Official Title: Sleep and Healthy Aging Research on Depression for Younger Women

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SPECIFIC AIMS

Depression is a major public health burden that is associated with increased morbidity and mortality,[1] and current antidepressant therapies have limited effectiveness[2]. Thus, biologically plausible models that would translate into new interventions are being pursued. Inflammation is hypothesized to play a significant role in the pathophysiology of depression in at least a subset of individuals. Approximately 30% of patients treated with high doses of interferon (IFN)-α develop major depressive episodes,[3] and when endotoxin is experimentally administered inducing robust systemic inflammation, there are substantial increases in depressive symptoms.[4] However, not all individuals exposed to increased inflammation develop depression.[5] Thus, investigating ***who*** becomes depressed at exposure to heightened inflammatory states—e.g., aging, obesity, infections, IFN-α, or endotoxin—and ***why*** one becomes depressed in such states informs future studies of depression prevention regarding identification of at-risk individuals and actual interventions. In particular, risk for depression is nearly twice as high in ***females*** as in males from puberty through adulthood,[6,7] while this female preponderance and the potentially related ***psychobiological mechanisms*** change in ***old age***. **The overarching objective of this study is to evaluate affective mechanisms underlying the sex- and age-related differences in inflammation-induced depression.**

We have demonstrated in younger adults that low-dose endotoxin induces transient increases in depressive symptoms,[8-12] which correlate with activation of brain regions implicated in depression[10,11,13] and predict subsequent depressive symptoms over the following 2 years (Preliminary Data). Notably, depressive responses to endotoxin are far more accentuated in females than males, which suggests one potential mechanism underlying enhanced risk for depression in females.[14,15]

A key unanswered question is whether female sex increases vulnerability to endotoxin-induced depression across the adult lifespan. The transition to late adulthood, particularly in women, is marked by a number of substantial psychobiological changes, including alterations in physiology (e.g., dopaminergic function[19], major hormonal shifts) and psychological processes (e.g., coping strategies[15]). Most notably, late adulthood is characterized by both *increased* systemic inflammation[20] and *decreased* depression prevalence[16]. Rates of depression in women drop precipitously after age 65[17]; one hypothesis is that this "aging paradox" may reflect age-related decreases in affective sensitivity to inflammation among women. However, no studies have directly compared effects of inflammation on affective processes in younger and older females.

Indeed, the psychobiological mechanisms underlying any differential risk conferred by sex and age in the context of an acute inflammatory challenge have not been systematically interrogated. Depression is increasingly recognized as a heterogeneous disorder comprised of dysfunction in a variety of domains, including increased negative affective processes (e.g., dysphoria) and decreased positive affective processes (e.g., anhedonia). While there is some evidence for sex- and age-related shifts in the predominance of such alterations, the extent to which inflammation differentially alters these domains is unclear. Characterizing these processes may clarify the mechanisms that contribute to the disproportionate prevalence of depression in women likely across the adult lifespan, which will inform prevention and intervention efforts.

The current study proposes to interrogate these mechanisms and how they differ as a function of sex and age. We will first leverage our ongoing experimental study examining endotoxin-induced depression in older adults (R01AG051944, PI Irwin) to test for sex differences in endotoxin-induced alterations in negative and positive affective processes among older adults. This study is expected to recruit 160 subjects aged 60-80 years (80 females vs. 80 males). We next propose to recruit an additional 40 healthy premenopausal women (age 30-40) to participate in this supplemental study, which will allow us a complementary test of age as a vulnerability factor for endotoxin-induced depression and associated affective responding mechanisms in females. Specifically, in this randomized placebo-controlled experimental study, we aim to:

1. Examine the effect of female sex on affective responses to endotoxin in older adults. We hypothesize that, compared to males, females will present with higher negative affective responses and lower positive affective responses to endotoxin.

2. Examine the effect of age on affective responses to endotoxin in females. We hypothesize that, compared to older females, younger females will present with higher negative affective responses and lower positive affective responses to endotoxin.

3. Examine the correlation between inflammatory response and affective response to endotoxin according to sex and age. We hypothesize that the correlations between circulating proinflammatory cytokines and affective responses following endotoxin administration will be stronger in older females than in older males and in younger females than in older females.

Exploratory Aim: Examine the prospective associations between acute affective responses to endotoxin and long-term development of depressive disorders over 2 years, extending our preliminary findings.

RESEARCH STRATEGY

A. SIGNIFICANCE

A.1. Female Sex: A Vulnerability Factor for Inflammation-Induced Depression?

Compelling evidence suggests that inflammation plays a significant role in the pathophysiology of depression.[16-18] Endotoxin is a model of systemic inflammation that can be used to experimentally interrogate the role of inflammation in inducing depressive symptoms. Endotoxin resembles a pathogen-induced, naturally occurring inflammatory immune response, which is driven by a complex interplay of various cytokines, all with distinct kinetics and locally differing concentrations.[19,20] Injection of a single cytokine does not model this response, nor does acute laboratory-based stress, which does not specifically target the inflammatory cascade and only induces modest inflammatory and depressive responses that are less robust than endotoxin. Increasingly, depression is viewed along a continuum of affective responses that lead to symptom expression, as opposed to being a categorical, diagnostic construct,[21,22] and endotoxin affects the two cardinal affective symptoms that constitute depression: depressed mood and anhedonia. Endotoxin induces depressed mood (Profile of Mood Scores[23], POMS>3) and mild depression (Montgomery-Asberg Depression Rating Scale[24], MADRS>7)[25] along with increased activity in neural areas related to depressed mood and threat detection.[11] Similarly, evidence suggests endotoxin also increases anhedonia, the lack of interest or pleasure,[25] and alters activity in reward-related brain regions like the ventral striatum (VS).[10] Thus, endotoxin can be used to probe for changes in both negative and positive affective processes and their associated neural substrates.

Despite the important contribution of inflammation to depression, not all individuals exposed to increased inflammation develop depression.[5] Less than 30% of patients treated with high doses of IFN-α develop major depressive episodes[3]; and even when endotoxin is experimentally administered inducing robust systemic inflammation, largely variable increases in depressive symptoms are found.[4] Indeed, we have shown that depressive responses to endotoxin are far more accentuated in females.[14] In line with our experimental data, the female preponderance in the prevalence of depression is a well-established finding, indicating a higher depression risk in females than males.[6,7] Moreover, women show higher levels of inflammatory markers,[26] and there are well-known sex differences in the prevalence of inflammatory disorders, with females being between 2 and 9 times as likely as males to develop autoimmune disorders.[27]

However, the female preponderance of depression prevalence decreases in the post-menopausal period,[28] and females show a nearly 3-fold decrease in depression incidence from ages 20 to 60. Rates of depression in males, by contrast, remain stable.[29] Likewise, levels of systemic inflammation are higher in females in males, but this sex gap decreases with age.[26] Thus, it is unknown whether the vulnerability to inflammation-induced depression evident among younger women remains in later life. If this study shows older females to be more vulnerable to endotoxin-induced depression than older males, the finding will be significant as it would direct prioritization of female older adults for depression prevention. If this study shows no sex differences, the finding will also be significant as it would suggest age-related changes in sex differences and inform future studies on underlying inflammatory and affective mechanisms.

