studies assessing the role of inflammation in psychologically-related disease risk have focused on circulating levels of proinflammatory mediators, including the cytokines interleukin (IL)-1, tumor necrosis factor-α (TNF-α), and IL-6. Locally, these cytokines are released by activated monocytes and macrophages in response to infection or injury and serve several functions, including stimulating the expression of epithelial adhesion molecules, increasing vascular permeability, and supporting leukocyte trafficking through chemotactic signaling. Proinflammatory cytokines also enter the peripheral circulatory system, and are involved in signaling a systemic inflammatory response. For example, IL-6 stimulates the production of acute phase proteins, such as C-Reactive Protein (CRP), by hepatocytes. It is widely accepted that circulating concentrations of IL-6 and CRP provide a marker of level of systemic inflammation.

While cytokine responses are vital to the coordination of immune responses that manage and eliminate invading pathogens, excessive and prolonged responses impose significant metabolic demand (Lochmiller & Deerenberg, 2000) and increase the potential for collateral damage and risk for chronic inflammatory disease (Finch, 2009). For this reason, magnitude of acute inflammatory response is tightly regulated by local and systemic negative feedback mechanisms. Locally, the release of proinflammatory cytokines from monocytes/macrophages

stimulates the production of anti-inflammatory cytokines, including IL-10, which act on IL-10 receptors on monocytes to shut down the inflammatory response. Increased circulating levels of peripheral inflammatory cytokines are also detected by the brain (reviewed by Dantzer, O'Connor, Freund, Johnson, & Kelley, 2008) and result in activation of the HPA axis and the release of glucocorticoids (GC's) from the adrenal cortex. GC-mediated signaling in the macrophages/monocytes results in the downregulation of peripheral inflammatory responses (reviewed by Sapolsky, Romero, & Munck, 2000), and thus provides a systemic mechanism for down-regulating peripheral inflammation.

1.3 ISSUES IN THE MEASUREMENT OF INFLAMMATION

Although it is widely accepted that circulating cytokines reflect an index of immune-derived inflammatory activity at the time of the blood draw, proinflammatory cytokines are released by a number of different cell types including endothelial cells and adipocytes, with adipocytes contributing 10-35% of total circulating IL-6 (Mohamed-Ali et al., 1997). Thus, it is possible that the inflammatory mediators that are detectable in peripheral circulation originate from non-immune sources, such as adipose tissue. In order to more directly examine the possibility that psychological factors impact innate immune processes, some studies have turned to an in vitro examination of the functional ability of immune cells to produce proinflammatory cytokines when exposed to immune stimulants. This provides a more direct method of capturing individual differences in the magnitude of inflammatory response, a process that is typically localized in vivo and may not be detectable using traditional systemic approaches. Furthermore, by

stimulating leukocytes with a ligand that activates specific leukocyte subsets, a higher degree of immune specificity is achieved. For example, lipopolysaccharide (LPS), a component of the extracellular matrix in gram-negative bacteria stimulates toll-like receptor 4 (TLR4), a surface receptor found predominantly on monocytes. LPS activation of TLR4 results in the expression of several proinflammatory cytokines including IL-6, TNF-α, and IL-1 beta (IL-1β). Individuals vary considerably in the magnitude of proinflammatory cytokine production in response to LPS, and that this variability is temporally stable (Wurfel et al., 2005). Thus, individual differences may reflect a stable propensity of the individual to mount an inflammatory response in the face of pathogenic challenge. The existence of such stable characteristics makes it conceivable that there is a meaningful distribution of differences in cytokine production that may form a physiological basis for associations between psychosocial factors and risk of inflammatory disease.

1.4 FACTORS ASSOCIATED WITH INDIVIDUAL DIFFERENCES IN INFLAMMATION

The negative health sequelae associated with chronic inflammation have stimulated interest in understanding its determinant and moderating factors. Currently, it is recognized that genetic factors play a significant role, with heritability estimates of LPS-stimulated IL-6, TNF-α, and IL-1b being 57%, 53%, and 86% respectively (de Craen et al., 2005). Although the other determinants of stimulated cytokine production are largely unknown, some studies have shown positive associations of LPS-stimulated cytokine production with age, male gender, and inflammatory disease status (Aulock et al., 2006; Nakamura, Saito, Kasanuki, Tamura, &

Yoshida, 1992), but not all results are consistent (Gardner & Murasko, 2002). Similarly, age, socioeconomic status (SES), adiposity, and physical activity are robust covariates of systemic inflammation (O'Connor, Motivala, Valladares, Olmstead, & Irwin, 2007; Worns, Victor, Galle, & Hohler, 2006). In addition, growing evidence suggests that psychosocial factors, particularly those associated with chronic psychological stress, may play an independent role (Hansel, Hong, Camara, & von Kanel, 2010). Here, a large body of literature shows an association of psychosocial risk factors, including chronic stress, lower SES, loneliness, and childhood adversity, with increased circulating markers of inflammation (e.g. Alley, Crimmins, Karlamangla, Hu, & Seeman, 2008; Danese, Pariante, Caspi, Taylor, & Poulton, 2007; Kiecolt-Glaser et al., 2003).

In addition to studies that show associations of stressful life situations with elevated inflammation, accumulating evidence suggests that individual characteristics, particularly negatively valenced emotional dispositions, such as depression, anxiety, anger, and hostility, covary positively with markers of systemic inflammation. For example, epidemiologic studies have shown that depressive symptoms and trait hostility predict increases in systemic IL-6 over a 6-year period of adulthood (Stewart, Rand, Muldoon, & Kamarck, 2009), and that greater depressive symptomatology at the time of a cardiac event predicts smaller decreases in CRP one month later (Shaffer et al., 2011). Laboratory studies also show that negative emotional responses to an acute stressor predict magnitude of task-related increases in IL-6 (Carroll et al., 2011), and that hostile individuals display greater post-stress elevations in IL-6 as compared to their less-hostile counterparts (Brydon et al., 2010). Taken together, evidence from epidemiologic and laboratory-based approaches links negative emotions to increased peripheral

inflammatory activity, raising the possibility that inflammation contributes to the risk for inflammatory disease that accompanies chronic stress and negative emotions.

The range of negative affective characteristics that accompany elevated inflammatory activity raises the possibility that an underlying factor reflecting common variance between these psychological constructs may play an active role. Supporting this notion, confirmatory factor analyses have shown that a single latent factor accounts for a substantial amount of variability common to individual negative emotions including fear, anger, guilt, and sadness (Watson & Clark, 1999). This shared variance is conceptualized to be trait Negative Affectivity (NA), reflecting individual differences in the propensity to experience negatively valenced affects (e.g., fear, anger, sadness, and anxiety), and is believed to contribute to shared health risks (Suls & Bunde, 2005; D. Watson & Clark, 1984). NA is a central component of the trait commonly labeled Neuroticism (or Negative Emotionality) in several taxonomies of personality and these traits may further reflect vulnerability towards feelings of general distress (Brief, Burke, George, Robinson, & Webster, 1988). To date, only one study conducted by Bouhuys, Flentge, Oldehinkel, and van den Berg (2004) has investigated associations stimulated cytokine production with a global construct of NA. Although individuals higher in negative emotionality showed greater in vitro production of IL-6 production in this study, current literature on associations of systemic inflammation and NA is mixed. For example, results of a recent study show that among 4923 individuals of varying ages (14-82 years), scores on the neuroticism subscale of the NEO Personality Inventory were positively associated with systemic levels of IL-6 (Sutin et al., 2010). Conversely, Nabi et al. (2008) show an association of trait NA with incident coronary heart disease over an approximately 10 year period, but no association with circulating levels of CRP or IL-6.

Reasons for inconsistences in the available literature linking NA with elevations in peripheral inflammatory activity are unclear. Given that infectious disease has driven the evolution of the immune system (Finch, 2009), it is plausible that a particularly plastic immune system that responds to environmental threat and associated negative emotional states by priming innate inflammatory mechanisms confers survival advantage in the face of acute challenge. Although beneficial in this capacity, an increased propensity towards inflammation may be detrimental in the long-term, contributing to the increased risk for inflammatory-related disease. In this regard, a malleable inflammatory phenotype with the capability to respond across a range of emotional experiences may confer a selective advantage in environments characterized by acute challenge, while simultaneously conferring a disadvantage among individuals who are disposed to negative emotional states. In light of these evolutionary forces, it is conceivable that there are individual differences in inflammatory susceptibility to negative emotional experience are heritable—a possibility that remains uninvestigated. The failure to consider the role of genetic factors that moderate the magnitude of these relationships may account for inconsistent findings the literature investigating associations of NA and inflammation.

1.5 PATHWAYS LINKING NA TO INFLAMMATION: THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS

One of the primary biological pathways connecting the brain to the immune system is the HPA axis, which is activated under conditions of psychological stress. Activation of this pathway begins in the hypothalamus with the release of arginine vasopressin and corticotropin releasing hormone (CRH) from the paraventricular nucleus into the pituitary portal vein system. This

signal stimulates release of adrenocorticotropin releasing hormone (ACTH) from the anterior pituitary into general circulation, ultimately resulting in the peripheral release of GC's from the adrenal cortex. In humans, the chief GC secreted by the adrenal cortex is cortisol. In the absence of external demands, cortisol follows a diurnal pattern of secretion characterized by an initial morning rise, peaking around 30 minutes after awakening, and a decline throughout the day, falling to a nadir in the evening hours (Kirschbaum & Hellhammer, 1989).

