Laureate Institute for Brain Research

Clinical Protocol

Response to inflammatory challenge in major depressive disorder

Protocol #2016-002-07

NCT03142919

LOT 94332B1 manufactured by LIST Biological Laboratories (E.coli O:113) under contract with the Clinical Center at NIH.

This study will be conducted under US Food & Drug Administration IND #17187.

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SYNOPSIS

The aim of this project is to understand the biological differences between two distinct subtypes of depression, patients with and without inflammation as defined by c-reactive protein (CRP). Using a double-blinded, parallel group, placebo-controlled design, participants will be phenotyped before and after a low-dose lipopolysaccharide (LPS) challenge designed to perturb the immune system and trigger a transient, mild inflammatory response. In a pilot study, 4 parallel groups comprising 100 depressed subjects with high and low CRP scores will be randomly assigned to receive either LPS (0.8 ng/kg of body weight; *Escherichia coli* group O:113) or placebo (same volume of 0.9% saline) administered as an intravenous bolus, yielding 4 groups of 25 subjects, each (Figure 5). We will also recruit 50 healthy individuals, 25 of whom will be randomized to LPS and 25 of whom will be randomized to saline. Prior to the experimental session, prospective participants will undergo safety screening comprising a physical exam, EKG, alcohol breathalyzer, urine toxicology screen, pregnancy test (in females), suicide screening (in the case of depressed participants), safety labs, and MRI scanning. The experimental session will take place within a week of the safety screen, at which time participants will complete additional safety procedures, provide blood and saliva samples for the measurement of immune and epigenetic markers, and complete rating scales to measure mood and fatigue. After placebo or LPS administration, vital signs will be monitored over a six hour period. Two hours after placebo or LPS administration, participants will provide blood samples, and will complete a second identical MRI session. One, 3, and 6 hours post-placebo or LPS, participants will provide additional blood and saliva samples and complete additional mood ratings. Subject to approval by a physician, participants will be discharged 6 hours post administration of endotoxin or placebo. Follow-up visits one day and one week post the experimental session will involve blood draws and mood ratings. This experimental design will allow for the delineation of the homeostatic mechanisms underlying sensitivity to inflammation-related depression. Low-dose LPS has been used by multiple groups to safely induce transient inflammatory responses in humans. The pilot study is expected to take approximately one year to complete.

TIME AND EVENTS SCHEDULE

	Visit 1	Visit 2 (Experimental Session)										Visits 3 and 4	
Time (hours)	-14 to -1 days	-1	0	0.5	1	1.5	2	3	4	5	6	1 day	1 week
Visit type	Screening Safety	Baseline	LPS / placebo	Post-LPS/placebo (hours)								Post-visit 2 safety	
Assessment/Activity													
PHQ9	X	X									X	X	X
PROMIS-D	X	X											
MADRS	X	X									X	X	X
HAM-A	X												
WRAT	X												
Appetite Weight Change Assessment	X												
C-SSRS	X	X									X	X	X
PSQI		X										X	X
ISI		X										X	X
VAS		X			X		X	X	X	X	X	X	X
SHAPS		X			X		X	X	X	X	X	X	X
POMS		X			X		X	X	X	X	X	X	X
Social Ladder		X					X		X			X	X
Feelings of Social Disconnection		X			X		X	X	X	X	X	X	X
Two-way Social Support		X							X			X	X
UCLA Loneliness Scale		X					X		X			X	X
Sleep Diary	X	X										X	X
TEPS		X			X		X	X	X	X	X		
FTT		X							X				
WAIS-IV Digit Symbol Coding		X							X				
EEfRT		X							X				
Imaging													
3-plane Localizer	X						X						
Asset Calibration	X						X						
MPRAGE (T1)	X						X						
Resting State	X						X						
Monetary Incentive Delay Task	X						X						
Interoceptive Attention Task	X						X						
DTI	X						X						
Safety Labs and Biomarkers													
HbA1c	X												
CRP	X	X			X		X	X			X		
TSH	X												
CBC	X												
CMP	X												
HIV	X												
HCV	X												
SARS CoVID-2	X	X											

Immune Panel		X			X		X	X			X	X	X
Epigenetics (saliva)		X						X					X
Physical Assessment													
Physical Exam	X												
EKG	X	X									X		
Drug Tox/EtOH	X	X											
Pregnancy	X	X											
Vital Signs		X		X	X	X	X	X	X	X	X	X	X
Sickness Scale		X		X	X	X	X	X	X	X	X	X	X
MD Discharge Assessment											X		

ABBREVIATIONS

ACC	anterior cingulate cortex
BMI	body mass index
BOLD	blood oxygenation level dependent
CBC	complete blood count
CCRE	clinical center reference endotoxin
CFR	code of federal regulations
CMP	comprehensive metabolic panel
CRP	c-reactive protein
CSF	cerebral spinal fluid
CSSRS	Columbia-Suicide Severity Rating Scale
DSM-V	diagnostic and statistical manual of mental disorders, fifth edition
DSMB	data safety monitoring board
DST	digit symbol coding task
DTI	diffusion tensor imaging
EefRT	Effort-Expenditure for Rewards Task
EKG	electrocardiogram
FDA	Food and Drug Administration
FTT	finger tapping task
HAM-D	Hamilton rating scale – depression
HAM-A	Hamilton anxiety rating scale
HbA1c	glycated hemoglobin
IAT	interoceptive awareness task
IIC	integrative immunology center
IL-6	interleukin 6
IND	investigational new drug
ISI	Insominia Severity Scale
LIBR	Laureate Institute for Brain Research
LPS	lipopolysaccharide
MADRS	Montgomery Asberg depression rating scale
MD	medical doctor
MDD	major depressive disorder
MID	monetary incentive delay task
MRI	magnetic resonance imaging
MPRAGE	magnetisation-prepared rapid gradient-echo
NIH	National Institutes of Health
PBMC	peripheral blood mononuclear cell
PET	positron emission tomography
PHQ9	patient health questionnaire
POMS	profile of mood states
PROMIS-D	patient reported outcomes measurement information system - depression
PSF	physical symptoms form
PSQI	Pittsburgh Sleep Quality Index
SHAPS	Snaith-Hamilton pleasure scale
TEPS	temporal experience of pleasure scale
TNF	tumor necrosis factor
TSH	thyroid-stimulating hormone
VAS	visual analog scale
VS	ventral striatum
VTS	ventral tegmental area
WIRB	Western Institutional Review Board

WRAT wide range achievement test

1. INTRODUCTION

About 1 out of 7 individuals experience Major Depressive Disorder (MDD) during their lifetime but only 1 out of 3 achieve remission with current treatment. MDD is the second largest source of disability and costs the economy $200 billion annually. MDD is a very heterogeneous disorder that affects how one processes events - 'what makes you feel good' (positive valence), 'what makes you feel bad' (negative valence), and how the brain processes body-relevant information (interoception). Some have proposed that inflammation plays a central role in a subset of depression that is characterized clinically by an increase in c-reactive protein (CRP). This proposal seeks to better understand the biological processes that underlie the inflammatory subtype of MDD by comparing subgroups of depressed individuals with low CRP (≤1mg/mL), and high CRP (≥3mg/mL). In particular, an experimental manipulation of the immune system will be used to contrast the clinical, immune, and neural function of depressed subjects with high and low CRP. Specifically, the low CRP MDD group (n=50) and the high CRP MDD group (n=50) will be divided to receive either normal saline or endotoxin (0.8 ng/kg) in order to induce a transient inflammatory response (Figure 5). Serial blood draws will be obtained during this time to quantify the pattern of inflammatory response using several inflammatory markers. At the same time, subjects will complete clinical ratings and undergo a pre- and post-endotoxin MRI scan to measure how the transient inflammatory response affects brain processing of anticipatory reward and interoception. In addition, in order to delineate the mechanistic pathways involved in the maladaptive response to LPS observed in MDD participants, we will randomize a group of 50 healthy controls (HC) to LPS (n=25) or Placebo (n=25). The same measures will be obtained on the HCs as the MDD subjects.

1.1. Background

Evidence for an inflammatory subtype of depression

Extant evidence indicates excessive activation of the innate immune system leading to low-grade inflammation in depression, with reports of: (a) depression-associated elevations of circulating pro-inflammatory cytokines [1,2], (b) differential expression of inflammation-related genes in monocytes or peripheral blood mononuclear cells of subjects with mood disorders [3,4], (c) the anti-inflammatory effect of certain classes of antidepressant medication [5], (d) prospective studies demonstrating a positive association between CRP or interleukin 6 (IL-6) concentrations at baseline and the development of *de novo* cases of MDD [6,7], (e) the epidemiological association between depression and diseases with an autoimmune or inflammatory component [8-10], and (f) a greater ratio of primed ("sensitized" to future inflammatory stimuli) over ramified ("resting") microglia in depressed samples at *postmortem* [11]. Nevertheless, depression is a heterogeneous disorder and not all patients have an inflammation-related depression. For instance, although the mean concentrations of CRP or pro-inflammatory cytokines, generally are found to be elevated in cross-sectional studies of depressed patients vs. healthy controls, this group difference usually is driven by a minority (in the order of 30%) of patients [12]. These data are consistent with the finding that only 30-40% of cancer and hepatitis C patients receiving treatment with interferon α or cytokines develop major depression [13,14], that a CRP score of <1 mg/L is predictive of therapeutic response to escitalopram, while a CRP score of >3 mg/L predicts response to nortriptyline [15], and that only depressed individuals with a CRP score of ≥ 5 mg/L show a therapeutic response to the TNF inhibitor, infliximab [16]. Yet, remarkably little is known about the behavioral, immunological, and neural differences between depressed individuals with and without "inflammation" which could have profound consequences for prognosis and treatment of this disorder - e.g. response to anti-inflammatory medications which have been reported to have some antidepressant efficacy [17,18] but may also be harmful [19,20]. Therefore, we intend to bridge this critical gap by examining individual differences at multiple phenotypic levels (clinical, molecular, systems) across patients with depression, both at baseline, and in response to an acute inflammatory (i.e. LPS) challenge.

Inflammation and alterations in symptoms and behavior related to depression

One of the cardinal symptoms of depression is impaired hedonic capacity and motivation (anhedonia). Between 30-50% of patients experience clinically significant anhedonia [21]. The term "anhedonia" initially was described as the inability to experience pleasure [22]. The DSM-V operationally defines anhedonia as diminished interest or pleasure in response to stimuli that were perceived as rewarding during the premorbid state. Anhedonia is comprised of two components, a motivational component (anticipatory anhedonia or a deficit in the capacity to anticipate pleasurable events, a "wanting" deficit) and a consummatory component (a deficit in the actual experience of pleasure or a "liking" deficit) [23]. It is anticipatory anhedonia ('wanting') that is primarily impaired in MDD [24,25]. The tight link between anhedonia and inflammation is derived in part from animal work showing that experimentally-induced immune system activation produces "sickness behavior", a motivational response to infection or disease that includes anhedonia-like behavior such as reduced sucrose preference [26,27]. In humans too, there is a striking overlap between the "psychological" symptoms associated with infection (e.g. depressed mood, irritability, social withdrawal, decreased concentration) and the DSM-V-defined symptoms of MDD [28]. Crucially, in hepatitis C patients receiving treatment with interferon α, there is a temporal disjunction between the "psychological" and "physical" manifestations of this immune-stimulating treatment, with the neurovegetative symptoms appearing within one week whereas the mood cognitive symptoms peak 8-12 weeks post-initiation of treatment [13,29]. Moreover, it is the mood and cognitive symptoms rather than the neurovegetative symptoms that are responsive to antidepressant treatment [13,29]. Similarly, administration of the typhoid vaccine or LPS to healthy volunteers results in an increase in depressive symptoms [30-34] but whether a subgroup of patients with depression will show a differential sensitivity to LPS that is characterized by a disproportionate increase in anhedonic symptoms relative to depressive symptoms, *per se*, is unclear. This question is significant because anhedonia and inflammation have independently been reported to be negative prognostic indicators [35-38] raising the possibility that the negative prognosis associated with anhedonia may be mediated in part by inflammatory processes and therefore that anti-inflammatory medications may have some benefit in treatment-resistant individuals.

