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Immune cell exhaustion and apoptotic markers in major depressive disorder: Effects of in vitro cannabidiol administration

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A B S T R A C T

Background: Immune dysregulation is a component of Major Depressive Disorder (MDD). Cannabidiol (CBD) has immunomodulatory and putative antidepressant effects. The relationship between apoptotic and exhaustion immune markers and the clinical features of MDD and the effects of CBD on these markers are still unknown.

Objectives: To assess pro-apoptotic (CD95) and T cell exhaustion (TIM3) markers on immune cells in patients with MDD, as well as the impact of in vitro CBD administration on these markers.

Methods: We recruited healthy controls and MDD patients and evaluated the immunophenotypes of T/B lymphocytes using flow cytometry in unstimulated and anti-CD3/CD28 stimulated conditions. We evaluated the immune profiles of M1 macrophages, immune-inflammatory response system (IRS), compensatory immunoregulatory system (CIRS), T cell proliferation, and immune-related neurotoxicity (IRN). We investigated the in vitro effects of CBD on immune cell subsets at concentrations of 0.1 μg/mL, 1 μg/mL, and 10.0 μg/mL.

Results: The stimulated $CD3^{+}CD95^{+}$ cell percentages were substantially correlated with the number of depressive episodes, recurrence of illness, and suicidal behaviors. The stimulated CD8+TIM3+ cell percentages were substantially and inversely associated with the M1, IRS, CIRS, T cell growth, and IRN immune profiles. TIM3+ bearing $CD3^{+}$, $CD4^{+}$ and $CD8^{+}$,cells were significantly suppressed by lower CBD concentrations (0.1–1 μg/mL). TIM3+ and $CD95^{+}$ bearing cells were significantly suppressed by the higher CBD concentrations (10.0 μg/mL).

Discussion: Aberrations in immune checkpoint molecular processes impact the features of MDD. CBD significantly impacts apoptotic and exhaustion processes thereby possibly interfering with immune homeostasis.

1. Introduction

Major depressive disorder (MDD) is among the most common psychiatric disorders globally. Recent studies indicate that MDD is associated with immunological dysregulation (Ruiz et al., 2022; Maes et al., 2022). MDD is linked to the activation of the immune-inflammatory response system (IRS), as indicated by elevated levels of pro-inflammatory cytokines, including interleukin (IL)-1β, IL-6,

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interferon (IFN)-γ, and tumor necrosis factor (TNF)-α (Maes and Carvalho, 2018; Więdłocha et al., 2018). MDD is also associated with heightened activity of the compensatory immune-regulatory system (CIRS) (Maes and Carvalho, 2018). The latter may mitigate excessive inflammation by downregulating the IRS and generating anti-inflammatory cytokines such as IL-4, IL-10, and transforming growth factor (TGF)-β(3). In the acute phase of severe MDD, the IRS is more activated than the CIRS, leading to net activation of the IRS (Maes and Carvalho, 2018). A prior flow cytometry investigation corroborates this hypothesis, demonstrating that individuals with MDD exhibit heightened activation of T helper ($CD4^+$) and cytotoxic T cells ($CD8^+$), accompanied by a reduction in T regulatory (Treg) cells (Rachayon et al., 2024). Treg cells are a crucial element of the CIRS functioning to suppress excessive T cell activation through various mechanisms (Gao et al., 2023). These include inhibition via CTLA-4 (CD152), which binds to the same antigen-presenting cell ligands as CD28, and the secretion of the anti-inflammatory cytokine TGF-β1(Workman et al., 2009). When Treg populations are affected, immune tolerance may be disrupted.

The dysregulation of these systems during MDD episodes may result in heightened activation of M1 macrophages, T helper (Th)1 and Th17 cells, elevated concentrations of their neurotoxic cytokines, and an expanded population of $CD4^+CD40L$ + T cells capable of inducing neurotoxicity (Maes and Carvalho, 2018; Rachayon et al., 2024). Moreover, increased numbers of activated T cells and lowered Treg cell numbers are significantly associated with key features of MDD, namely the number of depressive episodes and suicidal attempts, lifetime suicidal behaviors (that is a composite based on suicidal ideation and attempts), recurrence of illness (ROI, that is a composite based on the number of episodes, suicidal behavior and ideation), and the severity of the phenome of the index episode (a composite based on severity of depression, anxiety and suicidal behaviors) (Maes et al., 2024).

The activation of the IRS is governed not only by the CIRS pathways, involving Th2 and Treg cells, but also by apoptotic events and T cell exhaustion (Duman, 2009). Two key pathways that govern apoptosis and the exhaustion of immune cells are CD95 and TIM3 (Peter et al., 2015). CD95, or the Fas/APO-1 receptor, is essential for initiating apoptosis and significantly influences IRS activity. Upon activation by its ligand, CD95 ligand (CD95L), CD95 forms the death-inducing signaling complex (DISC). This complex comprises CD95, FADD, procaspase-8, procaspase-10, and c-FLIP, all of which are crucial for the initiation of apoptosis (Peter et al., 2015; Fouqué et al., 2014). CD95-mediated apoptosis is essential for immune surveillance, facilitating the removal of aberrant cells and averting illnesses such as cancer and autoimmune disorders (Risso et al., 2022). Patients with treatment-resistant depression exhibit a reduced fraction of $CD3^+CD8^+CD95^+$ T cells, suggesting a possible imbalance in apoptotic pathways and immunological function (Maes et al., 2021; Miller, 2010; Mohd Ashari et al., 2019).