An institute of medicine (IOM) report in 2001 stated: “Sex matters. Sex, that is, being male or female, is an important basic human variable that should be considered when designing and analyzing studies in all areas and at all levels of biomedical and health-related research … .The study of sex differences is evolving into a mature science. There is now sufficient knowledge… to allow the generation of hypotheses. The next step is to move from the descriptive to the experimental…”[30] *This supplemental study is significant by examining sex differences in inflammation-induced depression leveraging our highly controlled experimental study (R01AG051944, PI Irwin), and particularly because, while sex differences in depression and inflammation decrease with aging, it is unknown whether female sex remains a vulnerability factor for inflammation-induced depression in late life. Thus, this application addresses the following strategic goals of the new Trans-NIH Strategic Plan for the Health of Women "Advancing Science for the Health of Women": 1) To advance rigorous research that is relevant to the health of women; 2) To develop methods and leverage data sources to consider sex and gender influences that enhance research for the health of women.*

A.2. Using Age to Interrogate Sex Differences in Vulnerability to Inflammation-Induced Depression

No studies have specifically tested for age differences in vulnerability to inflammation-induced depression. Our prior work has shown that young females (i.e., below the age of 30) are more affectively sensitive to the effects of inflammation than young males, but it is not known if this vulnerability persists into late adulthood. Because late adulthood is characterized by both *increased* systemic inflammation[20] and

decreased depression prevalence,[16, 17] we hypothesize there may be potential age-related decreases in affective sensitivity to inflammation in older compared to younger females. Notably, major hormonal shifts (e.g., menopause) render older females more biologically similar to older males, which could help explain changes in affective sensitivity to inflammation across the lifespan. If results are consistent with this hypothesis, we will further characterize underlying affective mechanisms by testing sensitivity to both negative and positive affective processes in younger and older women.

If, conversely, results are not consistent with our hypothesis, and sex differences in vulnerability to inflammation induced depression remain evident in late adulthood, we will have identified a potentially vulnerable subgroup (i.e., female older adults) and we will be poised to interrogate affective mechanisms that enable this vulnerability to persist. Furthermore, it is also possible that *both* older female and older male adults will evidence *greater* vulnerability to inflammation-induced depression than younger women. Regardless of sex, there are alterations in cognition and dopaminergic function (e.g., the dopaminergic vulnerability hypothesis)[1931] that could confer increased sensitivity to inflammation and increased risk for inflammation-induced depression among older versus younger adults. The current study includes assessment of cognition and dopaminergic activity. *Thus, results from this study will facilitate identification of at-risk subgroups and potential mechanisms underlying these vulnerabilities.*

A.3. Negative and Positive Affective Mechanisms: A Dimensional Approach to Depression

Clinical presentation of depression is enormously heterogeneous, composed of both negative and positive psychological processes that have distinct neurobiological substrates that can further dynamically interact with the environment over time. Consistent with the articulated goals of the National Institute of Mental Health (NIMH), the current study adopts a dimensional approach to the study of depression, focusing on dysregulation in fundamental processes, such as excess negative affect and a deficit in positive affect. To date, the role of negative processes in depression has received a great deal of attention, but there is some evidence that among older adults, presence of anhedonia, but not dysphoria, is associated with elevated risk for disability and death.[32] Further, anhedonia predicts poor treatment response and is less successfully treated than other symptoms of depression, likely because therapies and pharmacology have traditionally addressed dysfunction in negative valence systems.[33] Indeed, dysregulation in the negative and positive valence systems likely benefit from different psychological and pharmacological treatment approaches.[34] Thus, addressing disruption in both negative and positive valence systems is critical and may have particular relevance for older adults. As detailed in the approach section below, we employ a vertically integrated assessment of dysregulation in the negative and positive valence systems, incorporating self-report questionnaires, observer ratings, and behavioral tasks.

A.4. Prospective and Clinical Validity of Endotoxin Model of Depression

Acute inflammatory reactivity predicts acute increases in depressive symptoms[35,36] as well as increases in depressive symptoms over the following year.[35] Our data also show that acute depressive reactivity (i.e., transient increases in depressed mood during 6 hours following endotoxin administration) predicts subsequent depressive symptoms over 2 years (Preliminary Data). However, the predictive ability of this experimental model—either inflammatory or depressive reactivity—for clinical depressive disorders has never been examined. Inflammation predicts the onset of depressive disorders.[37] Affective mechanisms such as hyposensitivity to reward[38,39] and negative bias in facial emotion recognition[40] also predict future depressive disorders. However, such affective mechanisms potentiated by an inflammatory challenge have never been examined for depression risk prediction. In our preliminary study, only depressed mood enhanced by endotoxin, but not depressed mood observed after placebo infusion, was predictive of subsequent depressive symptoms (Preliminary Data). Similar to a cardiac stress test for coronary heart disease, inflammatory and affective reactivity to endotoxin stress may be able to uncover latent vulnerability to depression that cannot be identified by conventional assessments. If the predictive ability of the endotoxin depression model for clinical depression is confirmed in this 2-year longitudinal design, we will have further justification to test interventions that target such inflammatory and affective mechanisms in prevention trials. *This experimental study followed by a 2-year prospective observation will be significant because of its potential to provide prior and future endotoxin studies of depression with empirical validation and clinical significance. Importantly, it also has the potential to provide further justification to test interventions that target inflammatory and affective mechanisms in large prevention trials of depression.*

A.5. Summary of Significance

The proposed study is significant by providing experimental insight into the impact of age and sex on

negative and positive affective mechanisms underlying vulnerability to inflammation-induced depression, which can inform the development of adjunctive psychological treatment components that target specific processes related to emotion processes (i.e., social rejection sensitivity, negative bias in facial emotion recognition, reward processing). Depression is a major public health burden across the lifespan, and effective screening methods and interventions for prevention are needed. Female sex may identify older adults at high depression risk to be prioritized for prevention efforts. Alternatively, aging may confer resilience among females; if so the current study assesses potential mechanisms that may underlie this resilience and could be leveraged at efforts for depression prevention and treatment. This study is also significant in using a 2-year longitudinal observation period to probe whether acute inflammatory and depressive responses as a function of sex and age predict depression risk over time. The findings will inform future trials to test pharmacologic and psychobehavioral interventions for depression prevention based on inflammatory and affective mechanisms.

B. INNOVATION

This research is highly innovative in using an experimental model of inflammation-induced depression to develop a framework for prevention of depression across the lifespan, integrating behavioral (i.e., reward, socio-emotional responses) and biologic (i.e., inflammation, inflammatory signaling, gene expression) processes to probe mechanisms that may underlie sex-differences in depression prevalence, focusing on how age may modulate affective sensitivity to inflammation. Furthermore, this study substantially advances our prior work for several reasons: 1) Older adults show higher rates of inflammation and depression, yet prior research using this experimental model of inflammation has been performed only in young- or middle-aged adults; 2) No studies have directly compared response to an inflammatory challenge in younger and older females, which will inform on sex differences in sensitivity to inflammation-induced depression; 3) Objective assessment of negative and positive affective responding informs the development of treatments that target these affective pathways, yet our prior research has relied exclusively on single item self-report measures and related neural substrates.

B.1. Use of a highly controlled experimental model to understand depression risk mechanisms

Low-dose endotoxin administration (0.8 ng/kg) is a highly innovative experimental model to understand the causal role of inflammation in the induction of depressive symptoms. Endotoxin induces increases in proinflammatory cytokines to levels similar to those found in an inflammatory disorder (e.g., rheumatoid arthritis) and chronic infections,[41] and induces replicable increases in depressive symptoms.[8-12,19,20,42] Other models such as interferon-α treatment and typhoid vaccination have also been proposed. However, the effects of interferon-α do not usually occur until 8-12 weeks of treatment,[43] and this model cannot be double-blind nor applied to healthy volunteers. Administration of the typhoid vaccine induces only slight increases in IL-6, and is not robustly associated with induction of depressed mood.[44,45] Laboratory stress (i.e., Trier Social Stress Task) can also activate inflammation, and such reactivity is associated with depressive symptoms 1 year later[35]; however, laboratory stress does not target the inflammatory cascade, and inflammatory responses are transient and less robust than endotoxin. Despite its advantages, endotoxin is a procedure that has only been implemented by a few laboratories in the world with only a small number of studies conducted to date (n=12).[19,20] Furthermore, no research has been conducted in older adults.[12] Moreover, no study has attempted to evaluate key clinical characteristics (i.e., sex and age) that might explain differential increases in depressed mood and anhedonic symptoms following inflammatory challenge.[19,20,46] *Here, we address these substantial gaps by evaluating differences in affective responding as a function of sex as well as age among females.*