Inflammation and alterations in neural circuits related to depression

Anticipatory anhedonia or 'wanting' has been linked to a variety of neural circuits and neurotransmitters, especially ventral tegmental area (VTA) dopaminergic projections terminating in the ventral striatum (VS) [39], and thus in the context of fMRI studies, anticipatory anhedonia manifests itself as a blunted hemodynamic response to reward stimuli in the VS [40-42]. Consistent with the proposed link between inflammation and anhedonia, hepatitis C patients receiving interferon α showed significantly reduced activation of the VS in the win vs. lose condition of a gambling task compared with patients awaiting interferon α treatment [43]. Similarly, healthy subjects exposed to LPS showed greater increases in self-reported and observer-rated depressed mood, along with reductions in VS response to monetary reward cues on the anticipation phase of the Monetary Incentive Delay (MID) task [44]. Further, the relationship between exposure to inflammatory challenge and increases in depressed mood was mediated statistically by differences in VS activity to anticipated reward, suggesting that inflammation altered reward-related neural responses and that these altered neural responses mediated the effects of inflammation on mood. This will be the first study to test whether depressed patients with high and low baseline levels of CRP will show differential neural sensitivity to anticipated reward in the context of an inflammatory challenge. This question is significant because conceivably depressed subjects with inflammation may respond preferentially to medications that modulate dopaminergic signaling and/or psychotherapies that target motivation such as behavioral activation therapy.

In addition to the specific effect on anticipatory reward, inflammation-related changes in motivated behavior are underpinned by immune-to-brain communication pathways that allow the brain to form a dynamic "image" of the body's internal state during the ongoing peripheral inflammatory response [45]. This capacity for internal representation of the body, also known as interoception [46,47], allows for the development of sickness behavior which can be conceptualized as a homeostatic response to an interoceptive perturbation aimed at conserving internal resources during an infection [28]. The brain region critical for the representation of the subjective feelings associated with interoceptive states is believed to be the anterior insula [46,47]. In theory, this insula-centric circuit may be differentially sensitive to inflammatory stimuli in people with depression. However, while

altered glucose metabolism or BOLD activity of the insula has been reported at rest in healthy individuals receiving LPS [31,48], the effect of LPS on the insula during an interoceptive attention task is unknown.

Inflammation fundamentally changes the internal state of the body leading to fundamentally different afferent signals to the brain, i.e. an altered brain-body connection. Therefore, it is conceivable that individuals with high and low inflammation will differ in interoceptive processing, involving – among other brain areas – the insular cortex. The results from this study will establish the link between the individual differences in inflammation - both at baseline and in response to immune perturbation - in depression and the degree of altered interoceptive processing. This link can help to: (1) delineate the pathophysiological (i.e. interoceptive) mechanisms underpinning the inflammatory subtype of depression, thus potentially identifying targets for the development of novel treatments, and (2) determine whether interoceptive dysfunction can serve as an outcome predictor.

1.1.1. Preliminary Studies

Effects of low-dose LPS on physiology, mood and cytokine production: Using the identical protocol to the one proposed herein, our collaborator, Dr. Irwin has demonstrated that LPS induces rapid but transient physiological (temperature, blood pressure), immunological (cytokine production), and mood effects that peak 2-3 hours post administration [49] (Figure 1).

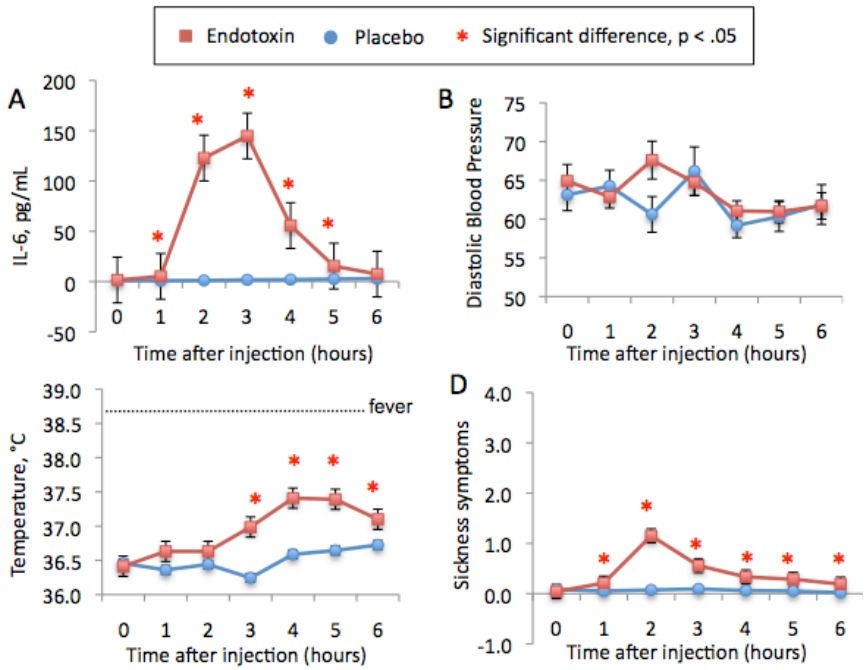

Figure 1. Physiological and self-reported symptoms following LPS administration (0.8 ng/kg).

Differences between depressed subjects and controls in neural response to monetary reward and the effects of LPS on this circuitry: We have reported reduced activity of the left nucleus accumbens (NAcc) in response to anticipated reward in unmedicated depressed patients vs. controls [50] (Figure 2). Further, we demonstrated the existence of 3 different subgroups of participants based on their neural responses to reward and loss that were found in equal proportions in depressed subjects and healthy controls [50], illustrating the heterogeneity of physiological response to reward. Secondly, healthy subjects exposed to LPS showed greater increases in self-reported and observer-rated depressed mood (43% of the subjects in the LPS group vs. 6% of subjects under placebo), increased IL-6 and TNF levels, and reductions in VS response to monetary reward

cues on the anticipation phase of the MID task [44] (Figure 3). Further, the relationship between exposure to inflammatory challenge and the increase in depressed mood was mediated statistically by differences in VS activity to anticipated reward, highlighting a neural circuit through which inflammation may contribute to the depressive syndrome [44].

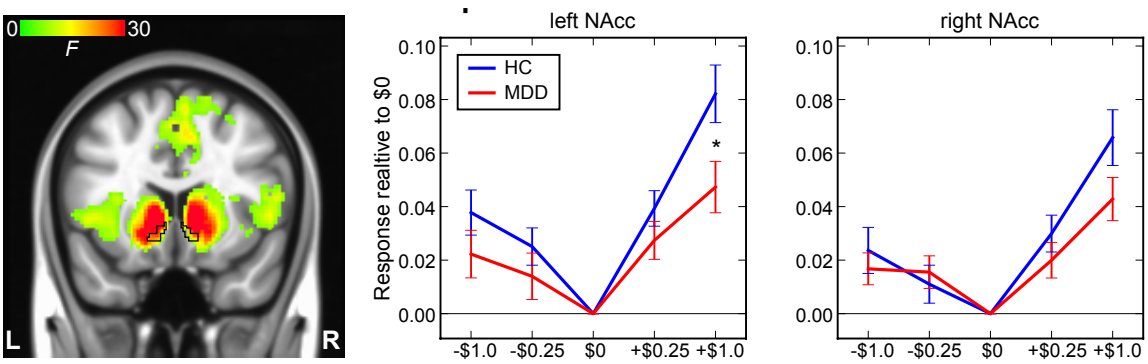

Figure 2. Left panel: *F*-values map of monetary condition effect and mean responses of the NAcc across both depressed and HC groups during anticipation of reward. Maps are obtained from a voxel-wise linear mixed-effect model analysis thresholded by *P* < 0.001 (uncorrected) and cluster size > 43 (*P* < 0.05) [50]. Middle and right panels show mean NAcc responses with standard errors in the left and right NAcc respectively for HC and depressed subjects.

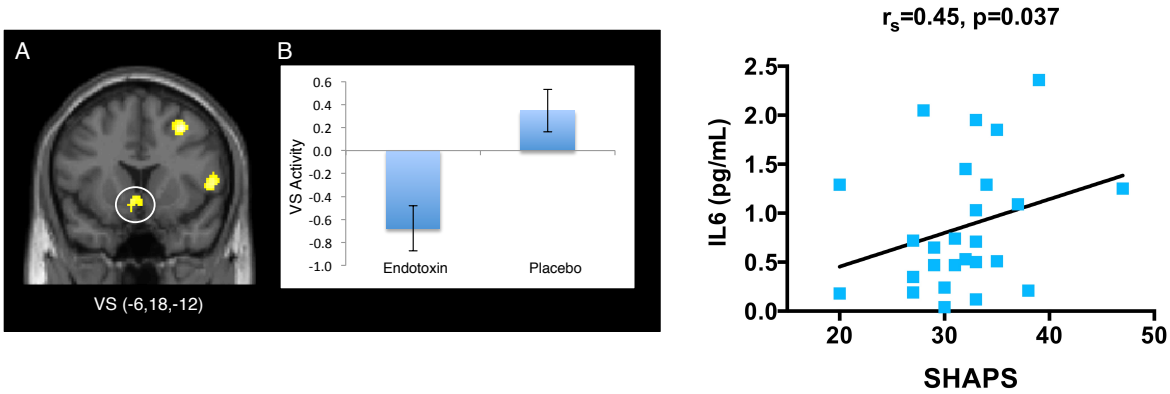

Figure 3. Left panel: VS activity from whole-brain analysis was stronger for placebo compared with endotoxin (p <.005, >10 voxels) [44]. Right panel: IL-6 concentration is positively correlated with the severity of anhedonic symptoms (SHAPS score) in unmedicated depressed subjects [51].

The relationship between IL-6 and anhedonia: We demonstrated that depressed subjects had significantly higher anhedonia (SHAPS) scores than healthy controls, and anhedonic, but not depressive symptoms *per se*, were positively correlated with IL-6 concentrations, indicating the salient link between inflammation and anhedonia [51] (Figure 3).

Differences between depressed subjects and controls in interoception: Our collaborator, Dr. Simmons showed that relative to controls, depressed subjects exhibited a decreased BOLD response of the insula when focusing on their heart beats, and this activity was negatively correlated with severity of depressive symptoms [52] (Figure 4).

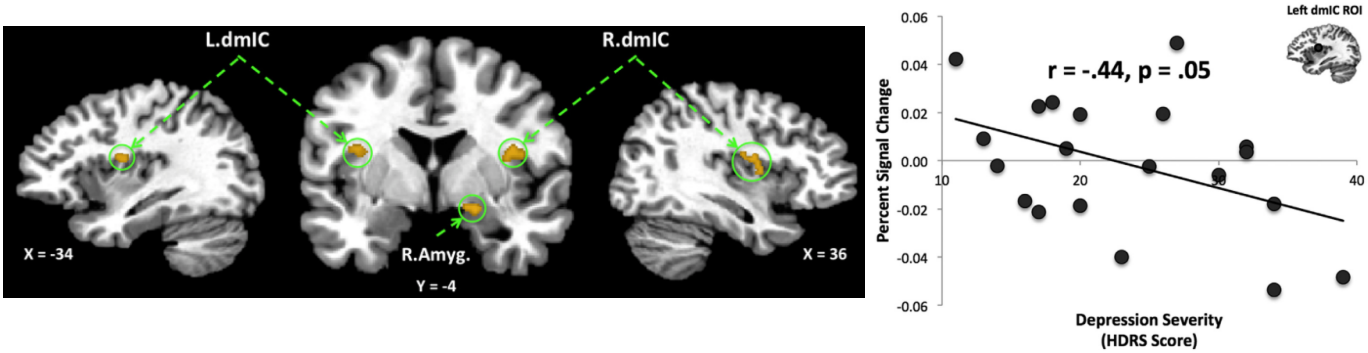

Figure 4. Left panel: depressed subjects exhibit decreased BOLD activity compared with healthy subjects bilateral dorsal mid-insula cortex and right insula [52]. Right panel: a significant negative correlation between depressed subjects' BOLD response during heartbeat interoceptive attention vs. exteroception and scores on the Hamilton Depression Rating Scale [52].

Other relevant publications: Dr. Savitz has a strong track record in delineating depression-associated immunological and neural abnormalities, and their interactions [4,51,53-58] (out of >50 first or senior author publications).

1.2. Overall Rationale for the Study

The value of an experimental probe that elicits inflammation

Since CRP is a general measure of inflammation, by definition an "inflammatory" subtype of depression can be identified based on CRP scores. Why then the need for an LPS study to understand the biology of inflammation-induced depression?

Central vs. peripheral inflammation: In contrast to the robust evidence for peripheral immune abnormalities in depression, the data on brain–immune abnormalities remains inconclusive [59,60]. For instance, while some studies have reported increases in inflammatory markers in *postmortem* samples from depressed patients, there also are data indicating a *decrease* in pro-inflammatory mediators in the brains of depressed individuals *postmortem* [61-63]. Further, there is a related question of how well peripheral measures of inflammatory mediators reflect central concentrations of inflammatory molecules. In both healthy volunteers [64] and depressed patients [65], the correlations between cytokine concentrations in the plasma and the CSF have been reported to be non-significant. Further, CRP does not cross the blood brain barrier and is in fact (non-significantly) inversely correlated with microglial activation measured with PET in MDD, raising the possibility that CRP is a better measure of peripheral adiposity than central inflammation [66]. Here we use an inflammatory challenge that has been demonstrated to induce microglial activation in humans [67], to evaluate the effect of inflammation on depressive symptoms and neural circuits in people with depression.

