Title: Adrenergic Attenuation of Immune Activity is Associated with Anhedonia in Adolescents

Running Title: Anhedonia and Adrenergic-Modulated Immune Activity

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Abstract

While inflammation has been implicated in the manifestation of psychiatric symptoms, the role of immune attenuation via sympathetic modulation in psychopathology remains unclear. Here, this study aimed to assess β_2-agonist clenbuterol (CBL) as a promising agent to model adrenergic-induced immune response following *ex vivo* immune activation with lipopolysaccharide (LPS). Relationships between CBL-modulated cytokine levels and symptom measures were then explored. Adolescents were evaluated with semi-structure interviews and self-reported depression, anxiety, and anhedonia levels. Fasting whole-blood samples were collected and stimulated with LPS in the presence and absence of CBL for 6 hours, with supernatants being subjected to Luminex multiplex assay for 41 cytokines. Cytokine levels between conditions were compared using Bonferroni-corrected nonparametric tests. Exploratory factor analysis reduced 41 cytokines into fewer factors in each experimental condition, and their relationships with psychiatric symptoms were examined with Spearman correlations controlling for age, sex, and BMI. Data from 130 participants (15.25 ± 2.16 years old, 59% female) were analyzed. 10 cytokines were significantly affected by CBL treatment compared to LPS alone. LPS+CBL factor 3 significantly correlated with both anticipatory (*rho* = -0.39, $p = 7.4 \times 10^{-5}$) and consummatory anhedonia (*rho* = -0.36, $p = 3.3 \times 10^{-4}$), and these correlations remained significant when controlling additionally for depression. There were no significant associations between immune factors with depression or anxiety severity. Findings support our hypothesis that CBL attenuates inflammatory effects thought to underlie psychiatric conditions in youth. Using a data-driven analytic method, distinctive

relationships between CBL-affected cytokines and dimensional anhedonia were reported, further elucidating the role of β_2-agonism in adolescent affective symptomatology.

Introduction

Inflammation has been implicated in numerous neuropsychiatric conditions (1, 2). While the majority of research has focused on adults, work by our group and others have consistently reported a link between peripheral inflammatory processes and psychiatric symptoms across the lifespan; this includes adolescence, a period when psychiatric symptoms often first emerge (3-7). The relationships between inflammation and psychopathologies are thought to reflect similar neurobiological mechanisms to those underlying “sickness behavior” in model animals (6). Much like many psychiatric illnesses, especially depression, sickness behavior is characterized by fatigue, malaise, and reductions in appetite, ability to concentrate, mood, social interactions, and pleasure-seeking behaviors (8-11). While sickness behavior is an adaptive process involving complex brain-body interactions, dysregulated immune activation is postulated to induce maladaptive changes in the central nervous system, which ultimately manifest as psychiatric disturbances (6).

As inflammation involves many intertwined and tightly orchestrated systems, it is crucial to examine processes modulating immune activity to delineate the brain-immune relationships. One prominent immune regulator is the adrenergic nervous system, which has been shown to halt overactivation of peripheral and central immune cells through β_2-adrenergic receptor transduction pathways (12). Among immune-inhibitory β_2 receptor agonists, clenbuterol (CBL) is a promising candidate for use in modeling adrenergic regulation of immune activation *ex vivo*. Especially relevant to psychiatric contexts, CBL readily crosses the blood brain barrier to act on central β_2 receptors, with preclinical evidence indicating that CBL reduces neuroinflammation associated with

strokes (13) and exhibits antidepressant-like effects (14) in rodent models. However, investigation of β_2 agonists adrenergic system regulating immune CBL's anti-inflammatory property using human blood samples is scarce, and available studies have only analyzed a limited number of inflammatory markers.

Here, using a comprehensive profile of 41 immune biomarkers including cytokines, chemokines, and growth factors (referred to throughout as "cytokines" for convenience), we sought to investigate whether adrenergic immune modulation could indeed be achieved in a functional immune response model using lipopolysaccharide (LPS) and CBL in whole blood samples of adolescents with diverse mood and anxiety symptoms as well as healthy adolescents. We focus our investigation on adolescence, a robust developmental period with increased vulnerability to systemic alterations, as findings will enhance our understanding of modifiable targets. Our participants were all psychotropic-medication-free. Based on our previous work, we did not expect differences in immune response between participants with psychiatric symptoms and healthy controls (15). Instead, our analyses focused on all participants regardless of clinical status to capture the full range of immune activity and clinical symptom severity, in line with the National Institute of Mental Health (NIMH) Research Domain Criteria (RDoC) guidelines (16). We hypothesized that CBL would attenuate immune activation following exposure to LPS. There were no *a priori* hypotheses regarding which specific LPS-stimulated cytokines would be downregulated by CBL, as we expected a general attenuating effect on cytokine production. Additionally, utilizing data-driven analytic methods, we explored the relationships between CBL-induced attenuation of inflammatory effects and severity of depression, anxiety, and anhedonia symptoms.

Methods

Study Participants

Adolescents ages 12–20 years in the New York City area were recruited via community advertisements and clinician referrals. On the first visit, adolescents underwent a comprehensive medical and psychiatric assessment to determine eligibility for study participation. Eligible adolescents were instructed to return to the study site within approximately 2 weeks to undergo study procedures, including vital sign measurements, a fasting blood draw, a urine toxicology screen, a pregnancy test if female, and completion of clinical questionnaires.

This study was approved by the Institutional Review Boards at Icahn School of Medicine at Mount Sinai and Albert Einstein College of Medicine. Participants ages 18 and older (n=24) provided written informed consent; participants younger than 18 years old provided assent, and a legal guardian provided signed consent.

Inclusion and Exclusion Criteria

Potential subjects were excluded from the study if they met any of the following criteria: a) a current or past DSM-IV diagnosis of schizophrenia, pervasive developmental disorder, or substance use disorder, b) history of immunological or hematological disorder, c) intake of any immune-affecting medication and supplements (e.g., steroids, non-steroidal anti-inflammatory drugs, omega-3 fatty acids) within 2 weeks prior to study enrollment, d) intake of any psychotropic medication within 30-90 days (depending on drug half-life) prior to the blood draw, e) history of chronic fatigue syndrome, f) any infection during the month prior to the blood draw (including the common cold), g) history of significant medical or neurological disorders, h) an

estimated IQ below 80 based on the Kaufmann Brief Intelligence Test (KBIT; 17), i) positive urine toxicology screens, and j) in females, a positive pregnancy test.

Psychiatric Symptom Measurements

Each participant received a thorough evaluation consisting of medical history as well as laboratory tests including complete blood count, metabolic panel, liver and thyroid function tests, and a urine toxicology test screening for amphetamines, barbiturates, benzodiazepines, cocaine, marijuana, methadone, opiates, phencyclidine, and propoxyphene. Both the participating adolescent and their accompanying parent were interviewed by a clinician to assess for possible presence of an infectious illness (including the common cold) within the month immediately prior to enrollment.