TIM3, or T cell immunoglobulin mucin-3, is an immune-regulatory protein which expression is elevated on exhausted T cells (Sakuishi et al., 2010a). Furthermore, TIM3 modulates apoptosis by interacting with its receptor, galectin-9, resulting in calcium influx into Th1 cells, which initiates apoptosis and suppresses Th1-mediated immune responses (Banerjee and Kane, 2018; Du et al., 2017). A study by Qin et al. indicates that TIM3 is associated with chronic stress, promoting autophagy, and leading to stress-induced immunosuppression (Qin et al., 2019). Nevertheless, the associations between CD95 and TIM3 bearing immune cells and the features of MDD, including suicidal behaviors, recurrence of illness (staging), severity of the index episode, have remained unknown.

Recent research indicates that cannabidiol (CBD), a phytocannabinoid derived from the cannabis plant, may possess antidepressant characteristics, as evidenced in animal models of depression (Silote et al., 2019; Campos and Guimarães, 2008). CBD functions via the endocannabinoid system, which comprises cannabinoid receptors, chiefly cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2) (Pertwee, 2006). CB1 receptors are predominantly located in the central nervous system, with lower levels in peripheral blood cells. In contrast, CB2 receptors are primarily located on peripheral immune cells, such as B lymphocytes (Piomelli, 2003). The regulation of CB1 and CB2 receptors is recognized to affect IRS functions (Massi et al., 2006; Kaplan, 2013; Maes et al., 2023). CB1 receptors, despite their lower prevalence in T cells, are crucial for T cell growth and death (Kaplan, 2013). Both CB1 receptor agonists and antagonists demonstrate anti-inflammatory effects, highlighting CB1's dual function in immune regulation (Smith et al., 2000; Lou et al., 2012). Exogenous CB1 agonists are recognized for their immunosuppressive and anti-inflammatory properties, although CB1 antagonists also have anti-inflammatory effects, highlighting the intricacy of CB1-mediated immune responses (Kaplan, 2013; Smith et al., 2000; Lou et al., 2012). CB2 receptors are present on peripheral blood mononuclear cells (PBMC) (Turcotte et al., 2016; Graham et al., 2010), and exogenous CB2 receptor agonists exert anti-inflammatory effects via multiple pathways, such as lowering pro-inflammatory cytokines, decreasing reactive oxygen species, attenuating T cell activation, and slowing microglial migration (Hegde et al., 2008; Wen et al., 2015; Romero-Sandoval et al., 2009; McCoy et al., 1999). Previous studies have also identified a dose-dependent effect of CBD, with higher doses exhibiting a more obvious immunosuppressive effect through CB1/CB2 receptors (Kaplan et al., 2008a; Rachayon et al., 2022). However, there is no research on CBD's effects on the number of immune cells bearing $CD95^+$ and TIM3+ markers.

Hence, this study is performed to examine a) the relationship between T cell apoptosis and exhaustion cell markers and MDD and its features; b) the effects of in vitro CBD administration on the number of T cells bearing $CD95^+$ and TIM3+; and c) the associations among $CD95^+$ and TIM3+ bearing cells and immune cytokine and chemokine profiles. The $CD95^+$ and TIM + cell subsets were assayed both in unstimulated (non-proliferating) and stimulated (proliferating) conditions (T cell activation via anti-CD3 and anti-CD28). The latter condition mimics the engagement of T cells with antigen-presenting cells resulting in activation and expansion of these cells (Rachayon et al., 2024).

2. Methodology

2.1. Participants

The research included subjects from Chulalongkorn University in Bangkok. Senior psychiatrists from the Department of Psychiatry enlisted outpatients diagnosed with MDD, while healthy controls were recruited via posters and referrals. All participants filled out questionnaires and submitted blood samples. The inclusion criteria for patients comprised persons aged 18 to 65 who comprehended Thai, had received a diagnosis of MDD from a psychiatrist utilizing DSM-5 criteria, and possessed a Hamilton Depression Rating Scale (HAMD) score exceeding 17, signifying moderate to severe depression. Patients were excluded if they had other psychiatric disorders such as schizophrenia, schizoaffective disorder, obsessive-compulsive disorder, post-traumatic stress disorder, psycho-organic disorders, or substance use disorders. Healthy controls were matched for age, sex and educational attainment and were not included if they had any axis 1 DSM-IV-TR condition or a familial history of MDD, BD, or psychosis. Patients and controls were excluded if they exhibited allergic or inflammatory responses in the preceding three months, had neuroinflammatory, neurodegenerative, or neurological disorders (e.g., epilepsy, Alzheimer's disease, multiple sclerosis, Parkinson's disease), (auto)immune diseases (e.g., COVID-19 infection, chronic obstructive pulmonary disease, inflammatory bowel disease, psoriasis, type 1 diabetes, asthma, rheumatoid arthritis), a history of immunomodulatory drug usage, therapeutic doses of antioxidants or omega-3 supplements within three months, or anti-inflammatory drug usage within one month prior to the study. Women who are pregnant or nursing were also excluded. A subset of MDD patients were administered psychotropic drugs, comprising sertraline (18 patients), various other

antidepressants (8 patients; fluoxetine, venlafaxine, escitalopram, bupropion, mirtazapine), benzodiazepines (22 patients), atypical antipsychotics (14 patients), and mood stabilizers (4 patients). The statistical analyses were adjusted for effects of the drug state (these drug state variables were entered as binary data in the multivariate analyses, see below).

All participants provided written informed consent, and the study was conducted in accordance with international and Thai ethical and privacy legislation. The Institutional Review Board of the Faculty of Medicine at Chulalongkorn University sanctioned the study (#528/63), in compliance with the Declaration of Helsinki, the Belmont Report, CIOMS Guidelines, and the International Conference on Harmonization in Good Clinical Practice (ICH-GCP).