B.2. Use of task based multi-dimensional methods to evaluate affective processes

Negative and positive affect mechanisms have been examined in young adults, but there is a paucity of such research in older adults; and experimental or longitudinal studies evaluating such mechanisms using task based multi-dimensional methods in older adults are even more limited. Given that social disconnection plays a critical role in the onset and perpetuation of depression,[36,47] we will examine distress following an experimental episode of social rejection, as a function of prior depression history and inflammatory challenge. We will examine the impact of sex and age and inflammatory challenge on accurate judgment of facial emotions with implications for perceived threat (i.e., angry face) or reward value (i.e., happy face).[48] The facial emotion recognition task is used to identify negatively biased processing of emotional faces and is associated with depression and risk of relapse in remitted patients.[40,49] Finally, this study is novel by evaluating positive affective responses, using a series of behavioral reward tasks that assess reward reactivity, learning, and motivation.[50-52] *Understanding how sex and age influence affective responding, at baseline and in response to inflammatory challenge, has the potential to refine psychobehavioral interventions to prevent depression, and*

to inform the design of neuroimaging studies aimed at identifying the neural sensitivities related to inflammation.

B.3. Use of systemic, cellular and genomic markers of inflammation: predictors of depression

The vast majority of research that has examined the associations between inflammation and depression has assessed C-reactive protein (CRP) and circulating levels of interleukin (IL)-6 and tumor necrosis factor (TNF).[53] We have pioneered the use of cellular and genomic methods to evaluate the upstream molecular pathways of inflammation: inflammatory gene expression,[54] inflammatory transcription factor, nuclear factor (NF)-κB,[55] and signal transducer and activator of transcription (STAT) family proteins,[56] which regulate IL-6 and the subsequent induction of CRP. *We will implement these novel methodologies to understand the impact of endotoxin administration, in the context of female sex and age, on genes involved in inflammation, and the extent that these genes and related transcriptional pathways map onto increases in inflammation and depressive symptoms. Integrated analyses of inflammatory and affective responding will inform targets of future translational studies.*

C. APPROACH

C.1. Preliminary Data

C.1.1. Inflammation induces increases in depressive symptoms.

Using an experimental inflammatory challenge (i.e., endotoxin vs. placebo), increases in proinflammatory cytokines are associated with increases in depressed mood and anhedonia.[8-12]

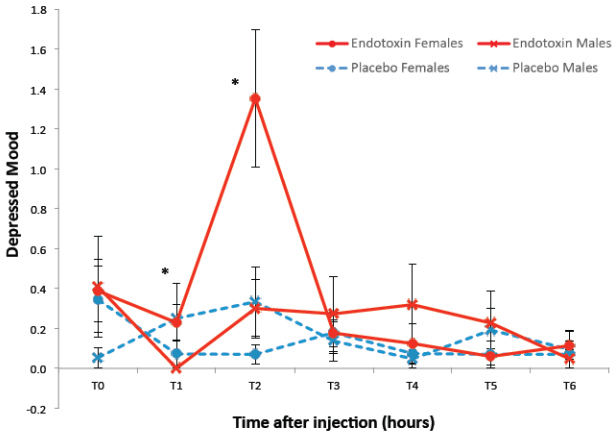

Figure 1. Changes in depressed mood over 6 hours following endotoxin administration (vs. placebo)

C.1.1.1 Inflammatory challenge induces increases in depressed mood, especially in females.[14] *Aim:* To evaluate the effects of endotoxin on depressed mood and sex differences in depressive responses to endotoxin. *Methods:* In a randomized, double-blind, placebo-controlled design, 115 participants (69 females, mean age 24.1 ± 6.3 years) received either low-dose endotoxin (0.8 ng/kg of body weight) or placebo (same volume of 0.9% saline). Blood sampling for IL-6 and TNF was obtained approximately every hour post-injection for the next six hours (T1-T6), along with assessment of social disconnection and depressed mood using the POMS. *Results:* Endotoxin (vs. placebo) led to significant increases in IL-6 ($P<0.001$) and TNF levels ($P<0.001$). Endotoxin (vs. placebo) also led to significantly greater increases in depressed mood ($P<0.001$), and these effects did not change when controlling for sickness symptoms ($P<0.05$) (Fig. 1). Additional analyses found that endotoxin induced increases in IL-6 and TNF which were similar between the two sexes, but suggested that women showed significantly greater increases in depressed mood than men in response to endotoxin ($P<0.05$). Similar results were found for feelings of social disconnection. *Conclusion:* Endotoxin induces increases in depressed mood, which are not due to sickness symptoms (i.e., headaches, muscle pain, shivering, nausea, breathing difficulties, and fatigue). Additional analyses suggested that women are more sensitive to an inflammatory challenge and show greater increases in depressed mood and social disconnection as compared to men. However, these analyses were limited to younger adults. Given the different hormonal milieu of younger vs. older women, and the regulation of inflammation by endocrine hormones, this study will evaluate inflammatory and affective responses in older adults stratified by sex and examine whether there are sex differences in inflammatory and affective responses.

C.1.1.2. Increases in inflammation correlate with depressive symptoms following endotoxin. *Aim:* To evaluate whether increases in inflammation account for increases in depressed mood following an inflammatory challenge. *Methods:* See C.1.3.1. *Results:* There were significant correlations between changes in IL-6 or TNF and social disconnection ($r = 0.27$, $P<0.05$; $r = 0.37$, $P<0\ 0.01$), and between changes in IL-6 or TNF and depressed mood ($r = 0.25$, $P<0.08$; $r = 0.30$, $P<0.05$). Additional analyses suggested that women showed more robust correlations than men. *Conclusion:* Levels of inflammation following endotoxin are associated with social affective responses, and these relationships appear to be stronger in younger women than men despite similar increases in inflammation in the two groups. This study will examine there are sex differences in affective sensitivity to inflammatory challenge in older adults.

C.1.1.3. Endotoxin safely induces increases in depressed mood in older females. *Aim:* To evaluate the safety and feasibility of endotoxin administration in older females. *Methods:* In our ongoing study (K23AG049085, PI Cho), healthy older females aged 60-70 years (N=35) were entered into the previously described endotoxin protocol, which included hourly assessments of depressed mood using the POMS. *Results:* None of the subjects had significant adverse events. Similar to prior results in younger adults, endotoxin was found to induce a significant increase in depressed mood (P=0.017; effect size 0.51), with peak increases in depressed mood at 2 hours; this timepoint of maximal increase is identical to our prior results (C.1.1.1). *Conclusion:* These data show that endotoxin can be safely administered to older females and induces increases in depressed mood with a similar time course of response.

C.1.2. Endotoxin-induced depressed mood predicts subsequent depressive symptoms over 2 years. *Aim:* To examine whether transient increases in depressed mood during 6 hours following endotoxin administration predict subsequent depressive symptoms over a 2-year follow-up. *Methods:* In our ongoing study (K23AG049085, PI Cho), healthy older females aged 60-70 years who completed the above-described endotoxin protocol (N=22) were followed up for 21 months with an assessment of depressive symptoms every 3 months using the telephone-administered 9-item Patient Health Questionnaire (PHQ-9). *Results:* Endotoxin and placebo groups did not differ in subsequent depressive symptoms over 21 months (P=0.37), indicating that the intervention itself did not affect subsequent depressive symptoms (Fig. 2). In the endotoxin group, depressed mood assessed during 6 hours of experimental session at baseline strongly correlated with subsequent depressive symptoms over 21 months (r = 0.71, P<0.0001) (Fig. 3). However, in the placebo group, depressed mood assessed during 6 hours of experimental session did not correlate with subsequent depressive symptoms (r = 0.07, P=0.74). *Conclusion:* Transient increases in depressed mood following endotoxin administration strongly predict subsequent depressive symptoms over 2 years.