The Schedule for Affective Disorders and Schizophrenia – Present and Lifetime version (K-SADS-PL; 18), an interview-based assessment tool commonly used in pediatric research settings, was conducted by a trained, licensed psychiatrist or clinical psychologist to assess presence of any psychiatric symptoms and lifetime history of psychiatric diagnoses per the Diagnostic and Statistical Manual of Mental Disorders(DSM). As the age range of “adolescence” may extend into early twenties due to continued development of the frontal lobe (19), the K-SADS-PL was conducted for all participants up to age 20 for consistency in this study. Evaluations were discussed between the interviewing clinician and the Principal Investigator, a board-certified child and adolescent psychiatrist, to enhance diagnostic reliability. Participants with any current psychiatric symptomatology were assigned to the psychiatric group (Psych) regardless of whether diagnostic criteria for DSM disorders were met, while participants

in the healthy control (HC) group had no psychiatric symptoms or lifetime history of any DSM diagnosis.

The self-rated, 39-item Multidimensional Anxiety Scale for Children (MASC; 20) was employed to quantify anxiety severity (scores ranging from 0 to 117). Participants self-reported their depression severity with the 21-item Beck Depression Inventory, 2nd edition (BDI-II; scores ranging 0 to 63; 21). Additionally, we utilized the Temporal Experience of Pleasure Scale (TEPS; 22) to capture anticipatory (TEPS-A; scores ranging 10 to 60) and consummatory (TEPS-C; scores ranging 8 to 48) components of anhedonia. Higher scores on the MASC and BDI indicate more severe anxiety and depression, respectively, while higher TEPS scores suggest lower levels of anhedonia.

Ex Vivo *Immune Stimulation with Lipopolysaccharide and Clenbuterol*

Blood samples were collected between 9 and 10 A.M. after an overnight fast lasting at least 12 hours. The samples were processed within 20 minutes of collection and stored at -80º C. Functional immune responses were assessed *in vitro* using a well-established LPS challenge protocol that reliably induces pro-inflammatory cytokine production from macrophages and monocytes in human tissue cultures as well as *in vivo* mouse models (23). Whole blood samples were cultured on standard growth medium for 6 hours in a tissue culture incubator under three conditions: in the presence of LPS (0.1 µg/ml) alone, in the presence of LPS along with the β_2-agonist CBL (10^{-6} M), and in the absence of both LPS and CBL (i.e. Control condition). CBL dosing was based on in-house preliminary data. The potency of LPS and CBL were authenticated on a quarterly basis using in-house reference whole blood samples collected from healthy donors.

Cytokines, Chemokines, and Growth Factors:

Levels of 41 immune biomarker analytes (cytokines, chemokines, and growth factors) were measured using a Luminex-200 system and the XMap Platform (Luminex Corporation). A full list of the 41 analytes examined in our study can be found elsewhere (24, 25). Acquired fluorescence data was analyzed by the Beadview software. Analyte levels were determined in duplicate from 25 μL volumes of plasma or serum collected from cultured whole blood samples using the multiplex cytokine panels (Multiplex High Sensitivity Human Cytokine Panel, Millipore Corp.). The lower and upper detection limits for these assays were 3.0 pg/mL and 15,000 pg/mL, respectively. Median fluorescent intensity (MFI) values were used for analyses, as these allow for increased statistical power compared to derived absolute concentration values (26). For each of the 3 blood culture growth conditions (i.e. Control, LPS, LPS+CBL), analyte MFI values were measured twice (once per duplicate sample) and averaged to obtain detailed subject-level immune profiles. One participant had missing MFI data for 9 analytes (fractalkine, G-CSF, IFN-α2, IL-7, IL-9, IL-13, MCP-1, MIP-1α, and PDGF-AB/BB) in the control and LPS conditions. A second participant had missing MFI data for 2 analytes (IL-7 and IL-13) in the LPS condition, and a third participant had missing MFI data for 1 analyte (IL-7) in the LPS condition; complete immune profiles were obtained for all other subjects and conditions. Laboratory staff, blind to participants' clinical status, performed assays at the Mount Sinai Human Immune Monitoring Center.

The overall study procedures are summarized in **Figure 1**.

[**Figure 1** here]

Statistical Analyses

Primary analyses were conducted in MATLAB R2020b (MathWorks, Natick, MA). An overview of our analytic protocol can be found in **Supplementary Figure 1**. Shapiro-Wilk tests indicated a non-normal distribution for most cytokines in our sample, necessitating a nonparametric statistical approach. We first utilized within-subject Friedman tests, controlled for family-wise errors using Bonferroni correction, to test for significant differences in cytokine levels across the three conditions: Control, LPS, and LPS+CBL. For any cytokines found to differ significantly, follow-up Wilcoxon signed-rank tests with Bonferroni correction were used to examine pairwise differences between conditions. Effect sizes for nonparametric tests were computed as the correlation coefficient, *r*, using the following equation:

$$r = \frac{\mathrm{z}}{\sqrt{\mathrm{N}}} \tag{1}$$

in which *z* is the Wilcoxon signed-rank test statistic as computed in MATLAB using the *signrank* function and *N* is the sample size (27-30).

For our secondary analyses, given the unknown relations between CBL-attenuated inflammatory markers and subjective psychiatric symptoms, we utilized an exploratory factor analysis (EFA) model to identify sources of shared variance within subjects' detailed immune profiles in a data-driven manner. Cytokine data were examined using 3 discrete EFA models corresponding to the 3 conditions (i.e. Control, LPS, LPS+CBL). EFA was performed in SPSS version 27.0 (IBM, Armonk, NY) using principal component analysis (PCA) dimensionality reduction and varimax orthogonal rotation. Finally, Bonferroni-corrected Spearman correlations controlling for age, sex, and body mass index (BMI) were used to assess the relationship between each

cytokine factor scores and BDI (depression), MASC (anxiety), TEPS-A (anticipatory anhedonia), and TEPS-C (consummatory anhedonia) scores. Despite being traditionally considered a core symptom of depression, anhedonia severity has been shown to vary across a group of moderately to severely depressed adolescents (31) and distinctively relate to inflammatory responses regardless of depression severity (25). As such, we conducted another set of Spearman correlations between cytokine factors and anhedonia measures controlling additionally for depression severity.

Based on the results of our secondary analyses, we suspected that differential inter-cytokine correlation structures may have contributed to the distinct associations with anhedonia across conditions. We therefore conducted a *post hoc* analysis contrasting cytokine relationships between LPS+CBL and LPS conditions. Spearman correlations (*rho*) between pairs of cytokines in the 2 conditions were computed, *z*-transformed, subtracted from each other, then transformed back to *rho* coefficients. This yielded a total of 820 pairwise correlation differences (Δ *rho*) per subject. Bias-corrected and accelerated (BCa) bootstrapping (32-34) with 10^6 resamples was conducted to determine differences in correlations of 820 ($\frac{41 \times 40}{2}$) possible cytokine-pairs between LPS+CBL and LPS conditions.

Results

Participants Characteristics

This study enrolled a total of 130 adolescents, including 97 in the Psych group (85 endorsed mood and anxiety symptoms; 12 with other externalizing behavioral symptoms without comorbid mood and anxiety symptoms) and 33 HC. As summarized in **Table 1**, participants were racially and ethnically diverse. Demographically, Psych and HC groups were similar ($p > 0.1$), with the exception of a significantly higher proportion of Hispanics among the Psych sample ($p = 0.012$). As expected, adolescents in the Psych group exhibited higher depression and anxiety severity compared to HC (see **Supplementary Table 1**). Depression and anhedonia scores were skewed, while anxiety scores were normally distributed (see **Supplementary Figure 2**). More than half of participants in the psychiatric group had at least two concurrent DSM diagnoses.

