



GDAŃSKI UNIWERSYTET MEDYCZNY

ROZPRAWA DOKTORSKA

**„Wpływ ketaminy na układ odpornościowy u pacjentów
z zaburzeniami depresyjnymi”**

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Podziękowania

Pragnę serdecznie podziękować wszystkim osobom, które przyczyniły się do powstania tej pracy.

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I. WYKAZ PRAC WCHODZĄCYCH W SKŁAD ROZPRAWY DOKTORSKIEJ

1. Praca pogładowa

Szałach ŁP, Lisowska KA, Cubala WJ. *The Influence of Antidepressants on the Immune System*. Archivum Immunologiae et Therapiae Experimentalis. 2019: vol. 67, nr 3. IF – 3,2; Punktacja MEiN – 140 pkt.

2. Praca oryginalna

Szałach ŁP, Cubala WJ, Lisowska KA. *Changes in T-Cell Subpopulations and Cytokine Levels in Patients with Treatment-Resistant Depression - A Preliminary Study*. International Journal of Molecular Sciences. 2023: vol. 24, nr 1. IF – 4,9; Punktacja MEiN – 140 pkt.

3. Praca oryginalna

Szałach ŁP, Ciesielska-Figlon K, Daca A, Cubala WJ, Lisowska KA. *The effect of ketamine on the immune system in patients with treatment-resistant depression*. International Journal of Molecular Sciences. 2025: vol. 26, nr 15. IF – 4,9; Punktacja MEiN – 140 pkt.

II. WYKAZ STOSOWANYCH SKRÓTÓW

ICD-10 – Międzynarodowa Statystyczna Klasyfikacja Chorób i Problemów Zdrowotnych, rewizja 10. / *International Statistical Classification of Diseases and Related Health Problems 10th Revision*

MDD – dosłownie: duże zaburzenie depresyjne, w domyśle: epizod depresyjny / *Major Depressive Disorder*

BD – zaburzenia afektywne dwubiegunowe / *bipolar disorder*

WHO – Światowa Organizacja Zdrowia / *World Health Organization*

TRD – depresja lekooporna / *treatment-resistant depression*

GABA – kwas gamma-aminomasłowy / *gamma-aminobutyric acid*

HPA – oś podwzgórze-przysadka-nadnercza / *hypothalamic-pituitary-adrenal axis*

IL – interleukina / *interleukin*

IFN – interferon / *interferon*

TNF – czynnik martwicy nowotworu / *tumor necrosis factor*

CD – antygen różnicowania komórkowego / *cluster of differentiation*

Th – limfocyty T pomocnicze / *helper T cells*

Tc – limfocyty T cytotoksyczne / *cytotoxic T cells*

ROS – reaktywne formy tlenu / *reactive oxygen species*

FoxP3 – skurfina / *forkhead box P3*

Treg – limfocyty T regulatorowe / *regulatory T cells*

sIL-6R – rozpuszczalny receptor dla interleukiny 6 / *soluble interleukin-6 receptor*

sTNFR1 – rozpuszczalny receptor 1 dla TNF / *soluble tumor necrosis factor receptor 1*

NMDA – kwas N-metylo-D-asparaginowy / *N-methyl-D-aspartate*

AMPA – kwas α -amino-3-hydroksy-5-metylo-4-izoksazolopropionowy / *α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid*

BDNF – neurotroficzny czynnik pochodzenia mózgowego / *brain-derived neurotrophic factor*

mTOR – ssaczy cel rapamycyny / *mammalian target of rapamycin*

G-CSF – czynnik stymulujący tworzenie kolonii granulocytów / *granulocyte colony-stimulating factor*

IP-10 – białko indukowane interferonem gamma-10 / *interferon gamma-induced protein 10*

MADRS – Skala Oceny Nasilenia Depresji Montgomery'ego-Asberga / *Montgomery-Asberg Depression Rating Scale*

PBMC – jednojądrzaste komórki krwi obwodowej / *peripheral blood mononuclear cells*

LPS – lipopolisacharyd / *lipopolysaccharide*

MINI – Mini-Międzynarodowy Wywiad Neuropsychiatryczny / *Mini-International Neuropsychiatric Interview*

DSM-5 – Diagnostyczny i statystyczny podręcznik zaburzeń psychicznych, edycja piąta / *Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition*

ATRQ – Kwestionariusz Odpowiedzi na Leczenie Przeciwdepresyjne / *Antidepressant Treatment Response Questionnaire*

BMI – wskaźnik masy ciała / *Body Mass Index*

EDTA – kwas etylenodiaminotetraoctowy / *ethylenediaminetetraacetic acid*

BPRS – Krótka Skala Oceny Stanu Psychicznego / *Brief Psychiatric Rating Scale*

CADSS – Skala Objawów Dysocjacyjnych Podawanych przez Lekarza / *Clinician Administered Dissociative Symptoms Scale*

NH₄Cl – chlorek amonu / *ammonium chloride*

KHCO₃ – wodorowęglan potasu / *potassium bicarbonate*

PBS – sól fizjologiczna buforowana fosforanami / *phosphate-buffered saline*

FITC – izotiocyanian fluoresceiny / *fluorescein isothiocyanate*

RPE-Cy5 – R-fikoerytryna-cyjanina 5 / *R-phycoerythrin-Cyanine 5*

APC-H7 – allofikocyjanina-H7 / *allophycocyanin-H7*

PE – fikoerytryna / *phycoerythrin*

HLA-DR – ludzki antygen leukocytny - DR / *Human Leukocyte Antigen - DR*

VPD450 – fioletowy barwnik proliferacyjny 450 / *Violet Proliferation Dye 450*

RPMI-1640 – podłoże RPMI-1640 / *Roswell Park Memorial Institute solution - 1640*

FBS – serum płodowe z cieląt / *fetal bovine serum*

NS – komórki niestymulowane / *non-stimulated cells*

S – komórki stymulowane / *stimulated cells*

LD – niska dawka (ketaminy) / *low dose (of ketamine)*

HD – wysoka dawka (ketaminy) / *high dose (of ketamine)*

CO₂ – dwutlenek węgla / *carbon dioxide*

BD (Pharmingen/Biosciences/Horizon) – Beckton Dickinson

CBA – Cytometric Bead Array

FACS – Fluorescence-Activated Cell Sorting

FSC – rozproszenie przednie / *Forward Scatter Channel*

SSC – rozproszenie boczne / *Side Scatter Channel*

ANOVA – analiza wariancji / *Analysis Of Variance*

ROC – krzywa charakterystyki operacyjnej odbiornika / *receiver operating characteristic curve*

CXCL8 – *CXC motif ligand 8*

III. STRESZCZENIE W JĘZYKU POLSKIM

Depresja jest poważnym zaburzeniem psychicznym występującym wśród ludzi na całym świecie. Szacuje się, że około 30% leczonych pacjentów nie osiąga remisji, mimo zastosowania różnych metod leczenia, co prowadzi do rozpoznania depresji lekoopornej. Pomimo szerokiego wachlarza dostępnych leków, lekooporność pozostaje poważnym wyzwaniem klinicznym. Konieczne jest zatem poszukiwanie nowych mechanizmów patofizjologicznych depresji oraz opracowywanie innowacyjnych metod leczenia działających odmiennie od klasycznych leków przeciwdepresyjnych.