2.2. Measurements

2.2.1. Clinical assessments

A psychiatrist performed semi-structured interviews. This interview included demographic data such as age, gender, body mass index, number of education years, smoking behaviors, and the incidence of depressive episodes. The diagnosis of Major Depressive Disorder (MDD) was established utilizing the DSM-5 criteria (Diagnostic and statistical manual of, 2013). Psychiatric illnesses were identified in each patient utilizing the Mini-International Neuropsychiatric Interview (M.I.N.I.) (Pettersson et al., 2018). The same physician administered the 17-item Hamilton Depression Rating Scale (HAM-D) to assess the severity of depressive symptoms (Zimmerman et al., 2013). The Thai version of the State–Trait Anxiety Inventory (STAI) was utilized to assess the intensity of state anxiety. The Columbia-Suicide Severity Rating Scale (C-SSRS) lifeline version was employed to evaluate the degree of current and lifetime suicidal ideation among participants, as well as any history of suicide attempts (Salvi, 2019).

According to Maes et al. (2024), lifetime suicidal behaviors were calculated as the first principal component derived from lifetime C-SSRS items concerning suicidal ideation (SI) and suicidal attempts (SA). A composite score was generated utilizing both SI and SA, termed lifetime suicidal behaviors (SB). ROI was determined by the first principal component derived from the quantity of depressive episodes, the C-SSRS item about "lifetime suicidal ideation," and the C-SSRS item concerning the total number of actual lifetime suicide attempts. The severity of the phenome of MDD was calculated as outlined by Maes et al. (Gao et al., 2023). To do this, we utilized the latent variable scores derived from the first principal component retrieved from the HAMD and STAI scores, along with current suicidal behaviors. The latter scores were calculated using 9 C-SSRS questions that evaluated recent (past month) reports of suicidal ideation and attempts. Tobacco use disorder was diagnosed based on DSM-5 criteria. Weight (in kilograms) was divided by height (in square meters) to get body mass index (BMI).

2.2.2. Methodology

Blood samples were collected from participants after an overnight fast, between 8:00 and 9:00 a.m. using BD Vacutainer® EDTA (10 mL) and BD Vacutainer® SST™ (5 mL) tubes from BD Biosciences (Franklin Lakes, NJ, USA). Serum was obtained by allowing the serum-separating tubes to clot at room temperature for 30 min, followed by centrifugation at 1100 g for 10 min at 4 °C. PBMCs were diluted with RPMI 1640 medium and isolated using density gradient centrifugation method. Heparinized blood samples were overlaid with Ficoll® Paque Plus Solution (GE Healthcare Life Sciences, Pittsburgh, PA, USA) and centrifuged for 30 min at 1500 rpm at ambient temperature. PBMCs were washed twice and counted for determining the viability with a hemocytometer and trypan blue (0.4 % solution, pH 7.2–7.3, Sigma-Aldrich Corporation, St. Louis, Missouri, United States), ensuring over 95 % cell viability under all conditions. For PBMC activation, 96-well plates were prepared by coating them overnight with 5 μg/mL of anti-human CD3 antibody (OKT3, eBioscience). Each well was then seeded with 3 $\times$ 10^5 PBMCs and 5 μg/mL of anti-human CD28 antibody (CD28.2, eBioscience). The cells were cultured in RPMI 1640 medium with L-glutamine, supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin-streptomycin (Gibco Life Technologies, Carlsbad, CA, USA), for 3 days at 37 °C in a 5 % CO_2 incubator. Unstimulated PBMCs cultured under the same conditions were used as a negative control. After the 3-day culture period, lymphocyte immunophenotypes were determined using flow cytometry. The natural CBD (99.89 % isolate) stock solution was prepared and its effect were determined with 3 concentrations: 0.1 μg/ml, 1 μg/mL, and 10 μg/mL. We assessed the percentage of 6 different phenotypes, namely $CD3^+CD95^+$, CD3+TIM3+, $CD4^+CD95^+$, CD4+TIM3+, $CD8^+CD95^+$, CD8+TIM3+cells and this in 5 conditions: unstimulated or baseline, anti-CD3 and anti-CD28 stimulated, stimulated +0.1 μg/mL CBD, stimulated +1.0 μg/mL CBD and stimulated +10.0 μg/mL CBD. The selected CBD concentrations were based on previous human studies, which have established a therapeutic dose range of 5–50 mg/kg/day, corresponding to plasma levels of 7.1–1200 ng/mL, with higher doses resulting in proportionally increased plasma concentrations (Millar et al., 2019). Therefore, we utilized CBD concentrations of 0.1–1.0 μg/mL. In rodent models, the concentrations in mesenteric lymph nodes exceed those in the spleen by over 50-fold and surpassed plasma levels by 250-fold (review: 36). Oral CBD combined with dietary lipids may significantly enhance the transport of CBD to the plasma and mesenteric lymphatic system (review: 36). Consequently, lymphatic PBMCs may encounter elevated CBD concentrations inside the 5–20 μg/mL range when employing this focused methodology, which may ultimately be utilized in individuals with inflammatory conditions (review: 36). Moreover, doses between 5 and 20 μg/mL suppress human PBMCs, and CBD has demonstrated effective anti-inflammatory properties at concentrations of 1–10 μM in immune cell studies (Zgair et al., 2017; Peyravian et al., 2020). Therefore, we included 10 μg/mL to represent CBD effects at higher concentrations.