[**Table 1** here]

Lipopolysaccharide and Clenbuterol Effects on Cytokines

Cytokine levels were not significantly different between HC and Psych participants for any condition (see **Supplementary Table 2**). Consequently, all analyses used combined data from the whole sample. Friedman tests (detailed in **Supplementary Table 3**) indicated significant differences in 21 cytokines across the three conditions after Bonferroni correction ($\alpha = \frac{0.05}{41} \approx 1.2 \times 10^{-3}$).

Pairwise follow-up analyses (i.e., Control vs. LPS, LPS vs. LPS+CBL, Control vs. LPS+CBL) of these 21 cytokines using Wilcoxon signed-rank tests are detailed in **Table 2**, with results considered significant at the Bonferroni corrected $\alpha = \frac{0.05}{21 \times 3} \approx 7.9 \times 10^{-4}$ level. Relative to the Control condition, LPS increased levels of 19 out of the 21

cytokines, with only PDGF-AB/BB and sCD40L not meeting significance. Levels of 17/21 cytokines (all except Fractalkine, G-CSF, IL-12P40, and sCD40L) were also elevated in the LPS+CBL condition relative to Control. For all but 2/21 cytokines (PDGF-AB/BB and sCD40L), effect sizes were smaller for LPS+CBL vs. Control than for LPS vs. Control, consistent with the immunosuppressant activity of CBL. Compared to LPS, levels of 9/21 cytokines were significantly reduced in the LPS+CLB condition, while only 1/21 (sCD40L) was found to be increased in the presence of CBL. Cytokines with the greatest differences in expression between LPS+CBL and LPS conditions included MIP-1β (r = -0.73), TNF-α (r = -0.70), MIP-1α (r = -0.65), and IL-6 (r = -0.62).

[**Table 2** here]

Cytokine Dimensionality Reduction Across Experimental Conditions

In each of the 3 examined conditions (see **Methods – Statistical Analyses**), EFA yielded an initial model containing 8 orthogonal cytokine factors with eigenvalues greater than 1, of which 5 factors were retained for our analyses on the basis of variance explained (≥5%) and scree plots. The factors explained 81.1%, 73.5%, and 74.1% variance of the original cytokine data for the Control, LPS, and LPS+CBL conditions, respectively. Loadings for all factors can be found in **Table 3.**

[**Table 3** here]

Relationships between Adrenergic Immune Modulation and Psychiatric Measures

Across the three experimental conditions, a similar cytokine factor (major loadings: PDGF-AB/BB, sCD40L, PDGF-AA, GRO, EGF, + RANTES for Control and LPS conditions, + G-CSF for LPS condition; see **Supplementary Table 4**) was identified to be significantly correlated with anticipatory anhedonia after Bonferroni

correction ($\alpha = \frac{0.05}{5\times4\times3} \approx 8.3 \times 10^{-4}$) and controlling for age, sex, and BMI. Specifically, correlations were found between anticipatory anhedonia and a) Control Factor (F) 4 (**Figure 2A**, *rho* = -0.35, $p = 3.6 \times 10^{-4}$), b) LPS F3 (**Figure 2B**, *rho* = -0.36, $p = 2.6 \times 10^{-4}$), and c) LPS+CBL F3 (**Figure 2C**, *rho* = -0.39, $p = 7.4 \times 10^{-5}$). In addition, LPS+CBL F3 significantly correlated with consummatory anhedonia (**Figure 2D**, *rho* = -0.36, $p = 3.3 \times 10^{-4}$).

[**Figure 2** here]

When depression severity was also included as a covariate, LPS+CBL F3 remained significantly correlated with anticipatory anhedonia (*rho* = -0.34, $p = 6.3 \times 10^{-4}$) and consummatory anhedonia (*rho* = -0.34, $p = 9.9 \times 10^{-4}$), while relationships between anticipatory anhedonia and Control F4 and LPS F3 did not meet the Bonferroni-corrected significant threshold $\alpha = \frac{0.05}{5\times2\times3} \approx 1.7 \times 10^{-3}$ (i.e. adjusting for number of factors, TEPS subscales, and conditions). No significant relationships were found between any cytokine factor and depression or anxiety. Results of all correlations are reported in **Supplementary Table 5**.

Exploring Clenbuterol-Induced Differences in Immune Profiles

Using stringent Bonferroni correction ($\alpha = \frac{0.05}{820} \approx 6.1 \times 10^{-5}$), we found that administration of CBL significantly affected 3 inter-cytokine correlations: RANTES and PDGF-AA (Δ *rho* = -0.18, $p = 3.36 \times 10^{-6}$); MIP-1α and MCP-1 (Δ *rho* = -0.29, $p = 3.5 \times 10^{-6}$); and MIP-1β and MCP-1 (Δ *rho* = -0.33, $p = 4.74 \times 10^{-6}$). At a relaxed exploratory significance threshold using false discovery rate (FDR) correction (35), we identified CBL-induced changes in correlations between 5 additional cytokine pairs: MIP-1β and IL-2 (Δ *rho* = 0.19, $p_{FDR} = 0.030$); MCP-1 and IL-6 (Δ *rho* = 0.29, $p_{FDR} = 0.036$); MCP-1

and IL-8 (Δ *rho* = -0.22, p_{FDR} = 0.021); MCP-1 and IP-10 (Δ *rho* = -0.26, p_{FDR} = 0.028); and RANTES and TNF-α (Δ *rho* = -0.20, p_{FDR} = 0.018). **Figure 3** visualizes the pairwise differences in *rho* (lower triangle) and corresponding significance (upper triangle) values.

[**Figure 3** here]

Discussion

To our knowledge, this study is the first to examine adrenergic modulation of *ex vivo* inflammatory responses using comprehensive immune biomarker assays. As hypothesized, our results indicated that CBL had a general attenuating effect on LPS-induced immune activation in a large sample of medically healthy adolescents with diverse psychiatric profiles. Further, utilizing data-driven factor analysis, we documented for the first time specific relationships between adrenergic mediation of cytokine production and psychiatric symptomatology in youth. We discuss our findings below.

Clenbuterol Functionally Modeled Adrenergic Immune Modulation

A major strength of this study was our use of multiplex immunofluorescence assays to simultaneously quantify 41 cytokines, chemokines, and growth factors. Whereas most studies investigating immune activity in psychiatric illnesses have assessed only a limited number of well-studied cytokines, our approach enabled a detailed characterization of immune profiles across multiple conditions. Using a conservative statistical approach with stringent correction for multiple comparisons, we identified nine immune analytes for which LPS-induced inflammatory responses were significantly attenuated by simultaneous administration of the β_2-adrenergic receptor agonist CBL, as well as one (sCD40L) for which CBL potentiated release compared to LPS alone. Notably, IL-1β, IL-6, and TNF-α, which have been consistently implicated in psychopathology (2, 36, 37), were among the cytokines most substantially attenuated by CBL in our study. While not previously reported in clinical studies, the observed post-CBL reduced levels of IL-1β, IL-6, and TNF-α mirror results from preclinical studies examining immune activity following CBL induction (38, 39).