Ketamina, od dawna stosowana w anestezjologii i leczeniu bólu, wykazuje również działanie przeciwdepresyjne, potwierdzone w licznych badaniach. W 2019 zarejestrowano esketaminę w formie donosowej jako nowoczesny, szybko działający lek przeciwdepresyjny. Jej główny mechanizm opiera się na modulacji przekazywania glutaminergicznego – blokadzie receptorów NMDA prowadzącej do wyrzutu glutaminianu, aktywacji receptorów AMPA i nasilenia synaptogenezy. Podejrzewa się jednak, iż za skutecznością ketaminy w depresji lekoopornej stoją także inne mechanizmy.

Jedną z hipotez jest immunomodulujące działanie ketaminy. Istnieją wcześniejsze doniesienia potwierdzające wpływ ketaminy na układ odpornościowy, choć są one nieliczne i ograniczone. Opisywano, iż ketamina zmniejsza poziomy cytokin prozapalnych we krwi. Jednakże sposób modulacji odpowiedzi immunologicznej, w tym zmiany w subpopulacjach komórek układu odpornościowego i wpływ tych zmian na odpowiedź przeciwdepresyjną, wciąż nie został szczegółowo poznany.

Projekt badawczy skupiał się na ocenie stanu wyjściowego układu immunologicznego, w szczególności profilu subpopulacji limfocytów T oraz poziomów cytokin w surowicy u pacjentów z depresją lekooporną i porównanie ich z profilem osób zdrowych. W kolejnym etapie sprawdzano, w jaki sposób ketamina wpływa na układ immunologiczny tych pacjentów – oceniano zmiany w odsetkach limfocytów pomocniczych, cytotoksycznych, ich aktywację oraz różnice w poziomach cytokin we krwi po podaży leku. Wyniki korelowano z oceną kliniczną objawów depresji. Jednocześnie sprawdzano, jak ketamina wpływa na wyizolowane limfocyty – ich aktywację, proliferację i produkowane przez nie cytokiny – w warunkach *in vitro* podczas inkubacji w obecności ketaminy. Wyniki badań zostały opublikowane w postaci dwóch prac oryginalnych.

IV. STRESZCZENIE W JĘZYKU ANGIELSKIM

Depression is a serious mental disorder affecting people worldwide. It is estimated that approximately 30% of patients do not achieve remission despite the use of various treatment strategies, which leads to the diagnosis of treatment-resistant depression (TRD). Despite the wide range of available antidepressant medications, treatment resistance remains a major clinical challenge. Therefore, it is essential to search for new pathophysiological mechanisms of depression and to develop innovative treatment methods that act differently from conventional antidepressants.

Ketamine, long used in anesthesiology and pain management, has also demonstrated antidepressant properties, confirmed in numerous studies. In 2019, esketamine in intranasal form was approved as a modern, rapidly acting antidepressant. Its primary mechanism involves the modulation of glutamatergic transmission – NMDA receptor blockade induces glutamate release, activation of AMPA receptors, and enhanced synaptogenesis. However, it is suspected that additional mechanisms may underlie ketamine's effectiveness in TRD.

One such hypothesis is its immunomodulatory effect. Previous reports, though limited, have indicated that ketamine influences the immune system, with evidence showing it reduces pro-inflammatory serum cytokine levels. Nevertheless, the exact mechanisms of immune modulation, particularly changes in immune cell subpopulations and their role in the antidepressant response, remain poorly understood.

The presented research project focused on assessing the baseline immune status, specifically the profile of T cell subpopulations and serum cytokine levels, in patients with TRD compared with healthy individuals. In the next stage, we examined how ketamine affects the immune system of these patients – evaluating changes in the proportions and activation of helper and cytotoxic T cells, as well as differences in serum cytokine levels after ketamine administration. These results were correlated with clinical assessments of depressive symptoms. Simultaneously, we examined how ketamine affects isolated lymphocytes – their activation, proliferation, and the cytokines they produce – in vitro during incubation in the presence of ketamine. The results of this research were published in the form of two original articles.

V. WPROWADZENIE

Depresja, zgodnie z definicją Międzynarodowej Klasyfikacji Chorób ICD-10 (z ang. *International Statistical Classification of Diseases and Related Health Problems, 10th Revision*) to stan, w którym przez minimum dwa tygodnie, przez większość czasu obecne są co najmniej dwa z trzech objawów osiowych, tj. obniżenie nastroju wyraźnie nieprawidłowe dla danej osoby, spadek energii i anhedonia (obniżona zdolność do odczuwania przyjemności). Ponadto wystąpić musi minimum jeden dodatkowy objaw, taki jak: obniżone poczucie własnej wartości, nieracjonalne wyrzuty sumienia, myśli dotyczące śmierci lub samobójstwa, spadek koncentracji, pobudzenie lub zahamowanie psychoruchowe, zaburzenia snu oraz apetytu, dopełniając w sumie liczbę objawów do minimum czterech. Wykluczyć należy wpływ substancji psychoaktywnych na wystąpienie tego stanu [1].

Epizod depresji (MDD – z ang. *major depressive disorder*), w klasyfikacji ICD-10 oznaczany kodem F32, może być jednostką chorobową samą w sobie, bądź też w przypadku wystąpienia minimum dwóch epizodów stanowić część zaburzenia depresyjnego nawracającego (F33), a także epizodem w przebiegu choroby afektywnej dwubiegunowej (BD – z ang. *bipolar disorder*) (F31), gdzie oprócz depresji pacjent doświadcza także epizodów hipomanii, manii i/lub epizodu o cechach mieszanych [1].

Szacuje się, że na depresję choruje obecnie około 4% ludzkości, co oznacza, iż ok. 332 miliony osób na świecie choruje na depresję [2]. Występuje ona około 1,5 raza częściej u kobiet niż u mężczyzn [2]. Światowa Organizacja Zdrowia (WHO – z ang. *World Health Organization*) zakłada, iż depresja do 2030 roku będzie najczęstszą przyczyną niepełnosprawności [3]. Depresja może być chorobą śmiertelną – rocznie ponad 700 tysięcy osób popełnia samobójstwo [2].

Leczenie depresji polega zarówno na stosowaniu psychofarmakoterapii, psychoterapii, jak i innych metod nefarmakologicznych, w tym elektrowstrząsów czy prądoczaszkowej stymulacji magnetycznej. Mimo dostępności wielu grup leków i substancji leczniczych, nawet 30% osób prawidłowo leczonych z powodu depresji nie osiąga remisji [4]. Taki stan określamy mianem depresji lekoopornej (TRD – z ang. *treatment-resistant depression*), którą definiujemy jako niewystarczającą odpowiedź na co najmniej dwie próby leczenia przeciwdepresyjnego prowadzone w odpowiednich dawkach i przez odpowiedni czas [5].