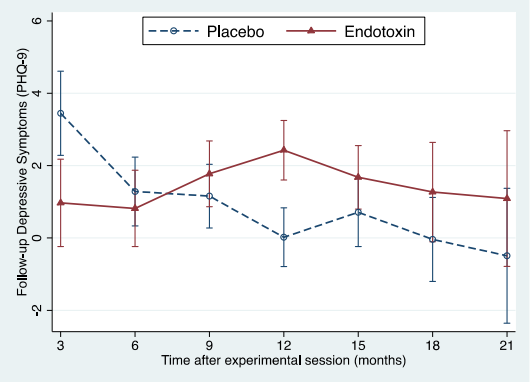

Figure 2. Changes in depressive symptoms over 2 years after endotoxin protocol (vs. placebo)

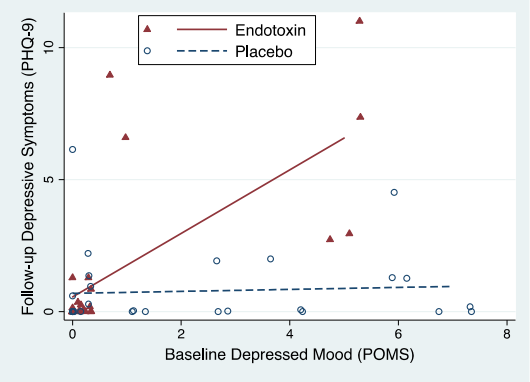

Figure 3. Correlations between endotoxin-induced depressed mood at baseline and subsequent depressive symptoms over 2 years (vs. placebo)

C.1.3. Increases in inflammation following influenza vaccine are associated with changes in reward processing. *Aim*: The aim of this observational study was to assess whether increases in the proinflammatory cytokine IL-6 would increase pre- to post-influenza vaccine, and whether these changes would correlate with changes in performance on reward tasks assessing motivation (monetary reward), learning (monetary reward) and sensitivity (monetary and general social reward). *Methods*: 41 healthy UCLA undergraduate students participated in this pre-post within-subjects study. Participants completed baseline assessment of reward tasks, a week of daily diary assessment, and were then scheduled to receive the annual influenza vaccine. Participants provided blood samples prior and 24-29 hours after the vaccine. Participants completed behavioral reward tasks 24-29 hours after the vaccine; this 24-29 hour period was based on prior research showing an IL-6 peak one day post-influenza vaccine. *Results*: Levels of IL-6 increased significantly from pre-to post-vaccine, but performance on the reward tasks did not significantly differ from pre- to post-vaccine. However, as hypothesized, increases in IL-6 were correlated with decreases in reward motivation for monetary reward, and decreases in attentional bias to positive faces (an index of sensitivity to general social reward). Changes in sensitivity to monetary reward, as operationalized through parameters in the reward motivation task, were not associated with changes in IL-6. Increases in IL-6 correlated with increases in reward responsiveness on a standardized learning task, although sample size for computational analyses was not sufficient to identify whether this was driven by increases in learning or sensitivity. *Conclusion*: Consistent with hypotheses, mild increases in IL-6 following influenza vaccination correlated with decreases in motivation for monetary reward, and decreases in sensitivity to general social stimuli. Contrary to hypotheses, increases in IL-6 were not correlated with change in sensitivity for monetary reward and were associated with increases in reward responsiveness on a reward learning task. These data suggest an association between the proinflammatory cytokine IL-6 and performance on reward tasks in multiple reward dimensions for both non-social and general social reward.

C.1.3. Summary of Preliminary Data

Inflammatory challenge induces depressed mood, especially in females; and increases in depressed mood correlate with increases in circulating markers of inflammation. Importantly, preliminary data suggest that older adults show similar increases in depressed mood in response to endotoxin as found in younger adult samples. Transient increases in depressed mood following endotoxin administration strongly predict subsequent depressive symptoms over 2 years. Prediction of clinical depression should be examined in a larger sample with appropriate diagnostic assessments in order to validate the endotoxin study procedures as a clinically relevant experimental model of depression.

C.2. RESEARCH DESIGN AND METHODS

C.2.1. Summary of the parent project

"Depression, one of the most common diseases in older adults, carries significant risk for morbidity and mortality. Because of the burgeoning population of older adults, the enormous burden of late-life depression, and the limited efficacy of current antidepressants in older adults, biologically plausible models that translate into depression prevention efforts are needed. Insomnia predicts depression recurrence, and is a modifiable target for depression prevention. Yet, it is not known how insomnia gets converted into biological- and affective risk for depression, which is critical for identification of molecular targets for pharmacologic interventions, and for refinement of insomnia treatments that target affective responding to improve efficacy. This study will use an inflammatory challenge (i.e., endotoxin) to probe acute inflammatory- and depression responses (primary outcome) in older adults as a function of insomnia. Older adults with insomnia show chronic inflammation; sleep disturbance also activates inflammatory signaling; chronic inflammation primes acute inflammatory responses; chronic inflammation, as well as acute inflammatory reactivity, predict depression over the following year; and finally, endotoxin induces acute inflammation along with depressive symptoms, with preliminary evidence that "two-hits" (i.e., sleep disturbance and inflammatory challenge) are associated with exaggerated increases in depression, especially in women. In this placebo-controlled, randomized, double-blind study of low dose endotoxin in older adults (60-80 y; stratified by sex) with insomnia (n=80) vs. comparisons without insomnia (n=80), we hypothesize that older adults with insomnia will show heightened inflammatory- and affective responding to inflammatory challenge as compared to those without insomnia. We aim to: 1) examine differences in depressive symptoms and measures of negative affect responding as a function of insomnia and inflammatory challenge; 2) examine differences in measures of positive affect responding as a function of insomnia and inflammatory challenge; and 3) examine differences in experimentally-induced inflammation in relation to depressive symptoms and measures of negative- and positive affect responding as a function of insomnia. If the hypotheses are confirmed, older adults with two "hits", insomnia and inflammation, would represent a high risk group to be prioritized for monitoring and for depression prevention efforts using treatments that target insomnia or inflammation. Moreover, this study will inform the development of mechanism-based treatments that target affect responses in addition to sleep behaviors, and which might also be coupled with efforts to reduce inflammation to optimize efficacy of depression prevention.**"**

C.2.2. Overview of study design

To address Aim 1, examining sex differences in affective sensitivity to inflammation among older adults, we will select a total of 80 healthy older adults aged 60-80 years (40 males and 40 females), who are absent of current psychiatric illnesses, insomnia, and medical conditions (e.g., inflammatory disorders), into a placebo-controlled, randomized, double-blind, experimental inflammatory challenge protocol. Participants will be randomly assigned to receive either endotoxin (n=40) or placebo (n=40) in a between-subjects manner, stratified by sex. To address Aim 2, examining age differences in affective sensitivity to inflammation among females, we will recruit an additional 40 healthy premenopausal adult women (age 30-40) to be randomly assigned to receive endotoxin (n=20) or placebo (n=20).

For all participants, administration of endotoxin (0.8 ng/kg body weight vs. placebo), will be followed by self-report measures of depressed mood, negative and positive affect, and sickness symptoms (e.g., fatigue) assessed hourly over a 12-hour period; objective ratings of depressive symptoms by an observer blind to condition will be made every 2 hours. Behavioral tasks will be administered to coincide with peak onset of depressed mood, which we have previously shown to occur at 2 hours in both older and younger adults. Our prior protocol duration was 6 hours, but the current proposal will test for the presence of delayed termination of response[57,58] and extend protocol duration to 12 hours. At baseline and starting two hours post-injection, at peak cytokine- and depressed mood responses, participants will complete the Probabilistic Reward Learning task[50-52], the Effort Expenditure for Reward Task,[59] the Social Choice task (i.e., intersection of reward

motivation and sensitivity for social reward),[60,61] the Emotional Face Recognition task,[62] and assessment of cognitive function.[63] At two hours post-injection only, participants will complete the Cyberball Social Exclusion task (i.e., self-reported sensitivity to social rejection).[11,64] Proinflammatory cytokines levels will be repeatedly assessed through hourly blood draws, and measures of activation of the inflammatory transcription factor (NF-κB) and inflammatory gene expression, as well as assessment of dopaminergic activity, will be obtained during the first half of the protocol prior to and immediately after peak cytokine responses. A between-subject parallel design will be used for the following reasons: 1) the proposed study involves endotoxin administration, and in a hypothetical crossover design, subjects who experience aversive sickness symptoms from endotoxin (possibly also from placebo due to expectation) might drop out, which would introduce bias and invalidate the results; 2) the effect of endotoxin on inflammation might not resolve between sessions; 3) repeated exposure to behavioral tests of social exclusion might alter responses; 4) two sessions have high subject burden; 5) distinct endotoxin and placebo groups are required for the 2-year follow-up.