A notable finding from our study is the pronounced changes in levels of MIP-1α and MIP-1β chemokines across *ex vivo* conditions. MIP-1α and MIP-1β have been implicated in multiple immune-related pathologies, including those affecting the central nervous system (40). Indeed, the detected increase of MIP-1α and MIP-1β levels following LPS stimulation in our study is consistent with previous reports (40), while their attenuated levels following CBL addition is a novel finding. Though infrequently investigated compared to other immune biomarkers in psychopathologic contexts, MIP chemokines have been assayed in a few studies recruiting adult psychiatric participants (40-42). One postmortem study found that MIP mRNA was expressed in brain tissue samples of 4 patients with schizophrenia and 1 patient with manic depressive illness but not in those of 8 patients with dementia, neurodegenerative disease, and brain tumor, suggesting a specific role in psychiatric vs. neurological illness (43). The handful of clinical studies to examine MIP levels in psychiatric cohorts have yielded inconsistent findings, with some reporting elevated serum MIP-1β concentrations in patients with more severe depression (41, 42) but others reporting lower or no difference in MIP levels in patients with major depression compared to healthy controls (44, 45). Here, we present new evidence supporting MIP chemokines as part of the general immune response to adrenergic stimulation in our adolescent participants with diverse mood and anxiety symptoms.

Although CBL attenuated the release of multiple pro-inflammatory cytokines, it appeared to have minimal impact on anti-inflammatory biomarkers, including IL-4 and IL-10 (46, 47). Animal literature has provided conflicting evidence regarding adrenergic modulation of IL-10; while some studies report IL-10 suppression following CBL

treatment (13, 48), others have documented IL-10 induction following administration of β_2 receptor agonists, including CBL (49, 50). As IL-4 and IL-10 were elevated in both the LPS and LPS+CBL conditions compared to Control in our study, one interpretation is that these cytokines were already being produced in response to LPS to limit the predominantly inflammatory effects of other induced cytokines and thus were not sensitive to the coadministration of CBL. Interestingly, the only biomarker enhanced by CBL exposure compared to LPS alone was sCD40L, a signaling molecule typically characterized as pro-inflammatory but which promotes the release of IL-10 and exhibits immunosuppressive activity in HIV infection and certain cancers (51, 52). Moreover, the mechanism behind sCD40L immunosuppression involves enhanced tryptophan degradation through induction of indoleamine 2,3-dioxygenase (51), the rate-limiting enzyme in the kynurenine pathway which itself produces both pro- and anti-inflammatory metabolites and which we have previously linked to anhedonia in adolescents (53). We note that these type of complex interactions between pro- and anti-inflammatory mechanisms are characteristic of the immune system resulting in varied observations using available measurement and analytic methods.

New Insights into Adrenergic-Modulated Immunologic Correlates of Anhedonia

The majority of work examining the role of inflammation in psychopathology measured only a few cytokine levels, thus did not fully describe immune activity in relationship with psychiatric diagnoses or symptoms. Indeed, because various, rather than individual, cytokines are produced in response to stimuli, it is likely that immune biomarkers act together to drive pathogenesis. In line with this understanding, our comprehensive assay of 41 cytokines allowed us to apply EFA to detect and sorted

highly correlated cytokines accordingly into immune clusters, showing immunologic patterns across different experimental conditions. Our data-driven analytic strategy revealed for the first time associations between sub-components of anhedonia and immune measures at baseline as well as in response to endotoxin and adrenergic challenge.

In our prior work, a comparable EFA method applied to data collected from 46 participants yielded 4, instead of 5, immune factors in the Control condition (24). The major loadings for Factor 4 previously identified are identical to those of Control F3 in this study (PDGF-AA/BB, sCD40L, PDGF-AA, GRO, EGF, RANTES, and sCD40L). Further, the major loadings of previously identified Factors 1, 2, and 3 closely correspond to those of Control F3, F2 + F5, and F1, respectively. Such similarities are expected as a subset of our participants were included in the previous study. Conversely, the differences can be explained by EFA being applied to only 40 cytokines (G-CSF was excluded) as well as a smaller sample size in the previous study.

In contrast with our current work, Factor 4 in Bradley *et al.* did not show any relationship with reward dysfunction as indexed by blood-oxygen level dependent (BOLD) signals during a functional magnetic resonance imaging (fMRI) reward flanker task. Rather, neural activation during reward attainment negatively correlated with Factor 2 (eotaxin, IL-12p40, IL-13, IL-15, IL-1α, IL-1Ra, IL-2, IL-5, IL-9, MCP-3, and TNF-β), while neural activity during anticipation negatively correlated with Factor 3 (FGF-2, Flt3-L, fractalkine, GM-CSF, IFN-α2, IFN-γ, IL-3, IL-4, IL-7, IL-17A, MDC, and VEGF). In a related study using the same fMRI task, we recently showed that C-reactive protein (CRP), a marker for generalized inflammation, did not correlate with clinical

symptoms yet had specific associations with neural reward function in adolescents (54). Taken together, these results suggest that the effects of inflammation on anhedonia severity in adolescents appear to be subtler than in adults, which we speculate may be related to more robust protective mechanisms in younger ages. Corroborating this interpretation, our current analysis showed that a factor derived from the LPS+CBL condition associated more strongly with anhedonia subcomponents than the comparable factor derived from the LPS condition. These findings thus complement earlier work by our group and others to understand the link between immune activity and adolescent reward dysfunction.

Our post-hoc analysis evaluating the effects of CBL on inter-cytokine relationships following LPS exposure provides further insight into these complex neuroimmune pathways. Specifically, we found that β_2-agonism had a pronounced impact on the coupling between chemokines, particularly MCP-1, MIP-1α, and MIP-1β, and other immune biomarkers in our sample. Future work should further examine the mechanisms behind how functional immune activity factors selectively influence clinical symptomatology.

Mechanisms of adrenergic modulation of the immune system in reward dysfunction

Findings from our study further support the theoretical interplay between the sympathetic and parasympathetic nervous systems in mediating immune-induced reward dysfunctions. While we cannot make inferences about precise molecular mechanisms of CBL's downregulating effect on LPS-induced immune dysregulation from the results of this study, intracellular cyclic adenosine monophosphate (cAMP) level elevation might serve as a mediation for such observed effect, as shown in a

previous *ex vivo* study using human peripheral blood mononuclear cells (39). CBL was also shown to suppress LPS-induced nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activity through prevention of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (IκB) phosphorylation in rat brains (55). Given that transcription factor NF-κB is ubiquitous in human cells (56), it is possible that CBL exerted its inflammatory-attenuating property through this modulator.

Further, the broad immune assay allowed us to pioneer examination of several hematopoietic growth factors' activity trends in the presence of CBL. We showed that hematopoietic growth factors (i.e. G-CSF, GM-CSF, PDGF-AA/BB, TGF-α) were responsive to LPS stimulus but did not appear to be affected by CBL. Few studies have examined if and how hematopoietic growth factors expression react to CBL, and virtually none addressed such questions in post-endotoxin exposure settings. A study in rats concluded that the expressions of growth factors TGF-β_1 and basic FGF, which are also known to be neurotrophic, could be enhanced by CBL, explaining its theoretical cerebroprotection property in brain ischemia (57). Additionally, several studies having detected differential peripheral levels of glial cell line-derived neurotrophic factor (GDNF), insulin-like growth factor-1 (IGF-1), and VEGF in participants with major depressive disorder compared to controls (58). Taken together, hematopoietic growth factors appear to be acting more centrally in psychopathologic contexts, which explain both of the following results of this study: a) the lack of substantial change observed peripherally in levels of hematopoietic growth factor following CBL induction, and b) the major loadings of the factors with significant relationship with anhedonia being growth factors PDGF-AA/BB, PDGF-AA, and EGF.