To study lymphocyte immunophenotypes, cultured PBMCs were labeled with monoclonal antibodies, including CD3-PEcy7, CD4-APCcy7, CD8-APC, CD95-PE/Dazzle594 and TIM3-PE (Biolegend, BD Biosciences and R&D Systems). We stained cells with all antibodies above which are surface markers for 30 min at 4 °C in the dark. Cells were washed 3 times by 1X PBS supplemented with 2 % FBS. The flow cytometry was performed using LSRII flow cytometer (BD Biosciences) to evaluate lymphocyte immunophenotypes. All data were analyzed using FlowJo X software (Tree Star Inc., Ashland, OR, USA). Electronic Supplementary File (ESF), Fig. 1 shows our gating strategies.

As previously described (Maes et al., 2023), we measured cytokines/chemokines/growth factors in unstimulated and stimulated diluted whole blood culture supernatant using the same blood samples as those employed for the flow cytometry. We used RPMI-1640 medium supplemented with L-glutamine, phenol red, and 1 % penicillin (Gibco Life Technologies, USA), with or without 5 μg/mL PHA (Merck, Germany) + 25 μg/mL lipopolysaccharide (LPS; Merck, Germany). On sterile 24-well plates, 1.8 mL of each medium was combined with 0.2 mL of 1/10-diluted whole blood. Using the Bio-Plex Pro human cytokine 27-plex assay kit (BioRad, Carlsbad, California, United States of America) and the LUMINEX 200 instrument (BioRad, Carlsbad, California, United States of America), the cytokines/chemokines/growth factors were measured. The intra-assay CV values were less than 11 %. The M1, IRS, CIRS, neurotoxicity, and T cell growth immune profiles were determined as z unit-based composite scores as explained previously (Rachayon et al., 2022). ESF, Table 1 lists the cytokines, colony stimulating factors, and growth factors determined in the current study. ESF, Table 2 shows how the immune profile composites were computed.

3. Statistics

Analysis of variance (ANOVA) was utilized to evaluate continuous variables across categories, whilst chi-square tests were conducted to ascertain associations among categorical variables. The various impacts

Table 1
Demographic and clinical data of in major depressed patients (MDD) and healthy controls (HC).

Variables	HC (n = 19)	MDD (n = 29)	F/X^2	df	p
Sex (Male/Female)	6/13	11/18	0.20	1	0.653
Age (years)	33.8 (8.2)	28.9 (8.6)	F = 3.94	1/46	0.053
Education (years)	16.0 (2.3)	15.7 (1.4)	F = 0.53	1/46	0.473
Smoking (Yes/No)	17/2	23/6	FEPT	–	0.366
BMI (kg/m^2)	21.5 (2.5)	25.0 (6.0)	F = 6.19	1/46	0.017
HAMD	1.0 (1.6)	23.4 (5.8)	MWUT	1/46	<0.001
STAI	37.7 (10.9)	56.7 (7.3)	51.83	1/46	<0.001
Number of depressive episodes	0	2.03 (0.94)	MWUT	–	<0.001
Number of suicidal attempts	0	1.24 (1.55)	MWUT	–	<0.001
Lifetime suicidal behaviors	−0.987 (0.0)	0.624 (0.758)	MWUT	–	<0.001
Recurrence of illness index	−1.089 (0.0)	0.731 (0.560)	MWUT	–	<0.001
Total phenome score	−1.131 (0.338)	0.747 (0.408)	277.14	1/46	<0.001

Results are shown as mean (±SD). FEPT: Fisher's exact probability test; X^2: analysis of contingency tables, F: results of analysis of variance, MWUT: Mann-Whitney *U* test; BMI: body mass index; HAMD: Hamilton Depression Rating Scale score.

of time or groups on immune profiles underwent a false discovery rate (FDR) p correction (Ni et al., 2024).

We utilized generalized estimating equations (GEE) analysis with repeated measurements to assess the effect of CBD administration on the immune cell subsets. The predetermined GEE analyses, employing repeated measures (unstructured correlation matrix, linear scale response, and maximum likelihood estimation for the scale parameter), incorporated fixed categorical effects of time (unstimulated, stimulated, and stimulated with the three CBD concentrations), diagnostic groups (depression versus controls), time-by-group interaction, sex, smoking, and continuous fixed covariates, specifically age and BMI. The repeated measurements of immune cell subtypes were the dependent variables. We calculated the estimated marginal mean (SE) values for the treatment and diagnostic groups, as well as the treatment × group interactions, and employed protected pairwise contrasts (least significant difference at p = 0.05) to assess differences between the treatment conditions and the interaction patterns between group and treatment. Additionally, we incorporated the drug state as binary variables into the GEE analysis as supplementary predictors to eliminate any influence of these potential confounders. The GEE technique allows for the consideration of significant confounders in the analysis of treatment effects at the individual level.

Multiple regression analysis was employed to identify the relationships between the features of depression (dependent variables) and the unstimulated and stimulated immune cell types, while allowing for the effects of age, sex, BMI, smoking and the drug state of the patients. To achieve this, we employed a manual and an automated method with a p-entry threshold of 0.05 and a p-removal threshold of 0.06 while evaluating the changes in R^2. Multicollinearity was assessed using tolerance and variance inflation factor, whilst multivariate normality was evaluated by Cook's distance and leverage, and homoscedasticity was examined via the White and modified Breusch-Pagan tests. The results of the regression analysis were consistently bootstrapped using 5000 bootstrap samples, and these are given if the findings were incongruent.