Because the administration of endotoxin in this experimental protocol is designed to mimic the increases in plasma cytokines reported in chronic low-grade inflammatory conditions (i.e., 2-10 fold increases in IL-6 and TNF), we will use low-dose endotoxin (0.8 ng/kg) similar to what we have used in our prior studies.[9,11,12,42,65] An endotoxin dose of 0.8 ng/kg yields significant increases in proinflammatory cytokines, increases in depressed mood and anhedonia.[9,11,12,42,65] Our preliminary data indicate that older adults show similar response to endotoxin without adverse events. We have extensive experience with all aspects of the endotoxin vs. placebo inflammatory challenge, and have administered endotoxin dose 0.8 ng/kg in over 170 participants with no adverse events, including older adults (20-70 years). We have also not observed any clinically significant fever or hyper- or hypotension at the 0.8 ng/kg dose. Of note, we have elected to use this low-dose rather than higher endotoxin dose (2.0 ng/kg; results in a 2.5-fold increase in proinflammatory cytokines) because the higher dose has been shown to induce greater fever responses, and hypotension in elderly subjects (60-69 years) as compared to young adults (20-27 years).[106,107]

C.2.3. Subjects

As of January 2019, a total of 74 participants have completed all the study procedures in the parent project, which is expected to complete the recruitment of 160 participants by July 2020. Thus, the parent project will include 40 insomniac older females, 40 non-insomniac older females, 40 insomniac older males, and 40 non-insomniac older males. For this supplemental study, we will select 40 non-insomniac older females and 40 non-insomniac older males to examine sex differences (Aim 1). We will use the basic demographic information in the sample of 40 healthy older females to guide recruitment of the younger female sample (Aim 2). Specifically, we will ensure that the samples are comparable in terms of BMI, years of education, and ethnicity. As with the parent project, compensation for completion of all study components will be $1,000.

Inclusion Criteria: Participants will be healthy older adults aged 60-80 years. For the younger sample, inclusion criteria will be healthy, biologically female adults between the ages of 30 and 40. By using this age range we will focus on women prior to the perimenopause or menopause transition who are also at high risk (median age of onset for mood disorders is 30; of note most studies using endotoxin have a mean age of participants well below age 30).[66,67]

Exclusion Criteria: Participants will be excluded with a current medical or psychiatric disorder or who report current use of medications such as antidepressants and anti-inflammatory drugs, consistent with prior endotoxin protocols involving elderly subjects.[41,58,68] For the younger sample, males will be excluded for the following reasons: feasibility, reduction of variability and subsequent enhanced power, and to build upon our prior observation of accentuated affective sensitivity to inflammation among women. Other exclusion criteria include pregnancy or planning to become pregnant; presence of chronic mental or physical illness, history of allergies, current and regular use of prescription medications, and nightshift work or time zone shifts (>3 hours) within the previous 6 weeks, or previous history of fainting during blood draws. See Protection of Human Subjects section for full details.

C.2.4. Experimental Procedures

Recruitment and Enrollment: Recruitment for the younger female sample will use existing infrastructure in place for the parent project, including identification of potential participants through the GENESYS Sampling System (Fort Washington, PA), which records telephone numbers and mailing addresses of households across the United States. This will allow us to select for households with at least one female person aged 30-40 years and living in the greater Los Angeles area; age information is based on known age-related data or a statistical estimate of age, predicted using individual household characteristics and Census demographic information.

Research staff are in place to send out recruitment letters and respond to inquiries by phone. Interested individuals will go through a two-step screening and assessment procedure to ensure that they are eligible for participation in the study. The initial screening process will be completed by phone; eligible and interested participants will then be scheduled for an in-person morning screening and assessment session (Visit 1).

Baseline Visit 1: Participants will be scheduled for this baseline session near the last day of their menstrual cycle to minimize variability due to hormonal status. They will have their height and weight measured, a urine sample to check for substance use, and an EKG to check for signs of heart disease. They will complete the Structured Clinical Interview for DSM-5 Disorders (SCID-5) with trained research staff. After this, participants will complete baseline assessment of several behavioral tasks, including tasks assessing cognition, reward, and emotion processing. These tasks will be administered between the hours of 10am and 2pm to match the time of administration during the experimental endotoxin vs. placebo session.

Experimental study session - Endotoxin vs. placebo administration: Similar to our previous research,[8-12] the experimental session will be conducted at the UCLA Clinical Translational Research Center (CTRC) using a randomized, double-blind, placebo controlled design. Before each session beginning at 8am, a CTRC nurse blind to the randomization schedule will assess height and weight as well as vital signs (blood pressure, pulse, temperature). Participants will be excluded if: (a) blood pressure less than 90/60 or greater than 160/120, (b) pulse less than 50 beats/minute, or (c) temperature greater than 99.5°F. An indwelling venous catheter with a heparin lock will be inserted into the participant's dominant forearm (right) for hourly blood draws and one into the non-dominant forearm (left) for a continuous saline flush (150 cc/h) for endotoxin vs. placebo administration. Baseline blood samples, self-report questionnaires, and experimental affective response tasks will then be completed. CTRC pharmacy will maintain the randomization assignment and prepare the endotoxin vs. placebo. After 90 minutes, participants will randomly receive either low-dose endotoxin (0.8 ng/kg of body weight) or placebo as an intravenous bolus over 30-60 seconds. NIH will provide reference endotoxin humans (E. coli group O:113).[68] Previous research has demonstrated the safe use of this reference endotoxin across many different samples[41,68 57,58]. Throughout the study protocol lasting 12 hours, vital signs and blood sampling will be obtained every half hour for the first two hours, and then hourly. Placement of an intravenous catheter for this duration does not induce nonspecific increases in circulating levels of IL-6.[9] Human Subjects section details safety monitoring. Although we are extending the protocol for 12 hours to accommodate a possible delayed termination of response in the older adult participants,[57,58] subject burden precludes in person follow-up the next day; however, participants will receive a safety follow-up phone call 1 and 7 days after the CTRC session.

C.2.5. Baseline Measures: Prior to the Experimental Protocol

Baseline assessment procedures (prior to experimental protocol): In a face-to-face interview, the following domains will be assessed: psychiatric history, insomnia, negative and positive valence, biobehavioral and medical factors (i.e., demographic; medical/medication histories; health status) and psychosocial stress and social support. The SCID-5 interview will determine the presence of past history of depression, and the absence of current depressive disorder and other psychiatric diagnoses. We have over two decades of experience in the administration of the SCID, train to criterion validity, obtain diagnoses in a weekly consensus meeting to maintain reliability and criterion validity, and use videotaped interviews for quarterly monitoring of validity/reliability of interviewers. Depressive/anxiety symptoms will be assessed using Beck Depression Inventory (BDI-II), Beck Anxiety Inventory (BAI),[69,70] and Inventory of Depressive Symptomatology.[71]

Negative and positive affect valence: Affect valence characteristics might be related to variable affect response following inflammatory challenge, and will be assessed by self-report questionnaires including the *Behavioral Inhibition and Activation Systems (BIS/BAS) Scale*[72] (i.e., punishment sensitivity; drive, fun seeking, reward responsiveness); *Temporal Experience of Pleasure Scale* [73] (i.e., anticipatory, consummatory pleasure); and *Response Style Questionnaire Ruminative Responses Scale*[74] (i.e., tendency to ruminate in response to symptoms of negative emotion).