It is widely accepted that GCs play an anti-inflammatory role in the peripheral immune system. By binding to intracellular glucocorticoid receptors (GR's) expressed in monocytes, lymphocytes, neutrophils, and macrophages, GCs orchestrate several intracellular pathways that down-regulate proinflammatory gene expression among other physiologic processes (reviewed by Barnes, 2011). Indeed, it is believed that 20% of genes expressed in human leukocytes are controlled by GC signaling (Galon et al., 2002). Among these gene-regulatory effects, it is well characterized that GC receptor activation elicits inhibition of NF-κB, a potent transcriptional activator that promotes TNF-α, IL-1β, and IL-6 gene induction (reviewed by Sapolsky et al., 2000), thus leading to a decrease in inflammatory activity. GC receptor signaling has also been shown to influence cell trafficking, trigger apoptosis of immature T and B cell precursors, and modulate cell adhesion molecules necessary for the localized inflammatory response (Cronstein, Kimmel, Levin, Martiniuk, & Weissmann, 1992; van de Stolpe, Caldenhoven, Raaijmakers, van der Saag, & Koenderman, 1993). Thus, cortisol plays a key physiologic role in the down-regulation of peripheral inflammatory responses. For this reason, GC's are clinically administered in the treatment of inflammatory disease (Barnes & Adcock, 2009).

It is generally accepted that negative emotional dispositions modulate activity of the HPA axis. Supporting this notion, several studies show elevated diurnal cortisol secretion in trait

negative individuals as compared to their more positive peers (e.g. Nater, Hoppmann, & Klumb, 2010; Polk, Cohen, Doyle, Skoner, & Kirschbaum, 2005; Portella, Harmer, Flint, Cowen, & Goodwin, 2005; Wetherell et al., 2006), but not all findings are consistent (e.g. Hauner et al., 2008; Schommer, Kudielka, Hellhammer, & Kirschbaum, 1999). Reasons for discrepant findings are unclear, but it has been suggested that temporal factors may play a role, with chronic stress eliciting an initial upregulation of HPA axis activity that is followed by a rebound to levels that are below normal (Miller, Chen, & Zhou, 2007). Thus, it is plausible that inconsistent associations of trait NA with cortisol levels reflect physiologic adaptation in response to persistent negative emotion. Recent evidence suggests that this adaptation involves down-regulation of the sensitivity of GC receptors, which may play a greater role in modulating health-related physiologic processes than absolute hormone levels per se (Cohen et al., 2012; Miller, Cohen, & Ritchey, 2002).

Given that GC's downregulate inflammation, elevations in inflammatory activity that have been shown to accompany NA appear paradoxical unless the role of GC sensitivity is considered. Here, it is proposed that chronic stress and negative emotional traits are accompanied by decreased GR sensitivity, possibly reflecting an adaptation to initially raised levels of cortisol (Miller et al., 2002). Early support for stress-related GC resistance came from murine models showing that when compared with rats kept in a stable social situation, rats exposed to the stress of frequent social disruptions (SDR) displayed decreased GC sensitivity of splenocytes, resulting in exaggerated immune responses to LPS (Stark et al., 2001). In parallel with murine studies, evidence for GC resistance has been found among non-human primates that show nervous temperaments (Capitanio, Mendoza, & Cole, 2011). In humans, consistent evidence shows an association of chronic stress with GC resistance. Here, evidence of GC resistance has been

shown in parents of children with cancer, spousal caregivers of patients with brain cancer, and individuals endorsing chronic psychological stress, social isolation, loneliness, vital exhaustion, and low SES (Bauer et al., 2000; Cohen et al., 2012; Cole, 2008; Corwin et al., 2013; Miller et al., 2002; Wirtz et al., 2003). Although the precise mechanism precipitating GC resistance is unclear, these findings raise the possibility that psychological factors including negative emotionality may play a role in modulating the functional propensity of target tissues to respond to cortisol. To the extent that activation of the HPA system assists in the down-regulation of inflammatory activity that accompanies NA, it is possible that diminished GR function may confer a subsequent increase in both immune-derived and systemic inflammatory activity.

1.6 THE POSSIBLE ROLE OF GENETIC FACTORS: THE GR-9β POLYMORPHISM

In addition to environmental challenge, recent studies suggest that genetic variability may contribute to individual differences in GC sensitivity. Functional polymorphic variation in the GC receptor is associated with clinical outcomes among those with inflammatory diseases, including rheumatoid arthritis, multiple sclerosis, cardiovascular disease, and asthma (Otte et al., 2010; Panek et al., 2012; van den Akker et al., 2008; van Winsen et al., 2009). Although fewer gene-linkage studies have focused on inflammation, evidence suggests that variation in a single-nucleotide polymorphism (SNP) in the glucocorticoid receptor gene (*NR3C1*), commonly referred as GR-9β (rs6198), may contribute to individual differences in GR sensitivity. Molecular studies show that the GR-9β minor G allele confers a stabilization of GR-β mRNA, the protein isoform of which reduces the capacity for cortisol to elicit its typical gene-regulatory

effects, including typical trans-repression of the pro-inflammatory genes (Kino, Su, & Chrousos, 2009; Oakley & Cidlowski, 2011). Thus, it is proposed that the GR-β isoform contributes to GC resistance and elevated peripheral inflammation (Li, Leung, Hall, & Goleva, 2006; van den Akker et al., 2006). In support of this possibility, studies show elevated GR-β mRNA expression in leukocytes from individuals with GC resistant forms of asthma, rheumatoid arthritis, and Crohn's disease (Goecke & Guerrero, 2006; Honda et al., 2000; Kozaci, Chernajovsky, & Chikanza, 2007; Sousa, Lane, Cidlowski, Staynov, & Lee, 2000). Particular significance of the GR-9β G allele in inflammatory disease has been demonstrated in studies of cardiovascular disease, rheumatoid arthritis, hypertension, and depression (Chung et al., 2009; Derijk et al., 2001; Koeijvoets et al., 2008; Otte et al., 2010; Szczepankiewicz et al., 2011). For example, a study conducted by van den Akker et al. (2008) showed higher levels of circulating IL-6 and CRP among homozygote GR-9β G allele carriers as compared to individuals of alternate genotypes. In this study, G allele homozygotes also displayed greater carotid-intima thickness, a subclinical measure of arterial atherosclerosis, along with increased risk for coronary heart disease and myocardial infarction. In vitro, the GR-9β G allele is also associated with increased resistance to dexamethasone suppression of IL-2 expression (van den Akker et al., 2006). Taken together, these converging findings suggest that polymorphic variation in *NR3C1* may play a functional role in individual differences in GC sensitivity. Consistent evidence shows that GR-9β G allele confers a decrease in GR function, which manifests as an increase in peripheral inflammatory activity.

1.7 THE CURRENT STUDY

Given that GC signaling normally assists in terminating the inflammatory cascade, diminished GR function is likely to manifest as an overall increase in inflammation, particularly in situations necessitating HPA activation in the down-regulation of inflammatory activity. To the extent that negative emotions elicit an increase in peripheral cortisol levels, it is possible that HPA-axis activation and GR sensitivity may play a role in the regulation of inflammatory activity that accompanies NA. Although diminished GR function conferred by the GR-9β G allele may exert a genetic main-effect on inflammatory activity, it is further plausible that this allele may manifest particularly among individuals high in NA, where recruitment of the HPA system is believed to play a role in the down-regulation of inflammation. Thus, the current study provides an initial investigation of whether NA covaries with peripheral inflammation as a function of polymorphic variation at the GR-9β locus. Accordingly, the specific aims were:

Specific Aim 1: To assess associations of dispositional NA with peripheral inflammatory activity, measured as circulating IL-6 levels and in vitro lipopolysaccharide (LPS)-stimulated production of proinflammatory cytokines: IL-6, IL-8, TNF-α, and IL-1β. Based on existing evidence (e.g. Marsland, Prather, Petersen, Cohen, & Manuck, 2008; Marsland, Sathanoori, Muldoon, & Manuck, 2007; Sutin et al., 2010), it was hypothesized that trait NA would be positively associated with circulating IL-6 and magnitude of proinflammatory response.

Specific aim 2: To examine associations of the GR-9β polymorphism with inflammation. Prior studies show elevated levels of systemic inflammation among GR-9β G allele homozygotes (Otte et al., 2010; van den Akker et al., 2008). Thus, it was hypothesized that the GR-9β G allele would be associated with higher circulating IL-6 and stimulated production of proinflammatory cytokines.

Specific aim 3: To Investigate whether associations between dispositional NA and proinflammatory cytokines are moderated by allelic variation at the GR-9β locus. It was hypothesized that the magnitude of association between NA and markers of inflammation would be greater among GR-9β G homozygotes as compared to individuals of alternate genotypes.

2.0 METHODS

2.1 SAMPLE

Study data were derived from two samples of mid-life community adults recruited via mass-mail solicitation from Southwestern Pennsylvania (primarily Allegheny County) who participated in the University of Pittsburgh Adult Health and Behavior (AHAB) projects. Similar information was collected on participants in both projects, providing a compendium of behavioral and biological measurements, including socio-demographic measurements; indices of personality, temperament, and psychopathology; health-impairing attributes of habit and lifestyle; biological measurements germane to cardiovascular, metabolic, endocrine, autonomic, immune, and central nervous system functioning; and DNA for the study of genetic variation associated with registry phenotypes. The AHAB-1 project recruited a total of 1,379 participants between the ages of 30 to 54 years. Exclusion criteria for this sample included: age <30 or >54 years; a reported history of atherosclerotic cardiovascular disease, chronic kidney or liver disease, cancer treatment in the preceding year, and major neurological disorders, schizophrenia, or other psychotic illness. Participants were also excluded if they were using insulin and glucocorticoid, antiarrhythmic, psychotropic, and prescription weight-loss medications. Other exclusion criteria included pregnancy and working nightshifts exclusively. The AHAB-2 project included 490 participants. In addition to exclusion criteria employed in AHAB-1, the AHAB-2 study also excluded

individuals taking fish-oil, DHA supplements, or the fat blocker Alli; those with reading skills below the 8th grade level; shift workers; and persons whose employment situation would not permit momentary interruptions required for electronic diary and ambulatory data collection. Because the AHAB-2 study included an fMRI assessment, individuals were also excluded if they were claustrophic; had metal objects in or on the body that could not be removed; or if their body habitus would not permit them to fit into the MR scanner. Data collections in both phases of the AHAB study occurred over multiple laboratory sessions and informed consent was obtained in accordance with approved protocols and guidelines of the University of Pittsburgh Institutional Review Board.