Przyczyny występowania depresji są złożone i wieloczynnikowe. Obecnie depresję traktuje się jako złożoną reakcję organizmu na różnorodne czynniki genetyczne, biologiczne, psychologiczne i społeczne [6]. W kwestii czynników biologicznych opisuje się głównie teorię monoaminergiczną, polegającą na dysregulacji w obrębie neuroprzekaźników, takich jak serotonina, noradrenalina czy dopamina, teorię dysregulacji układu glutaminergicznego i GABA oraz zaburzenia neuroplastyczności, teorię dysregulacji osi podwzgórze-przysadka-nadnercza (HPA – z ang. *hypothalamic-pituitary-adrenal axis*) i teorię zapalną depresji, w której główną rolę odgrywają zaburzenia w układzie immunologicznym. Teorie te wzajemnie przenikają się i uzupełniają w swoich modelach.

Hipoteza zapalna depresji została opisana i potwierdzona w licznych badaniach naukowych [7]. Wykazano, że stężenia cytokin prozapalnych, głównie interleukin (IL) IL-1, IL-6, IL-12, IFN- γ (z ang. *interferon-gamma*) i TNF- α (z ang. *tumour necrosis factor-alpha*), są podwyższone w surowicy pacjentów cierpiących na zaburzenia depresyjne [8-10]. Cytokiny prozapalne są produkowane między innymi przez pomocnicze limfocyty T CD4+ typu 1 (Th1), które wraz z makrofagami, komórkami dendrytycznymi i cytotoksycznymi limfocytami T CD8+ uczestniczą w odporności komórkowej niezbędnej do eliminacji patogenów wewnątrzkomórkowych. Opisywano również podwyższone stężenia innych cytokin, m.in. IL-8 [11] czy IL-10 [10]. Cytokiny te biorą udział w rozwoju odpowiedzi humoralnej, m.in. przeciwko patogenom zewnątrzkomórkowym, wspieranej przez limfocyty pomocnicze typu 2 (Th2).

Zmiany w układzie odpornościowym u pacjentów z depresją są również zauważalne w liczbie i proporcji populacji leukocytów. Opisywano wzrost liczby komórek zaangażowanych w odpowiedź wrodzoną, tj. monocytów, makrofagów i neutrofilii [12], a także zwiększoną produkcję reaktywnych form tlenu (ROS – z ang. *reactive oxygen species*) [13]. Obserwowano spadek liczby i aktywności komórek NK odpowiedzialnych za naturalną cytotoksyczność [14] czy wzrost stosunku limfocytów T CD4+ do limfocytów T CD8+ [15,16]. Wykazywano również, że limfocyty T u pacjentów z rozpoznaniem epizodu depresyjnego prezentują zwiększoną ekspresję markerów aktywacji, takich jak CD95 [17,18], CD69 [19,20] czy CD25 [19,21]. Liczba limfocytów Th17, podgrupy prozapalnych pomocniczych limfocytów T, również wzrasta w depresji, najprawdopodobniej w związku z podwyższonym stężeniem IL-6 w surowicy [22,23]. Pacjenci z MDD charakteryzują się także obniżonym odsetkiem komórek CD4+CD25+FoxP3+, czyli limfocytów regulatorowych (Treg), odpowiedzialnych za utrzymanie tolerancji wobec auto-antygenów, a tym samym zapobieganie reakcjom autoimmunologicznym [22,24].

W depresji lekoopornej opisywano zarówno wyższe stężenia IL-6, IL-8, TNF- α [25] oraz sIL-6R (z ang. *soluble IL-6 receptor*) [26,27], jak i niższe poziomy IL-6 i TNF- α [28] w porównaniu do pacjentów z rozpoznaniem depresji bez lekooporności. Inne badanie wykazało, że pacjenci z BD i TRD mieli wyższe stężenia IL-6 i TNF- α oraz niższe poziomy sTNFR1 (z ang. *soluble TNF receptor 1*) niż pacjenci z MDD [29]. U pacjentów z BD podwyższony poziom IL-1 β został zaproponowany jako potencjalny marker predykcyjny oporności na leczenie [30]. Brakuje natomiast danych dotyczących zmian w populacjach limfocytów T w tej grupie pacjentów.

Ketamina, pochodna fencyklidyny zsyntetyzowana po raz pierwszy w 1962 roku, jest lekiem szeroko stosowanym w anestezjologii celem indukcji i podtrzymania znieczulenia ogólnego oraz zniesienia bólu [31]. Używana jest w postaci roztworu do wstrzyknięć dożylnych lub domięśniowych. Historia badań nad ketaminą w leczeniu depresji sięgają 2000 roku, kiedy to Berman i wsp. [32] opisywali znaczne zmniejszenie się objawów depresyjnych u pacjentów cierpiących na depresję lekooporną już po niecałych dwóch godzinach od jednorazowej podaży dożylnej leku w dawce 0,5 mg/kg m.c. Co ciekawe, efekt leczenia utrzymywał się u 35% pacjentów nawet po tygodniu od iniekcji, co nie mogło być wytłumaczone obecnością substancji w organizmie, gdyż jej okres półtrwania to 2 godziny oraz 5 godzin dla jej aktywnego metabolitu norketaminy [32]. Wielokrotnie powtarzane badania z ketaminą wśród pacjentów cierpiących na depresję, głównie lekooporną, wykazały szybki i długotrwały efekt przeciwdepresyjny ketaminy [33], co doprowadziło do rejestracji lewoskrętnej formy cząsteczki – esketaminy – w formie donosowej, jako leku przeciwdepresyjnego [34].

Główny mechanizm działania ketaminy to niekompetycyjny antagonizm receptora NMDA (N-metyl-D-asparaginowego) dla glutaminianu – ketamina wiążąc się z nim blokuje go, co prowadzi do zahamowania aktywności tych receptorów, zwłaszcza tych zlokalizowanych na interneuronach GABAergicznych (GABA – kwas gamma-aminomasłowy). Skutkuje to zahamowaniem aktywności GABAergicznej, odhamowaniem neuronów glutamatergicznych i zwiększeniem uwalniania glutaminianu. Blokada receptorów NMDA prowadzi również do nadaktywacji receptorów AMPA (kwas α -amino-3-hydroksy-5-metylo-4-izoksazolopropionowy) [35]. To z kolei uruchamia wewnątrzkomórkowe kaskady sygnalizacyjne obejmujące m.in. czynnik neurotroficzny pochodzenia mózgowego (BDNF – z ang. *brain-derived neurotrophic factor*) oraz kinazę mTOR (z ang. *mechanistic target of rapamycin*), które sprzyjają synaptogenezie i zwiększają neuroplastyczność mózgu [35].