Cause vs. effect: Obesity, which leads to elevations in peripheral inflammatory mediators, and co-morbid illnesses with an inflammatory component are over-represented in people with depression. Although elegant preclinical studies and the interferon α literature suggests that inflammation is a cause rather than an effect of depression [13,68], the issue of cause vs. effect is still unclear in humans with non-medical illness-related depression. This will be the first study to directly test the effect of an experimental inflammatory stimulus on mood and neurophysiology in the context of primary depression. This approach will move the field beyond simple correlational designs involving single time-point group comparisons of inflammatory markers, thus providing a more rigorous test of the hypothesis that inflammation is involved in the development of depression and is not simply a surrogate marker of obesity and poor health behaviors.

Immune system priming: Chronic low-grade inflammation and/or stress may sensitize the immune system to secondary inflammatory triggers, resulting not only in an overall peripheral immune response that is greater in magnitude than the sum of responses to the individual stimuli alone, but at least in animal models, elicits a discordant central immune response as a result of microglial "priming" (changes in the reactive state of microglia that prime the cells to respond more aggressively to subsequent stimuli) [69-71]. Static, single time-point measurements of CRP do not capture acute inflammatory responses that regulate neural response to perturbation of the immune system. In this study, we examine acute inflammatory responses to LPS, and whether baseline CRP differentially primes these inflammatory- and associated neural responses to LPS challenge. Thus the use of an experimental probe is important because it will allow us to test whether depressed individuals with inflammation show a more vigorous immune response to LPS than depressed individuals with low levels of inflammation thus potentially providing a parallel with the preclinical literature on macrophage and microglial priming as well as a *postmortem* study reporting a significant increase in the ratio of primed over ramified ("resting") microglia in the dorsal anterior cingulate cortex (ACC) of depressed suicides [11]. This is significant because in animal studies microglial priming has been associated with prolonged depressive behavior, cognitive impairment, neuropathology, and degenerative disorders [72] and there is a longstanding debate in the literature over whether mood disorders are developmental or degenerative illnesses [73].

2. OBJECTIVES

Aim 1: To delineate a multi-level (symptoms, behavior, neural circuits, and immune and genetic markers) phenotype of an inflammatory subtype of MDD thereby identifying the biological pathways associated with affective sensitivity to LPS.

Hypothesis 1.1. Relative to the low CRP MDD group, the high CRP MDD group will show higher levels of anhedonia at baseline. Relative to the placebo groups, the LPS groups will show higher levels of anhedonia. The high CRP MDD group will be differentially sensitive to LPS, showing a greater LPS-induced increase in anhedonic symptoms than the low CRP MDD group and controls receiving LPS.

Hypothesis 1.2. Relative to the low CRP MDD group, the high CRP MDD group will show higher levels of IL-6 and TNF at baseline. Relative to the two placebo groups, the two LPS groups will show higher levels of IL6 and TNF. The high CRP MDD group will display a greater LPS-induced increase in IL-6 and TNF than the low CRP MDD group and controls receiving LPS.

Hypothesis 1.3. Relative to the low CRP MDD group, the high CRP MDD group will show reduced activity of the ventral striatum (VS) in response to anticipated reward vs. no reward at baseline. Relative to the placebo groups, the LPS groups will show a reduced BOLD response to anticipated reward at two hours post-LPS challenge (T_2). The high CRP MDD group will display a greater LPS-induced decrease in VS BOLD activity than the controls and the low CRP MDD group receiving LPS.

Hypothesis 1.4. Relative to the low CRP MDD group, the high CRP MDD group will show reduced activity of the insular cortex during interoception vs. exteroception at baseline. Relative to the placebo groups, the LPS groups will show a reduced BOLD response during interoception at T_2. The high CRP MDD group will display a greater LPS-induced decrease in insular BOLD activity than the controls and the low CRP MDD group receiving LPS.

Aim 2: To examine whether HCs and MDD participants differ in their acute and prolonged immunological, and neural responses to LPS vs. saline (placebo).

Hypothesis 2: Relative to HC, the MDD group will show the following changes during LPS vs placebo:
A. A greater increase in pro-inflammatory cytokines (primary outcome is IL-6) from baseline.
B. Greater activity of the insula and posterior cingulate cortex (PCC) in response to interoceptive stimuli (focusing attention on the heart and stomach) vs. exteroceptive stimuli (focusing attention on font color); such difference would indicate a deficit in regulating the interoceptive circuitry during LPS as compared to HCs.

C. Reduced functional connectivity between the insula and the mid-cingulate evaluated with resting state fMRI.

Aim 3: To identify epigenetic predictors of response to endotoxin in the MDD group.

Hypothesis 3.1: Individuals with reduced methylation of the promoter regions of both the IL-6 and TNF genes will show greater expression (gene and protein) of IL-6 and TNF both at baseline and subsequent to endotoxin challenge.

3. OVERVIEW OF STUDY DESIGN

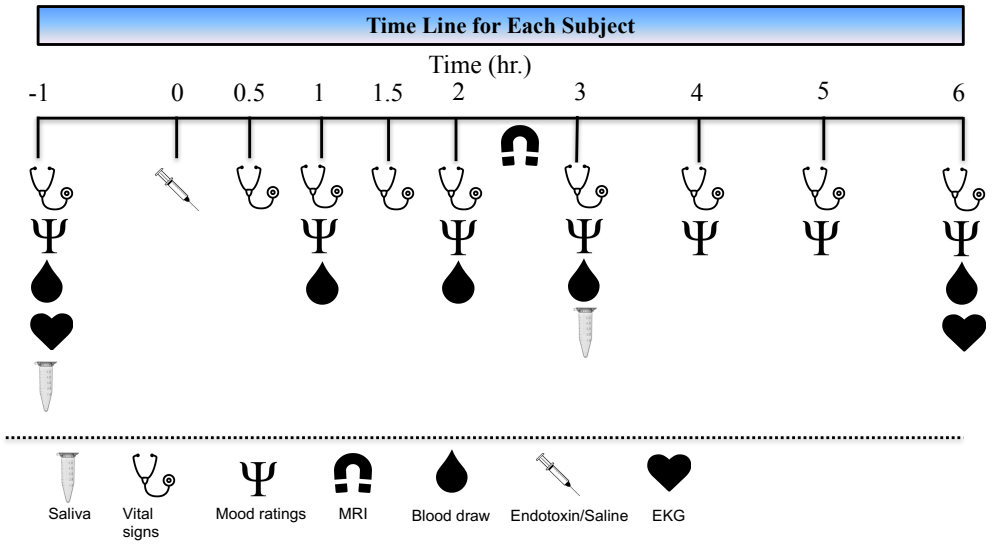

Figure 5: Experimental Design.

Four parallel groups comprising 100 medicated or unmedicated depressed subjects with high and low CRP scores (50/50) will be randomly assigned to receive either LPS (0.8 ng/kg of body weight; *Escherichia coli* group O:113) or placebo (same volume of 0.9% saline) administered as an intravenous bolus, yielding 4 groups of 25 subjects, each. “Low” CRP is defined as ≤1 mg/L and “high” CRP as ≥3 mg/L, according to standard cutoffs recommended by the Centers of Disease Control and the American Heart Association. We also will recruit a group of 50 healthy individuals, 25 randomized to LPS and 25 randomized to placebo for comparison purposes.

3.1. Summary of Research Schedule

Initially, subjects will complete a psychiatric and medical evaluation to determine eligibility for the study. This evaluation takes place under a separate screening protocol (WIRB # 20101611). Briefly, the purpose of LIBR Screening protocol is to allow for the careful screening of potential research participants (ages 8 – 65) in order to determine eligibility for inclusion in research protocols at the Laureate Institute for Brain Research (LIBR). This protocol will serve as an entry point for individuals with schizophrenia, mood and/or anxiety disorders, substance use disorders and healthy volunteers to proceed further into other investigative research protocols. Participants will be interviewed regarding any of the following: demographics, medical and psychiatric history. The screening evaluation may include the following: psychiatric interview, diagnostic interview, questionnaires and rating scales, medical history, physical exam, substance use testing and request for medical records.

Upon conclusion of the screening process, participants will either be offered participation in a research protocol or will be considered not appropriate for participation in LIBR research and will be referred back into the community. The screening protocol thus serves as an entry point for individuals with psychiatric disorders or healthy volunteers to enter IRB approved LIBR protocols, including the current protocol. Participants will have their data saved in the LIBR database to be contacted for future studies when they give verbal agreement during the phone screen.

Once the subject is deemed eligible to participate in the study, they will be scheduled for Visit 1 of this study (safety screening visit). Upon arrival at LIBR for Visit 1, each participant will be consented, receive a urine drug screen and alcohol breathalyzer test, complete self-report questionnaires, have vital signs (temperature, pulse, blood pressure), weight and height measured by a nurse, complete a physical exam and an EKG, provide a blood sample for CRP and safety labs, and undergo a one hour baseline MRI scan. A cardiologist will evaluate the EKG. Study Visit 2 will be scheduled within 1 week of Visit 1. Visit 2, the experimental session, will include vital signs measured by a nurse who is blind to condition; a 1 hour MRI scan to measure BOLD activity at rest and during monetary reward/loss and interoceptive attention tasks; blood draws; saliva sampling; and mood and neuropsychological testing. 500cc of normal saline will be administered prior to LPS or saline injection to ensure adequate hydration. We may also administer up to 500cc of additional saline subsequent to the LPS/saline infusion either to assist in maintaining an open line for blood draws or for additional hydration at the discretion of a clinician. Vital signs will be assessed every 30 min post injection of LPS/placebo for the first 2 hours (and thereafter every hour). EKGs will be performed at baseline and prior to discharge, and subjects also may have single-lead cardiac monitoring between experimental procedures (i.e. blood draws, neuroimaging, and cognitive testing). Blood samples will be taken at baseline (T_{-1}), T_1, T_2, and T_3 (~ peak response) and prior to discharge (T_6; i.e. 5 times altogether). Saliva will be taken at baseline and at T_3. Participants also will complete hourly measures of symptoms of sickness (e.g., fatigue, pain), depression, and anhedonia. Two hours post-injection at the approximate peak of the inflammatory response, the participants will complete the same MRI scans as at baseline. Subject to the judgment of a physician, the participants will be discharged 6 hours post injection so that the total experimental procedure will take ~ 8 hours to complete. Subsequently, two follow-up visits will be conducted one day and one week after Visit 2. These interviews will include the C-SSRS, PHQ-9, ISI, MADRS, a modified sickness scale which includes questions about alcohol and medication use, a blood draw and saliva sample collection. If the participant is unable to return in person, they will be interviewed by phone.

3.2. Study Design Rationale

LPS (as opposed to other inflammatory stimuli such as typhoid vaccination) is used to induce an inflammatory response because (a) it elicits a robust, reliable, and temporally well-characterized immune response that peaks 2-3 hours after administration, (b) there is an extensive preclinical literature on endotoxin-induced anhedonia [27] and (c) it has been shown to induce central microglial activation in both non-human primates [74] and humans [67] at the doses used herein.

Participants will be stratified by CRP score because individuals with chronic inflammation display priming of the innate immune system such that macrophage and microglia cells respond more vigorously to a secondary inflammatory stimulus [75,76]. It is thus plausible that the high CRP group will be more sensitive to the depressogenic effects of endotoxin, a hypothesis that will be tested here.

The placebo groups are necessary to control for non-specific effects. For instance, Dr. Irwin has demonstrated that ~5-10% of healthy participants show elevations in depressive symptoms after saline administration [44]. In the context of a within-subject design in which participants receive endotoxin on one occasion and saline on another, it is clear to most participants when they are receiving endotoxin vs. placebo, and thus a between-subjects design will be used to ensure control for placebo-related confounds.

All participants in this study will be free of psychiatric medication for at least 3 weeks (6 for fluoxetine) or will be taking a stable dose of only one type of anti-depressant medication.

4. STUDY POPULATION

4.1. General Considerations

A total of 150 human participants will be involved in the proposed research – 50 healthy controls and 100 depressed patients. The participants will be recruited through the Screening protocol WIRB # 20101611. Participants will be unmedicated or medicated males or females between the ages of 18 and 65 with symptoms of depression (mild to severe) in the case of the depressed group. To enhance generalizability and maximize the clinical diversity of the study, depressed participants will be included based on a threshold score on the PHQ-9 that is indicative of mild depression. A clinical diagnosis with the MINI (using WIRB 20101611) will be performed to obtain a DSM-V diagnosis for secondary analyses.