Given that allele frequencies in the glucocorticoid receptor differ between ethnic groups (e.g. Hawkins et al., 2004), the current study analyses were limited to the non-Hispanic Caucasian participants in both studies. Out of the total available sample of 1,869 participants, 1,500 were non-Hispanic Caucasians (1099 and 401 participants in AHAB-1 and AHAB-2, respectively). For circulating IL-6 measures, data were aggregated across both phases of the AHAB project. Among the combined sample of 1,500 participants, 173 were excluded due to history of chronic inflammatory disease (hepatitis, asthma, and/or rheumatoid arthritis) or use of inhaled corticosteroids, and 33 participants were excluded for endorsing signs of acute infection at the time of the blood draw. For circulating measures, 57 additional individuals with serum IL-6 concentrations above 10pg/mL were excluded from analyses, due to heightened likelihood of an acute inflammatory process, creating a final sample of 1237 individuals. Stimulated cytokine assays were only conducted in AHAB-2. Of the 401 non-Hispanic Caucasians in that sample, 33 individuals were excluded due to chronic inflammatory-related disease or inhaled corticosteroid use, creating a final sample of 368 individuals.

2.2 GENOTYPING

The GR-9β SNP was amplified using unique sequence primers, and genotyped by the fluorescence method of Chen et al. (1999). Forward and reverse primer sequences were CCTACGCAGTGAAATGTC and CAGATTGGACAATCGGAAC respectively. Genotypes were assigned by direct comparison to sequence confirmed genotypes of each genotype. A 5% resample was genotyped independently to check for reproducibility, and all genotypes were confirmed.

2.3 NEGATIVE AFFECTIVITY

A number of measures of trait NA were available in AHAB-1 and AHAB-2. In both samples, measures included the NEO Personality Inventory (NEO-PI-R; Costa & MacCrae, 1992) and the Positive and Negative Affect Schedule (PANAS; Watson, Clark, & Tellegen 1988). In addition, for AHAB-2 only, participants completed an electronic diary across 4 days, which included momentary assessment of 7 negative affective states, which were averaged to produce an index of trait affectivity.

The NEO-PI-R consists of 240-items designed to measure 5 broad domains—neuroticism, extraversion, openness, agreeableness, and conscientiousness—that are believed to reflect the basic structure of personality. For each item, participants were asked to indicate their level of agreement (1=strongly disagree, 2=disagree, 3=neutral, 4=agree, 5=strongly agree). Sum scores for the neuroticism domain were computed, comprised of 6 facets including anxiety, angry hostility, depression, self-consciousness, impulsiveness, and vulnerability to stress.

Supporting the notion that these facets may reflect a common construct of NA, internal consistency of the neuroticism domain is high, with reliability coefficients ranging form .89-.95 in non-psychiatric populations (Costa, McCrae, & Dye, 1991). Validity of the neuroticism domain has been demonstrated by its association with similar constructs and convergence with peer reports (Costa & McCrae, 1992; McCrae & Costa, 1987).

The PANAS is a 60-item adjective checklist believed to reflect general positive and negative affective tendencies (Watson & Clark, 1984). The negative affect scale is comprised of 10 items: afraid, scared, nervous, jittery, irritable, hostile, guilty, ashamed, upset, and distressed. Here participants were instructed to rate each adjective on a 1-5 scale describing how they generally feel, and sum scores were computed. Internal consistency of the PANAS NA scale is high (reliability coefficients ranging from .85-.93), and construct validity has been shown by convergence with peer report (Watson & Clark, 1999).

In addition to the paper and pencil scales, participants in AHAB-2 completed an electronic diary each hour after awakening across 4 separate days (3 work days and 1 weekend day) during a 2-week period. On each occasion, the diary included 7-adjectives taken from the PANAS that assessed current negative mood: upset, hostile, nervous, afraid, angry, lonely, and sad (David Watson et al., 1988). Participants were required to rate the extent that each item described their current mood on a 7-point Likert scale, ranging from 0 (not at all accurate) to 6 (extremely accurate). The use of aggregated EMA data to characterize individual differences in NA may present and advantages over traditional retrospective self-report questionnaires administered in laboratory settings (Shiffman, Stone, & Hufford, 2008). For example, it is well established that retrospective recall can be inaccurate (Bradburn, Rips, & Shevell, 1987) and recall concerning affective dispositions is likely influenced by current affective states. These

issues are minimized with EMA, where participants make momentary, real-time assessments of current affect. Additionally, the use of repeated assessments over several days may provide a more accurate characterization of subjects' "typical" state than single measurement occasions. However, the extent that mood over a 4-day period represents more stable affective characteristics is unclear.

2.4 PROCEDURES

Blood samples for measurement of circulating levels of IL-6 and stimulated production of inflammatory cytokines were collected on a different day 2-4 weeks before the assessment of NA. On this occasion, participants were asked to fast for 8 hours, to avoid exercise for 12 hours, and alcohol for 24 hours before coming into the laboratory. Blood was drawn through antecubital venipuncture into sodium-heparin treated Vacutainer tubes and serum separator tubes during morning hours.

2.5 SERUM IL-6 ASSESSMENT

Serum IL-6 concentrations were determined using a high sensitivity quantitative sandwich enzyme immunoassay kit (R&D Systems) according to manufacturer's directions. The assay standard range is 0.156 to 10 pg/mL. IL-6 levels were extrapolated from a standard curve with linear regression from a log-linear curve. Log transformation was applied to normalize raw score distributions of circulating IL-6 values.

2.6 STIMULATED CYTOKINE PRODUCTION AND MULTIPLEX ASSAY

Whole blood was diluted 10:1 in saline solution, and stimulated with LPS at a final concentration of 2.5ug/mL under sterile conditions and incubated at 37 degrees Celsius with 5.0% CO_2 for 24 hours. Blood was then centrifuged at 1000g for 10 minutes and plasma was frozen at -80 degrees Celsius for batch analysis. Following study completion, samples were assayed using a multiplex analysis system. Multiplex bead kits (Biosource, Camarillo, CA), based on the principle of solid phase sandwich immunoassays, were employed and stimulated levels of IL-6, IL-1β, TNF-α, and IL-8 were determined using Bio-Plex Manager Software (Bio-Rad Corporation, Hercules, CA), interpolating from a standard curve (Logistic-5PL curve fit). Levels of stimulated cytokines fell within the range of reliable detection for 332, 330, 327, and 292 individuals on assessment of IL-1β, IL-6, TNF-α, and IL-8, respectively. To normalize raw score distributions, log transformations were applied to stimulated IL-6 and IL-8 concentrations. Square root transformations were applied to stimulated IL-1β and TNF-α values.

2.7 ROLE OF DEMOGRAPHICS AND LIFESTYLE FACTORS

Several biobehavioral covariates that might confound associations of NA and inflammatory activity were assessed. These variables included age, sex, years of formal schooling, body mass index (BMI), physical activity, and smoking status. Here, the Paffenbarger Physical Activity Questionnaire (Paffenbarger, Blair, Lee, & Hyde, 1993) was used to provide an indicator of physical activity. Height and weight data collected at the time of blood draw were utilized to compute BMI scores, and self-report data were used to determine age, years of school, and

smoking status. To normalize raw distributions, BMI and physical activity scores were log and square root transformed respectively.

2.8 ANALYTIC STRATEGY

Specific Aim 1: Hierarchical linear regression was used to examine associations of NA with the inflammatory measures. Demographic characteristics (age, sex, years of school) were entered in step 1, followed by the different measures of NA in a second step of separate models predicting the inflammatory outcomes. Additional analyses examined whether lifestyle factors (BMI, physical activity, smoking status, and alcohol use) contributed to any variance in inflammatory factors associated with NA.

Specific Aim 2: To determine whether inflammatory measures covaried with GR-9β genotype, a series of hierarchical regression analyses was conducted, comparing 1) GG individuals against a combined GA and AA group, 2) a combined GG and GA group against AA individuals, and 3) all three genotypes in an additive G allele model. To initially determine whether any of the relevant participant demographics (age, gender, years of school) and lifestyle factors (BMI, physical activity, smoking status, and alcohol consumption) varied by genotype, a series of analysis of variance, Chi-squared, and t-tests were conducted for each genotype comparison. In analyses of NA, genotype terms were entered in step 2, with age, gender, and years of school entered in step 1 of separate models predicting the different inflammatory measures.

Specific Aim 3: To address the possibility that associations of NA and inflammatory activity covary as a function of GR-9β genotype, hierarchical regression was utilized. For these analyses, NA and genotype scores were centered to reduce potential collinearity issues. Age, gender and years of school were entered in step 1 of the model, with genotype and NA scores entered in step 2, and the interaction product terms entered in step 3. Analyses were again repeated adjusting for lifestyle factors as well as demographics in step 1.

3.0 RESULTS

3.1 DEMOGRAPHIC CHARACTERISTICS AND LIFESTYLE FACTORS

For both phases of the AHAB project (AHAB-1 and AHAB-2), demographics and lifestyle factors are reported in Table 1. Participants in AHAB-1 were older, less educated, and less physically active than their AHAB-2 counterparts. For this reason, subsequent analyses of circulating IL-6 in the combined dataset were conducted with the inclusion of study phase (AHAB-1 vs. AHAB-2) as a covariate.