Dotychczasowe, choć nieliczne, wyniki badań w populacji pacjentów cierpiących na depresję dowodzą, iż ketamina wykazuje również działanie immunomodulujące. Zaobserwowano, iż początkowo podwyższone stężenia IL-6 i G-CSF (z ang. *granulocyte colony-stimulating factor*), a także IL-1 i IP-10 (z ang. *interferon gamma-induced protein 10*) ulegały obniżeniu po 4 godzinach od pojedynczej dożylniej dawki ketaminy (0,5 mg/kg m.c.) [36]. Zmiany w stężeniach cytokin nie korelowały jednak ze zmianami w wynikach skali MADRS (z ang. *Montgomery-Asberg Depression Rating Scale*) – szeroko stosowanej skali w badaniach klinicznych i praktyce psychiatrycznej jako narzędzie do oceny nasilenia objawów depresji; im wyższa punktacja w skali MADRS, tym silniejsze nasilenie objawów depresji. Obserwowano również spadki stężeń IL-6 i TNF- α już po 40 minutach od podaży ketaminy dożylniej. Spadek TNF- α pomiędzy punktem wyjściowym a 40. minutą po infuzji był dodatnio skorelowany ze spadkiem wyników MADRS w czasie [37].

Przeciwwzapalne działanie ketaminy badano również w innych grupach pacjentów otrzymujących ketaminę jako lek anestetyczny lub analgetyczny. W jednej z metaanaliz opisujących wpływ ketaminy na poziomy IL-6 u pacjentów po zabiegu operacyjnym z użyciem ketaminy wykazano znaczący spadek stężenia IL-6 do 6 godzin po zakończeniu operacji [38]. Inne badanie przeprowadzono w grupie pacjentów poddawanych ortotopowemu przeszczepowi wątroby. Stężenia TNF- α i IL-6 u pacjentów, którzy otrzymali ketaminę przed fazą anhepatyczną i we wczesnym okresie pooperacyjnym, były znacząco niższe niż w grupie kontrolnej [39].

Badania dotyczące wpływu ketaminy na ludzkie limfocyty są in vitro ograniczone. W jednym z badań, choć przeprowadzonym w populacji pacjentów z rakiem żołądka, ich jednojądrowe komórki krwi obwodowej (PBMC – z ang. *peripheral blood mononuclear cells*) inkubowano przez 24 godziny z różnymi stężeniami ketaminy (25, 50 i 100 μ M, co odpowiada 6, 9, 13,7 i 27,4 ng/ml). Stwierdzono, że stosunek limfocytów CD4⁺/CD8⁺, a także odsetek Treg istotnie wzrastał wraz ze wzrostem stężenia ketaminy [40]. Inne badanie analizujące proliferację progenitorowych komórek nerwowych pochodzących ze zdrowych ludzkich komórek wykazało istotny statystycznie wzrost proliferacji po 24 godzinach inkubacji z ketaminą. Efekt ten był najbardziej wyraźny przy stężeniu ketaminy 0,5 mM (około 137 ng/ml) i był odwrotnie proporcjonalny do dalszego wzrostu stężenia ketaminy [41]. W kwestii produkcji cytokin in vitro zaobserwowano, iż podczas stymulacji zdrowych komórek krwi obwodowej przy użyciu LPS (lipopolisacharydu) w obecności 100 ng/ml ketaminy, hamowała ona bezpośrednio syntezę TNF- α , IL-6 oraz IL-8 [42].

Powyższe doniesienia dotyczące wpływu ketaminy na układ immunologiczny człowieka sugerują, iż może ona poprzez immunomodulujący wpływ wykazywać swoje działanie przeciwdepresyjne.

VI. CELE PRACY

Celem pracy była ocena profilu immunologicznego pacjentów z depresją lekooporną, zbadanie wpływu ketaminy na komórki układu odpornościowego, w szczególności na subpopulacje limfocytów T z uwzględnieniem ich fenotypu powierzchniowego oraz stężenia cytokin w surowicy u pacjentów z rozpoznaniem epizodu depresji. Kolejnym celem było skorelowanie wyników badań z efektem przeciwdepresyjnym ketaminy ocenianym za pomocą skal psychometrycznych oraz próba znalezienia biologicznego markera depresji lekoopornej i odpowiedzi terapeutycznej. Cele te osiągnięto poprzez realizację następujących celów szczegółowych:

1. Analiza podstawowych parametrów immunologicznych, w szczególności odsetków subpopulacji pomocniczych i cytotoksycznych limfocytów T krwi obwodowej oraz stężeń wybranych cytokin w surowicy u pacjentów z depresją lekooporną oraz osób zdrowych i skorelowanie otrzymanych wyników pacjentów z punktacją w skali MADRS oraz klinicznymi parametrami, takimi jak BMI, wiek, płeć, czy długość epizodu depresyjnego.
2. Ocena wpływu ketaminy na te same parametry u pacjentów zakwalifikowanych do leczenia oraz skorelowanie ich z wynikami leczenia ocenianymi za pomocą skali MADRS.
3. Ocena wpływu ketaminy na wyizolowane od pacjentów z depresją lekooporną oraz osób zdrowych limfocytów T w warunkach hodowli in vitro, w szczególności na ich zdolność do aktywacji oraz produkcji cytokin w różnych stężeniach ketaminy.

Cele pracy zostały zrealizowane w cyklu **dwóch** spójnych tematycznie prac oryginalnych, co zostało opisane w poniższym skrócie.

VII. MATERIAŁY I METODY

1. Grupy badane

Grupę badaną stanowiło 20 dorosłych pacjentów, hospitalizowanych w Oddziale Psychiatrycznym z rozpoznaniem depresji lekoopornej w przebiegu dużej depresji (MDD – z ang. *major depressive disorder*) lub epizodu depresji lekoopornej w przebiegu zaburzeń afektywnych dwubiegunowych (BD – z ang. *bipolar disorder*) bez objawów psychotycznych. 18 pacjentów z tej grupy zostało zakwalifikowanych do podania ketaminy dożylniej. Diagnozy potwierdzano na podstawie wywiadu MINI (z ang. *Mini-International Neuropsychiatric Interview*) oraz kryteriów DSM-5 (z ang. *Diagnostic and Statistical Manual of Mental Disorders*), a nasilenie objawów depresyjnych oceniano za pomocą skali MADRS. Wszyscy uczestnicy zostali zakwalifikowani jako pacjenci spełniający kryteria lekooporności w odniesieniu do aktualnego epizodu depresyjnego. W przypadku MDD lekooporność zdefiniowano jako niewystarczającą odpowiedź na co najmniej dwie próby leczenia przeciwdepresyjnego prowadzone w odpowiednich dawkach i przez odpowiedni czas i oceniano z użyciem kwestionariusza ATRQ (z ang. *Massachusetts General Hospital Antidepressant Treatment Response Questionnaire*). W przypadku depresji dwubiegunowej, lekooporność zdefiniowano jako klinicznie niezadowalającą odpowiedź na co najmniej dwie próby zastosowania różnych leków mających udowodnione działanie przeciwdepresyjne w chorobie dwubiegunowej, podawanych w adekwatnych dawkach i przez wystarczający czas.

Grupę kontrolną w badaniach *ex vivo* stanowiło 13 osób zdrowych w podobnym wieku, ze zbliżonym BMI i podobnym rozkładem płci do grupy badanej.