4.2. Inclusion criteria

Both healthy controls and depressed participants will be required to be in good general health (as evaluated during Visit 1, including EKG) and to be 18-65 years of age. Depressed participants will be required to have symptoms of depression (i.e. a PHQ-9 score ≥10). Half the depressed participants (N=50) will be required to have a high-sensitivity C-Reactive Protein (CRP) score of ≥3 mg/L, and half the participants will be required to have a CRP score of ≤1 mg/L.

4.3. Exclusion criteria

General Exclusion Criteria:

- Pregnancy
- Previous history of fainting during blood draws.

Medical Conditions:

- A history of a head injury with loss of consciousness.
- Presence of co-morbid medical conditions not limited to but including cardiovascular (e.g., history of acute coronary event, stroke) and neurological diseases (e.g., Parkinson's disease), as well as pain disorders.
- Presence of co-morbid inflammatory disorders such as rheumatoid arthritis or other autoimmune disorders.
- Presence of an uncontrolled medical condition that is deemed by the investigators to interfere with the proposed study procedures, or to put the study participant at undue risk.
- Presence of chronic infection that may elevate pro-inflammatory cytokines.
- Presence of an acute infectious illness or receipt of a vaccination in the two weeks prior to an experimental session.
- Positive SARS CoVID-19 antigen test

Psychiatric Disorders:

- Current severe suicidal ideation or attempt within the past 12 months.
- Psychosis
- Bipolar disorder
- Substance abuse or dependence within the previous 6 months
- Age of onset of depression >40 years

Contraindications for MRI:

- Severe claustrophobia
- Bodily implants of unsafe paramagnetic materials such as pace-makers and aneurysm clips.

Medications:

- Current and/or past regular use of hormone-containing medications (excluding contraceptives)
- Current use of non-steroid anti-inflammatory drugs that is deemed by the investigators to potentially confound the results of the study
- Current and/or past regular use of immune modifying drugs that target specific immune responses such as TNF antagonists
- Current use of analgesics such as opioids or history of addiction to opioids or other analgesics
- Current and/or past regular use of cardiovascular medications, including antihypertensive, antiarrhythmic, anti-anginal, and anticoagulant drugs (does not apply where medications are taken for different purpose e.g. anti-hypertensives for migraine).
- Evidence of recreational drug use from urine test.
- Lifetime use of methamphetamine

Health Factors:

- BMI > 35 because of the effects of obesity on pro-inflammatory cytokine activity
- Clinically significant abnormalities on screening laboratory tests
- Abnormal EKG
- In addition, participants who on arrival to the study, show any of the following symptoms will not be allowed to complete the study:
    (a) screening supine systolic blood pressure >140 mmHg or <100 mmHg
    (b) screening supine diastolic blood pressure >90 mmHg or <60 mmHg
    (c) 12-lead EKG demonstrating a PR interval > 0.2 msec QTc >450 or QRS >120 msec (Bazett) If the QTc exceeds 450 msec, or QRS exceeds 120 msec, the EKG will be repeated 2 more times and the median value will be used
    (d) pulse less than 50 beats/minute or greater than 100 beats/minute
    (e) temperature greater than 99.5°F.

Non-English speaking participants:

- The majority of the assessments proposed for this study have not been translated from English, thus, non-English speaking volunteers will be excluded.

4.4. Gender / Minority / Pediatric Inclusion for Research

Women and minorities will be included in the study without prejudice according to their representation in the study population. Adult subjects will be recruited from the greater Metro Tulsa area and should thus share the racial and ethnic composition of this area. All efforts will be made to ensure that our subject population closely resembles the gender, ethnic, and racial composition of the greater Tulsa area.

Children under age 18 will not be included in this protocol.

5. TREATMENT ALLOCATION AND BLINDING

Individuals who are eligible for the study will be randomly assigned based on a computer-generated randomization schedule prepared by LIBR. It will assign low- and high-CRP participants to placebo or endotoxin groups through the use of a block randomization scheme to ensure the inclusion of equal numbers of participants with low and high CRP in each group. HC will be randomized to LPS or placebo in a 1:1 ratio regardless of their CRP concentrations.

Two different group blinding codes will used. One code will be assigned to the LPS and placebo treatments, and one code to the high and low CRP groups. These codes will be available to the investigators for participant assignment. Blinding codes will not be broken except in medical emergencies when LIBR clinical or medical staff requires knowledge of the intervention for proper management of the participant. The key to the code will be held by 2-3 staff members at LIBR who are not involved in the study. After completion of the study, blinding codes will not be broken for analyses until all participants' data have been evaluated for inclusion and documented.

6. DOSAGE AND ADMINISTRATION

6.1. Dosage

Study drug dosage is dependent on body weight (0.8 ng/kg), which will be recorded on Visit 1 and double-checked on Visit 2. The individualized preparation of the LPS or normal saline will be performed under sterile conditions by staff at All Saints Home Medical pharmacy which is affiliated with Saint Francis Hospital. NIH will provide endotoxin (LPS) that is called LOT 94332B1 manufactured by LIST Biological Laboratories (E.coli O:113) under contract with the Clinical Center at NIH. It is GMP grade material suitable for Phase I trials in humans. LPS has been shown to be safe for studies of experimental inflammation in humans at the doses proposed herein [34,44,77]. Normal saline solution will be obtained from All Saints Home Medical pharmacy.

6.2. Administration

Intact vials of LPS will be stored in the refrigerator (2˚C - 8˚C; 36˚F - 46˚F) at the All Saints Home Medical pharmacy.

LPS will be received from the NIH in clear glass, 2 mL vial containing a sterile, white, lyophilized powder. Each vial contains 1.0% lactose plus 0.1% polyethylene glycol 6000 (PEG6000). The quantities of LPS are: 1 mcg/vial (white vial label) or 10,000 ± 5,000 EU/vial. Vials will be reconstituted with 1 mL of sterile water for injection. Since the endotoxin does not go into solution readily, though it appears to be dissolved, the vials will be shaken for 15 minutes on a vortex shaker or a Metamix 100 (or equivalent). When reconstituted with 1 mL of water, the concentration of lactose will be 0.5%, PEG6000 will be 0.05%, and endotoxin will be 1 microgram/mL.

LPS will be administered at a dose of 0.8 ng/kg of body weight. Saline solution (0.9%) will be administered in the same volume.

A nurse will insert a catheter intravenous line with a heparin lock into a forearm for blood draws and a continuous saline flush and one into a forearm for LPS or saline administration. The administration of the LPS or saline will be performed at the Laureate Institute of Brain Research, 6655 S. Yale Ave, Tulsa OK, 74136.

Laboratory description: The experimental setup involves the intravenous delivery of bolus infusions of LPS or normal saline. All participants are informed they may receive LPS or normal saline during the experiment, but that neither they nor the experimenter will know the identity of any particular infusion. All participants' physiological parameters will be continuously monitored during the infusions (single-lead ECG to provide heart rhythm and heart rate), and vital signs will be measured before and after each infusion session. All participants are pre-screened with a 12 lead EKG, urine pregnancy (when applicable) and drug screening, vital sign measurement and a careful medical history (including medication use, family history of cardiovascular disease) is taken and a physical exam is performed to evaluate for the presence of exclusionary conditions. On the day of testing, several of these measures are again repeated (urine pregnancy, urine drug screening, vital sign measurement).

7. TREATMENT COMPLIANCE

The study drug will be administered by LIBR clinical staff.

8. PRESTUDY AND CONCOMITANT THERAPY

Treatment of Physical Symptoms

If any physical symptoms are particularly distressing, they will be relieved by acetaminophen, aspirin or ibuprofen.We have had to administer acetaminophen on one occasion thus far (out of approximately 50 subjects). Treatment of physical symptoms will be documented.

9. STUDY EVALUATIONS
9.1. Study Procedures
9.1.1. Overview

The study is divided into 4 phases: the general LIBR screening process performed through a different protocol in which preliminary eligibility for the study is established, a secondary Screening/Safety Phase specific to this study in which eligibility is confirmed - (Visit 1), an Experimental Phase (Visit 2), and a Follow-up Phase (Visits 3/4). During Visit 1, prospective participants will have a physical exam, EKG, a baseline MRI scan, and blood drawn for safety labs (CBC, CMP, HbA1C, HCV, HIV, TSH). Participants will be tested for SARS COV-2 at either the general screening session or at visit 1. In the case of a negative test, SARS CoVID-2 testing will be repeated prior to the infusion of LPS or saline at visit 2. During Visit 2, baseline assessments, an EKG assessment, and a blood draw are performed and subsequently study drug or placebo is administered. Thereafter participants undergo several vital sign, EKG, mood, neurocognitive, and clinical assessments, and blood draws. An MRI scan also is performed. The Time and Events Schedule summarizes the frequency and timing of the assessments, EKGs, blood draws, and MRI scans.

Visit	# Blood Tubes	# ml of Blood*	# TBSP of blood *
Visit 1	7	46	3
Visit 2	25	220	15
Visit 3	5	44	3
Visit 4	5	44	3
Total for All	42	354	24
		*approximately	*approximately

At visit 2 participants may be asked to provide a baseline saliva sample T_{-1}, a second sample 3 hours post-administration of LPS/placebo, as well as another saliva sample at visit 4.

9.1.2. Screening Phase (Pre-screening and Visit 1)

Recruitment and Consent Procedures

The participants will be recruited through the WIRB # 20101611 (see appendix).

Subsequent to general LIBR screening, prospective participants who pass first-round screening will be informed about the LPS study, in particular the fact that they may be exposed to a bacterial toxin that could induce mild flu-like symptoms and that they will have multiple blood draws. Individuals will be asked if they are still interested. If interested, participants will then be scheduled for Visit 1, an in-person screening session in which informed consent will be obtained, an EKG and physical exam will be performed, blood will be drawn for

additional screening tests, and a baseline MRI scan obtained. Saliva and/or a nasal swab may be used to test for evidence of active viral infections. The participants will be informed regarding the study procedures and the potential side effects of LPS. Participants will be told their participation is completely voluntary and that they are free to discontinue their participation at any time. After reading the consent form, each participant will be encouraged to ask any questions that they may have about the procedure. Each participant will be asked if they are comfortable that they understand the procedure to their full satisfaction. Under no circumstances will coercion be applied to obtain informed consent, and participants will be thanked for their participation in the study regardless of whether they choose to continue in the main study. In the event that the participant has signed the consent, but wishes to terminate the study at any time before completion they will be allowed to do so. Dr. Savitz, a trained member of Dr. Savitz's lab, or the LIBR assessment team will conduct the informed consent.

9.1.3. Treatment Phase (Visit 2)

Visit 2 procedures and assessments are specified in the Time and Events Schedule. Briefly, prospective participants will have a complete a series of blood draws, mood rating scales, neuropsychological tests, an MRI scan, and will receive either endotoxin or normal saline.

Measures to decrease coercion of participants

The researcher will remind the subject that participation is strictly voluntary and remind them that they have the right to withdraw at any time without penalty. Family members will be allowed to be present and discuss the consenting process with the participant if requested.

9.1.4. Follow-up Phase (visits 3 and 4)

The follow-up phase will include two visits or telephone interviews (if participant is unavailable to return) at one day and one week after Visit 2 to assess suicidality (C-SSRS), depression (PHQ-9, MADRS, VAS, SHAPS), physical symptoms, sleep disturbance, questions about alcohol and new medication usage (modified sickness scale), and one blood draw per visit. A saliva sample also will be collected at visit 4.

9.2. Screening Evaluations

9.2.1. Psychiatric and Medical Screening

An unstructured psychiatric interview and the MINI International Neuropsychiatric Interview (M.I.N.I.) will be administered as part of the determination of eligibility. Subjects will be asked a series of standardized questions about psychiatric or medical symptoms they have experienced during their lifetime. These assessments will be obtained through the LIBR Screening protocol WIRB # 20101611. At visit 1, prospective participants will have a physical exam administered by a RN. They also will complete a drug and alcohol screen, an EKG, a CBC, comprehensive metabolic panel, HbA1C, and TSH test to in order to exclude individuals with medical conditions that may increase the risk of LPS-induced side-effects. They will complete additional psychiatric rating scales.

Female participants will undergo urine-screening pregnancy tests at Visit 1 and again prior to administration of endotoxin/placebo at Visit 2. The subject and a member of the clinical assessment team will "read" the pregnancy tests.