3.2 AIM 1: DOES NEGATIVE AFFECT COVARY WITH MEASURES OF INFLAMMATION?

3.2.1 Stimulated cytokines & negative affect

In Aim 1, it was hypothesized that NA would positively covary with stimulated production of IL-6, IL-1β, TNF-α, and IL-8 levels. Bivariate correlations among study variables are reported in Table 2. Simulated levels of IL-6, TNF-α, and IL-1β were highly intercorrelated (*r*'s = .58-.80). Stimulated IL-8 associated positively with IL-6 (*r* = .14), and inversely with IL-1β and TNF-α (*r* = -.18; *r* = -.20, respectively). Stimulated levels of all three proinflammatory cytokines were

inversely associated with age (*r*'s = -.15 - -.18), and lower levels of IL-6 and TNF-α were found in females (*r*'s = -.26 & -.14 respectively). Higher IL-8 was associated with fewer years in school (*r* = -.13), higher BMI (*r* = .14), male sex (*r* = -.14), and current smoking (*r* = .14). Scores on the different measures of NA (NEO Neuroticism, PANAS NA, and EMA NA) covaried significantly (*r*'s = .34 - .66). However, no significant bivariate associations of stimulated proinflammatory cytokine concentrations with NA measures were observed.

To investigate the possibility that NA may relate to stimulated cytokine levels independently of demographics and lifestyle factors, a series of regression analyses were conducted. No independent associations between any of the NA measures and cytokine production was seen in models that controlled for demographic characteristics (age, gender, years of school) alone or for demographic characteristics and lifestyle factors (BMI, physical activity, current smoking status, and alcohol use; Table 3).

3.2.2 Circulating IL-6 & negative affect

It was hypothesized that NA would covary positively with circulating levels of IL-6. In the combined sample of 1237 individuals with reliable IL-6 measures, bivariate correlations between study variables are reported in Table 4. Consistent with prior literature, higher circulating IL-6 was associated with older age (*r* = .13, *p* < .01), higher BMI (*r* = .35, *p* < .01), fewer years of school (*r* = -.16, *p* < .01), physical inactivity (*r* = -.18, *p* < .01), and current smoking (*r* = .11, *p* < .01). In the larger sample, an association of NEO Neuroticism and PANAS NA scores was observed (*r* = .65 *p* < .01). However, neither was significantly associated with circulating IL-6 concentration in bivariate analyses or in multivariate models that adjusted for demographics and lifestyle factors (Table 5). In the smaller sample whom EMA data were available, circulating IL-

6 levels showed no significant association with EMA-derived NA scores in bivariate analyses (r = .025, p = .63), and in multivariate models adjusted for demographics (β = .011, p = .83). and lifestyle factors (β = -.010. p = .81). Analyses of this combined dataset were repeated with the inclusion of study phase (AHAB-1 vs. AHAB-2) as a covariate and produced virtually identical results.

3.3 AIM 2: DOES GR-9B GENOTYPE COVARY WITH MEASURES OF INFLAMMATION?

3.3.1 GR-9β & stimulated cytokines

Among the sample with stimulated cytokine data, 362 individuals (98%) were successfully genotyped. The GR-9β minor G allele was found at a frequency of 17%, and the distribution of genotypes conformed to Hardy Weinberg equilibrium (GG = 10, GA = 102, AA = 250, p > .05).

To assess associations of GR-9β genotype with participant characteristics and cytokine production, analyses were conducted using independent t-tests, chi-squared tests, and analysis of variance to compare (1) individuals with the GG genotype to those with GA and AA genotypes, (2) individuals with the GG and GA genotypes to the AA genotype, and (3) all three genotypes in an additive G allele model. Results of these analyses are presented in Table 6. None of the demographics characteristics (age, gender, years of school) varied significantly as a function of GR-9β genotype (Table 6). Of the lifestyle factors, subjects homozygous for the A allele consumed more alcoholic beverages per week prior to study entry $t(359) = 2.19$, $p < .05$. However, no other lifestyle factor (BMI, physical activity, current smoking status) differed as a

function of GR-9β genotype. There were no differences across the GR-9β genotypes in scores on the NA measures (NEO Neuroticism, PANAS NA, or EMA NA; Table 6). In Aim 2, it was hypothesized that stimulated production of proinflammatory cytokines would vary as a function of GR-9β genotype. Analyses examining stimulated production of IL-6, IL-1β, TNF-α, IL-8 across the genotype groups provided no support for this hypothesis (Table 6). Individuals with the GG genotype were not significantly different from those with the alternate genotypes. Similarly, no significant associations of genotype with cytokine production were observed in multivariate models that controlled for demographic characteristics and lifestyle factors (Table 7).

3.3.2 GR-9β & circulating IL-6

Of the 1,237 individuals with reliable IL-6 data, 1,208 were successfully genotyped (98%). In this sample, the G allele was observed at a frequency of 18%, and again the distribution of genotypes conformed to Hardy Weinberg equilibrium (GG=43, GA=338, AA=827; $p>.05$). Analyses were conducted as described for the stimulated cytokine sample, using a series of independent t-tests, chi-squared tests, and analysis of variance to compare participant characteristics and circulating levels of IL-6 among (1) the GG group compared to the other genotypes, (2) the GG and GA group compared the AA genotype, and (3) the 3 separate genotype groups in an additive G allele model (GG, GA, AA). Results are presented in Table 8 and show no significant difference in demographic or lifestyle factors, or NA (NEO Neuroticism or PANAS NA) as a function of GR-9β genotype.

Aim 2 also hypothesized that GR-9β G allele homozygotes would display elevated levels of circulating IL-6 as compared to other genotypes. Consistent with this possibility, a marginal

effect of G allele homozygosity emerged, with G allele homozygotes tending to show higher levels of circulating IL-6 than individuals of alternate genotypes $t(1026) = 1.85$, $p = .065$ (Table 8). In hierarchical regression models that controlled for age, gender, and years of school, this trend reached statistical significance ($\beta = .057$, $p = .045$, $R^2 = .047$). However, the effect was not significant in the full model that added lifestyle factors (BMI, physical activity, current smoking status, and alcohol use ($\beta = .033$, $p = .238$, $R^2 = .194$). Although these analyses raise the possibility that lifestyle factors contribute to the association of GR-9β G allele homozygosity and increased IL-6 levels, in isolation, none of these lifestyle charactierstics sigificantly attenuated this association. These results remained significant after inclusion of study phase (AHAB-1 vs. AHAB-2) as a covariate.

3.4 AIM 3: DOES GR-9B GENOTYPE MODERATE ASSOCIATIONS OF NA AND INFLAMMATION?

In Aim 3, it was hypothesized that GR-9β genotype would moderate associations of NA and peripheral cytokine activity, with GR-9β G allele carriers showing greater inflammatory susceptibility to negative emotionality. To address this possibility, an interaction term was entered into the final step of regression models that included NA scores and GR-9β genotype terms as main-effects. Results are shown in Tables 9-11, which display results of models comparing (1)GG vs. GA+AA, (2) GA+GA vs. AA, and (3) additive G alleles, respectively.

In support of the hypothesis, on analysis of stimulated IL-1β, a marginally significant interaction of PANAS NA and GR-9β G allele homozygosity emerged in unadjusted models ($\beta = .106$, $p = .06$, $\Delta R^2 = .011$) (Table 9). This interaction reached significance after controlling for

demographics (β = .120, p = .032, ΔR^2 = .014), and persisted after subsequent adjustment for lifestyle factors (β = .127, p = .027, ΔR^2 = .015). When stratified by GR-9β genotype, a positive association of PANAS NA and stimulated IL-1β was observed among GR-9β G allele homozygotes (r = .631, p < .05), but this association was non-significant among GA and AA individuals (r = -.050, p > .05) (Figure 1).

An interaction of NA with GR-9β genotype was also observed on analysis of TNF-α production. However, here, the interaction was between NEO Neuroticism and GR-9β A allele homozygosity. This interaction term was significant in unadjusted models (β = -.141, p = .038, ΔR^2 = .014), but did not persist after controlling for demographics (β = -.113, p = .089, ΔR^2 = .009) and lifestyle factors (β = -.110, p = .103, ΔR^2 = .008). As shown in Figure 2, when stratified by GR-9β genotype, an unexpected inverse association of NEO Neuroticism and stimulated TNF-α emerged in the combined GG and GA group (r = -.233, p < .05) as compared to the AA group (r = -.020, p > .05). There was no significant interaction of NA with GR-9β genotype in the prediction of stimulated or circulating IL-6 or stimulated IL-8 levels (see Tables 9-11).

4.0 DISCUSSION

It is widely suggested that dispositional NA is associated with an increase in systemic inflammation that confers risk for a range of chronic diseases of aging. The HPA axis is one biological pathway that may mediate associations of negative emotionality with inflammation, raising the possibility that individual differences in inflammatory susceptibility to HPA signaling may play a role. In this regard, it was hypothesized that the GR-9β polymorphism—the minor G allele of which confers a decrease in GC sensitivity—would moderate associations of NA and peripheral inflammation. More specifically, it was hypothesized that GR-9β G allele homozygotes would show increased susceptibility to affect-related elevations in inflammatory activity. The presented findings provide mixed support for this hypothesis. Consistent with our expectations, we observed a significant interaction of GR-9β G allele homozygosity and PANAS NA scores in the prediction of stimulated levels of IL-1β. To the extent that GG individuals reported greater levels of NA as assessed by the PANAS, they showed greater stimulated production of IL-1β—an association that was not observed among the GA and AA genotypes. These findings are consistent with the possibility that diminished GC-mediated signaling conferred by the GR-9β G allele resulted in resistance to the anti-inflammatory effects of GCs, and increased inflammation in the context of NA. For GR-9β A allele carriers, however, elevations in inflammation that accompany negative emotions may be down-regulated by intact GC-mediated anti-inflammatory signaling.