Kryteriami wyłączenia były: wiek poniżej 18 lat, objawy psychotyczne, niekontrolowane nadciśnienie tętnicze oraz choroba wieńcowa, podwyższone ciśnienie śródczaszkowe, ostre i przewlekłe choroby zakaźne, choroby zapalne, autoimmunologiczne, metaboliczne i nowotworowe. W grupie kontrolnej kryterium wyłączenia stanowiła przebyta choroba psychiczna w przeszłości.

2. Zgody

Niezależna Komisja Bioetyczna ds. Badań Naukowych wyraziła zgodę na przeprowadzenie badania (decyzja nr NKBBN/398/2017 z dnia 12 października 2017 r.). Grupę badaną stanowili pacjenci włączeni do naturalistycznego, obserwacyjnego protokołu

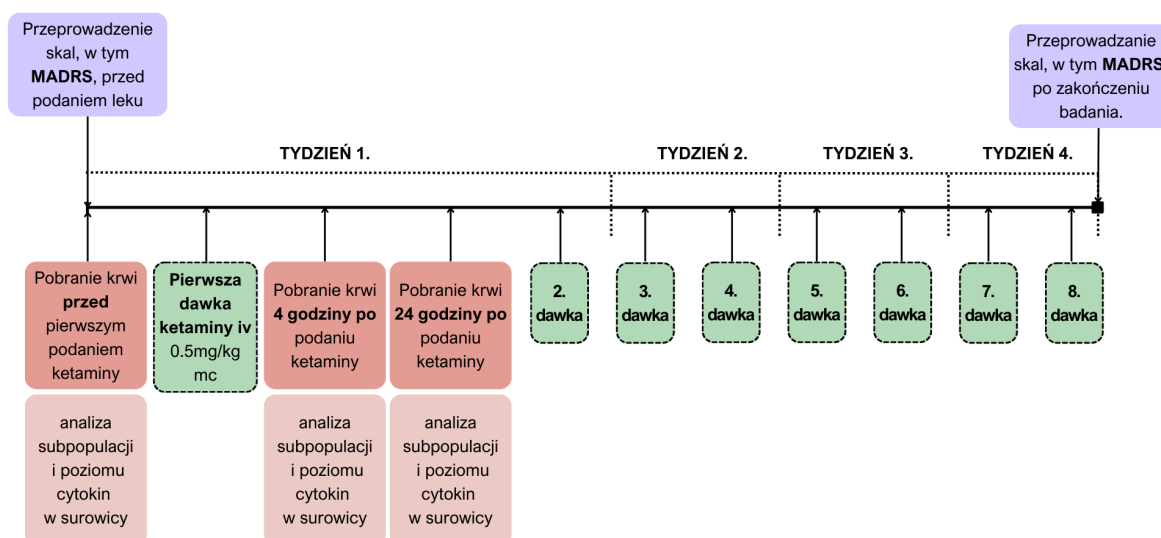
rejestracyjnego dotyczącego dożylnego leczenia ketaminą w depresji lekoopornej: A Naturalistic Study of Ketamine for Treatment-Resistant Mood Disorders (GDKet) (NCT04226963). Wszyscy uczestnicy przed rozpoczęciem udziału w badaniu wyrazili świadomą, pisemną zgodę. Zgody uzyskano po szczegółowym przedstawieniu i omówieniu wszystkich procedur.

3. Materiał badany

Do badań immunologicznych *ex vivo* w każdym z punktów czasowych pobierano 5 ml krwi żyłnej do probówek z EDTA (z ang. *ethylenediaminetetraacetic acid*) celem oznaczenia i analizy subpopulacji limfocytów oraz 5 ml do probówek bez antykoagulantu w celu oznaczenia stężenia wybranych cytokin. Surowicę przechowywano w temperaturze -80°C . Celem przeprowadzenia hodowli komórkowych *in vitro* pobierano do 20 ml krwi żyłnej do probówek z EDTA.

4. Schemat badań

W pierwszym etapie badania oceniano parametry immunologiczne pacjentów z depresją lekooporną przed podaniem ketaminy (Rycina 1) i porównywano je z parametrami grupy kontrolnej (Publikacja nr 2). W drugim etapie oceniano zmiany w subpopulacjach limfocytów po podaniu pierwszej dawki ketaminy dożylniej w czasie, porównując parametry immunologiczne przed podaniem, po 4h oraz po 24h od podania ketaminy (Publikacja nr 3).



Rycina 1. Schemat badania przedstawiająca punkty czasowe dożylnego podania ketaminy, pobrania próbki krwi i oceny MADRS.

Badanie miało charakter obserwacyjny – pacjenci kontynuowali dotychczasowe leczenie psychiatryczne. Ketaminę podawano dożylnie w dawce 0,5 mg/kg masy ciała przez 40 minut (preparat Ketalar 50 mg/ml). Lekarz psychiatra monitorował bezpieczeństwo przed, w trakcie i po infuzji, co 15 minut przez 1,5 godziny, obejmując pomiary parametrów życiowych (tętno, temperatura, oddech, ciśnienie, saturacja), oraz ocenę objawów psychiatrycznych, w tym objawów dysocjacyjnych za pomocą skal BPRS (z ang. *Brief Psychiatric Rating Scale*) i CADSS (z ang. *Clinician-Administered Dissociative States Scale*), przed podaniem i 1 godz. po podaniu ketaminy.

5. *Badania laboratoryjne*

5.1. *Oznaczanie ex vivo subpopulacji limfocytów T*

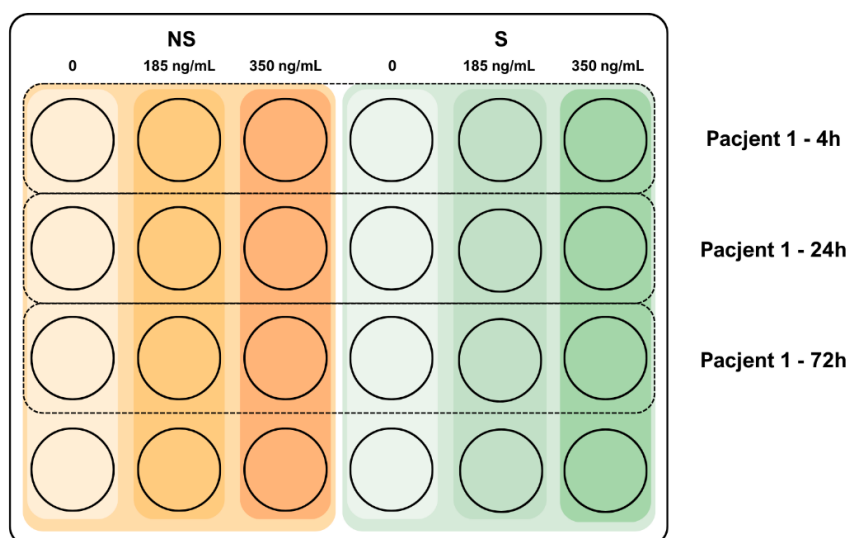
Po 100 µl krwi z każdej próbki przenoszono do probówek, gdzie barwiono komórki przeciwciałami monoklonalnymi i przeprowadzano lizę erytrocytów za pomocą buforu z 0,8% NH₄Cl i 0,1% KHCO₃. Komórki płukano za pomocą buforu PBS (sól fizjologiczna buforowana fosforanem) a następnie inkubowano z monoklonalnymi przeciwciałami skoniugowanymi z fluorochromami: FITC (CD3, CD95), RPE-Cy5 (CD4), APC-H7 (CD8), PE (CD28, CD25, CD69, HLA-DR) (BD Pharmingen, USA) przez 30 minut w 4°C w ciemności. Następnie komórki płukano za pomocą PBS i zawieszano w PBS do momentu odczytu cytometrycznego.