Clinical Implications of Adrenergic Modulating Interventions in Depression

Our findings suggested that targeting β_2-adrenergic receptors might be a promising anti-inflammatory approach for interventions in a subset of patients suffering from depression, as β_2-agonism could dampen the effects of peripheral inflammation modulating psychiatric symptoms. Particularly, we speculated that adrenergic-targeting interventions might be favorable for patients experiencing anhedonia. This approach should be cautiously considered as attempts to develop adrenergic-specific psychopharmacologic therapy targeting depression have been met with cardiovascular and electrolytic challenges (59), and data to date have yet to suggest a clinically meaningful effect of beta-blockers in treating anxiety disorders (60). One previous trial of CBL as a potential anti-depressant treatment was conducted in 5 female patients with MDD, and all patients experienced systemic side effects, prompting the authors to concluded CBL was not a viable treatment for depression (61). Indeed, we do not advocate for administering CBL as a primary or stand-alone psychotherapeutic agent, but possibly considered as an adjunct to other treatment modalities. Alternatively, this study provides early neurobiological evidence to further support existing interventions that engage sympathetic and parasympathetic systems, including deep breathing exercises, meditation, and physical exercises.

Limitations and Future Directions

This study has several limitations. First, as cytokine levels in our experiment were measured at only one time point (6 hours post-exposure), temporal cytokine behaviors following treatment of LPS with and without CBL remain unclear. Future research might measure cytokine levels at several post-exposure time points to gain

more comprehensive insights in CBL-affected cytokine behaviors. Second, correlations were found between immune modulation and anhedonia, thus we cannot make further inferences regarding underlying neural mechanisms that might modulate this relationship. As we also collect fMRI data on many of these participants as part of our on-going research program, we aim to incorporate measurements of reward dysfunctions in our future studies to examine CBL-affected immune response and reward-related neural activity. Third, our study design is cross-sectional, limiting our ability to infer stability of these associations over time. In our research program, we invite participants back for follow-up studies, thus we expect future work to incorporate longitudinal analyses allowing insights into the role of immune activity throughout adolescent psychopathologic development. Finally, our analysis did not account for several factors influencing the immune system and processes that modulate it, including menstrual cycle stage, exercise, diet, stress, and sleep. These data should be collected in future studies to further control for latent confounders and parse out more specific relationships between immune attenuation and symptomatology in adolescents.

Conclusions

The results of this study further inform the research and clinical communities about CBL's anti-inflammatory effects that can potentially target neuroinflammation implicated in adolescent psychopathology. More broadly, the results of this study can pave the way for further understanding of the role of β_2-agonism underlying reward-related constructs that contribute to mood and anxiety symptomatology.

Disclosure/Conflict of Interest

All authors declare no conflict of interest.

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Figure 1

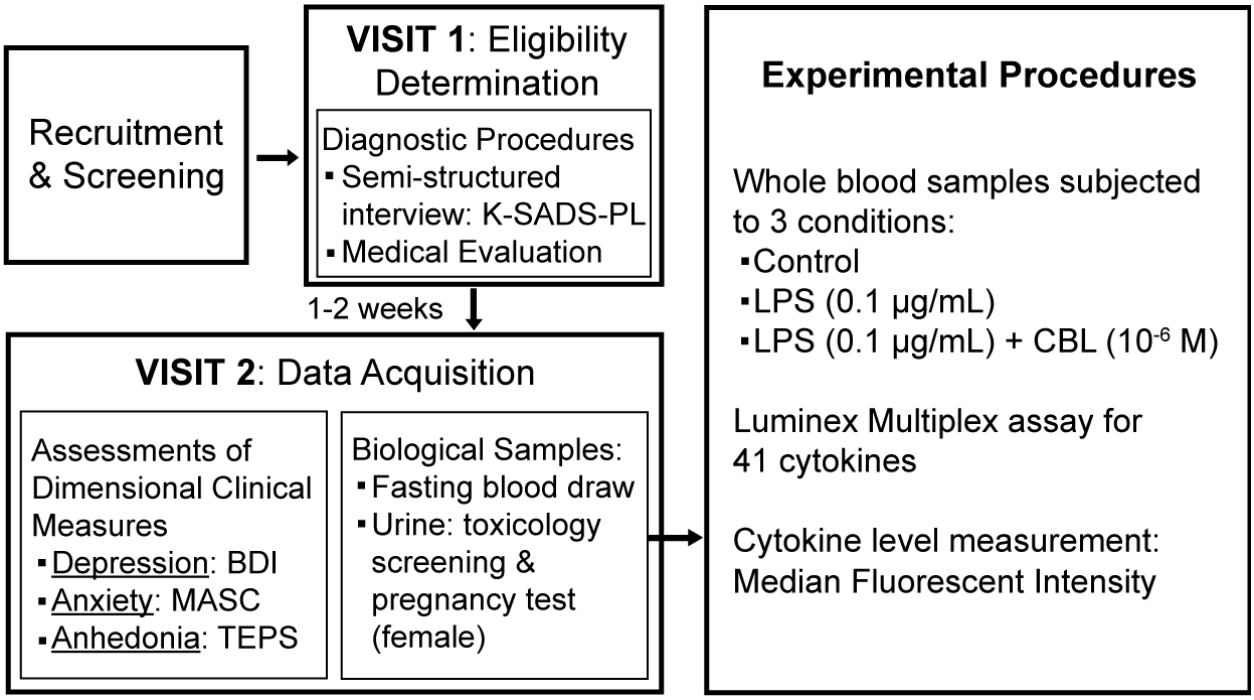

Figure 1. Summary of study procedures
Abbreviations: BDI: Beck Depression Inventory; CBL: Clenbuterol; K-SADS-PL: Schedule for Affective Disorders and Schizophrenia – Present and Lifetime version; LPS: Lipopolysaccharide; MASC: Multidimensional Anxiety Scale for Children; TEPS: Temporal Experience of Pleasure Scale.

Table 1

Measure	HC (n = 33)	Psych (n = 97)	Whole Sample (N = 130)
Demographics			
Age (Mean ± SD)	15.06 ± 2.41	15.31 ± 2.08	15.25 ± 2.16
Sex	F = 17, M = 16	F = 60, M = 37	F = 77, M = 53
Race [a]	W = 15, B = 13, A = 0, O = 5	W = 41, B = 28, A = 5, O = 23	W = 56, B = 41, A = 5, O = 28
Ethnicity [b]	H = 9, N = 24	H = 51, N = 46	H = 60, N = 70
Clinical Profile			
BDI (Mean ± SD)	2.06 ± 2.54 (n = 33)	14.67 ± 12.31 (n = 94)	11.39 ± 12.01 (N = 127)
MASC (Mean ± SD)	27.81 ± 12.89 (n = 31)	44.29 ± 17.58 (n = 92)	40.14 ± 17.98 (N = 123)
TEPS-A (Mean ± SD)	48.20 ± 8.52 (n = 30)	44.24 ± 9.50 (n = 74)	45.38 ± 9.36 (N = 104)
TEPS-C (Mean ± SD)	36.76 ± 8.10 (n = 29)	33.54 ± 7.28 (n = 72)	34.47 ± 7.62 (N = 101)
BMI (Mean ± SD)	24.27 ± 6.02 (n = 32)	25.60 ± 7.19 (n = 97)	25.27 ± 6.92 (N = 129)
Most Common DSM Diagnoses			
Mood Disorders [c]	0	58	58
Anxiety Disorders [c]	0	54	54
ADHD [c]	0	35	35
ODD [c]	0	12	12
PTSD [c]	0	9	9
Comorbidity [c, d]	N = 33, Y = 0	N = 43, Y = 54	N = 76, Y = 54