Although findings from the analysis of IL-1β are consistent with the possibility that GR-9β G allele homozygotes show elevated inflammatory susceptibility to negative emotionality, this pattern was not observed on analysis of circulating levels of IL-6, or stimulated production of IL-8 or IL-6. Although a significant interaction of allele group and NA was observed on analysis of TNF-α production, the direction of effects was inconsistent with expectations. Here, G allele carriers showed lower levels of TNF-σ with increasing NA. However, this was a small effect that did not achieve significance once demographic characteristics were considered, which raises caution about its interpretation. Although TNF-α production may differ from the other cytokines investigated, it is also possible that this association is spurious, particularly given the number of analyses conducted.

While the HPA system is speculated to play a role in associations of psychosocial risk factors and inflammation, other candidate pathways have been shown to link the central nervous and peripheral immune systems, and may also play a role in associations of negative affecitivty and inflammation. For example, it is well known that the sympathetic nervous system (SNS) modulates peripheral immune function, with stimulation of beta-adrenergic receptors on monocytes resulting in an upregulation of proinflammatory gene activity (see Irwin & Cole, 2011). To the extent that negative emotions have been shown to modulate SNS activity, it is possible that the SNS and other psychophysiologic pathways operate in concert with the HPA axis in mediating associations of NA and inflammation. Thus, the lack of consistency in the current findings may be attributable to alternate biological pathways that play a greater role in associations of negative affectivity and inflammation. Future investigation into the genetic polymorphisms that confer inflammatory susceptibility within these systems is warranted, and

this approach may shed further light on the psychophysiologic processes involved in associations of negative emotionality and inflammation.

Strategies for the detection of gene by environment interactions have been overviewed by Moffitt, Caspi, and Rutter (2005). Of relevance to the current study, Moffitt et al. (2005) suggest consulting quantitative behavioral-genetic models demonstrating heritability of the outcome of interest. In accordance with this notion, heritability estimates of circulating IL-6 have been previously shown to be 17-24%, while heritability estimates of LPS-stimulated IL-6, TNF-, and IL-1b are 57%, 53%, and 86%, respectively (de Craen et al., 2005). Second, Moffitt et al. (2005) suggest identifying a candidate environmental pathogen with a plausible effect on biological systems. As previously discussed, negative emotionality has been shown to activate the HPA axis and upregulate innate inflammatory activity, raising the possibility that GC-mediated signaling may mediate associations of negative affectivity and inflammation. Of noteworthy consideration, Moffitt et al., (2005) also raise the importance of optimizing risk measurements by selecting environmental measures that are proximal to the outcome of interest. In this regard, the current study focused on trait negative emotionality. However, no overall association of trait NA with the inflammatory measures was observed. It is possible that a measure of emotional state taken at the time of the blood draw may have shown stronger associations with inflammation. Alternatively, future investigation should consider the possibility that unique aspects of specific negative emotions, such as hostility or anxiety are more closely related to inflammation than their shared variance.

The existing literature provides mixed support for an association of trait NA with systemic inflammation. To date, three studies of neuroticism have shown positive associations with IL-6. First, in a population-based study of 4923 Italians age 14-102 years, Sutin et al. (2010)

found that higher Neuroticism scores on the Revised NEO Personality Inventory, were related to elevated levels of IL-6. Similarly, Mottus, Luciano, Starr, Pollard, and Deary (2013) observed an association of Neuroticism scores on the NEO Five Factor Inventory (NEO-FFI), with higher IL-6 levels during a 3-year follow-up of a British cohort of 592 individuals aged 70 years. Finally, in a cross-sectional study of 666 individuals, Millar et al. (2013) showed a positive association of IL-6 with neuroticism scores on the Eysenck Personality Questionnaire, particularly among low SES participants. Although these studies provide some evidence for an association between a global construct of NA and circulating IL-6 levels, several others, including the current study, find no or even inverse associations. For example, Nabi et al. (2008) found no association between IL-6 and Negative Affectivity on the Bradburn Affect-Balance Scale among 6396 individuals participating in the Whitehall II Study. Similarly, Chapman et al. (2011) found no evidence of a relationship between NEO-FFI Neuroticism scores and IL-6 over a 34-week follow-up period in a sample of 200 older adults. Finally, a recent study of 1054 individuals living in the American Midwest found an unexpected inverse association of Neuroticism with circulating IL-6 levels (Turiano, Mroczek, Moynihan, & Chapman, 2013). It is possible that inconsistent findings indicate lack of an association, however, it is also possible that heterogeneity of sample characteristics across studies may obscure otherwise significant associations, which are typically small in magnitude.

In addition to hypothesizing that NA would be associated with a marker of systemic inflammation, the current study also examined markers of inflammatory response. Here, it was hypothesized that dispositional negative affect would be associated with increased LPS-stimulated production of proinflammatory mediators. Contrary to expectations and the existing literature (Bouhuys et al., 2004), we did not observe any significant associations of negative

affectivity, as measured by using standard questionnaires or ecological momentary assessment, with stimulated production of IL-6, IL-1β, TNF-α, or IL-8. These results are inconsistent with an emerging literature linking specific negative emotional dispositions to higher proinflammatory cytokine production. Reasons for discrepant findings are unclear; however, it is possible that results may vary as a function of distinct affective elements, rather than their shared affective variance. For example, several studies show positive associations of hostile dispositions with stimulated production of proinflammatory cytokines (Janicki-Deverts, Cohen, & Doyle, 2010; Marsland et al., 2007; Mommersteeg, Vermetten, Kavelaars, Geuze, & Heijnen, 2008; Suarez, Lewis, Krishnan, & Young, 2004; Suarez, Lewis, & Kuhn, 2002). In contrast, findings from studies investigating stimulated production and depression are largely mixed (e.g. Cyranowski et al., 2007; Suarez, Krishnan, & Lewis, 2003; Suarez et al., 2004). It is possible that inconsistencies across studies reflect heterogeneity of specific emotional constructs, with results varying as a function of different affective, attitudinal, and expressive elements. In this regard, the global construct of NA assessed in the current study may not have adequately addressed the specificity of emotions involved in the upregulation of inflammatory processes. Thus, further investigation into the individual emotions comprising negative affectivity is warranted, and may yield more positive findings, particularly in the case of hostile dispositions.

Consistent with expectations, we found higher levels of circulating IL-6 among GR-9β G allele homozygotes in comparison to the GA and GG genotypes after adjustment for demographic factors. These results are consistent with findings from two previous studies (Otte et al., 2010; van den Akker et al., 2008), which also showed elevated markers of systemic inflammation among GG carriers. Taken together, these results complement molecular studies of GC-mediated signaling, where it is suggested that the GR-9β G allele results in stabilization of

GR-β mRNA, a splicing variant that fails to bind to cortisol and reduces the capacity for cortisol to elicit its typical gene-regulatory effects, including typical trans-repression of the pro-inflammatory genes (Kino, Su, & Chrousos, 2009; Oakley & Cidlowski, 2011). Thus, our findings raise the possibility that elevated IL-6 levels observed among G allele homozygotes are explained by an attenuation in anti-inflammatory signaling typically imparted by the HPA system and cortisol release. The current findings lend further support to the possibility that GR-9β genotype confers an elevation in systemic inflammation, which may, in turn, contribute to increased risk for chronic inflammatory disease.

In contrast to the elevated levels of circulating IL-6 observed among GR-9β G allele homozygotes, the current findings showed no association of GR-9β genotype with stimulated production of IL-6, IL-1β, TNF-α and IL-8 levels. To our knowledge, this is the first study to investigate associations of GR-9β with stimulated cytokine production. It is possible that null findings relate to the in vitro stimulation approach utilized in the current study. Although the expression of proinflammatory cytokines in peripheral leukocytes is largely contingent on localized factors, integration of systemic signaling conferred by the HPA and sympathetic nervous systems is widely acknowledged to modulate the magnitude of immune responses. While the stimulated cytokine method utilized in the current study provides a clearer assessment of leukocyte-derived proinflammatory activity as compared to systemic IL-6 levels, this in vitro response reflects an artificially induced state of immune reactivity in the absence of systemic regulation. Given that the GR-9β polymorphism likely moderates inflammatory activity in the face of HPA axis activation and cortisol release, it is possible that the observed findings are attributable to the separation of blood leukocytes from HPA signaling resulting from the ex-vivo methodology. Supporting this notion, a study by van den Akker et al. (2006) showed that

elevations in inflammatory activity observed in GR-9β G allele homozygotes were observed only in the presence of dexamethasone, a synthetic analogue of cortisol with similar anti-inflammatory properties. Quantification of cytokine activity following co-incubation of blood leukocytes with a range of cortisol levels likely provides a more direct assessment of GC sensitivity (see Burnsides et al., 2012), and this approach may shed light on potential differences in inflammatory activity in GR-9β G allele carriers.

5.0 LIMITATIONS

There are several noteworthy limitations of the current study that warrant attention in future investigations. First, the cross-sectional design was limited to single assessments of circulating IL-6 and stimulated cytokine levels. While these assessments are relatively stable over time, a more reliable indicator of inter-individual variability in inflammatory activity may be derived by aggregating across multiple assessment occasions. Second, the methodology utilized to assess acute infection differed across both phases of the AHAB Study. While participants were actively screened for acute illness in AHAB-2, this proceedure was not conducted in AHAB-1, and thus participants with IL-6 concentrations above 10 pg/mL were excluded from analyses. It is possible this proceedure may have obscured associations of negative affectivity and circulating IL-6 levels, further contributing to our null results. Third, analyses in the current study were limited to a sub-sample of individuals of European-American ancestry, limiting the generalizability of our findings to other ethnic populations.