9.2.2. MRI Safety Screening

An MRI safety-screening questionnaire will be completed by each subject immediately upon completion of the consent form. The MRI screening questionnaire is developed and distributed by the Institute for Magnetic

Resonance Safety, Education, and Research (IMRSER) in Los Angeles, CA*. IMRSER is a non-profit organization sponsored by major MRI-related corporations, including GE (the manufacturer of LIBR's 3T magnet), to "disseminate information regarding current and emerging MR safety issues" and "to develop and provide materials and resources to facilitate MR safety- related education and training". The safety-screening questionnaire probes for possible occupational exposure to metal slivers or shavings (remnants of which may remain lodged in a subject's head or neck), surgical clips or shrapnel, cochlear implants, or any other form of ferrous metal implanted in or on the subject's body. Subjects answering in the affirmative to any of these conditions will be excluded. All subjects with any form of implanted wires, metal, or electronic devices will be excluded. All persons involved in this protocol will receive MR safety training conducted at the Laureate Institute for Brain Research by their MR safety officer.

*INSTITUTE FOR MAGNETIC RESONANCE SAFETY, EDUCATION, AND RESEARCH 7511 McConnell Avenue, Suite 100, Los Angeles, CA 90045, http://www.imrser.org

Although there are no known risks of MR to pregnant women, there may be unknown risks. Therefore, females who are pregnant will not participate in this study. To ensure that female subjects are not pregnant at the time of the MRI procedure, all female participants will undergo a standard urine-screening pregnancy test prior to participation in any MRI scanning. Subjects will then receive the safety-screening questionnaire.

9.3. Pharmacodynamic Evaluations

9.3.1. MRI

Overview

MRI scans will be acquired on a 3-tesla GE Discovery MR750 scanner (GE Medical Systems) and 32 channel phased-array coil system (Nova Medical).

All MRI scanning in this protocol will be non-invasive (i.e. no intravenous or other forms of external contrast agents will be used) and subjects will be provided with earplugs as protection from scanner sounds. Subjects will be placed in the magnet with the investigator ensuring that the subject is both comfortable, able to fully see the stimuli, able to comfortably manipulate any required response apparatus, and trained to use the scanners emergency call button. The emergency call button is a hand-held response apparatus that allows the patient to request the investigator/MR Technologist's immediate attention. Each of the two MRI scanning sessions (T_0 and T_2) will last for an hour.

Scans

Each MRI session will last for approximately one hour and may include the following scans:

- Clinical Scans: T2-weighted FLAIR images that are sensitive to CNS and white matter abnormalities.
- A T1-weighted magnetization-prepared rapid gradient-echo sequence will be collected for anatomical reference.
- fMRI: Resting State to measure functional connectivity.
- fMRI: Monetary Incentive Delay Task to measure anticipated reward.
- fMRI: Interoceptive Attention Task to measure interoceptive attention.
- DTI to obtain measures of white matter integrity and free water content.

Imaging Preparation

At the beginning of any scan session a series of 15-second MRI localizer scans will be obtained in order to prescribe locations of the subsequent anatomical and functional scans. Prior to task-relevant functional scans,

subjects will be reminded of the task instructions. During the periods between scanning runs the subject's comfort level will be assessed and instructions will again be provided.

Physiological Monitoring

Psychophysiological (heart rate, respiration, skin conductance, eye blink electromyogram) may be acquired concurrently with fMRI, allowing for enhanced physiological noise and movement correction.

9.3.2. Behavioral Tasks

The Monetary Incentive Delay (MID) task is one of the "gold standard" measures of neural response to monetary reward and loss in the context of depression [78-80] and previously has been demonstrated by Dr. Irwin to be sensitive to the inflammatory effects of endotoxin in healthy subjects [44] (Figure 3). Gain cues elicit positive affect that increases activity in the VS while loss cues elicit negative affect that increases activity in the anterior insula and medial caudate [81]. Further, in subjects tested twice over 2 years, the MID showed significant test-retest reliability with intra-class correlations >0.50 [82]. Nevertheless, inconsistent results have been reported in depressed populations [80,83,84], raising the possibility that only a specific subtype of depression is characterized by a deficient neural response to anticipatory reward.

The Interoceptive Awareness Task (IAT) was developed by Dr. Simmons at LIBR and has been shown to effectively map interoceptive regions in the insula in healthy controls [85], and to distinguish depressed subjects from healthy controls [52]. Specifically, relative to controls, depressed subjects exhibited a decreased BOLD response of the insula when focusing on their heart beats, and this activity was negative correlated with depression and somatic symptoms severity [52].

The EEfRT task is a multi-trial game in which participants are given an opportunity on each trial to choose between two different task difficulty levels in order to obtain monetary rewards [86]. The task was developed by Michael Treadway at Emory and is currently being used by over 70 different research groups. Treadway et al. have shown that administration of the DA agonist, *d*- amphetamine produces a dose-dependent increase in motivation for rewards as assessed by the EEfRT, a finding that parallels the animal literature [87,88]. For all trials, participants make repeated manual button presses within a short period of time. Each button press raises the level of a virtual ''bar'' viewed onscreen by the participant. Participants are eligible to win the money allotted for each trial if they raise the bar to the ''top'' within the prescribed time period. Each trial presents subjects with a choice between two levels of task difficulty, a 'high- effort' and 'low-effort' task that require different amounts of speeded button pressing. Reward magnitudes for the high effort task vary between $1.24 and $4.33, while reward magnitudes for the low effort task remain constant ($1.00). Trials also vary in terms of 3 levels of probability of winning the amount associated with the choice selected. Subjects participate in the task for 20 minutes and the first 50 trials are used for analysis. For statistical analyses the proportion of hard-task choices across each level of probability is calculated. Lower proportions of hard task choices indicate decreased motivation for monetary rewards. The task has excellent test-retest reliability (test-retest r > 0.85), and has been successfully used in prior multi-session studies [89,90].

The Finger Tapping Task uses a specially adapted tapper that the subject taps as fast as possible using the index finger. The subject is given 5 consecutive 10-second trials for the preferred and non- preferred hands. The finger tapping score is the mean of 5 trials and is computed for each hand. Performance norms have been established, and scores have been shown to be stable over time [91]. The FTT is designed to assess subtle motor impairment and is altered in subjects with basal ganglia disorders and lesions [92].

The Digit Symbol Task is a subtest of the Wechsler Adult Intelligence Scale (WAIS) and consists of rows of blank squares, each printed with a randomly assigned number. The test involves graphimotor speed, visual scanning and memory, with about half of the variance being accounted for by graphimotor speed, a third by visual scanning and 4-5% by memory [93]. This test is one of the most frequently used in neuropsychology and

relevant norms and test-retest reliability have been well established [91]. Performance on the DST has been found to correlate with subcortical atrophy in disorders involving the basal ganglia including Huntington's disease and multiple sclerosis [94,95].

A wearable Fitbit Ionic device may also be used to track a variety of metrics including step count, floors climbed, distance, calories burned, active minutes, sleep time and stages, and heart rate. The device will be worn continuously on the subject's wrist. Data collected from the Fitbit Ionic device will be stored with code numbers on Fitabase, a cloud-based data management platform.

9.3.3. Questionnaires

Measures of psychiatric health and mood will include common measures of suicidal ideation, emotional wellbeing, social adjustment, anhedonia, cognition, sleep, and screeners for psychiatric illness (Please see Assessment/Activity list in Time and Events Schedule).

9.4. Pharmacodynamic Evaluations

CRP will quantified with an immunoturbidometric assay performed with an FDA-cleared, CE compliant, point of care platform – the Diazyme SMART. The coefficient of variation is <10% and the lowest level of quantification (LLOQ), 0.47 mg/L. Cytokines such as IL-6 and TNF will be measured by high-sensitivity multiplexed EIA technology, i.e. the Meso Scale Discovery (MSD) MESO QuickPlex SQ 12 instrument and V-PLEX assay kit, which has recently been shown to be superior to Cytometric Bead Array (CBA) multiplex cytokine assay platforms with respect to accuracy of quantification [96].

9.5. Biomarkers

The blood samples may be used to obtain immune cells, and serum for various immune and metabolic assays such as CRP, cytokines, cellular function assays, flow-cytometry, autoantibodies, kynurenine metabolites, glucocorticoid and TLR4 receptor function and expression, and inflammatory gene expression.

9.6. Pharmacogenomic Evaluations

Genomic Storage for Future Research

The blood and saliva samples may be stored and used to obtain DNA for genotyping and epigenetic assays associated with this study.

9.7. Sample Collection and Handling

A nasal swab may be collected and used to test for evidence of active viral infections. Samples collected for this procedure will be discarded immediately after use. Blood samples will be collected in appropriate Vacutainer tubes and transported to the University of Oklahoma Integrative Immunology Center (IIC) laboratories. Saliva samples also will be sent to IIC for storage. Processing of the plasma, serum, and PBMCs will be performed by staff at the IIC less than two hours after collection. Several aliquots of plasma and serum will be stored in secured -80ºC freezers that are kept on emergency backup power and have automated alarms for temperature fluctuations. PBMC aliquots will be stored in a secure room with liquid nitrogen-filled dewars equipped with liquid level monitors and alarms.

10. SUBJECT COMPLETION / WITHDRAWAL

10.1. Completion

Participants will be considered to have completed visit 2 when a medical doctor evaluates and discharges the participant. Participants will be considered to have completed the study when visit 4 has been completed.

10.2. Discontinuation of Study

Individual Safety Limits: The protocol will be stopped for any of the following reasons:

- At least one symptom at Grade 3 level (severe) listed in tables 1-4, below.

Group Safety Limits:

We will halt the study, submit a report to the Western IRB and DSMB, and reassess the dosage if:

- Two or more individuals each experience at least one symptom of Grade 3 (severe) symptoms in tables 1-4, below. A grade 3 symptom/sign listed in tables 2-4 will be designated as an adverse event if it is still present six hours post administration (at typical discharge time).

- If there are two or more serious adverse events possibly, probably or definitely related to endotoxin administration.

- If one or more subjects report clinically significant suicidal ideation, undergo psychiatric hospitalization, and/or display psychosis or other serious psychiatric events following administration of endotoxin

If we have one participant who experiences a Grade 4 (potentially life-threatening) symptom, the entire study will be stopped until evaluation by Western IRB, DSMB, FDA, and NIH.

If the participant discontinues the study they will be monitored until MD discharge is granted. Further, we will arrange follow-up visits (or telephonic interviews) at one-day and one-week post administration of LPS/saline.

10.3. Withdrawal From the Study

A participant will be withdrawn from the study if they withdraw consent or if a study physician believes that it is the interest of the participant to withdraw.

11. STATISTICAL ANALYSIS PLAN

11.1. Statistical Hypotheses

Aim 1: To delineate a multi-level (symptoms, behavior, neural circuits, and immune makers) phenotype of an inflammatory subtype of MDD thereby identifying the biological pathways associated with affective sensitivity to LPS.

Hypothesis 1.1. Relative to the low CRP group (n=50), the high CRP group (n=50) will show higher levels of anhedonia at baseline. Relative to the two placebo groups, the two LPS groups will show higher levels of anhedonia. The high CRP group will be differentially sensitive to LPS, showing a greater LPS-induced increase in anhedonic symptoms than the low CRP group receiving LPS.

Analysis: Linear mixed effects model with time (T_0 through T_6), CRP group (high vs. low), and condition (LPS vs. saline) as factors, time*group*condition as the primary interaction term of interest, SHAPS score as the dependent variable, and sex and PHQ-9 score at baseline as covariates.

Hypothesis 1.2. Relative to the low CRP group, the high CRP group will show higher levels of IL-6 and TNF at baseline. Relative to the two placebo groups, the two LPS groups will show higher levels of IL6 and TNF. The high CRP group will display a greater LPS-induced increase in IL-6 and TNF than the low CRP group receiving LPS.

Analysis: Two separate linear mixed-effects models with time (T_0, T_2, T_3, T_6), CRP group (high vs. low) and condition (LPS vs. saline) as factors, time*group*condition as the primary interaction term of interest, IL-6 or TNF as the dependent variable, and sex and PHQ-9 score at baseline as covariates.

Hypothesis 1.3. Relative to the low CRP group, the high CRP group will show reduced activity of the VS in response to anticipated reward vs. no reward at baseline. Relative to the two placebo groups, the two LPS groups will show a reduced BOLD response to anticipated reward at two hours post-LPS challenge (T_2). The high CRP group will display a greater LPS-induced decrease in VS BOLD activity than the low CRP group receiving LPS.