There were no missing values in any of the clinical, immune, or sociodemographic data. The tests were two-tailed, with statistical significance established at $p < 0.05$. The statistical studies were conducted using IBM SPSS version 28 for Windows.

Employing a two-tailed test with a significance level of 0.05 and assuming a power of 0.80, an effect size of 0.2, two groups, and five measurements with intercorrelations of around 0.4, the estimated sample size for a repeated measures ANOVA would be approximately 38.

4. Results

4.1. Demographic and clinical data

Table 1 displays the sociodemographic and clinical information of the patients and the control group in the study. The two groups exhibited no significant differences in age, gender distribution, educational attainment, or smoking behaviors. Nevertheless, patients exhibited a somewhat elevated BMI in comparison to the controls. We controlled all findings for potential confounding variables, including age, BMI, sex, smoking, and medication status. However, no significant impacts from these potential confounding factors were detected. The patients had markedly elevated average HAMD and STAI scores compared to the controls, signifying that the majority of patients were undergoing moderate to severe clinical depression and anxiety. All clinical characteristic ratings for depression were considerably elevated in MDD compared to controls.

4.2. Baseline and stimulated lymphocyte population in MDD

As shown in ESF Table 3, no significant differences in immune profiles were observed between MDD patients and healthy controls, including $CD3^+$, $CD4^+$, and $CD8^+$ cells expressing $CD95^+$ or TIM3+, under both baseline and stimulated conditions.

Table 2
Results of multiple regression analyses with the clinical features of major depression as dependent variables.

Dependent Variables	Explanatory Variables	Estimates			Model			
		B	t	p	F	df	p	R^2
Number of depressive episodes	Model $CD3^+CD95+$%	−0.303	−2.16	0.036	4.65	1/46	0.036	0.092
Number of suicidal attempts	Model $CD3^+CD95+$%	−0.318	−2.28	0.028	5.18	1/46	0.028	0.101
Lifetime suicidal behaviors	Model $CD3^+CD95+$%	−0.394	−2.91	0.006	8.46	1/46	0.006	0.155
Recurrence of illness index	Model $CD3^+CD95+$%	−0.352	−2.55	0.014	6.49	1/46	0.014	0.124
Total phenome score	Model $CD3^+CD95+$%	−0.324	−2.32	0.025	5.39	1/46	0.025	0.105

%: prevalence of anti-CD3/CD28 stimulated immune cell populations.

Table 3
Results of multiple regression analyses with immune profiles as dependent variables and immune cell expression markers as explanatory variables.

Dependent Variables	Explanatory Variables	Estimates			Model			
		β	t	p	F	df	p	R^2
#1. M1 macrophage	Model S_CD8+TIM3+ %	−0.286	−2.03	0.049	4.1	1/46	0.049	0.082
#2. IRS	Model S_CD8+TIM3+ %	−0.301	−2.14	0.037	4.6	1/46	0.037	0.091
#3. CIRS	Model S_CD8+TIM3+ %	−0.298	−2.12	0.040	4.49	1/46	0.040	0.089
#4. T cell growth	Model S_CD8+TIM3+ %	−0.291	−2.06	0.045	4.25	1/46	0.045	0.085
#5. Immune-linked neurotoxicity	Model S_CD8+TIM3+ %	−0.366	−2.63	0.012	6.94	1/45	0.012	0.134

%: prevalence on immune cell populations; S: anti-CD3/CD28 stimulated, U: unstimulated. The automatic multiple regression methods could include one predictor only.
IRS: immune-inflammatory response system; CIRS: compensatory immunoregulatory system.

4.3. Associations between MDD features and number of CD95 bearing cells

Table 2 presents the outcomes of multiple regression analysis with clinical features as dependent variables. We identified substantial correlations between the proportion of $CD3^+CD95^+$ cells under stimulated circumstances and several clinical characteristics. The quantity of depressive episodes had a significant negative correlation with $CD3^+CD95+$%, which explained 9.2 % of the variance. A comparable trend was noted with the frequency of suicide attempts, exhibiting a significant negative impact that accounted for 10.1 % of the variance. $CD3^+CD95+$% accounted for 15.5 % of the variance in lifetime suicidal behaviors, demonstrating an inverse correlation. A higher percentage of $CD3^+CD95^+$ cells was correlated with a lower ROI. The severity of the phenome exhibited a notable negative correlation with the $CD3^+CD95^+$ T cells, accounting for 10.5 % of the variance.

Overall, the proportion of $CD3^+CD95^+$ cells under stimulated conditions consistently acted as a substantial negative predictor in all models, underscoring its potential impact on the severity of clinical features of MDD. The results for TIM3 expressing cells showed no association with depressive symptoms.

4.4. Regression analyses of immune profiles on numbers of $CD95^+$ and TIM3+ cells

We conducted multiple regression analysis with immunological profiles (M1, IRS, T cell growth, and neurotoxicity) as dependent variables and the percentages of CD95+/TIM3+ lymphocytes as the explanatory variable under both stimulated (S) and unstimulated (U) circumstances.

Table 3 indicates that, under stimulated conditions, the fraction of CD8+TIM3+ cells adversely affected the immunological profiles across the different models. First, elevated percentages of activated CD8+TIM3+ cells correlated with diminished expression of cytokine profiles, including M1, IRS, and CIRS, accounting for 8.2 %, 9.1 %, and 8.9 % of the variation, respectively. The activated percentage of CD8+TIM3+ cells had a negative correlation with T cell proliferation and immune-related neurotoxicity (IRN), explaining 8.5 % and 13.4 % of the variation, respectively. In depressed subjects, there were no significant associations between both factors.