5.2. *Izolacja PBMC i stymulacja w obecności ketaminy*

Zebrano 15 ml krwi żyłnej od 4 zdrowych osób i 4 pacjentów z depresją (2 z BD, 2 z MDD) do probówek z EDTA. PBMC izolowano metodą wirowania na gradiencie Histopaque™ (Sigma Aldrich, USA). Komórki barwiono barwnikiem VPD450 (BD Horizon™ Violet Proliferation Dye 450) przez 12 minut w ciemności w 37°C, a następnie płukano za pomocą PBS i zawieszano w pożywce hodowlanej (RPMI-1640 + 10% FBS + glutamina, penicylina i streptomycyna) w stężeniu 1 mln komórek/mL.

Komórki podzielono na dwie grupy: niestymulowane (NS) oraz stymulowane (S) za pomocą monoklonalnego przeciwciała anty-CD3, według schematu poniżej (Rycina 2). W obrębie każdej z grup utworzono podgrupy, w zależności od użytego stężenia ketaminy: 0 ng/ml (kontrola), 185 ng/ml (niska dawka - LD) i 350 ng/ml (wysoka dawka - HD). Komórki inkubowano w standardowych warunkach (5% CO₂, 37°C, 100% wilgotności) przez 3 dni. Komórki pobierano po 4, 24 i 72 godzinach inkubacji, a następnie barwiono

przeciwciałami (CD4, CD8, CD25, CD28, CD69, HLA-DR, CD95) (BD Pharmingen, USA) i analizowano cytometrycznie.



Rycina 2. Schemat płytki hodowlanej.

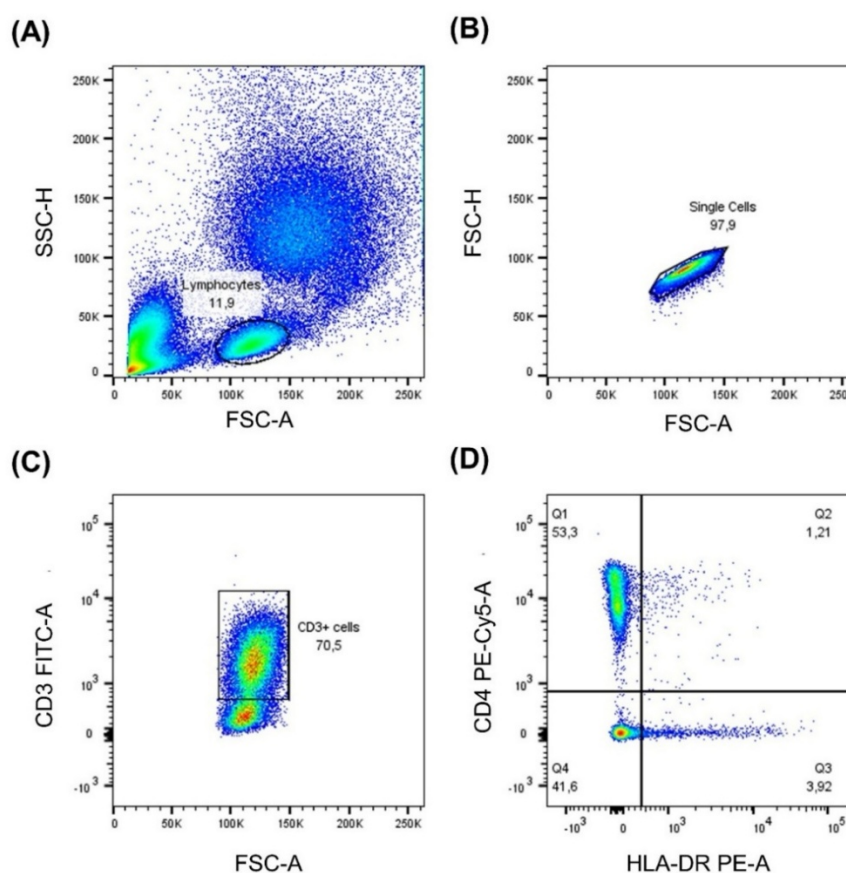
5.3. Pomiar cytokin w próbkach surowicy oraz supernatantach z hodowli

Do oznaczenia poziomu IL-12p70, TNF, IL-10, IL-6, IL-1 β oraz IL-8 w próbkach surowicy pacjentów z lekooporną depresją oraz w supernatantach hodowlanych zastosowano zestaw Cytometric Bead Array (CBA) Human Inflammatory Cytokines Kit (BD Biosciences, USA), zgodnie z protokołem producenta. Zakres wykrywalności dla cytokin wynosił od 20 do 5000 pg/mL, a granice detekcji wynosiły: IL-8 – 3,6 pg/mL; IL-1 β – 7,2 pg/mL; IL-6 – 2,5 pg/mL; IL-10 – 3,3 pg/mL; TNF- α – 3,7 pg/mL; IL-12p70 – 1,9 pg/mL. Wyniki cytokin analizowano metodą regresji nieliniowej z transformacją logarytmiczną $X = \log(X)$ przy użyciu oprogramowania GraphPad Prism 9 (GraphPad Software, USA).

5.4 Odczyt cytometryczny

Próbki odczytywano na cytometrze FACSVerse lub FACSARIA (Becton Dickinson, USA). Dla każdej próbki cytometrycznej analizowano 20 000 zdarzeń odpowiadających żywym limfocytom. Najpierw limfocyty identyfikowano na podstawie parametrów rozproszenia światła (FSC/SSC), limfocyty T – na podstawie ekspresji CD3, pomocnicze limfocyty T na podstawie antygenu CD4, cytotoksyczne – na podstawie antygenu CD8, a na

końcu subpopulacje z ekspresją markerów aktywacji (np. HLA-DR), zgodnie z poniższą Ryciną nr 3.



Rycina 3. Schemat analizy cytometrycznej: (A) limfocyty wybrane na podstawie wielkości oraz ziarnistości, (B) wybrane tzw. single cells (wykluczenie dupletów komórkowych), (C) limfocyty T wybrane na podstawie ekspresji antygenu CD3, (D) pomocnicze – na podstawie antygenu CD4 i oznaczona ekspresja HLA-DR (antygeny aktywacyjnego).

Do oceny proliferacji limfocytów analizowano 30 000 zdarzeń. Wzorce podziałów komórek śledzono za pomocą barwnika VPD450 (Publikacja nr 3) – podczas podziału barwnik rozdziela się równomiernie między komórki potomne, tracąc intensywność fluorescencji o połowę przy każdym podziale.