Analysis: Method 1. For each individual, beta-weights for the average activity across the VS as a whole will be extracted at T_0 and T_2. Subsequently, a linear mixed effects model will be used for the analysis with time (T_0 and T_2), CRP group (high vs. low), and condition (LPS vs. saline) as factors, time*group*condition as the primary interaction term of interest, beta-weights for the average activity across the VS as the dependent variable and sex and PHQ-9 score as covariates. Method 2. The AFNI program 3dANOVA3 will used to evaluate the main effects of condition (LPS vs. saline) and group (high vs. low CRP) and their interaction on both striatal and voxel-wise activity. Sex and PHQ-9 scores will be used as covariates. Additionally, the magnitude of the increase in cytokine levels (from baseline to 2h post-injection) will be entered as regressors into a random-effects, whole-brain group analyses (for those in the LPS condition only) in order to identify the neural circuitry associated with cytokine-induced alterations of anticipated reward. For the whole-brain analyses, statistical contrast maps will be corrected using the false-discovery rate (FDR) method [97]. A voxel-wise threshold of $p<0.005$ will be set within the VS, corrected for multiple comparisons at a cluster-size threshold of $p<0.05$ with 3dClustSim.

Hypothesis 1.4. Relative to the low CRP group, the high CRP group will show reduced activity of the insular cortex during interoception vs. exteroception at baseline. Relative to the two placebo groups, the two LPS groups will show a reduced BOLD response during interoception at T_2. The high CRP group will display a greater LPS-induced decrease in insular BOLD activity than the low CRP group receiving LPS.

Analysis: Method 1. For each individual, beta-weights for the average activity across the insula will be extracted at T_0 and T_2. Subsequently, a linear mixed effects model will be used with time (T_0 and T_2), CRP group (high vs. low) and condition (LPS vs. saline) as factors, time*group*condition as the primary interaction term of interest, beta-weights for the average activity across the insula as the dependent variable, and age, sex, and PHQ-9 score as covariates. Method 2. 3dANOVA3 will used to evaluate the main effects of condition (LPS vs. saline), group (high vs. low CRP) and their interaction on both insular and voxel-wise activity. Age, sex, and PHQ-9 scores will be used as covariates. Additionally, the increase in cytokines (from baseline to 2 h post- injection) will be entered as regressors into random-effects, whole-brain group analyses (for those in the LPS condition only) in order to identify the neural circuitry associated with cytokine-induced alterations of interoception. Statistical thresholds are as described in Aim 1c. Note that for analyses 1 A-D we will not use BMI as a covariate since its mean value differs non-randomly across the target group of interest (i.e. high vs. low CRP) making it an inappropriate covariate [98]. In contrast, sex will be used as a covariate because of evidence that females are more sensitive to the effects of LPS than males [34,99].

Aim 2: To examine whether HCs and MDD participants differ in their acute and prolonged immunological, and neural responses to LPS vs. saline (placebo).

Hypotheses 2A-C: There will be a diagnosis*drug interaction such that relative to the HC LPS group, the MDD LPS group will display increased cytokine production (T_2-T_0), a blunted response of the insula and cingulate

during internally-focused attention (V_2-V_1), and a relative reduction in connectivity between the insula and the cingulate cortex at rest (V_2-V_1).

Analysis: General linear model (GLM) with diagnosis (MDD vs. HC) and drug (LPS vs. saline) as factors, diagnosis*drug as the primary interaction term of interest. Age, sex, BMI, and where applicable, head motion, will be evaluated as covariates using Bayesian Information Criterion (BIC). Dependent variables are shown in table 2. Exploratory outcomes at different time points will be tested using linear mixed-effects models (LMM). Within-subject dependency will be captured by random subject intercept and/or slope if the response pattern appears to be linear with time and/or within-subject correlation structures.

Aim 3: To identify epigenetic predictors of response to endotoxin in the MDD group.

Hypothesis 3.1: Individuals with reduced methylation of the promoter regions of both the IL-6 and TNF genes will show greater expression (gene and protein) of IL-6 and TNF both at baseline and subsequent to endotoxin challenge.

Analysis: Linear regression with percent methylation in the IL-6 and TNF gene promoter regions as the independent variable and mRNA expression levels and protein concentrations with dependent variables.

11.2. Sample Size Determination

Statistical Power: No data are available on the effect sizes of the hypothesized phenotypic differences between depressed subjects with high and low CRP at baseline or after LPS. With regard to healthy subjects receiving LPS, Irwin et al. demonstrated a 3-fold increase in depressive symptoms measured with the POMS and 50-150 fold increase in concentrations of IL-6 and TNF vs. baseline (total n=40) [34,49] indicating effect sizes (Cohen's d) of ~ 0.6 and 1.2, respectively. Regarding the MID task, Irwin and colleagues showed a decreased BOLD response in the left VS during anticipated reward in LPS (n=23) vs. placebo (n=16) groups with an effect size (*d*) of ~0.7 (for ROI analysis) [44] while the IAT was sensitive to differences in insula activity during interoception in 20 MDD subjects vs. 20 healthy controls, also with an effect size of ~0.7 [52]. Thus assuming that the results obtained in healthy individuals can be extrapolated to depressed populations, our pilot study sample should be large enough to adequately test the hypotheses specified above.

11.3. Pharmacodynamic Analyses

<u>Neuroimaging</u>

All fMRI data analyses will be conducted with Analysis of Functional Neuroimaging (AFNI) software. At the subject-level, fMRI data pre-processing will include slice-time correction, motion correction by registering all time points to the first volume of the first functional run, spatial normalization to Talairach space, smoothing with a 6mm FWHM Gaussian kernel (approximately twice the voxel size to optimize statistical power [100]), and scaling of signal intensity at each voxel to reflect percent signal change from the voxel's mean. Multiple regression will be used to analyze individual subjects' data, with predictors in the model constructed by convolving each column of the task design matrix with a canonical hemodynamic response function. Regressors of non-interest will be included in all models to account for each run's signal mean, linear, quadratic, and cubic signal trends, as well as motion parameters.

Voxelwise statistical tests of group differences will use mixed-effects modeling on aggregations of the maps of the subjects' beta-weights and standard deviations of the estimate of their beta-weights (AFNI's *3dMEMA*). This approach has the advantage of taking into account in the group analysis *both* effect estimates as well as their within- and between-subjects variances [101]. In the case of statistically significant results from a mixed-effects analysis that includes more than two groups, subsequent *post-hoc* tests of the simple effects will be conducted with random-effects two-sample t-tests to determine which comparisons are driving the omnibus effect. Sex and PHQ-9 score will be included as covariates in the analyses. Corrections for multiple

comparisons will be implemented in two ways. First, we will use independently defined regions of interest (ROI)-based analyses with small volume-corrected statistical thresholds of p<0.05 within the ventral striatum (VS) (MID task) and insula (IA task). Masks of the insula and VS in each brain hemisphere will be anatomically defined using the TT-N27 atlas brain within AFNI. Secondly, within the VS and insula, Monte Carlo simulations implemented in AFNI's *3dClustSim* will be used to identify the required cluster-size threshold, given a voxel-wise probability of *p* <.001 and the volume of the ROI.

11.3. Safety Analyses

Adverse Events

Participants that discontinue the study due to an adverse event will be recorded and summarized by treatment group.

12. ADVERSE EVENT REPORTING

12.1. Definitions

12.1.1. Adverse Event Definition and Classifications

Adverse Event

Adverse event means any untoward medical occurrence associated with the use of a drug in humans, whether or not considered drug related (definition per FDA, CFR Title 21 312.32).

Reportable Adverse Event

An adverse event is considered reportable if all three following criteria are met:

- The adverse event is unexpected.
- The adverse event is related or possibly related (a reasonable possibility) to participation in the research.
- The adverse event suggests the research places participants or others at a greater risk of harm or discomfort than was previously known or recognized.

12.2. Procedures

Reporting mechanisms for adverse events to the IRB: Any reportable adverse event or deviation will be reported within one business day to the Laureate Institute for Brain Research Human Protection Administrator at (918) 502-5155 or via email at hpa@laureateinstitute.org. A full report of the reportable adverse events and/or deviation will be reported to the Western IRB within 5 business days.

Stop/Holding Rules:

Grade 3 symptoms are severe, clinically significant symptoms that substantially interfere with normal activity and do not resolve by 6 hours post-endotoxin including: chills, headache, malaise, fatigue, myalgia, nausea with vomiting, dizziness, and/or inability to take fluids by mouth.

If at least one Grade 3 symptom (tables 1-4) occurs that is related to the protocol (i.e., occurring during the protocol period), then an Adverse Event form will be completed and we will notify the DSMB Safety Officer (Dr. Robert Dantzer) as well as the Western IRB within 5 working days. A grade 3 symptom/sign listed in tables 2-4 will be designated as an adverse event if it is still present six hours post administration (at typical discharge time). No action will be taken to halt the study unless the DSMB Safety Officer requests that the study be halted until formal review.

If two or more individuals each experience at least one of the above symptoms at a Grade 3 severity during the protocol period, we will halt the study until review of the Grade 3 Adverse Events by the DSMB and WIRB. Following review by the DSMB, the study could be re-instated with revision of the inclusion/exclusion criteria, protocol procedures, dosage of endotoxin.

Grade 4 symptoms (tables 1-4) are those Adverse Events that are life-threatening and occur during the protocol period. Precautionary hospitalization for the monitoring of a severe symptom will not be classified as a grade 3 event if it is not life-threatening. If we have one participant who experiences a life-threatening symptom at a Grade 4 severity--life threatening, the entire study will be stopped until evaluation by regulatory bodies. We will notify the DSMB Safety Officer (Dr. Robert Dantzer) as well as the DMSB, WIRB, FDA, and NIH within 24 hours.

Table 1. Clinical Abnormalities: Local Reaction to Injectable Product

	Mild (Grade 1)	**Moderate (Grade 2)**	**Severe (Grade 3)**	**Potentially Life Threatening (Grade 4)**
Pain	Does not interfere with activity	Repeated use of non-narcotic pain reliever > 24 hours or interferes with activity	Any use of narcotic pain reliever or prevents daily activity	Severe, potentially life-threatening reaction that warrants hospitalization
Tenderness	Mild discomfort to touch	Discomfort with movement	Significant discomfort at rest	Severe, potentially life-threatening reaction that warrants hospitalization
Erythema/Redness *	2.5 - 5 cm	5.1 - 10 cm	> 10 cm	Necrosis or exfoliative dermatitis that requires hospitalization
Induration/Swelling **	2.5 - 5 cm and does not interfere with activity	5.1 - 10 cm or interferes with activity	> 10 cm or prevents daily activity	Necrosis that requires hospitalization

* In addition to grading the measured local reaction at the greatest single diameter, the measurement should be recorded as a continuous variable.
** Induration/Swelling should be evaluated and graded using the functional scale as well as the actual measurement.

Table 2. Clinical Abnormalities: Vital Signs

	Mild (Grade 1)	**Moderate (Grade 2)**	**Severe (Grade 3)**	**Potentially Life Threatening (Grade 4)**
Fever (°C) ** (°F) *	38.0 – 38.4 100.4 – 101.1	38.5 – 39.1 101.2 – 102.0	39.2 – 40 102.1 – 104	> 40 > 104
Tachycardia - beats per minute	101 – 115	116 – 130 ****	> 130 ****	Hospitalization for arrhythmia
Bradycardia - beats per minute***	50 – 54	45 – 49 ****	< 45 ****	Hospitalization for arrhythmia
Hypertension (systolic) - mm Hg	141 – 150	151 – 155	> 155	Hospitalization for malignant hypertension
Hypertension (diastolic) - mm Hg	91 – 95	96 – 100	> 100	Hospitalization for malignant hypertension
Hypotension (systolic) – mm Hg	85 – 89	80 – 84	< 80	Hospitalization for hypotensive shock
Respiratory Rate – breaths per minute	17 – 20	21 – 25	> 25	Intubation

* Subject should be at rest for all vital sign measurements.
** Oral temperature; no recent hot or cold beverages or smoking.
*** When resting heart rate is between 60 - 100 beats per minute. Use clinical judgment when characterizing bradycardia among some healthy subject populations, for example, conditioned athletes.
**** accompanied by clinical symptoms (i.e., chest discomfort, acute changes in mental status, shortness of breath). For an episode of bradycardia to be identified as a Grade 3 symptom, it should be sustained despite verbal or manual stimulation.