4.5. Effects of cannabidiol on lymphocyte and pro-apoptotic markers percentages

Table 4 illustrates the impact of CBD on the proportions of $CD95^+$ and TIM3+ T cells. GEE analysis was employed to assess all subjects across five experimental conditions: unstimulated, stimulated with anti-CD3/anti-CD28 antibodies, and incubated with three varying doses of CBD. The findings underscore the impact of CBD on these immune cell subgroups. At lower concentrations (0.1 μg/mL and 1.0 μg/mL), CBD inhibited TIM3-expressing cells, encompassing the CD3+TIM3+, CD4+TIM3+, and CD8+TIM3+ subsets. Nevertheless, although significant, the effects showed modest decrements of around 5.0 % (at the 1.0 μg/mL range). At a concentration of 10 μg/mL, CBD exhibited significant suppressive effects on TIM3+ and $CD95^+$ expressing $CD3^+$, $CD4^+$, and $CD8^+$ cells. GEE analysis showed that the overall stimulated number of $CD3^+CD95^+$ cells was significantly ($p = 0.041$) lower in MDD (estimated marginal mean: 69.50 ± 1.28) than in controls (74.33 ± 1.82). There were no significant effects of CBD on the $CD3^+CD95^+$ and $CD4^+CD95^+$ cell subsets.

5. Discussion

5.1. Associations between pro-apoptotic markers and MDD

This study's first major finding is the negative correlation between CD95 expression on $CD3^+$ T cells under stimulated conditions and several clinical outcomes in depression, including ROI, total phenome score, frequency of depressive episodes, and lifetime suicidal behaviors. A reduced expression of CD95 was associated with elevated scores in

Table 4
Effects of cannabidiol (CBD) on anti-CD3/CD-28 induced changes in various $CD95^+$ or TIM3+ bearing immune cells.

Variables	Unstimulated	Stimulated (St)[a]	St + CBD 0.1 μg/mL[b]	St + CBD 1.0 μg/mL[c]	St + CBD 10 μg/mL[d]	Wald ×2 (df = 4)	p Value
$CD3^+CD95+$%	13.93 (2.96)	93.11 (0.56)[d]	92.83 (0.61)[d]	92.71 (0.78)[d]	67.02 (2.49)[abc]	361.54	<0.001
CD3+TIM3+%	4.25 (1.09)	61.03 (1.17)[bcd]	59.57 (1.27)[acd]	57.24(1.30)[abd]	15.40 (1.25)[abc]	1501.52	<0.001
$CD4^+CD95+$%	18.24 (3.21)	94.30 (0.54)[d]	93.95 (0.67)[d]	94.13 (0.91)[d]	69.47 (2.64)[abc]	331.85	<0.001
CD4+TIM3+%	2.95 (1.04)	54.91 (1.30)[bcd]	53.36 (1.45)[acd]	50.89(1.50)[abd]	11.33 (1.01)[abc]	1468.82	<0.001
$CD8^+CD95+$%	8.93 (2.94)	98.59 (0.18)[bd]	98.83 (0.17)[acd]	98.66 (0.23)[bd]	92.31 (1.41)[abc]	334.23	<0.001
CD8+TIM3+%	5.23 (1.66)	77.63 (1.38)[bcd]	76.28 (1.49)[acd]	73.63 (1.60)[abd]	20.96 (1.34)[abc]	828.95	<0.001

All results of GEE analysis examining the effects of time (unstimulated versus stimulated) and cannabidiol (CBD) effects on immune cell subsets bearing $CD95^+$ and TIM+. Results are shown as estimated marginal mean values (SE). All unstimulated immune cell numbers are significantly lower than the 4 stimulated values. [a,b,c,d]: protected post-hoc differences among the 4 stimulated values (at $p = 0.05$).

these outcomes, indicating that CD95 may have protective effects attenuating depressive symptoms and reduce the likelihood of relapse and suicidal behaviors.

It is well established that the acute phase of major depression is closely linked to immune system dysfunction (Maes et al., 2022; Miller, 2010). Studies have shown increased activation of T effector cells, including $CD4^+$ CD40L+, $CD8^+$ CD40L+, and $CD8^+$ HLA-DR+, accompanied by a reduction in T regulatory cell expression (Rachayon et al., 2024). Additionally, numerous studies have reported elevated production of pro-inflammatory cytokines, which are associated with both T cell activation and the clinical manifestation of depression (Rachayon et al., 2024; Miller, 2010; Stolfi et al., 2025). Notably, an increased expression of T helper 17 (Th17) cells has also been linked to a higher risk of suicidality (Schiweck et al., 2020). Peripheral immune cell dysfunction can disrupt the blood-brain barrier and induce microglia hyperactivation and neuroinflammation. This increase in immune-inflammatory response may contribute to neuronal cell loss, which may play a role in the pathophysiology of major depressive disorder (Maes and Carvalho, 2018; Maes et al., 2024).

The CD95 (Apo-1/Fas) and CD95 ligand (CD95L) interaction is a well-established mechanism of apoptosis induction in T cells (Krammer, 2000). CD95 functions as a death receptor that triggers apoptosis upon interaction with CD95L, an essential mechanism for maintaining immune tolerance and homeostasis (Li-Weber and Krammer, 2003; Yamada et al., 2017). Research by Paulsen et al. demonstrates that increased CD95L levels facilitate death, whilst reduced levels encourage T cell activation (Paulsen and Janssen, 2011). Thus, abnormal operation of the CD95/CD95L complex may lead to immunological dysregulations observed in several diseases (Li et al., 2014; Cullen Sean et al., 2013). However, limited research has investigated the associations between MDD and CD95 expression. One study found that patients with treatment-resistant depression (TRD), a subgroup likely experiencing severe depression, exhibited decreased frequencies of $CD3^+CD8^+CD95^+$ T cells, implying dysregulated T-cell apoptosis and impaired immune homeostasis (Szałach et al., 2022). This finding aligns with our results, which demonstrate a negative correlation between CD95 expression on $CD3^+$ cells and both the severity of the phenome of depression and suicidal behaviors.