6. Analiza i statystyka

Analizę danych cytometrycznych przeprowadzono w programie FlowJo wersja 10 (Becton Dickinson, USA). Analizy statystyczne przeprowadzono w programie GraphPad Prism 9. Rozkłady analizowanych zmiennych sprawdzano testami Kołmogorowa–Smirnowa i Shapiro–Wilka. W związku z nienormalnym rozkładem zmiennych celem analizy porównawczej subpopulacji limfocytów oraz stężeń cytokin pomiędzy pacjentami

a osobami zdrowymi użyto testu Manna-Whitneya. Do analiz odsetków subpopulacji oraz poziomu cytokin w czasie (przed i po podaży ketaminy) używany był test Wilcozona. Analizy hodowli komórkowych przeprowadzono z użyciem testu ANOVA Friedmana z testem wielokrotnych porównań Dunna. Korelację przeprowadzono z użyciem testu Spearmana. Za istotny statystycznie przyjęto poziom $p < 0,05$.

VIII. OMÓWIENIE PUBLIKACJI 1

Szałach ŁP, Lisowska KA, Cubała WJ. *The Influence of Antidepressants on the Immune System*. Archivum Immunologiae et Therapiae Experimentalis. 2019: vol. 67, nr 3.

Pierwszym artykułem otwierającym cykl prac jest artykuł przeglądowy pt. „The Influence of Antidepressants on the Immune System”. Celem artykułu było wprowadzenie do koncepcji psychoneuroimmunologii oraz przedstawienie dotychczasowych danych opisujących relację leków przeciwdepresyjnych i układu immunologicznego. Dotychczas skupiano się głównie na bezpośrednim działaniu leków przeciwdepresyjnych na transportery neuroprzekaźników lub też ich receptory. Obecnie jednak poszukuje się innych możliwych mechanizmów, w tym immunomodulacji, mogących wpływać na odpowiedź przeciwdepresyjną tych leków.

We wstępie pracy został sprecyzowany termin „depresja”, obejmujący zarówno epizod depresyjny w chorobie afektywnej jedno-, jak i dwubiegunowej. Przytoczone zostały statystyki dotyczące lekooporności depresji, wskazujące na potrzebę poszukiwania kolejnych leków i mechanizmów mogących wpłynąć na osiągnięcie remisji. Wprowadzony został też termin „psychoneuroimmunologia”, pokazujący wzajemne relacje między stanem psychicznym, układem neuroendokrynnym oraz układem immunologicznym.

W kolejnym akapicie zostały przytoczone dotychczasowe badania opisujące zmiany w układzie immunologicznym, zarówno w obrębie populacji komórek odpornościowych, jak i stężeń cytokin u pacjentów z rozpoznaniem depresji. Zmiany te zostały podsumowane w postaci schematu.

Następny akapit to główna część artykułu, w którym zostały podsumowane wyniki badań naukowych opisujące relacje między odpowiedzią na leki przeciwdepresyjne a zmianami w układzie immunologicznym. Na początku przedstawiono wyniki metaanaliz oraz dużych randomizowanych badań, następnie zaś poszczególne, nieuwzględnione poprzednio badania, doniesienia dotyczące poszczególnych grup leków lub konkretnych substancji na parametry immunologiczne pacjentów z depresją. Pod koniec akapitu wspomniano ketaminę jako ówczesnie potencjalny lek przeciwdepresyjny charakteryzujący się pewnymi immunomodulacyjnymi właściwościami. Z powodu niewielkiej ilości badań, dotychczasowe doniesienia opierano głównie na wynikach w populacjach pacjentów anestezjologicznych.

W kolejnych, podsumowujących akapitach, zaprezentowano koncept nowej grupy leków przeciwdepresyjnych opierających się na immunomodulacji. Przytoczone zostały badania, które pokazują działanie przeciwdepresyjne substancji o charakterze przeciwzapalnym, takich jak niesteroidowe leki przeciwzapalne, kurkumina oraz leki biologiczne. Podkreślono też złożoność czynników wpływających na układ immunologiczny, niejednoznaczne, często sprzeczne wyniki dotychczasowych prac oraz potrzebę kontynuowania badań w sferze psychoneuroimmunologii.

IX. OMÓWIENIE PUBLIKACJI 2

Szałach ŁP, Cubała WJ, Lisowska KA. *Changes in T-Cell Subpopulations and Cytokine Levels in Patients with Treatment-Resistant Depression - A Preliminary Study*. International Journal of Molecular Sciences. 2023: vol. 24, nr 1.

Celem pierwszej pracy z cyklu prac oryginalnych była ocena profilu immunologicznego pacjentów z depresją lekooporną, ze szczególnym uwzględnieniem subpopulacji limfocytów T oraz stężeń cytokin we krwi krążącej pacjentów. Wyniki zostały porównane z grupą kontrolną, którą tworzyły osoby zdrowe, bez historii zaburzeń psychicznych, w podobnym wieku oraz podobnym rozkładzie płci.

We wstępie przedstawiono dotychczasowe doniesienia na temat odchyień zmian w układzie immunologicznych osób cierpiących na depresję, zarówno jedno-, jak i dwubiegunową. Podkreślono wagę lekooporności jako istotnej kwestii, która powinna skłaniać do poszukiwania kolejnych patomechanizmów depresji, między innymi w immunopsychiatrii.

Uzyskane wyniki zostały opisane w trzech podrozdziałach. W pierwszym podrozdziale przedstawiono wyniki profili immunologicznych pacjentów cierpiących na depresję lekooporną, tj. odsetki populacji pomocniczych (CD4+) oraz cytotoksycznych (CD8+) limfocytów T z uwzględnieniem obecności antygenów aktywacyjnych na ich powierzchni (CD28, CD25, CD69, CD95, HLA-DR) oraz poziomy cytokin biorących udział w regulacji procesów zapalnych, tj. IL-12p70, TNF- α , IL-10, IL-6, IL-1 β i IL-8, w krwi krążącej. Na początku została przedstawiona charakterystyka pacjentów oraz osób w grupie kontrolnej biorących udział w badaniu oraz schemat analizy wyników uzyskanych metodą cytometrii przepływowej.

Wyniki badań ukazały znamienne różnice w subpopulacji CD4+CD25+ oraz CD8+CD95+. Wykazano, iż pacjenci TRD cechowali się znacznie niższymi odsetkami tych populacji w stosunku do osób zdrowych. W kwestii cytokin, zauważalne były istotnie niższe stężenia IL-12p70 oraz TNF- α u pacjentów oraz znacząco wyższe poziomy IL-8. Ponadto, analiza krzywej ROC wykazała, że poziom IL-8 w surowicy powyżej 19,55 pg/mL wiązał się z 10,26-krotnie zwiększonym ryzykiem rozwoju lekoopornej depresji, przy czułości wynoszącej 78,95% i swoistości 92,31%.

Drugi akapit w tej sekcji opisuje korelacje między parametrami immunologicznymi a obserwacjami klinicznymi, takimi jak BMI, wiek, płeć, długość epizodu, palenie tytoniu.