In genetic analyses, we observed significant interactions of GR-9β and negative affectivity in the prediction of stimulated IL-1β and TNF-α. However, directionality of these interactions differed between cytokines, and given the number of models analyses conducted, the possibility of Type 1 Error should be considered. Finally, the current study investigated a SNP located in *NR3C1* gene previously associated with increased systemic inflammation. It is

possible that associations of NA and inflammation may have been moderated by other functional variants located in *NR3C1*.

6.0 SUMMARY AND FUTURE DIRECTIONS

Although it is speculated that genetic variaiblity may play a role in physiologic susceptibility to psychosocial risk factors, few empirical studies have investigated this possibility. The current study utilized a candidate gene approach and found limited evidence that the GR-9β G allele, previously associated increased levels of systemic inflammation, moderated associations of negative emotionality and inflammatory activity. Although the HPA system may play a role in associations of psychosocial risk factors and inflammation, the psychologically-elicited modulation of inflammatory processes is likely reflective of an intricate process, involving both the integration and interaction of several systems. In this regard, future studies investigating genetic variants conferring inflammatory sensitivity to other intermediary psychobiological pathways, including the autonomic nervous system, are warranted. To the extent that the psychological specificity of negative emotions may play a role in the upregulation of inflammatory processes, future studies should also consider assessing individual negative affects. The proximity of these emotions may also increase the likelihood of uncovering gene-environment interactions.

7.0 TABLES & FIGURES

Table 1. Demographics and lifestyle factors of the AHAB-1 and AHAB-2 samples. Absolute T-values are reported.

	AHAB-1 Mean (SD)	AHAB-2 Mean (SD)	AHAB-1 vs. AHAB-2
Age (years)	44.7 (6.8)	42.4 (7.3)	$\mathbf{t_{(1235)} = 5.43^{**}}$
Gender (% female)	51.2	51.1	$\chi_1^2 < 1$
Years in school	16.0 (2.3)	17.1 (2.8)	$\mathbf{t_{(1235)} = 6.57^{**}}$
BMI	27.1 (5.4)	26.4 (5.0)	$t_{(1235)} = 1.91$
Weekly kilocalorie expenditure	12326.7 (1780.3)	2772.6 (2041.1)	$\mathbf{t_{(1235)} = 2.70^{**}}$
% Currently smoking	13.0	11.3	$\chi_1^2 < 1$
# Alcoholic beverages in past week	4.1 (7.8)	3.4 (4.8)	$t_{(1048)} = 1.50$

* = p<.05
**=p<.01

Table 2. Bivariate correlations between stimulated cytokine levels, negative affect measures, demographics, and lifestyle factors in the AHAB-2 sample.

		Stimulated Cytokine Levels				Negative Affectivity Measure			Demographics and Lifestyle Factors							
	Mean (SD)	**IL-6**	**IL-1β**	**TNFα**	**IL-8**	NEO Neuroticism	PANAS	EMA	Age	Gender	Years in school	BMI	Weekly kilocalories	Smoking	# Alcoholic beverages	Sleep Quality
Stimulated IL-6 (pg/mL)	51306 (35461)	--	.582**	.590**	.140*	-.064	-.013	-.054	-.159**	-.256**	.032	.019	.010	.087	.063	.004
Stimulated IL-1β (pg/mL)	12110 (7558)	--	--	.795**	-.182**	-.020	-.021	-.072	-.146**	.032	.053	-.057	.040	-.010	-.018	-.070
Stimulated TNF-α (pg/mL)	7222 (6155)	--	--	--	-.200**	-.047	-.040	-.064	-.180**	-.144**	.084	-.105	.053	-.013	-.094	-.021
Stimulated IL-8 (pg/mL)	679749 (2870583)	--	--	--	--	-.031	.062	-.027	.009	-.140*	-.132*	.126*	-.076	.140*	.063	.022
Negative Affectivity																
NEO Neuroticism	74.86 (23.20)	--	--	--	--	--	.664**	.355**	-.069	.052	-.100	-.007	-.065	.120*	.059	.328**
PANAS Negative Affect	15.53 (5.15)	--	--	--	--	--	--	.340**	.047	-.097	.017	-.014	-.033	.128*	.151**	.313**
EMA Negative Affect	1.89 (0.71)	--	--	--	--	--	--	--	.034	-.035	-.049	.066	-.051	.119*	.126*	.206**
Age (years)	42.81 (7.37)	--	--	--	--	--	--	--	--	.142**	-.051	.095	-.019	.045	.051	.052
Gender (% female)	52.2	--	--	--	--	--	--	--	--	--	-.148**	-.089	-.042	-.016	-.121*	-.052
Years in school	17.00 (2.96)	--	--	--	--	--	--	--	--	--	--	-.146**	.156**	-.144**	.016	-.061
BMI	26.94 (5.19)	--	--	--	--	--	--	--	--	--	--	--	-.200**	.103*	.048	.090
Weekly kilocalorie expenditure	2753.11 (2108.71)	--	--	--	--	--	--	--	--	--	--	--	--	-.072	-.066	-.103*
% Currently smoking	14.1	--	--	--	--	--	--	--	--	--	--	--	--	--	.180**	.097
# Alcoholic beverages in past week	2.99 (4.29)	--	--	--	--	--	--	--	--	--	--	--	--	--	--	.203**

* = p<.05
**=p<.01

Table 3. Results of hierarchical regression analyses examining associations between negative affect measures and stimulated cytokine concentrations after controlling for demographic and lifestyle factors in the AHAB-2 sample.

	Predictor [a]	Stimulated IL-6 (n=332)			Stimulated IL-1β (n=336)			Stimulated TNF-α (n=333)			Stimulated IL-8 (n=292)		
Model 1 [b]		**ß**	***p***	**R^2**	**ß**	***p***	**R^2**	**ß**	***p***	**R^2**	**ß**	***p***	**R^2**
	NEO Neuroticism	-.067	.216	.086	-.034	.547	.030	-.054	.333	.057	-.046	.435	.050
	PANAS Negative Affect	-.044	.412	.086	-.028	.610	.027	-.069	.210	.056	.043	.464	.046
	EMA Negative Affect Score	-.066	.215	.089	-.062	.262	.028	-.062	.258	.056	-.050	.395	.034
Model 2 [c]		**ß**	***p***	**R^2 Change**	**ß**	***p***	**R^2 Change**	**ß**	***p***	**R^2 Change**	**ß**	***p***	**R^2 Change**
	NEO Neuroticism	-.089	.130	.007	-.023	.709	.000	-.052	.377	.002	-.059	.352	.003
	PANAS Negative Affect	-.068	.231	.004	-.020	.734	.000	-.064	.269	.004	.036	.559	.001
	EMA Negative Affect Score	-.077	.163	.006	-.052	.364	.003	-.048	.393	.002	-.075	.215	.000

[a] Each NA measure was entered in a separate model.

[b] Covariates entered in this step of the regression equation: age, gender, and years of education.

[c] Covariates entered in this step of the regression equation include those entered in Model 3 in addition to BMI, physical activity, current smoking status, and weekly alochol use.

Table 4. Bivariate correlations between circulating IL-6, negative affect measures, demographics, and lifestyle factors in the combined AHAB sample.

			Negative Affectivity Measure		Demographics and Lifestyle Factors						
	Mean (SD)	**IL-6**	NEO Neuroticism	PANAS	Age	Gender	Years in school	BMI	Weekly kilocalories	Smoking	# Alcoholic beverages
Circulating IL-6 concentration (pg/mL)	.50 (.16)	--	.038	-.023	.134**	.050	-.160**	.349**	-.178**	.105**	.039
		--									
Negative Affectivity		--									
NEO Neuroticism	74.84 (23.54)	--	--	.654**	-.119**	.057*	-.120**	.064*	-.099**	.068*	.065*
PANAS Negative Affect	14.97 (5.12)	--	--	--	-.113**	-.065*	.009	.040	-.048	.064*	.138**
		--	--	--							
Age (years)	44.09 (7.09)	--	--	--	--		-.088**	.060*	-.054	-.004	.034
Gender (% female)	51.2	--	--	--	--	--	-.089**	-.161**	-.064*	-.026	-.212**
Years in school	16.38 (2.83)	--	--	--	--	--	--	-.096**	.123**	-.140**	-.018
BMI	26.89 (5.32)	--	--	--	--	--	--	--	-.165**	-.027	.004
Weekly kilocalorie expenditure	2558.7 (1864.9)	--	--	--	--	--	--	--	--	-.090**	.057
% Currently smoking	12.5	--	--	--	--	--	--	--	--	--	.206**
# Alcoholic beverages in past week	3.88 (6.89)	--	--	--	--	--	--	--	--	--	--

* = p<.05
**=p<.01

Table 5. Results of hierarchical regression analyses examining associations between negative affect measures and circulating IL-6 concentration after controlling for demographic and lifestyle factors in the combined AHAB sample

		Circulating IL-6 (n=1237)		
Model 1 [b]		**ß**	***p***	**R^2**
	NEO Neuroticism	.040	.158	.046
	PANAS Negative Affect	-.013	.647	.047
Model 2 [c]				
	NEO Neuroticism	.017	.548	.194
	PANAS Negative Affect	-.015	.593	.197

[a] Each NA measure was entered in a separate model.

[b] Covariates entered in this step of the regression equation: age, gender, and years of education.

[c] Covariates entered in this step of the regression equation include those entered in Model 3 in addition to BMI, physical activity, current smoking status, and weekly alochol use.

Table 6. Univariate participant characteristics by GR-9β genotype in the AHAB-2 stimulated cytokine sample. Absolute T-values are reported.