A study by Szuster-Ciesielska et al. investigated apoptosis levels in the blood leukocytes of individuals with depression, revealing an increase in apoptosis correlated with heightened expression of CD95 (Szuster-Ciesielska et al., 2008). Similarly, CD95 dysregulation is apparent in other immune-related illnesses that demonstrate considerable comorbidity with MDD. For example, genetic mutations in CD95 or CD95L have been associated with systemic lupus erythematosus (SLE)—a condition frequently accompanied by depression as a neuropsychiatric manifestation (Risso et al., 2022). Additionally, significant decrease in Fas receptor expression has been seen in multiple sclerosis (MS) patients, with receptor levels rising following treatment, indicating that T cells in MS displayed reduced sensitivity to apoptosis prior to intervention (Božič and Rozman, 2006).

The cumulative effects of compromised Treg functions and dysfunctional apoptotic mechanisms together with the heightened T cell activation in MDD may exacerbate the pronounced IRS activation in that illness.

5.2. TIM3, immunoregulation and MDD

The second major finding is the robust negative connection between the number of TIM3 bearing $CD8^+$ cells and cytokine production, encompassing that of M1 macrophages, IRS, CIRS, Th17, T cell proliferation, and IRS immune cell profiles.

It is known that TIM3 attenuates T-cell receptor signaling in $CD8^+$ T cells by blocking nuclear-factor κB (Szklarczyk et al., 2023). In addition, TIM3 serves as a hallmark of T cell exhaustion and operates as a co-inhibitory immune checkpoint protein which attenuates IRS and autoimmune responses, regulates macrophage functions, and promotes immunological tolerance (Jones et al., 2008; Jin et al., 2010; PHARMACOLOGY TCLmoAoIBGt). In rheumatoid arthritis, TIM3 has been identified as having a negative connection with disease severity, indicating its involvement in the regulation of Treg cells to promote immunotolerance (Lee et al., 2011). Furthermore, in MS patients, the impaired negative regulation of TIM3 and diminished expression relative to healthy individuals was restored following treatment (Yang et al., 2008). Numerous investigations suggest that TIM3 exerts an inhibitory role, especially in chronic inflammatory situations (Jones et al., 2008; Jin et al., 2010). Given the negative relationship between the immunological profiles assessed in the current study and TIM3, it can be posited that a diminished inhibitory action via this exhaustion mechanism may result in enhanced activation of immune cells.

Consequently, the cumulative effects of T cell activation, and compromised Treg functions coupled with dysfunctional apoptotic processes in MDD may be further aggravated by loss of TIM3 checkpoint regulation.

5.3. CBD suppressed expression of pro-apoptotic markers

The third major finding of the current study is that CBD doses ranging from 0.1 to 1.0 μg/mL exhibited an inhibitory effect on TIM3+ expressing $CD3^+$, $CD4^+$, and $CD8^+$ cells. In addition, at a dosage of 10 μg/mL, CBD demonstrated a strong inhibitory impact on CD3+/CD4+/CD8+ TIM3+ and $CD95^+$ cells. Although these findings correspond with CBD's established immunomodulatory characteristics, they contrast with its anticipated anti-inflammatory effects.

Numerous studies indicate that impairing CD95 activity can lead to immunological dysregulation (Galli et al., 2022; Gerasimova et al., 2022). Consequently, elevated levels of CBD inhibit CD95 expression on lymphocytes, potentially diminishing apoptotic checkpoint control thereby disturbing immunological homeostasis and fostering inflammation (Paulsen and Janssen, 2011). Furthermore, at lower dosages (0.1–1.0 μg/mL), CBD exhibited an inhibitory effect on TIM3 expression in immune cells, and this effect was more pronounced at the higher concentrations. We hypothesize that the varying response to CBD's concentrations regarding TIM3+ T cells may be ascribed to the suppressant effect of CBD on the JAK/STAT pathway (starting at a concentration of 0.3 μg/ml) (Peyravian et al., 2020; Mujib et al., 2012). In contrast, the expression of CD95 is associated with activation of nuclear factor-κB which may be suppressed by CBD at higher concentrations (1.5–3 μg/ml) (Just et al., 2016; Kozela et al., 2010). A prior investigation into the impact of CBD on cytokines demonstrated that CBD exerts an immunological modulatory effect in a concentration-dependent way on several cytokines, with concentrations of 1.0 μg/mL or greater potentially inhibiting the production of CIRS activity coupled with increased growth factor production, while higher doses suppressed Th1, Th17, IRS, and CIRS cytokine profiles, but elevated growth factor and T cell growth profiles (Rachayon et al., 2022).