Biobehavioral and medical factors will be assessed that might be associated with inflammation, depression risk, or both, including *demographic information*; *biobehavioral confounds of inflammation* [75] such as alcohol use, smoking, BMI, and physical activity (i.e., *Godin Leisure-Time Exercise Questionnaire*[76]); and *medical factors* (i.e., *Charlson Co-Morbidity Index, Chronic Disease Score*[77]) and health functioning (i.e., *Medical Outcomes Study Short-form [SF-36]*[78-80]). We also assess menstrual history and status.

Psychosocial factors will be assessed including number and perceived threat of recent life stresses (i.e., *Perceived Stress Scale*[81]) and subjective social isolation that is associated with depression, sleep

disturbance and inflammation (i.e., *10-item Revised UCLA Loneliness Scale*,[82] *Interpersonal Support Evaluation List,*[83] *short form Social Support Questionnaire [SSQ]*[84]).

C.2.6. Experimental Measures

Negative Affect Responding

Self-report and observer based measures of depressed mood: Depressed mood, a core symptom of depression, will be assessed hourly using the Profile of Mood States (POMS).[23] The Depression Adjective Check List (DACL) will also be administered hourly as the DACL, used in another endotoxin study,[65] correlating well with the Beck Depression Inventory (BDI). We will also use the Montgomery-Asberg Depression Rating Scale (MADRS)[24] as it has been found to be sensitive to acute changes in depressed mood and anhedonia following endotoxin.[19] Because feelings of social disconnection and loneliness predict depression and co-occur with depressed mood following inflammatory challenge, feelings of social disconnection (Feelings of Social Disconnection Scale[9]) and loneliness (adapted UCLA Loneliness Scale[82]) will also be assessed. To complement prior research that perceived social support is a risk factor for cytokine-induced depression, participants will complete the short form of the SSQ[84] at baseline and 2-hours post injection. Finally, an interviewer, blind to the experimental condition, will make ratings of items of depressed mood, feelings of guilt, loss of interest, retardation/agitation, anxiety, and somatic symptoms using items from the Hamilton Depression Rating Scale,[85] one of the most widely used scales in depression research, which will be adapted to evaluate acute changes in depressive symptoms.

Experimental task: subjective sensitivity to social rejection (Cyberball Social Exclusion Task) Socio-emotional mechanisms of depression will be examined in this study as they are particularly significant to older adults, who often experience objective and subjective social isolation, which is associated with depression risk.[86] Feelings of 'social disconnection' play a critical role in the onset and perpetuation of (non-inflammatory forms of) depression.[87] Inflammatory processes can trigger social withdrawal[88] and increase feelings of distress and related neural sensitivity (i.e., activation of the dorsal anterior cingulate cortex).[9] Using the Cyberball Social Exclusion Task,[64] we will examine feelings of social distress. Briefly, participants will be told that they will play a virtual ball-tossing game with two other players over the Internet. In reality, they will play with a preset computer program. In the first round (inclusion), participants will play with the two other players for the entire period. In the second round (exclusion), participants will receive the ball for seven throws and then will be excluded for the rest of the round when the two players will stop throwing the ball to the participant. Following this task, they will complete a self-report measure of social distress in response to social exclusion.[89]

Experimental task: negative bias to emotional face recognition (Emotion Recognition Task) Depressed subjects have a negative bias in the perception of emotions expressed in faces as they tend to interpret neutral faces as sad and happy faces as neutral.[49] Furthermore, such a bias appears to play a role in the etiology and maintenance of depression as it predicts relapse in currently depressed and remitted patients.[40] Emotional Face Recognition Task[62] consists in showing participants a series of black and white photographs (Ekman Pictures of facial affect*)*, in which facial expression is morphed from Neutral to either Sad, Angry, or Fearful.[90] For each image, they will be asked to make a forced choice about the emotion expressed, and rate their certainty on a 0-9 scale. Earlier recognition of a Sad face with certainty would be indicative of negative bias.

Positive Affect Responding

Self-report measures of positive mood. Participants will self-report positive mood each hour using items from the Profile of Mood States (POMS), and items that assess anticipatory (i.e., future oriented) and consummatory (i.e., present oriented) pleasure and interest in activities and social interaction (e.g., the Snaith Hamilton Pleasure Scale; the Affective Forecasting Questionnaire).[91]

Experimental task: intersection of reward motivation and sensitivity for social and non-social reward (Social Choice Task and cartoon task). A social choice[61] and semi-structured social interaction[60] task will be used to assess motivation and sensitivity for social reward. The social choice task will be administered at baseline (visit 1) and during the experimental session (2 hour post-injection). Participants will be asked to rate their desire to engage in three 10-minute activities on a 1-10 Likert scale. These include a social activity (talking with another person) and two solitary activities (solving word problems, sitting quietly). Participants are told that this preference along with an element of chance will determine which they actually do. At the baseline visit, a neutral option will always be chosen. During the experimental session, the social interaction will always be selected. For this semi-structured social interaction task, participants will be asked to spend 5 minutes talking about an important person in their life to a research assistant trained in reflective listening. The

discussion will be recorded and later transcribed and scored for percentage of positive and negative emotional words using Linguistic Inquiry and Word Count Software. Perceptions of the RA will also be assessed (e.g., "S/he was truly interested in me"). A "Cartoon Effort Task"[41] that has been used in samples with depressed adults as well as healthy controls, will be used to assess the degree to which reward motivation and sensitivity coincide. Past work suggests dissociation is a feature of depression. The task uses humorous and nonhumorous single-panel cartoons as reward and non-reward stimuli. Motivation is the amount of effort participants are willing to exert to view funny vs non-funny cartoon. Participants also rate their enjoyment when viewing funny cartoons. The task is 20-30 minutes long, and has been piloted in our lab previously.

Experimental tasks: reward learning (Probabilistic Reward Task, PRT; Probabilistic Selection Task), motivation (Effort Expenditure for Rewards Task (EEfRT), and sensitivity (Emotional Dot Probe, Face Morphing Task) risk (BART). Anhedonia is a core feature of major depressive disorder and includes a reduction in experienced pleasure (liking reward), motivation (wanting reward) and reward learning.[50-52] We have found that inflammatory challenge reduces neural sensitivity to reward anticipation[10], and endotoxin has also been shown to alter reward motivation.[92,93] We will use a series of laboratory based tasks to probe these dimensions of reward. To assess reward learning, we will use the PRT, which objectively measures participants' ability to modulate behavior as a function of reward, because this task identifies reduced reward learning in depressed patients which is state-dependent and also predicts the persistent diagnosis of depression in the midst of treatment.[50-52] This computerized reward-learning task is extensively described in Pizzagalli et al.[94] The *probabilistic selection task* is a 15-minute trial and error (procedural) implicit learning task that assesses reinforcement learning for both positive and negative monetary cues; this task has also been used by the Pizzagalli group and was developed by Michael Frank (Science, 2004). Prior work suggests that dopaminergic function is associated with learning from both types of cues via different neurobiological mechanisms. Lower dopamine levels are associated with decreased sensitivity to positive reinforcement, but increased sensitivity to negative reinforcement. A similar trend is expected following the inflammatory stimulus.