Table 1. Demographic and clinical characteristics of study participants
[a] A = Asian, B = Black, O = Other/Mixed Race, W = White
[b] H = Hispanic, N = Non-Hispanic
[c] Includes past and/or subthreshold symptoms
[d] N = 0-1 diagnosis, Y = ≥ 2 diagnoses
Abbreviations: ADHD: Attention-deficit/hyperactivity disorder; BDI: Beck Depression Inventory; BMI: body mass index (kg/m²); DSM: Diagnostic and Statistical Manual of Mental Disorders; HC: healthy control group; MASC: Multidimensional Anxiety Scale for Children; ODD: Oppositional Defiant Disorder; Psych: psychiatric group; PTSD: Post Traumatic Stress Disorder; SD: standard deviation; TEPS-A: Temporal Experience of Pleasure Scale – Anticipatory; TEPS-C: Temporal Experience of Pleasure Scale – Consummatory.

Table 2

Cytokine	LPS vs. Control		LPS+CBL vs. LPS		LPS+CBL vs. Control	
	r	*p* value	r	*p* value	r	*p* value
Fractalkine	0.40	**3.5×10^{-6} ***	- 0.21	1.7×10^{-2}	0.24	5.3×10^{-3}
G-CSF	0.34	**9.5×10^{-5} ***	- 0.10	2.3×10^{-1}	0.23	1.0×10^{-2}
GM-CSF	0.40	**3.5×10^{-6} ***	- 0.15	6.9×10^{-2}	0.30	**6.3×10^{-4} ***
GRO	0.42	**1.0×10^{-6} ***	0.22	1.1×10^{-2}	0.42	**6.9×10^{-7} ***
IL-10	0.54	**1.2×10^{-10} ***	- 0.01	7.7×10^{-1}	0.54	**1.1×10^{-10} ***
IL-12P40	0.36	**3.7×10^{-5} ***	- 0.22	1.0×10^{-2}	0.23	9.5×10^{-3}
IL-1RA	0.73	**5.2×10^{-21} ****	- 0.47	**3.7×10^{-8} ***	0.57	**8.2×10^{-12} ****
IL-1α	0.41	**2.2×10^{-6} ***	- 0.21	1.2×10^{-2}	0.31	**3.1×10^{-4} ***
IL-1β	0.82	**7.8×10^{-29} ****	- 0.51	**1.0×10^{-9} ***	0.77	**3.4×10^{-24} ****
IL-4	0.43	**4.4×10^{-7} ***	0.039	6.6×10^{-1}	0.39	**6.8×10^{-6} ***
IL-6	0.82	**8.2×10^{-29} ****	- 0.62	**2.6×10^{-14} ****	0.76	**7.0×10^{-23} ****
IL-7	0.42	**1.6×10^{-6} ***	- 0.12	1.8×10^{-1}	0.36	**3.5×10^{-5} ***
IL-8	0.82	**3.4×10^{-28} ****	- 0.26	2.8×10^{-3}	0.79	**3.0×10^{-25} ****
IP-10	0.71	**3.5×10^{-19} ****	- 0.56	**2.4×10^{-11} ****	0.35	**4.8×10^{-5} ***
MCP-1	0.71	**2.6×10^{-19} ****	- 0.60	**4.8×10^{-13} ****	0.37	**2.3×10^{-5} ***
MIP-1α	0.77	**3.7×10^{-23} ****	- 0.65	**8.3×10^{-16} ****	0.64	**3.0×10^{-15} ****
MIP-1β	0.82	**1.2×10^{-28} ****	- 0.73	**2.4×10^{-20} ****	0.72	**8.7×10^{-20} ****
PDGF-AB/BB	0.23	8.0×10^{-3}	0.21	1.5×10^{-2}	0.33	**1.8×10^{-4} ***
sCD40L	0.21	1.5×10^{-2}	0.33	**2.0×10^{-4} ***	0.28	1.4×10^{-3}
TGF-α	0.65	**8.6×10^{-16} ****	- 0.36	**2.7×10^{-5} ***	0.54	**1.1×10^{-10} ***
TNF-α	0.84	**8.6×10^{-32} ****	- 0.70	**1.0×10^{-18} ****	0.78	**1.7×10^{-24} ****

Table 2. Pairwise comparisons of cytokine levels across 3 *ex vivo* conditions.
*****: significant at $\alpha = \frac{0.05}{21 \times 3} \approx 7.9 \times 10^{-4}$ (Bonferroni correction)
******: significant at $\alpha = 1.0 \times 10^{-10}$
r: effect size computed from nonparametric test statistics