Table 3. Clinical Abnormalities: Systemic (General)

	Mild (Grade 1)	**Moderate (Grade 2)**	**Severe (Grade 3)**	**Potentially Life Threatening (Grade 4)**
Nausea/vomiting	No interference with activity or 1 - 2 episodes/24 hours	Some interference with activity or > 2 episodes/24 hours	Prevents daily activity, requires outpatient IV hydration	Hospitalization for hypotensive shock
Diarrhea	2 - 3 loose stools or < 400 gms/24 hours	4 - 5 stools or 400 - 800 gms/24 hours	6 or more watery stools or > 800gms/24 hours or requires outpatient IV hydration	Hospitalization for life-threatening dehydration
Headache	No interference with activity	Repeated use of non-narcotic pain reliever > 24 hours or some interference with activity	Significant; any use of narcotic pain reliever or prevents daily activity	Hospitalization for extreme headache in the context of a clinically significant medical condition
Fatigue	No interference with activity	Some interference with activity	Significant; prevents daily activity	Hospitalization for extreme fatigue in the context of a clinically significant medical condition
Myalgia	No interference with activity	Some interference with activity	Significant; prevents daily activity	Hospitalization for myalgia in the context of a clinically significant medical condition
Malaise	No interference with activity	Some interference with activity	Significant; prevents daily activity	Hospitalization for malaise in the context of a clinically significant medical condition
Chills	No interference with activity	Some interference with activity	Significant; prevents daily activity	Hospitalization for chills in the context of a clinically significant medical condition

Table 4. Psychiatric Abnormalities: Mood and Suicidality

	Mild (Grade 1)	**Moderate (Grade 2)**	**Severe (Grade 3)**	**Potentially Life Threatening (Grade 4)**
Mood	Mild increase in anxiety, depression	Moderate increase in anxiety, depression	Severe, clinically significant increase anxiety, depression	Suicidal ideation
Neurological	Mild somnolence, agitation	Moderate somnolence, confusion, disorientation, agitation	Severe, clinically significant somnolence, lethargy, delirium, agitation, hallucinations	Obtundation, coma, seizures, toxic psychosis

13. STUDY DRUG INFORMATION

For nearly two decades, LPS derived from *Escherichia Coli* has been used safely in the United States to examine the effects of experimental inflammation in humans [102-106]. Outside of the United States, researchers have conducted similar studies safely with the use of *Salmonella abortus equi* LPS [107,108]. NIH will provide endotoxin (LPS) that is called LOT 94332B1 (not CCRE) manufactured by LIST Biological Laboratories (E.coli O:113) under contract with the Clinical Center at NIH. It is GMP grade material suitable for Phase I trials in humans.

Risks Associated with the Administration of LPS

A previous study examined the effects of the CCRE LPS in healthy subjects [106]. In this study, twenty healthy participants were randomly assigned to receive either placebo or 1, 2, or 4 ng/kg of CCRE. (*Note: We will be using a dosage of LPS that is much lower than any of these amounts, namely 0.8 ng/kg, posing less of a risk to our participants.)* Results revealed that sickness symptoms (e.g., chills, myalgia, nausea, vomiting) were most prominent with the highest dose of CCRE, although sickness symptoms were also present in the placebo condition (number of sickness symptoms: placebo = 6; 1 ng/kg = 9; 2 ng/kg = 8; 4 ng/kg = 12). An increase in temperature occurred in each of the LPS groups and increased in a dose-dependent manner (placebo = 98.5°F; 1 ng/kg = 100.4°F; 2 ng/kg = 100.8°F; 4 ng/kg = 101°F). Diastolic but not systolic blood pressure showed a modest but statistically significant decrease in the LPS groups (placebo = 66; 1 ng/kg = 63; 2 ng/kg = 59; 4 ng/kg = 66). Heart rate rose marginally in all three LPS groups compared to placebo (placebo = 83; 1 ng/kg = 80; 2 ng/kg = 84; 4 ng/kg = 89). With regard to immune and other physiological variables, the LPS groups, compared to the placebo group, showed increases in total leukocytes, cytokines (interleukin-1 receptor antagonist, interleukin-6, interleukin-8, and tumor necrosis factor), and cortisol levels. There were no clinically significant adverse events, and the authors of the study concluded that CCRE is safe when given to healthy subjects.

For the current proposal, we will be using a low-dose of LPS (0.8 ng/kg) with a 6 hour follow-up so that symptom levels in our participants are minimal and will securely return to pre-LPS levels within the study session in LIBR. In a previous study performed by our collaborator, Dr. Irwin using an LPS dose of 0.8 ng/kg, with a follow-up observation for 6 hours, no clinically significant adverse events in the evaluation of over 150 healthy subjects. The potential risks associated with LPS administration are the discomfort associated with the indwelling catheter (for LPS/placebo administration) and the side effects (physiological and affective) of LPS.

a) *Discomfort due to indwelling catheter.*
The procedures for the indwelling catheter – for the purpose of the blood draws and injection of low-dose LPS/placebo – may be annoying or mildly upsetting to some participants. Prospective participants will be told during the initial phone screening interview and the face-to-face screening that multiple blood draws are part of the protocol (5 blood samplings by IV plus one injection by IV at visit 2), and so any individual for whom this is a highly stressful experience will likely withdraw at that time. Only experienced, registered nurses will draw the blood and administer the injection, which should minimize adverse consequences, such as physical discomfort or lightheadedness. Still, bruising or discoloration of the skin may occur after the catheter has been withdrawn from the skin; this usually resolves within 2-3 days.

b) *Safety concerns regarding the physiological and affective effects of LPS.*
Although we will be administering low doses of LPS (0.8 ng/kg of body weight), participants may experience mild side effects, such as a slight increase in temperature and pulse, reduced blood pressure, or mild nausea. In Dr. Irwin's previous study, using the identical dose of LPS (0.8 ng/kg of body weight), they found significant increases in pro-inflammatory cytokine levels as a function of LPS (Figure 2, above), no significant changes in systolic blood pressure, or diastolic blood pressure, a significant but not clinically meaningful increase in pulse over time (average pulse at peak = 88 beats/minute), and a significant increase in temperature, but not a febrile response (> 38.6°C; Figure 2). In addition, at the time of peak response, participants, on average, reported experiencing "mild" sickness symptoms (e.g., fatigue, nausea) in response to LPS (around a '1' on a scale ranging from 0-no symptoms to 4-very severe symptoms). The

highest level of self-reported sickness symptoms observed in any one subject was “moderate” (a ‘2’). All self-reported symptoms returned to baseline by the end of the study. With regard to affective changes, although subjects exposed to LPS (vs. placebo) have been shown to demonstrate a significant increase in depressed mood,[34,107] Dr. Irwin’s previous work has shown that these changes are mild, with no induction of severe depression or suicidality. In addition, although subjects exposed to LPS showed a significant increase in self-reported feelings of social disconnection, these changes were relatively moderate (with self-reports of feeling “moderately” socially disconnected at the time of peak response; a ‘3’ on a scale ranging from 1-not at all to 5-very much so) and resolved by the end of the study.[109] In order to ensure subject safety, throughout the infusion protocol and for several hours following infusion, subjects will be carefully monitored with repeated vital signs, single-electrode cardiac monitoring, and behavioral observations including clinical assessment of suicidality if identified by self-report (see section on *Minimizing risks associated to the administration of LPS*).

Minimizing Risks Associated to the Administration of LPS.

In order to ensure adequate hydration of subjects, 500cc of normal saline will be administered to the participants prior to endotoxin/placebo administration. We may also administer up to 500cc of additional saline subsequent to endotoxin/placebo administration at the discretion of a clinician in the case of signs and symptoms of hydration. Clinical staff at the LIBR will be educated about all side effects that can occur following LPS, and along with study personnel, will monitor the subjects for any clinically significant behavioral or physical symptoms, including repeated vital signs. For example, nurses involved in the project will be instructed to notify a LIBR physician (Drs. Khalsa or Paulus) if any of the following post-infusion symptoms are observed: (a) temperature greater than 101°F, (b) systolic or diastolic blood pressure drops of 10 mmHg or more from baseline values, or (c) heart rate increases of 15 or more points from baseline (or if heart rate is above 120). In addition, a LIBR physician will be notified if the participant begins to experience any of the following symptoms to a degree that they interfere with participant’s normal activities: chills, myalgia, headache, nausea, shortness of breath, or inability to tolerate fluids by mouth. Symptoms are time-limited and if present, are likely to be most prominent for two to three hours after the LPS dose and will then lessen considerably by six hours after the dose is given. If any symptoms are particularly distressing, they will be relieved by acetaminophen, aspirin or ibuprofen, although Dr. Irwin has not had to take these actions in their study of over 150 subjects.

The LIBR clinical staff who will be involved in the study have worked previously on projects that have involved the assessment of depressive symptoms and blood or cerebrospinal fluid sampling. In addition, Dr. Savitz will meet with each clinician involved in the project ahead of time to outline the present study and to detail the side effects and possible behavioral and physiological symptoms associated with LPS administration. All procedures (e.g. blood draw, LPS/placebo exposure) will be performed by qualified medical professionals. Moreover, participants will be reminded that they can withdraw from the study at any time if they no longer feel like participating.

In addition, a physician will be on call throughout each experimental session. All reportable adverse events resulting from participation in the research study will be reported in writing to the DSMB Safety Officer (Dr. Robert Dantzer) as well as the Western IRB within 5 working days. If an adverse event occurs, the investigators will assist the participant.

Subjects will not be released from LIBR until about 6 hours following LPS administration, at a point where all physical, behavioral, and emotional effects of the infusion should have fully resolved. Participants will only be discharged from LIBR with approval from a LIBR physician. If in the opinion of the physician, the participant cannot be discharged due to grade 3 or 4 psychiatric symptoms, the participant will be taken to the Laureate Psychiatric Clinic and Hospital for assessment. If the participant cannot be discharged due to potential grade 3 or 4 physical symptoms, they will be taken to the Saint Francis Trauma Emergency Center (SFTEC) for

assessment. Dr. Scott Grantham, MD is the Medical Director of Laureate Psychiatric Clinic and Hospital. He has agreed to be the contact person for Laureate Institute for Brain Research if a participant needs to be assessed at the SFTEC. All of these facilities are located on a common campus.

14. ETHICAL ASPECTS

14.1. Study-Specific Design Considerations

Importance of Knowledge to be Gained

Given the major public health burden imposed by depression, the limited effectiveness of current antidepressant therapies, and the lack of available biomarkers that predict response to existing treatments, alternative models for understanding depression are critical. Inflammation-induced depression is a promising alternative biological model of depression. This project focuses on inflammatory mechanisms of depressive symptoms to establish a theoretical and mechanistic basis for future translational research of depression prevention. The findings of this project may guide future clinical studies in identifying individuals at risk of developing depression when exposed to heightened inflammatory states, which may not only contribute to the development of new behavioral or pharmacological therapies, but may predict which patients are likely to respond to existing types of medication.

Risks Reasonable in Relation to Benefits

There is no immediate personal or medical benefit derived from participation. The results of these studies, however, will further the scientific community's understanding of mood disorder pathophysiology. By increasing understanding of pathophysiology, these research experiments have the potential to improve treatment modalities, diagnostic capabilities, and classification systems. They may aid in de-stigmatizing psychiatric illness and convincing noncompliant patients that medications are likely to be helpful. As such, participants may derive personal satisfaction from their contribution to the discovery process.

The risk classification for this study is greater than minimal due to the use of LPS in depressed participants. We have carefully considered several potential safety limitations of this project, all of which indicate that the likelihood of harm is very low, and that the project is feasible in the clinical populations proposed: (a) care will be taken to exclude any individuals who might respond adversely to LPS based on a thorough medical assessment, including EKG, and safety labs and (b) there is a follow-up observation period of 6 hours as well as one day and one week follow-up in order to ensure that all participants return to their pre-LPS levels of physical and emotional symptoms. MRI procedures generally hold few risks to persons, other than to individuals listed in the exclusion criteria.

14.2. Regulatory Ethics Compliance

14.2.1. Investigator Responsibilities

The Principal Investigator will be responsible for maintaining quality control over the data collection process and for securing its confidentiality. Specifically, the PI will oversee the data collection process and ensure that data from blood assays, the physical examination, and clinical evaluation are reviewed for completeness and accuracy. During each scheduled experimental session, we will carefully monitor any potential threats to the safety and psychological well-being of participants, and a LIBR physician will be on call for each of the Visit 2 sessions. If, for example, there is any adverse change in the medical condition of participants that is revealed during the course of the day (through vital signs or self-report assessments), project staff and/or LIBR nurses will contact a LIBR physician for immediate medical treatment.

14.2.2. Data Safety Monitoring Board

A Data Safety Monitoring Board (DSMB) will be responsible for data and safety monitoring. The DSMB will be responsible for reviewing study procedures, AEs, safety mailings (if applicable), enrollment, active subject progress, drop-out rates, and ongoing conduct of the research. The DSMB members can ask questions and make comments and/or recommendations to the investigators. The Western Institutional Review Board (WIRB) is notified of significant findings by way of the DSMB meeting minutes after each meeting. DSMB members will consist of physicians and scientists not listed as site investigators on this study. Data on the number of subjects enrolled and the number of AEs will be reviewed by the DSMB at least bi-annually and more frequently if needed. Any unanticipated events will be immediately directed to the Compliance Manager at LIBR who will follow WIRB's reporting procedures.