To assess reward motivation, we will employ the EEfRT,[59] an established task in the depression literature that assesses motivation to work for monetary reward at varying degrees of probability of winning and varying degrees of potential reward magnitude. Reduction in reward sensitivity/liking will be assessed with two common emotion processing tasks, the emotional dot probe and face morphing task,[95,96] which assess sensitivity to positive social reward cues; specifically, reduced sensitivity to rewarding social stimuli is indicated by slower identification of happy emotional faces or less attentional vigilance for happy emotional faces. The Balloon Analogue Risk task (BART) is used to assess reward sensitivity and risk. In this brief task, participants view a computer screen with three items: a balloon with a button labeled Click here to pump, button labeled Collect $$$, and a box where total earnings were tallied in every trial. The goal during the BART is to earn as many dollar points as possible. Participants are shown a mock list of high scores to provide a frame of reference for their performance, but no monetary reward was offered. Every time the subject clicked on the "pump" button, the balloon increased slightly in size. When the Collect $$$ button was pressed, the total earnings display added 5 cents for the current balloon. Each balloon in the 30 trials was set to explode at a random pump. If a balloon was pumped past its individual explosion point, a "pop" sound effect played and the participant did not earn any money for that balloon. At any point during a trial, participants could cash out by clicking the Collect $$$ button and their earnings would be updated while a slot machine "payoff" sound emphasized the payment. The number of pumps before an explosion occurred ranged from 1–128. For every balloon, the first pump had 1/128 probability of exploding and a potential gain of 100% (i.e., from 5 cents to 10 cents), the second pump had a 1/127 probability of exploding and a potential gain of 50% (i.e., from 10 cents to 15 cents), and so on until the 128th pump which carried a 1/1 probability of exploding and a potential gain of 0%. Thus, with each additional pump on a particular balloon the risk of losing increased and the relative gain decreased. In this way, some risk taking was necessary to make gains but excessive risk was associated with diminishing returns.

Dopaminergic Function. Given that inflammation can influence dopaminergic function, which underlies reward motivation and learning, this proposal includes an assessment of *resting state eye blink rate (EBR).* EBR is used as an indicator of striatal dopaminergic (D2 receptor) activity and correlates with performance on reward motivation and learning tasks. Alterations in dopaminergic function activity have been proposed to play a key role in mediating effects of inflammation on reward motivation, and EBR has been shown to decrease with greater age. However, EBR has not been assessed following endotoxin administration. With the addition of this brief and simple measure, we will test for changes in central dopaminergic activity following endotoxin, and examine whether these changes are correlated with reward task performance among women in early and late adulthood. EBR will be assessed prior to infusion, and 1.5, 3, 4.5, and 6 hours post-infusion. Eye blinks will

be video recorded for a 5-minute period. The participant will be asked to sit quietly, look ahead, and refrain from visually fixating on any objects. The video will be coded by two research assistants (blinded to condition).

Daily Diary Assessment

Daily diaries will be completed for 7 days prior to the experimental session and 7 days after the session. Participants will receive prompts by text or email that will direct them to the survey site at 6 random points during the day (range of time between prompts = 30 to 180 minutes). Participants will have 1 hour to respond to a single prompt; this is to ensure that participants are given sufficient time to complete an assessment during a working environment (e.g., business meetings) or family obligations (e.g., driving children to school). Data from a second or third submission in the same 30-minute window will be discarded. Completing each prompt will take 2 minutes. Participants first indicate the extent to which they enjoyed 10 types of activities since the last prompt (or since waking) on a 0-100 visual analogue scale. From the same list of activities, participants then rate how much they are currently looking forward to each activity. For each activity, they rate their motivation (hedonic, eudaimonic, or not applicable if their "wanting" is sufficiently low). Each evening, participants will complete one additional prompt assessing positive and negative mood, daily rumination and sleep quality (estimated time: 2 minutes).

Cognition and Executive Function. Behavioral tasks will be administered by computer to assess key aspects of executive function at baseline (Visit 1) and 3-hours post-injection during the experimental session.[63] The *Spatial 2-Back* task is used to assess Updating, the ability to monitor and replace information in working memory. The *Color-Shape* task is used to assess Shifting, or the ability to switch between mental sets. The *Antisaccade* task is used to assess Inhibition. Participants are tasked with inhibiting a reflexive response towards a visual cue in order to correctly identify a target stimulus presented elsewhere.

Emotion Regulation. Participants will complete questionnaires assessing dispositional and situational emotion regulation strategies and attitudes towards emotions. Participants will also complete a 20-30 minute standardized emotion regulation task[44]. The task includes two phases: a reactivity phase and a regulation phase, and assesses the ability to down-regulate negative emotional response to negative images and/or film clips using reappraisal strategies. Participants receive instructions as to how to reappraise (e.g., thinking about the "silver lining" or imagining oneself at a distance from the negative emotion). The dependent variable is the degree to which self-reported emotion changes when reacting to versus reappraising negative stimuli. The task also includes assessment of the ability to up-regulate positive emotion using cognitive strategies (e.g., thinking about a positive image/film clip in such a way that more positive emotion is felt). The stimuli used in the emotion regulation task are drawn from standardized databases and have been used in many previous studies, including studies conducted in our group with younger breast cancer survivors.

Physical Sickness Symptoms (i.e., muscle pain, shivering, nausea, breathing, difficulties, and fatigue) will be assessed hourly by self-report, and controlled in analyses to ensure that the social or affective consequences of inflammatory challenge are not due simply to individuals feeling more 'sick.'

Inflammatory Cytokine, Signaling Pathway, Gene Expression Analysis: Blood samples will be collected at baseline, every half hour for the first two hours, and hourly for the remainder of the 12 h. Samples will be immediately processed and stored at -80^{0}C. Plasma samples (all timepoints) will be assayed for pro- and anti-inflammatory cytokines (IL-1, IL-6, IL-8, TNF, and IL-10) by means of a high sensitivity bead-based multiplex immunoassay (Performance High Sensitivity Human Cytokine, R& D Systems, Minneapolis, MN) and a Bio-Plex 200 (Luminex) Instrument, with excellent intra- and inter-assay reproducibility[97] and very strong correlations ($r \geq 0.94$) with high sensitivity ELISA.[98] We are examining IL-1β, TNF, and IL-6 as these cytokines are increased in response to endotoxin and correlate with increases in depressed mood. IL-8 is also assayed because endotoxin activates the TLR-4 receptor and IL-8 is a proinflammatory cytokine that is released with TLR-4 activation. We also explore changes in IL-10, an anti-inflammatory cytokine that is thought to counter-regulate the inflammatory responses; no study has examined IL-10 responses to endotoxin and determined whether its expression might alter magnitude/duration of inflammatory response. Baseline levels of CRP will be assessed, as CRP is a robust marker of systemic inflammation. Baseline levels of CRP may moderate increases in inflammation and affective responding. However, repeated measures of CRP will not be obtained. Increases in IL-6 induce CRP over several hours or longer; hence, the experimental protocol is too short in duration to evaluate changes in CRP. Peripheral blood mononuclear cell (PBMC) nuclear extracts (baseline, 30 min, 1 h, 2 h or peak cytokine response) will be prepared and assayed to quantify the amount of activated NF-κB p65 present in the nucleus utilizing recombinant p65 (Active Motif, Carlsbad, CA) as the reference

standard (range 0.08-5.00ng),[99] as we have previously described.[100] At baseline, 2- and 4 hours post-injection, blood samples will be drawn in PaxGene RNA tubes, which preserve RNA integrity. Expression of genes involved in proinflammatory pathways (*IL1B, IL6, IL8, CD83, CCL3, TNFAIP3*, and NF-κB/Rel family) will be assayed by quantitative real-time RT-PCR using established TaqMan Gene Expression Assays. Genome-wide transcriptional profiling will be performed using Illumina HT-12 BeadArrays.