Table 3

Cytokine	Control					LPS					LPS + CBL				
	F1	F2	F3	F4	F5	F1	F2	F3	F4	F5	F1	F2	F3	F4	F5
IL-3	**0.956**	0.071	0.021	-0.004	0.047	**0.871**	0.084	0.084	0.093	0.029	**0.882**	0.101	0.108	0.082	0.002
IL-12P70	**0.945**	0.193	0.104	-0.049	0.056	**0.778**	0.466	0.061	0.071	-0.004	**0.754**	0.447	0.108	0.203	0.041
Flt-3L	**0.943**	0.083	0.092	-0.012	0.058	**0.737**	0.277	0.144	0.035	-0.021	**0.663**	0.312	0.214	0.179	0.016
FGF-2	**0.942**	0.137	0.095	0.077	0.045	**0.747**	0.401	0.285	0.013	-0.032	**0.710**	0.366	0.372	0.142	0.015
IFN-α2	**0.919**	0.192	0.152	0.061	0.103	**0.735**	0.367	0.234	0.087	0.076	**0.704**	0.330	0.275	0.130	0.146
IL-7	**0.916**	0.189	0.135	0.165	0.111	**0.653**	0.425	0.370	0.192	0.093	**0.537**	0.520	0.333	0.134	0.148
VEGF	**0.913**	0.332	0.124	0.070	0.093	**0.761**	0.537	0.164	0.087	0.093	**0.724**	0.540	0.220	0.163	0.113
IL-15	**0.877**	0.277	-0.024	-0.068	0.150	**0.687**	0.364	-0.028	-0.022	0.245	**0.697**	0.411	-0.017	-0.106	0.191
IL-12P40	**0.870**	0.306	0.043	-0.097	0.146	**0.764**	0.297	-0.127	0.101	0.204	**0.747**	0.386	-0.063	-0.041	0.178
IL-2	**0.863**	0.249	0.039	0.098	-0.005	**0.859**	0.139	0.099	-0.024	0.013	**0.882**	0.122	0.143	0.073	0.004
GM-CSF	**0.856**	0.353	0.264	0.020	0.117	0.562	**0.718**	0.082	0.202	0.051	0.541	**0.729**	0.129	0.179	0.094
G-CSF	**0.825**	0.097	0.070	0.317	0.133	-0.047	0.106	**0.269**	-0.022	0.028	**0.551**	0.513	0.469	0.119	0.099
IFN-γ	**0.820**	0.185	0.222	0.006	0.051	**0.734**	0.247	0.017	0.182	0.029	**0.734**	0.223	0.062	0.295	0.047
IL-10	**0.809**	0.173	0.046	-0.093	0.093	**0.622**	0.183	-0.175	0.366	0.163	**0.625**	0.316	-0.109	0.252	0.092
IL-17A	**0.762**	0.399	0.120	-0.038	-0.025	**0.621**	0.531	-0.005	0.116	-0.058	**0.648**	0.425	0.045	0.254	-0.036
Fractalkine	**0.694**	0.152	0.049	0.163	0.045	**0.476**	0.230	0.300	0.015	-0.004	**0.391**	0.197	0.333	0.050	0.200
MDC	**0.202**	0.055	0.049	0.100	-0.053	0.186	**0.644**	0.079	-0.008	-0.098	0.103	**0.474**	0.056	-0.071	-0.086
MCP-3	0.269	**0.938**	-0.011	0.025	0.100	0.426	**0.816**	0.053	-0.032	0.221	0.483	**0.794**	0.016	-0.083	0.211
IL-13	0.293	**0.930**	-0.033	0.048	0.172	0.419	**0.797**	0.080	-0.097	0.337	0.486	**0.756**	0.036	-0.121	0.317
TNF-β	0.249	**0.912**	-0.035	0.036	0.294	0.392	**0.759**	0.085	-0.110	0.442	0.456	**0.717**	0.031	-0.125	0.439
IL-1RA	0.311	**0.910**	0.056	0.067	0.153	0.489	**0.722**	0.052	0.082	0.257	0.540	**0.709**	0.012	0.000	0.261
IL-5	0.315	**0.891**	-0.053	0.072	0.145	0.433	**0.748**	0.113	-0.104	0.299	0.496	**0.697**	0.051	-0.138	0.287
TGF-α	0.467	**0.761**	0.089	-0.051	-0.012	0.424	**0.845**	-0.006	0.070	0.015	0.428	**0.840**	0.025	0.110	0.024
Eotaxin	0.304	**0.718**	0.227	0.079	-0.016	0.500	**0.517**	0.090	-0.072	0.107	**0.497**	0.494	0.016	-0.072	0.078
IL-8	0.125	**0.692**	0.398	-0.039	-0.052	0.192	**0.695**	-0.034	0.237	-0.090	0.143	**0.762**	0.009	0.242	-0.057
MIP-1β	0.019	0.070	**0.960**	0.010	0.045	0.069	-0.003	-0.122	**0.860**	0.014	-0.008	0.120	-0.058	**0.750**	0.049
TNF-α	0.164	0.005	**0.948**	0.010	0.005	0.231	0.006	0.085	**0.859**	-0.047	0.280	0.000	0.021	**0.837**	-0.052
MIP-1α	0.010	0.021	**0.911**	0.054	0.041	0.086	0.019	-0.067	**0.905**	0.033	0.053	0.106	-0.008	**0.792**	0.042
IL-1β	0.392	0.131	**0.836**	-0.058	0.034	0.287	0.072	-0.170	**0.762**	-0.043	0.202	0.067	-0.131	**0.751**	-0.041
IL-6	0.099	0.305	**0.762**	-0.049	0.489	0.291	**0.747**	-0.011	0.450	0.046	0.308	**0.793**	0.017	0.352	0.069
MCP-1	0.097	0.617	**0.527**	-0.032	-0.014	0.064	**0.638**	-0.026	0.620	-0.069	0.084	**0.752**	0.048	0.449	-0.037
IP-10	0.118	0.005	**0.362**	-0.079	-0.092	-0.169	0.087	-0.028	**0.677**	-0.024	0.093	-0.034	-0.046	**0.768**	-0.037
PDGF-AA	0.114	0.021	0.018	**0.942**	-0.032	0.262	-0.116	**0.881**	-0.051	-0.059	0.202	-0.099	**0.896**	-0.020	-0.018
sCD40L	0.089	-0.066	-0.058	**0.928**	-0.032	0.165	-0.168	**0.893**	-0.065	-0.054	0.097	-0.157	**0.915**	-0.060	-0.040
PDGF-AB/BB	0.065	-0.003	-0.045	**0.928**	-0.032	0.142	-0.080	**0.913**	-0.081	-0.051	0.031	-0.048	**0.920**	-0.059	-0.049
GRO	0.071	0.030	-0.007	**0.902**	-0.026	0.160	0.192	**0.826**	-0.039	-0.070	0.074	0.263	**0.851**	-0.021	-0.029
EGF	0.156	0.430	-0.057	**0.731**	0.022	0.140	0.237	**0.700**	-0.112	0.103	0.173	0.259	**0.718**	-0.145	0.075
RANTES	-0.200	-0.100	-0.033	**0.387**	0.326	-0.250	-0.067	**0.457**	0.040	0.294	**-0.397**	0.034	0.371	-0.044	0.294
IL-1α	0.090	0.147	-0.012	-0.034	**0.962**	0.023	0.089	0.005	-0.007	**0.951**	0.032	0.059	-0.018	-0.015	**0.965**
IL-9	0.213	0.219	-0.004	-0.047	**0.931**	0.099	0.119	-0.020	-0.021	**0.951**	0.109	0.110	-0.021	-0.040	**0.950**
IL-4	0.317	0.155	0.129	0.008	**0.861**	0.179	0.107	0.042	-0.026	**0.882**	0.188	0.082	0.037	0.059	**0.917**

Table 3. Factor loadings for 5 factors (F1 – F5) derived from cytokine levels in the control, LPS, and LPS + CBL conditions using principal component analysis (Rotation method: Varimax with Kaiser Normalization). These 5 factors explained 81.1%, 73.5%, and 74.1% data variance, respectively. Major loadings (>0.3) for each factor are bolded.