14.2.3. Privacy of Personal Data

Measures instituted to protect the privacy and/or confidentiality of participant PHI

Data will be collected by trained staff who have completed training in human subjects protections in research and research integrity, training on research data management and confidentiality, and training to criterion on the project protocol. To protect confidentiality, no personally identifying information will be coded on questionnaires, interviews, bioassays, brain imaging data, or other scoring forms. Unique subject identification numbers are assigned to each participant. The file that links names with subject numbers will be stored separately from the de-identified data. All data will be stored on password-protected computers located behind a secure and maintained firewall. Any paper documents will be scanned and uploaded to the REDCAP database using LIBR computer access.

14.2.4. Risks

1. **Risks Associated with Screening and Evaluation**: The risks and discomforts of the screening evaluations are minimal. Some of the questions in the interviews may be painful or uncomfortable to answer.

2. **Risks Associated with Blood Draw**: The risks of blood drawing are minimal. Possible mild side effects include mild pain or bruising at the site of venipuncture. There is a very small risk of fainting or infection in the area of the needle insertion.

3. **Risks Associated with MRI**: MRI uses powerful magnetic fields and weak radio frequency pulses (electromagnetic radiation), neither of which has been associated with adverse effects in patients or laboratory animals when studied under clinical imaging protocols. However, as in the clinical setting, subjects must be free of any external or implanted ferrous material. People are at risk for injury from the MRI magnet if they have pacemakers or other implanted electrical devices, brain stimulators, some types of dental implants, aneurysm clips (metal clips on the wall of a large artery), metallic prostheses (including metal pins and rods, heart valves, and cochlear implants), permanent eyeliner, implanted delivery pump, or shrapnel fragments.

Because MRI is performed in confined quarters, the feeling of being isolated or confined may cause some healthy subjects and patients to request termination of their participation. It is expected that a very low percentage of subjects will be unable or unwilling to complete participation.

Because the noise from the scanner is loud enough to damage hearing, participants will be fitted with hearing protection prior to MRI scanning and will wear hearing protection devices at all times during scanning.

4. **Risks Associated with Delaying Treatment:** MDD subjects will NOT be asked to discontinue any medications for the purposes of this study and will be informed that they should not participate in the study if it will be difficult for them to delay treatment.

14.2.5. Procedures for Protecting against or Minimizing Potential Risks

1. **Minimizing Risks Associated with Screening and Evaluation**: The subject may decline to answer any specific questions or discontinue the psychiatric interview at any time.

2. **Minimizing Risks Associated with Blood Draw:** The physician or trained phlebotomist will utilize sterile technique to draw blood by venipuncture.

3. **Minimizing Risks Associated with MRI**: Throughout scanning the following physiological signals will be continuously monitored in all subjects: heart rhythm, via MRI compatible ECG leads (GE systems), heart rate via MRI compatible pulse oximeter, respiratory rate via scanner compatible belt.

We intend to minimize claustrophobia using a series of procedures: 1) by giving a detailed explanation of the environment prior to scanning, 2) allowing subjects the opportunity to habituate to the scanner environment by resting in LIBR's mock scanner prior to their real scan, 3) maintaining voice contact with the subjects at all times, 4) maintaining visual contact of the patient in the scanner using observational cameras placed inside the scanner room, and 5) providing subject an emergency squeeze ball to signal the MRI technician to stop scanning.

To minimize the risk of hearing damage, all participants will be fitted with hearing protection and be required to wear the hearing protection for the duration of the MRI scanning.

All scans will be read by a neuroradiologist. Upon detection of incidental findings during MRI scanning a medical doctor will communicate the discovery in a verbal and/or a written communication to the subject. The communication will guide the subject to make the discovery known to their primary-care physician and that the Laureate Institute for Brain Research will provide a digital copy of the suspect MR scans to the primary-care physician upon request.

4. **Minimizing Risks Associated with Delaying Treatment**: If subjects need alternative or additional medications prescribed during the study they will not have this treatment delayed, but instead will be dropped from the study and referred to a physician for follow-up treatment. If this treatment is needed on an emergent basis, then the additional medication may be initiated by a physician during transfer of care. Medications will NOT be discontinued to allow participation in the study. A LIBR physician will stop participation in the study if it becomes clear that continued participation is not in the patient's best medical interest.

5. **Contingency plans for monitoring suicidality**

All volunteers who are deemed a serious suicide risk will be excluded from the study. All research volunteers will undergo routine, state-of-the-art screening and diagnostic assessments at LIBR. The screening will be conducted by a clinician interviewer (who holds either a nursing or MD degree, or a degree in clinical or counseling psychology and has received extensive training and experience in the evaluation and management of patients with major psychiatric disorders). Participants also will complete the self-report version of the Columbia Suicide scale at Visit 1, at Visit 2, prior to administration of endotoxin, and at Visit 2, prior to discharge. During the follow-up phase interviews, participants also will complete the C-SSRS. The C-SSRS is currently used for clinical triage by first responders in ER settings and crisis call centers, for non-mental health users like teachers or clergy, or in situations where frequent monitoring is required (e.g. inpatient shift monitoring, day programs). In the evaluation of suicidal risk, we specifically will exclude from participation any volunteer who endorses having developed a plan or intent to attempt suicide or has a lifetime history of a suicide attempt. Any volunteer who is excluded from participation for these reasons will be referred for emergency care according to written LIBR policies for managing potentially suicidal patients, as described below.

While participating in this study at LIBR, subjects initially will undergo the clinical evaluation and screening interviews, a baseline MRI scan, and then within a week complete an 8-hour long experimental session,

comprising, a 1-hour-long MRI scan, a battery of clinician and self-administered depression rating scales, and 5 blood draws. Participants must complete these study procedures within one week of starting the study. This limit is intended to reduce the length of time that participants remain untreated (using medication) during their participation in this research protocol. While the participants are in this study they will be monitored for the development of suicide risk or for worsening in their clinical illness as follows:

If concerns arise when the subject is physically present at LIBR, psychiatrist, Martin Paulus, M.D., is available on site to address any concerns that arise. Dr. Paulus is a board-certified psychiatrist with 25 years of experience in assessing and managing patients with mood disorders including suicide assessment and management. In the event that Dr. Paulus is unavailable, psychiatrist Sahib Khalsa, M.D., serves as back-up for Dr. Paulus.

For patients who are at the LIBR facility and are deemed to constitute a serious risk for suicide, the LIBR policy requires that they be escorted by two clinicians to the onsite, 24-hour emergency facility at Laureate Psychiatric Clinic and Hospital (which is located approximately 100 yards down a sidewalk from the LIBR facility). If participants refuse to be escorted to this facility and leave the LIBR premises, the study clinician will contact the Community Outreach Psychiatric Emergency Services (COPES – 918-744-4800), which is available 24 hours per day to send a mobile unit to the person's home.

For participants who develop serious suicidal ideation while not on the LIBR premises, these participants will be instructed to call 911 or to go to the nearest emergency room if they feel they are a threat to themselves or others. They will also be given the contact details of the Tulsa Community Outreach Psychiatric Emergency Services (COPES – 918-744-4800).

For participants who have clinical issues that do not reflect a psychiatric emergency, they will be given a telephone number where they can reach a LIBR clinician during those hours when LIBR is officially staffed by clinicians (weekdays between 0800 and 1700). For weekends and off-hours (evenings and nights) they are provided a second telephone number where they can reach the 24-hour per day on call service for LIBR, which is provided through the Call Center of Laureate Psychiatric Clinic and Hospital.

Upon study completion or early withdrawal, patients who are currently under treatment will be referred to their own clinician. Patients who complete the study and are not under the care of a clinician, will be provided a referral to a psychiatrist or other mental health professional at one of the following clinics:

Outpatient:	
Insured: Laureate Psychiatric Clinic and Hospital 6655 South Yale Ave Tulsa OK, 74136 (918) 481-4000	*Uninsured (sliding scale):* Tulsa Center for Behavioral Health 2323 South Harvard Ave Tulsa OK, 74114 (918) 293 2100
Sliding scale:	
Department of Psychiatry University of Oklahoma College of Medicine Tulsa OK, 74136 (918) 619 4400	Associated Centers for Therapy 7010 South Yale Ave, Suite 215 Tulsa OK, 74136 (918) 492 2554
Inpatient:	
Laureate Psychiatric Clinic and Hospital 6655 South Yale Ave	Shadow Mountain Behavioral Health System 6262 S. Sheridan Road

Tulsa OK, 74136 (918) 481-4000	Tulsa, OK 74133 (918) 492 8200

Individually tailored referrals will be made for subjects who reside outside of the Tulsa region, so that they will be referred to psychiatric services that are located near their home or workplace.

6. **Emergency protocol**

The study will be performed at the Laureate Institute of Brain Research, 6655 S. Yale Ave, Tulsa OK, 74136.

In the instance of an adverse event, the following standard CTRC procedures will be followed:

1. The subject will be evaluated and the severity of the adverse event will be determined. A medical evaluation will be performed if the study personnel has concerns about the health of the individual. Depending on the severity of the adverse event, the study will be stopped.

2. In the case of an anaphylactic reaction, an epinephrine injection (Epi-Pen) will be administered and the subject will be taken to the ER.

3. For hypotension, the patient will be placed in the trendelenburg position to quickly enhance blood pressure and cerebral perfusion. We may also orally and/or intravenously hydrate the subject if positioning changes do not help with hypotension.

4a. If this procedure achieves resolution of hypotension or return of consciousness, the patient will be excused from the study

4b. If this procedure does not result in resolution of hypotension or return of consciousness, then the remainder of the emergency protocol will be instituted (see #5 below). Similarly, the following procedures will also be employed if the patient is found to be in asystole, or is pulseless and unresponsive, or reports any other potentially adverse outcome (e.g. chest pain).

5. A Basic Life Support (BLS) certified member of the study nursing staff will immediately begin performing cardiopulmonary resuscitation (CPR). They will rotate CPR with other Basic Life Support (BLS) certified members of the study staff including the study physician (MD).

6. Simultaneously, a member of the study staff will call for emergency response services by dialing 911. Based on prior experience, the response time is approximately 5 minutes for the Emergency Medical Services Authority (EMSA).

7. Immediately after calling for EMSA, the same study staff will bring the Automated External Defibrillator (AED) to the scene. LIBR possesses three AEDs. Each AED is positioned in the participant testing areas, immediately outside of testing rooms. There is an AED positioned 30 feet outside of the infusion room.

8. The study staff member will subsequently activate the LIBR emergency response alert, triggering a notification via overhead speakers throughout the building. This will call all other BLS certified staff members (MDs, and RNs) to the scene.

9. While awaiting EMSA responders (estimated response time: 5 minutes), all LIBR study staff present (BLS certified) will follow BLS protocol including performance of CPR if applicable, and AED deployment if applicable.

10. EMSA responders will determine the need for implementing the appropriate ACLS protocol.

11. Emergency medical services responders will determine the need for transfer to the Saint Francis Healthcare System Emergency Department (located across the street) for further evaluation and management.

14.2.6. Plan of Action for Incidental Findings

In addition to standard MP-RAGE and EPI sequences used for the research component of the study, we will also obtain T2-weighted FLAIR images which are sensitive to CNS and white matter abnormalities. For each subject, a set of T1- and T2 weighted images will be sent via PACS and evaluated by a neuroradiologist (Integris Radiology Group, Oklahoma City, OK) for the presence of unanticipated abnormalities (e.g., tumors). The neuroradiologist will recommend referral to a physician, if incidental findings are discovered which warrant follow-up evaluation. Upon detection of such incidental findings during MRI scanning, a physician researcher from LIBR will verbally communicate the discovery to the subject. The physician researcher will also provide written communication of the incidental finding to the subject. The written communication will guide the participant to make the discovery known to their primary-care physician. In addition, once the participant provides written consent authorizing the release of his/her medical records and MR images, LIBR will provide a digital copy of the MRI scans to the primary-care physician. Additionally, detection and disclosure of incidental findings will be documented in a database contained on the LIBR computer cluster.

14.2.7. Compensation

Visit 1	Approximate Time in Hours	Compensation	Incentive Earned
Consent	0.5		
Blood draw, Physiological measures & EKG	0.5	$25	
Interviews & behavioral evaluations	2	$20	
MRI scan	1	$50	
Incentive Tasks in scanner	0		up to $30
Total for Visit 1	4	$95	up to $125
Visit 2			
Blood draw x5, Physiological measures & EKG x2	1	$25	
IV placement x2	0.5	$50	
Interviews & behavioral evaluations x5	4	$40	
LPS or Placebo injection	2	$100	
Lunch & Rest provided	1	$0	
MRI scan	1	$50	
Incentive Tasks in scanner			up to $30
Total for Visit 2	9.5	$265	up to $295
Visits 3 and 4 (x2)			
Blood draw	0.5	$25	
Interviews & behavioral evaluations	0.5	$20	
Total per Visit		$45	
Total for visits 3 and 4		$90	

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