C.2.7. Two-Year Longitudinal Follow-up

Participants who complete the experimental session will be followed up by telephone over 2 years for the assessment of depressive symptoms and disorders. A research assistant will call participants every 3 months for administration of the PHQ-9. If their responses suggest depression (i.e., PHQ-9 ≥ 5), the project coordinator will conduct the SCID-5-RV over telephone. We have successfully conducted such a telephone follow-up in an ongoing depression prevention Study (R01AG026364, PI Irwin). Telephone administration of the SCID has been found acceptable and comparable to in-person administration.[101,102]

C.2.8. Statistical Analyses

All measured variables will be assessed for distributional qualities and transformed if necessary for use in the selected statistical models. Adequacy of random assignment and matching will be tested using t-tests and chi-square tests as appropriate. All pre-classification variables (age, BMI, insomnia, medical comorbidity, prior depression history) and baseline values of outcomes will be compared; those that are significantly different between females and males (at $P<0.15$) will be considered for inclusion as covariates in the main analyses. Missing data patterns will be analyzed to assess the potential for biased parameter estimates; missing data will not be imputed as the methods selected allow for missing data under common missingness assumptions. The basic design for Aim 1 is a 2 group (females vs. males) by 2 condition (endotoxin vs. placebo) independent balanced analysis of variance (ANOVA) with one or repeated outcome measures (times: T_{hr} where hr = 0 for baseline, 0.5 to 12 post-infusion; peak cytokine concentration at T_2^{12}), conducted with linear mixed models (LMM). The number of repeated measures varies by assessment. The key results are the main effects of sex, condition, and their interaction; for those analyses with repeated measures, the two main effects and interaction will further interact with the time variable. Other planned analyses include lagged (time series) relationships between cytokine outcomes and affect measures via general linear modeling (GLM). Standard pharmacokinetic parameters (e.g., AUC, C_{MAX}, T_{MAX}) may also be used to create summary cytokine and affect profile outcomes. Finally, covariates (i.e., pre-classification variables such as prior depression history, insomnia, or baseline CRP) will be tested if appropriate (i.e., $p<0.15$) to assess the robustness of the results and further explicate the findings. **Aim 1** is tested with LMM for the following outcomes (timepoints): POMS Depression, DACL, Social Disconnection (T_0, $T_1 – T_{12}$), Observer rated depression (T_0, $T_{2,4,6,8,12}$), Cyberball Social Exclusion Task (T_2), Emotion Tasks (T_0, T_2), Social Support Questionnaire (T_0, T_2), Social Choice Task (T_0, T_2), Reward Response (T_0, T_2), Reward Learning (T_0, T_2), and Reward Motivation (T_0, T_2). The basic design for **Aim 2** is identical to Aim 1 but using age as the group variable (young females vs. older females) instead of sex. **Aim 3** is restricted to the endotoxin condition to examine the correlations between inflammatory responses and affective responses to endotoxin according to sex and age. These correlations will be examined using mixed-effects model linear regression, and cytokines and depressed mood, including excess negative affect and deficits in positive affect, will be entered in the regression models in their longitudinal format, thus reflecting all the measurements performed during the study (T_0, $T_1 – T_{12}$). **Exploratory Aim** is tested using mixed-effects logistic regression, separately in the endotoxin and placebo groups, because the outcome (depressive episodes) is a binary variable repeatedly measured over 2 years. The predictor variables include the sum of POMS depression during 12 hours of experimental session, Cyberball Social Exclusion Task (T_2), Emotion Tasks (T_2), Social Choice Task (T_2), and Reward Tasks (Response, Learning, Motivation, Sensitivity) (T_2).

Finally, we will also evaluate the changes of gene expression in response to endotoxin as a function of sex and age and evaluate *a priori* whether changes in the inflammatory transcriptome are associated with affective response; these analyses will focus upon specialized genomic analyses (T_0, T_2, T_4), and utilize methods previously reported by us.[54,103,104] Quantile-normalized gene expression values will be log2-tranformed and subject to GLM analysis to provide maximum likelihood point estimates of differential transcript abundance across group (females vs. males; younger females vs. older females) and condition (endotoxin vs. placebo), which provide maximally replicable inputs into the higher-order set-based bioinformatics analyses. TELiS (Transcription Element Listening System www.telis.ucla.edu) promoter-based bioinformatics analyses will test the hypothesis that PBMC will show alterations by group and condition in global gene expression profiles consistent with increased activity of the proinflammatory transcription factors NF-κB (assessed by

prevalence of the TRANSFAC V$NFKAPPAB65_01 nucleotide motif in differentially expressing promoters) and AP-1 (V$AP1FJ_Q2) in the female groups and the endotoxin condition. We will explore whether female sex, younger age, and endotoxin decrease activity of Type I interferon signaling pathways (V$ISRE_01, V$IRF2_01); decrease activity of the anti-inflammatory glucocorticoid receptor (GR) (V$GR_Q6); and increase activity of CREB transcription factors involved in β-adrenergic signaling by the sympathetic nervous system (V$CREB_01). Both depression and inflammation are implicated in the regulation of these pathways.[105,106] Moreover, the human immune system appears to have developed a conserved transcriptional response to adversity (CTRA) that involves up-regulating pro-inflammatory gene expression (e.g., cytokine genes) while conversely down-regulating anti-viral gene expression (e.g., interferon response genes) whenever environmental conditions are experienced as threatening, stressful, or uncertain for an extended period of time.[107] The ratio of response element frequencies in the promoters of up- vs. down-regulated genes are assumed to be a measure of differential activity of transcription control pathways, and (log) ratios will be averaged over 9 different parametric combinations of promoter length (-300, -600, and -1000 to +200 bp upstream of RefSeq-designated transcription start site) and motif detection stringency (TRANSFAC mat_sim values of .80, .90, and .95) to ensure robust results.[108] To identify the primary cellular sources of differentially expressed genes, we will carry out Transcript Origin Analysis.[109] In both TELiS and Transcript Origin Analyses, standard errors will be estimated by 2000 cycles of bootstrap resampling of residual vectors from the linear models used to estimate differential gene expression across groups (sex and age) and condition (controlling for correlated expression across genes).

C.2.9. Justification of Sample Size

In our endotoxin study of younger adults, the effect of endotoxin on depressed mood was large in females (d=0.70) while minimal in males (d=0.003).[14] Among older adults, we expect to see a smaller but still substantial difference of effect between sexes. In our endotoxin study of older females (K23AG049085, PI Cho), as shown in C.1.1.3, the effect size of endotoxin on depressed mood was still large but expectedly smaller (d=0.51) than in younger females. For Aim 1, based on these estimates, the assumed minimal effect size in older males (d=0.003), and the use of matching and multivariate regression analyses, with 40 older healthy females and 40 older healthy males, we expect a power >80% (α=.05, two-tailed). For Aim 2, assessing differences between younger and older females, our previous work with several of the behavioral reward tasks has yielded moderate effect sizes when assessing the relationship between increases in IL-6 and task performance (e.g., f^2 = 0.22, 0.32). Given this range of effect sizes, a multiple regression analysis with three predictors of interest (age, group, age x group interaction) and two covariates (BMI, education level) would require a sample size of 38 to 75 to detect effects with 0.80 power. This work has relied on very mild increases in IL-6, and we expect endotoxin to yield stronger effects. Indeed, prior work with the endotoxin model has shown significant differences on a reward motivation task with a sample of 29 participants (15 saline placebo, 14 endotoxin),[92] and greater feelings of social disconnection following endotoxin vs. placebo were observed in a small sample of women (n=20; age 18-36) in our previous study. Thus, the current study aims to recruit 40 premenopausal women with equal randomization between the endotoxin and saline group, and an additional 40 females will be drawn from the parent study for a total sample of 80.

C.3. Interpretation of Results and Potential Concerns

Endotoxin elicits sickness symptoms (e.g., nausea, achiness) that will be assessed as potential covariates in statistical analyses. The battery of administered tasks could lead to excessive fatigue and non-compliance. This is not currently a concern in the parent study, but we will monitor participants' subjective response to the tasks, as well as current fatigue, and assess these variables either as potential covariates or as indicators that the experimental session requires modification. While we do not anticipate strong emotional response to any of the tasks, there is the potential for carryover effects without task counterbalancing. However, since the order is the same for the experimental and the control group, such effects should not impact the primary research questions assessing group differences.

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