	GR-9β Genotype		
	GG vs. GA+AA	GG+GA vs. AA	GG vs. GA vs. AA
Demographic Fators			
Age (years)	$t_{360}=1.56$	$t_{360}<1$	$F_{2,359}=1.32$
Gender (% female)	$\chi_1^2=3.50$	$\chi_1^2=3.37$	$\chi_2^2=5.49$
Years in school	$t_{360}=1.37$	$t_{360}<1$	$F_{2,359}<1$
Lifestyle Factors			
BMI	$t_{360}<1$	$t_{360}<1$	$F_{2,359}<1$
Weekly kilocalorie expenditure	$t_{360}=1.946$	$t_{360}<1$	$F_{2,359}=2.15$
Currently smoking	$\chi_1^2<1$	$\chi_1^2<1$	$\chi_2^2=1.01$
# Alcoholic beverages in past week	$t_{359}<1$	**$t_{359}=2.19$***	$F_{2,358}=2.41$
Negative Affect Measures			
NEO Neuroticism	$t_{357}<1$	$t_{359}=1.72$	$F_{2,356}=1.48$
PANAS Negative Affect	$t_{359}<1$	$t_{359}<1$	$F_{2,358}<1$
EMA Negative Affectivity	$t_{356}=1.06$	$t_{356}<1$	$F_{2,355}<1$
LPS-stimulated Cytokine Production			
Stimulated IL-6	$t_{325}<1$	$t_{325}<1$	$F_{2,324}<1$
Stimulated IL-1β	$t_{323}<1$	$t_{323}<1$	$F_{2,322}<1$
Stimulated TNF-α	$t_{321}<1$	$t_{321}<1$	$F_{2,320}<1$
Stimulated IL-8	$t_{283}<1$	$t_{283}<1$	$F_{2,282}<1$

* = p<.05
**=p<.01

Table 7. Results of regression analyses examining associations between GR-9β genotype and stimulated cytokine concentrations after controlling for demographic and lifestyle factors in the AHAB-2 sample.

		Stimulated IL-6 (n=332)			Stimulated IL-1β (n=330)			Stimulated TNF-α (n=327)			Stimulated IL-8 (n=292)			Circulating IL-6 (n=1208)		
	Mode of Genetic Influence [a]															
Model 1 [e]		ß	*p*	R^2	ß	*p*	R^2	ß	*p*	R^2	ß	*p*	R^2	ß	*p*	R^2
	GG vs. GA+AA [b]	.068	.209	.086	.059	.292	.036	-.027	.627	.049	-.045	.446	.052	**.057**	**.045**	**.047**
	GA+GG vs. AA [c]	.058	.280	.084	.013	.813	.033	.023	.675	.049	.010	.866	.050	.032	.257	.045
	Additive [d]	.072	.180	.086	.030	.585	.033	.011	.839	.049	-.005	.937	.050	.046	.100	.043
Model 2 [g]																
	GG vs. GA+AA [b]	.068	.213	.093	.062	.272	.040	-.028	.614	.070	-.052	.371	.077	.033	.238	.194
	GA+GG vs. AA [c]	.058	.284	.092	.016	.781	.037	.015	.787	.070	.000	.994	.074	.018	.516	.194
	Additive [d]	.073	.183	.093	.034	.549	.037	.004	.946	.070	-.016	.781	.075	.027	.344	.194

[a] Reported values correspond to genotype codes that were created for recessive, dominant, and additve modes of genetic influence.

[b] AA, GA, and GG genotypes were coded as 0, 0, and 1 respectively.

[c] AA, GA, and GG genotypes were coded as 0, 1, and 1 respectively.

[d] AA, GA, and GG genotypes were coded as -1, 0, and 1 respectively.

[e] Covariates entered in this step of the regression equation: age, gender, and years of education

[g] Covariates entered in this step of the regression equation include those entered in Model 1 in addition to BMI, physical activity, current smoking status, and weekly alochol use.

Table 8. Univariate participant characteristics by GR-9β genotype in the combined AHAB sample. Absolute T-values are reported.

	GR-9β Genotype		
	GG vs. GA+AA	GG+GA vs. AA	GG vs. GA vs. AA
Demographic Factors			
Age (years)	$t_{1206}<1$	$t_{1206}<1$	$F_{2,1205}<1$
Gender (% female)	$\chi_1^2<1$	$\chi_1^2<1$	$\chi_2^2<1$
Years in school	$t_{1206}<1$	$t_{1206}<1$	$F_{2,1205}<1$
Lifestyle Factors			
BMI	$t_{1204}<1$	$t_{1204}<1$	$F_{2,1203}<1$
Weekly kilocalorie expenditure	$t_{1203}<1$	$t_{1203}<1$	$F_{2,1202}<1$
% Currently smoking	$\chi_1^2<1$	$\chi_1^2<1$	$\chi_2^2<1$
# Alcoholic beverages in past week	$t_{1028}<1$	$t_{1028}=1.77$	$F_{2,1027}=1.58$
Negative Affect Measures			
NEO Neuroticism	$t_{1198}<1$	$t_{1198}<1$	$F_{2,1197}<1$
PANAS Negative Affect	$t_{1197}<1$	$t_{1197}<1$	$F_{2,1196}<1$
Circulating IL-6	$t_{1206}=1.85$	$t_{1206}<1$	$F_{2,1205}=1.82$

* = p<.05
**=p<.01

Table 9. Results of regression analyses examining interactions between rs6198 genotype and NA measures predicting stimulated cytokines and systemic IL-6, after adjustment for demographic characteristics and lifestyle factors. GG, GA, and AA genotypes were coded

		Stimulated IL-6 (n=332)			Stimulated IL-1β (n=330)			Stimulated TNF-α (n=327)			Stimulated IL-8 (n=292)			Circulating IL-6 (n=1208)		
		ß	SE	*p*	ß	SE	*p*	ß	SE	*p*	ß	SE	*p*	ß	SE	*p*
Unadjusted models																
	NEO Neuroticism X GG vs. GA+AA	-.016	.005	.778	.020	.614	.721	.039	.616	.495	.005	.008	.936	-.004	.001	.881
	PANAS Negative Affect X GG vs. GA+AA	.047	.021	.402	.106	2.730	.062	.074	2.759	.192	.022	.041	.717	-.012	.005	.681
	EMA Negative Affect X GG vs. GA+AA	.014	.134	.822	.046	16.952	.445	.074	17.181	.225	-.046	.279	.486	--	--	--
Model 2 [a]																
	NEO Neuroticism X GG vs. GA+AA	-.034	.005	.533	.039	.610	.493	.042	.607	.464	-.027	.008	.649	-.020	.001	.488
	PANAS Negative Affect X GG vs. GA+AA	.041	.021	.453	**.120**	**2.702**	**.032**	.080	2.708	.154	-.001	.040	.984	-.017	.005	.549
	EMA Negative Affect X GG vs. GA+AA	.005	.128	.935	.052	16.806	.387	.073	16.866	.222	-.053	.274	.411	--	--	--
Model 3 [b]																
	NEO Neuroticism X GG vs. GA+AA	-.024	.005	.668	.049	.629	.407	.042	.621	.472	-.005	.008	.930	-.014	.001	.632
	PANAS Negative Affect X GG vs. GA+AA	.053	.021	.341	**.127**	**2.774**	**.027**	.078	2.754	.167	.019	.041	.747	.009	.005	.750
	EMA Negative Affect X GG vs. GA+AA	.007	.130	.908	.052	17.105	.395	.066	17.021	.271	-.029	.281	.662	--	--	--

[a] Covariates in this model include age, gender, and years of education,

[b] Covariates in this model include those entered in Model 2 in addition to BMI, physical activity, current smoking status, and weekly alcohol use.

Table 10. Results of regression analyses examining interactions between rs6198 genotype and NA measures predicting stimulated cytokines and systemic IL-6, after adjustment for demographic characteristics and lifestyle factors. GG, GA, and AA genotypes were coded

		Stimulated IL-6 (n=332)			Stimulated IL-1β (n=330)			Stimulated TNF-α (n=327)			Stimulated IL-8 (n=292)			Circulating IL-6 (n=1208)		
		ß	SE	*p*	ß	SE	*p*	ß	SE	*p*	ß	SE	*p*	ß	SE	*p*
Unadjusted models																
	NEO Neuroticism X GG+GA vs. AA	-.113	.001	.092	-.071	.177	.284	**-.141**	**.178**	**.038**	.046	.002	.526	.055	.000	.113
	PANAS Negative Affect X GG+GA vs. AA	-.040	.006	.537	.008	.832	.901	-.069	.842	.294	-.018	.012	.796	.039	.002	.264
	EMA Negative Affect X GG+GA vs. AA	-.129	.045	.056	-.035	5.714	.608	-.051	5.820	.454	-.068	.080	.351	--	--	--
Model 2 [a]																
	NEO Neuroticism X GG+GA vs. AA	-.086	.001	.187	-.069	.176	.303	-.113	.175	.089	.054	.002	.446	.037	.000	.278
	PANAS Negative Affect X GG+GA vs. AA	-.027	.006	.664	.006	.824	.929	-.064	.825	.319	-.004	.011	.957	.031	.002	.376
	EMA Negative Affect X GG+GA vs. AA	-.112	.043	.083	-.023	5.679	.734	-.037	5.712	.584	.076	.078	.289	--	--	--
Model 3 [b]																
	NEO Neuroticism X GG+GA vs. AA	-.085	.001	.199	-.063	.179	.352	-.110	.177	.103	.054	.002	.453	.033	.000	.325
	PANAS Negative Affect X GG+GA vs. AA	-.029	.006	.654	-.030	.399	.614	-.069	.836	.292	-.007	.012	.924	.023	.002	.504
	EMA Negative Affect X GG+GA vs. AA	-.115	.043	.081	-.019	5.769	.784	-.036	5.753	.598	-.081	.079	.259	--	--	--

[a] Covariates in this model include age, gender, and years of education,

[b] Covariates in this model include those entered in Model 2 in addition to BMI, physical activity, current smoking status, and weekly alcohol use.