The mechanism by which CBD regulates inflammation is highly complex, involving multiple pathways with dose-related effects (Nichols and Kaplan, 2020). One key mechanism is its ability to modulate immune-inflammatory processes by downregulating the NF-κB signaling pathway, a central intracellular pathway that governs the transcription of numerous inflammatory genes (Carrier et al., 2006). This suppression leads to a reduction in pro-inflammatory cytokines, including IL-1β, IL-6, and IFN-β, thereby mitigating the inflammatory response (Kozela et al., 2010). Furthermore, CBD at a dose 0.3–3 μg/ml exerts anti-inflammatory effects by modulating the JAK/STAT pathway, interfering with Adenosine A_2A receptor activation, and regulating immune responses through CB1 and CB2 receptors (Peyravian et al., 2020; Carrier et al., 2006; Kaplan et al., 2008b; Malfait et al., 2000). However, a biphasic response to CBD has been observed in several studies, with CBD also exacerbating inflammation under certain conditions (Chen et al., 2012; Karmaus et al., 2013; Burstein, 2015).

Studies on endogenous cannabinoids, including anandamide (AEA) and 2-arachidonoylglycerol (2-AG), have shown that both compounds exhibit anti-inflammatory and pro-inflammatory effects (Atalay et al., 2019; DeMorrow et al., 2007). AEA demonstrates considerable affinity for both CB1 and CB2 receptors, functioning as a partial agonist with greater affinity for CB1 compared to CB2. Conversely, 2-AG functions as a complete agonist at both CB1 and CB2 receptors, but with moderate to low affinity for both receptors (Pertwee et al., 2010; Zou and Kumar, 2018). While AEA is acknowledged for its anti-inflammatory capabilities (Atalay et al., 2019; Pflüger-Müller et al., 2020), the involvement of 2-AG in inflammation is more complex and may also show pro-inflammatory effects by recruitment of leukocyte and stimulation production of pro-inflammatory cytokines (Sido et al., 2016; Turcotte et al., 2015).

Therefore, our findings suggest that one of the mechanisms by which CBD modulates immune dysregulation in depression is through the attenuation of apoptotic signaling pathways and mitigating T-cell exhaustion.

6. Limitations

A first limitation is that this study did not measure other death cell markers, including PD-1 (programmed cell death-1). Similar to TIM3, the expression of PD-1 is elevated in exhausted T cells, and its expression has been found to be reduced in individuals with MDD (Sakuishi et al., 2010b; Huang and Huang, 2023). Moreover, subsequent studies ought to examine these death, apoptosis and checkpoint markers during the partial remission and full remission stages, as well as in different MDD phenotypes. Furthermore, the intracellular mechanisms underlying CBD's effects on $CD95^+$ and TIM3+ T cell expression remain to be elucidated. While our ex vivo study employed CBD concentrations reflecting plasma levels, in vivo studies are needed to examine how administration routes and pharmacokinetics may influence circulating CBD concentrations. Such investigations would provide a more comprehensive understanding of CBD and its immunomodulatory effects.

7. Conclusions

The stimulated percentage of $CD3^+CD95^+$ cells is inversely correlated with essential characteristics of MDD, whether considering lifetime aspects (ROI, lifetime suicidal behaviors) or evaluations during the acute phase of MDD (severity of the phenome). The stimulated percentage of CD8+TIM3+ cells is negatively correlated with the activation of M1, IRS, CIRS, T cell proliferation, and IRN immunological profiles. These findings may indicate that reduced levels of checkpoint (apoptotic and exhaustion) markers in MDD may have diminished the negative feedback on immune activation pathways. Consequently, disruptions in apoptotic mechanisms in immune cells greatly influence the phenotype of MDD, whereas abnormalities in immunological exhaustion indicators may lead to diminished immunoregulatory effects. Consequently, CD95 and TIM3 may represent novel pharmacological targets for the treatment of the acute phase of MDD. In cancer immunotherapy, both receptors have been extensively studied as therapeutic targets (Bhat et al., 2025; He et al., 2018). Modulation of these receptors has shown potential to enhance cancer cell apoptosis and improve effector T cell function (Bhat et al., 2025; Friedlaender et al., 2019). However, their specific roles in MDD and potential therapeutic applications in this context require further investigation.

Administering lower concentrations of CBD, specifically between 0.1 and 1.0 μg/mL, significantly suppressed TIM3+ expressing $CD3^+$, $CD4^+$, and CD8+cells. Elevated doses of CBD had inhibitory effects on both TIM3+ and $CD95^+$ bearing cells. This suggests that therapeutical concentrations of CBD may significantly affect apoptosis and exhaustion markers during T cell activation and expansion. These inhibitory actions may diminish the negative feedback of the apoptosis and exhaustion checkpoint proteins on immunological activation in MDD, hence contributing to the pathophysiology of the acute phase of this disorder. Therefore, it cannot be recommended to use CBD as an anti-inflammatory compound.

CRediT authorship contribution statement

Muanpetch Rachayon: Project administration, Methodology, Investigation, Formal analysis, Data curation, Writing – original draft, Conceptualization. **Ketsupar Jirakran:** Resources, Methodology, Investigation. **Pimpayao Sodsai:** Methodology, Investigation. **Chavit Tunvirachaisakul:** Supervision, Methodology, Writing – review & editing. **Atapol Sughondhabirom:** Supervision, Conceptualization, Writing – review & editing. **Jing Li:** Writing – review & editing, Validation. **Yingqian Zhang:** Writing – review & editing, Validation, Visualization. **Michael Maes:** Methodology, Project administration, Supervision, Writing – review & editing, Conceptualization.

Ethical statement

All participants provided their written informed consent before their involvement in the study. The research was performed in compliance with the Declaration of Helsinki of 1975, as revised in 2013, and received approval from the Ethics Committee of the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (#528/63).

Data availability statement

The dataset produced and/or examined in this study will be accessible from the corresponding author (M.M.) upon reasonable request, following the complete utilization of the dataset by the authors.

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Declaration of competing interest

The authors do not report any conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2025.101066.

Data availability

Data will be made available on request.

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