Figure 2

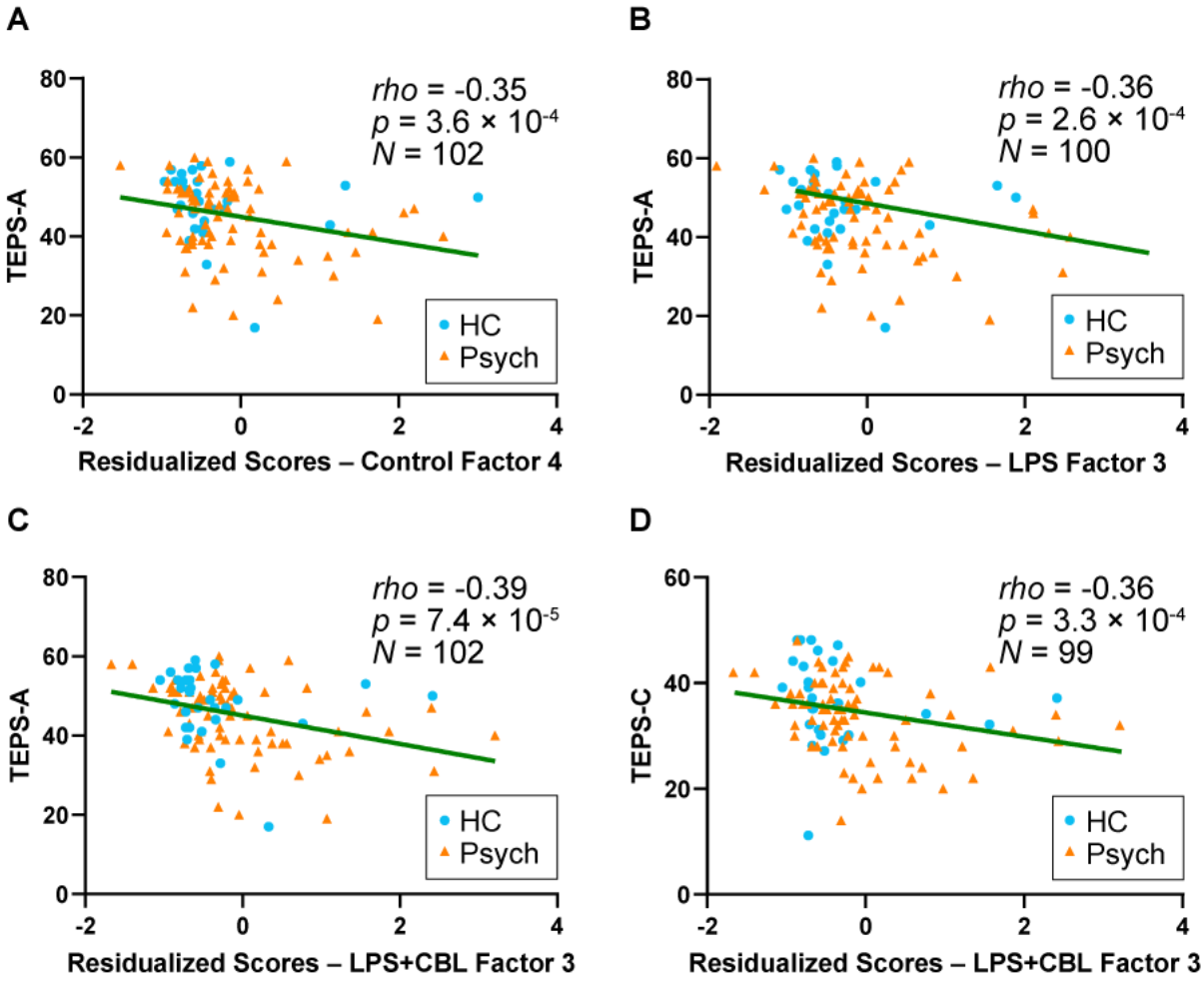

Figure 2. Partial correlations controlling for age, sex, and body mass index (BMI) between **(A)** Control Factor 4 and Anticipatory Anhedonia; **(B)** LPS Factor 3 and Anticipatory Anhedonia; **(C)** LPS+CBL Factor 3 and Anticipatory Anhedonia; **(D)** LPS+CBL Factor 3 and Consummatory Anhedonia. All are significant at Bonferroni-corrected significant threshold $\alpha = \frac{0.05}{5\times2\times3} \approx 1.7 \times 10^{-3}$.

Abbreviations: CBL: clenbuterol; HC: healthy control participants; LPS: lipopolysaccharide; Psych: participants with psychiatric symptoms and/or diagnoses. TEPS-A: Temporal Experience of Pleasure Scale – Anticipatory; TEPS-C: Temporal Experience of Pleasure Scale – Consummatory.

Figure 3

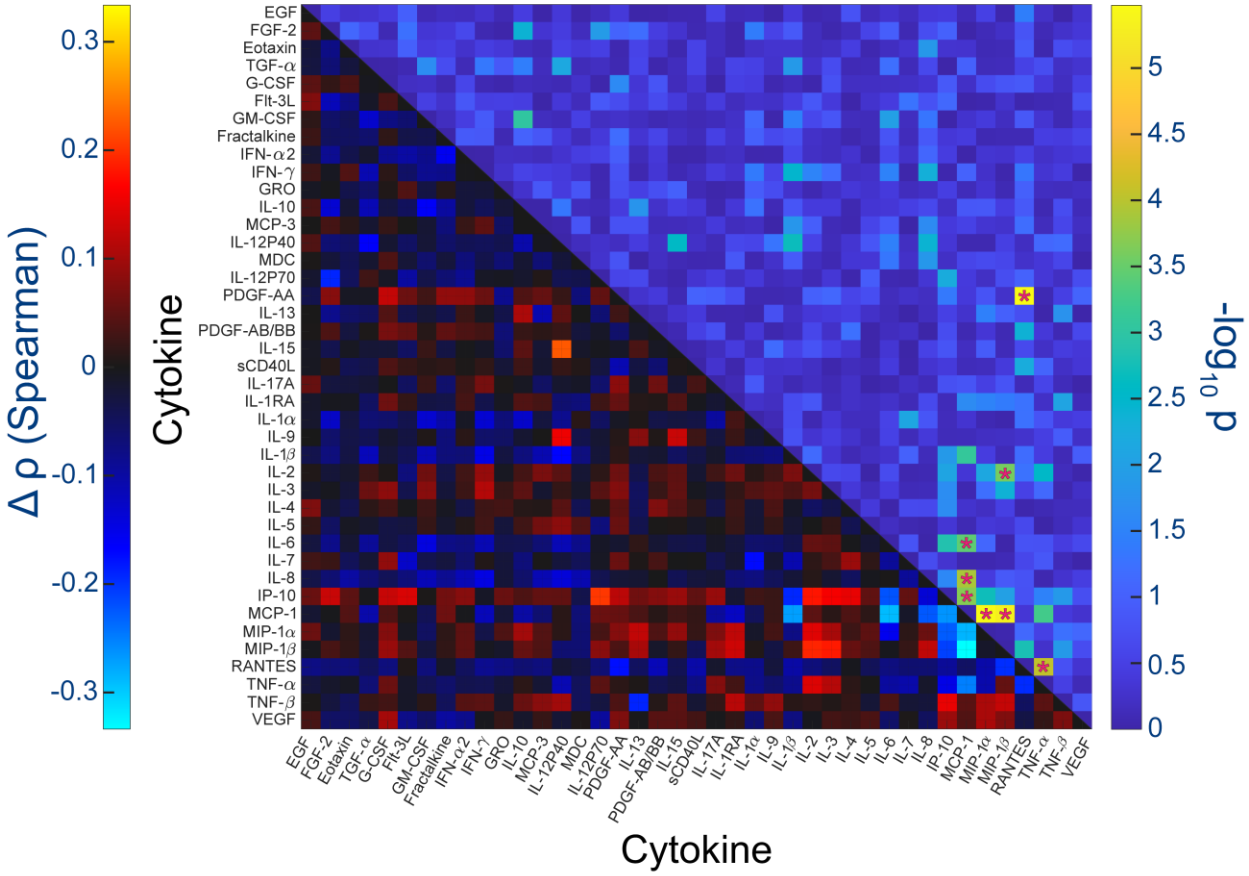

Figure 3. Change in cytokine correlations in the LPS+CBL condition relative to the LPS condition (LPS+CBL – LPS; lower half triangle), and their corresponding log-transformed p values (upper half triangle). Significant differences after false discovery rate (FDR) correction are marked by asterisks (*).
Abbreviations: CBL: clenbuterol; LPS: lipopolysaccharide