Table 11. Results of regression analyses examining interactions between rs6198 genotype and NA measures predicting stimulated cytokines and systemic IL-6, after adjustment for demographic characteristics and lifestyle factors. GG, GA, and AA genotypes were coded

		Stimulated IL-6 (n=332)			Stimulated IL-1β (n=330)			Stimulated TNF-α (n=327)			Stimulated IL-8 (n=292)			Circulating IL-6 (n=1208)		
		ß	SE	*p*	ß	SE	*p*	ß	SE	*p*	ß	SE	*p*	ß	SE	*p*
Unadjusted models																
	NEO Neuroticism X Additive G Allele	-.107	.001	.106	-.075	.161	.263	-.114	.163	.090	.042	.002	.555	.056	.000	.208
	PANAS Negative Affect X Additive G Allele	-.022	.006	.739	.039	.750	.546	-.037	.760	.566	-.010	.011	.880	.037	.002	.409
	EMA Negative Affect X Additive G Allele	-.107	.039	.109	-.012	5.019	.855	-.027	5.105	.693	-.079	.072	.271	--	--	--
Model 2 [a]																
	NEO Neuroticism X Additive G Allele	-.089	.001	.167	-.051	.160	.440	-.089	.160	.178	.039	.002	.574	.029	.000	.516
	PANAS Negative Affect X Additive G Allele	-.012	.006	.842	.041	.742	.522	-.032	.744	.617	-.004	.010	.951	.025	.002	.574
	EMA Negative Affect X Additive G Allele	-.094	.038	.142	.001	4.989	.985	-.012	5.012	.851	-.088	.071	.210	--	--	--
Model 3 [b]																
	NEO Neuroticism X Additive G Allele	-.085	.001	.192	-.044	.164	.516	-.087	.163	.194	.046	.002	.516	.030	.000	.505
	PANAS Negative Affect X Additive G Allele	-.010	.006	.876	.050	.761	.451	-.037	.756	.570	-.001	.011	.988	.030	.002	.496
	EMA Negative Affect X Additive G Allele	-.095	.038	.142	.005	5.059	.941	-.013	5.042	.844	-.087	.072	.222	--	--	--

[a] Covariates in this model include age, gender, and years of education,

[b] Covariates in this model include those entered in Model 2 in addition to BMI, physical activity, current smoking status, and weekly alcohol use.

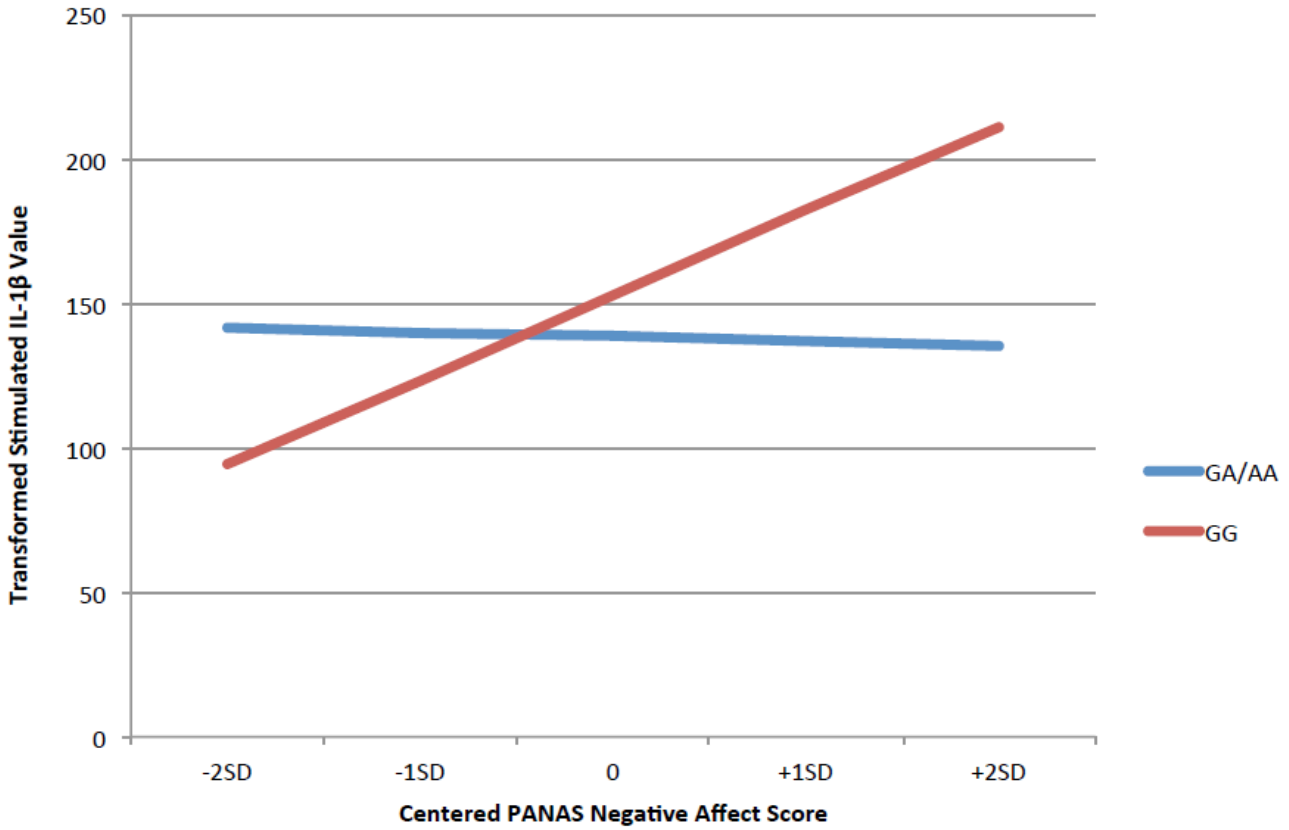

Figure 1. Stimulated IL-1β values estimated from the interaction of GR-9β genotype and PANAS Negative Affect scores.

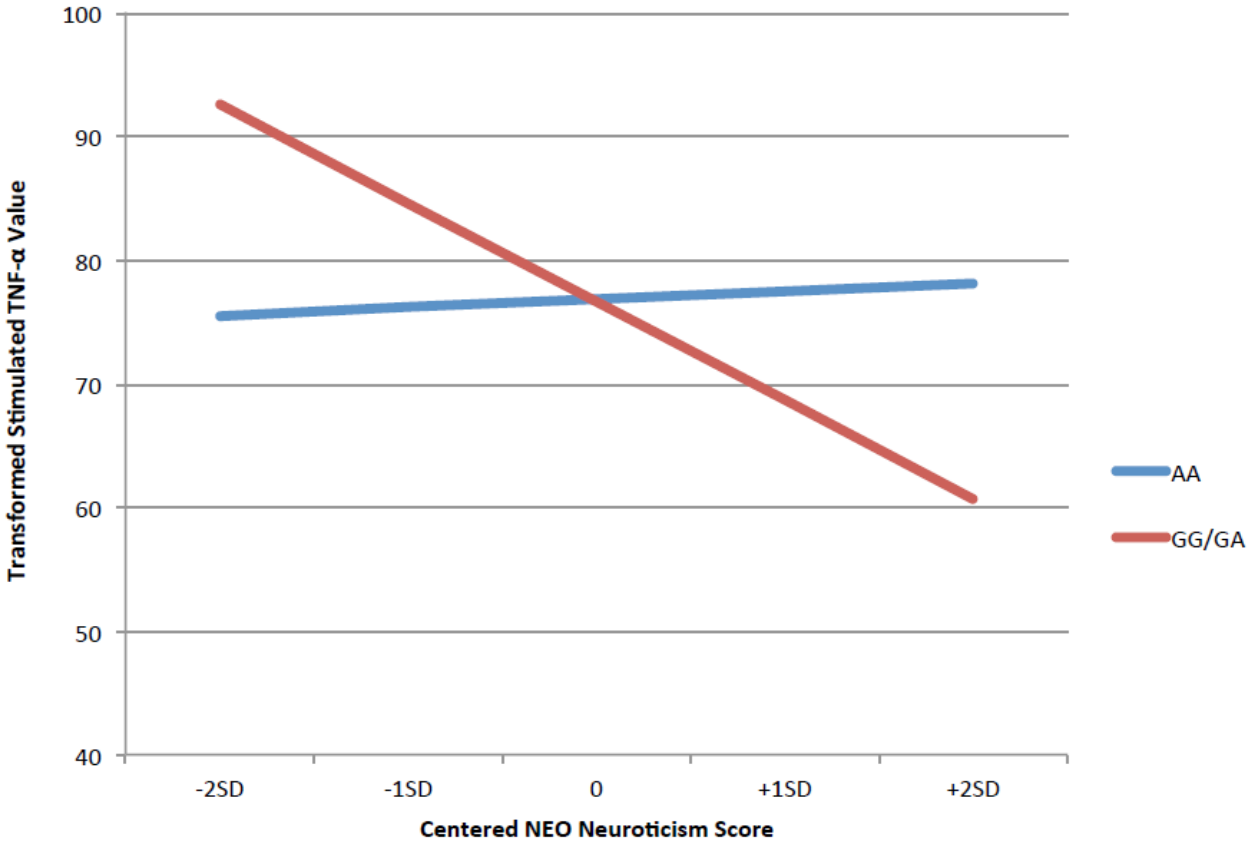

Figure 2. Stimulated TNF-α values from the interaction of GR-9β genotype and PANAS Negative Affect scores.

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