Nie znaleziono powiązań między profilem immunologicznym a wynikiem MADRS. Uwidoczniono ujemną korelację między wynikiem MADRS a BMI. Wiek pacjentów był ujemnie skorelowany ze stężeniem IL-1 β i IL-8. Zaobserwowano również dodatnią korelację między długością obecnego epizodu depresyjnego a odsetkiem limfocytów CD4+ i stosunkiem CD4+/CD8+ oraz ujemną między długością epizodu a odsetkiem populacji CD8+.

Trzeci akapit części wynikowej poświęcony został korelacjom pomiędzy odsetkami subpopulacji limfocytów T oraz poziomami cytokin pacjentów. Zaobserwowano, że wyższe stężenia IL-10 wiążą się z wyższym odsetkiem komórek CD8+, ale mniejszym odsetkiem limfocytów CD4+ i niższym stosunkiem limfocytów CD4+/CD8+. Wyższe stężenie IL-6 wiązało się z większym odsetkiem limfocytów CD8+CD95+, zaś wyższe stężenie IL-1 β było powiązane z większym odsetkiem subpopulacji CD4+CD25+ oraz CD4+CD69+. Stężenie IL-8 rosło wraz ze wzrostem odsetków subpopulacji CD4+CD69+ oraz CD4+HLA-DR+.

W części poświęconej na dyskusję na temat otrzymanych wyników przytoczone zostały wyniki innych badań, często odmienne od naszych. Podjęto próbę wyjaśnienia mechanizmów mogących odpowiadać za taki stan rzeczy, m. in. przewlekłość stanu zapalnego u pacjentów lekoopornych. Podkreślono również obecność istotnych różnic w wynikach badań *ex vivo*, z krwi bezpośrednio pobranej od ludzi, *versus in vitro*, które nierzadko ukazują zupełnie inne tendencje. Pokazuje to złożoność funkcjonowania układu immunologicznego człowieka i trudności w ujednoliceniu badań nad nim.

W dyskusji zaakcentowano wagę naszych wyników w kontekście IL-8. Pacjenci biorący udział w badaniu charakteryzowali się prawie siedemnastokrotnym wzrostem stężenia tej interleukiny w porównaniu do osób zdrowych. Podobne wyniki zaobserwowali Strawbridge i wsp. [43], którzy zasugerowali wysokie stężenia IL-8 jako wskaźnika lekooporności depresji. W chwili obecnej nie jest znany dokładny mechanizm działania IL-8 w układzie nerwowym. IL-8 jest kluczowym mediatorem, który jest produkowany przez monocyty, makrofagi i neutrofile i odpowiada głównie za migrację neutrofili. Niektóre wcześniejsze odkrycia wskazują, że komórki mikrogleju mogą syntetyzować IL-8 w odpowiedzi na bodźce prozapalne.

W ostatniej sekcji tego akapitu przedstawiono ograniczenia naszego badania, w szczególności niewielką grupę badaną, co uniemożliwiło podział statystyczny na podgrupy uwzględniające np. stosowane leki psychiatryczne czy też diagnozę jedno-/dwubiegunowości.

W podsumowaniu ponownie zaakcentowano, iż waga udziału cytokin i limfocytów T w patofizjologii lekoopornej depresji jest wciąż nie do końca jasna i niezbędne są dalsze, szczegółowe badania w tym obszarze. Przetawione wyniki pokazują, że pacjenci z depresją lekooporną cechują się zmniejszoną aktywnością układu odpornościowego, zaś podwyższone poziomy IL-8 mogą przyczyniać się do utrzymywania przewlekłego stanu zapalnego w przebiegu depresji. Celem wysnucia ostatecznych wniosków, w jaki sposób układ immunologiczny wpływa na występowanie lekooporności w depresji, wymagane jest przeprowadzenie dalszych badań z udziałem większej ilości osób.

X. OMÓWIENIE PUBLIKACJI 3

Szałach ŁP, Ciesielska-Figlon K, Daca A, Cubala WJ, Lisowska KA. *The Effect of Ketamine on the Immune System in Patients with Treatment-Resistant Depression*. International Journal of Molecular Sciences. 2025: vol. 26, nr 15.

Trzecim artykułem w cyklu prac doktorskich jest praca oryginalna pt. „The Effect of Ketamine on the Immune System in Patients with Treatment-Resistant Depression”. Jest to główna praca zwięzająca badania oryginalne nad wpływem ketaminy na układ odpornościowy pacjentów z depresją lekooporną. Opisywany w niej jest immunomodulujący efekt ketaminy zarówno *ex vivo*, czyli bezpośrednio na komórki krwi krążącej pacjentów, jak i *in vitro*, a więc w warunkach laboratoryjnych, w hodowlach komórkowych.

We wstępie pracy podkreślono wagę zrozumienia mechanizmów lekooporności w depresji. Wymieniono również różne możliwe przyczyny mogące powodować lekooporność, stawiając akcent na zaburzenia w funkcjonowaniu układu immunologicznego. Opisywana jest ketamina wraz z szeregiem mechanizmów działania mogących przyczyniać się do jej skuteczności przeciwdepresyjnej, takich jak wpływ na układ glutaminergiczny, synaptogenezę, czy szlak kynureninowy. Podkreślono, iż wcześniejsze badania wskazują na immunomodulacyjny wpływ ketaminy, zwłaszcza w odniesieniu do cytokin prozapalnych, jednak brak jest jednoznacznego konsensusu co do jej działania na układ odpornościowy i znaczenia tego mechanizmu w efekcie przeciwdepresyjnym.

Celem pracy było zbadanie zmian w profilu immunologicznym pacjentów po podaniu ketaminy dożylniej. Krew od pacjentów pobierano przed podaniem leku oraz po 4 i 24 godzinach po podaniu ketaminy. U części pacjentów była możliwość pobrania krwi również w kolejnych tygodniach trwania podaży ketaminy. Przeprowadzano zarówno ocenę subpopulacji limfocytów T, jak i stężeń cytokin w powyższych punktach czasowych. Przeanalizowano odsetki limfocytów T CD4⁺ i limfocytów T CD8⁺ pod kątem ekspresji antygenów CD28, CD69, CD25, CD95 oraz HLA-DR. Oznaczono również stężenia cytokin zapalnych w surowicy: IL-12p70, TNF, IL-10, IL-6, IL-1β i IL-8. Dodatkowo przeprowadzone zostały eksperymenty *in vitro*, tzn. hodowle komórkowe PBMC pobranych od pacjentów i osób zdrowych, aby zbadać, jak limfocyty reagują na niskie (185 ng/ml) oraz wyższe (350 ng/ml) stężenia ketaminy. Dobór stężeń do hodowli komórkowych odbył się na

podstawie doniesień naukowych dotyczących korespondujących stężeń ketaminy w krwi krążącej po podaniu jej dożylnie w dawce 0,5mg/kg mc. [44]. Następnie przeanalizowano, w jaki sposób ketamina wpływa na fenotyp powierzchniowy limfocytów, ich zdolność do proliferacji oraz produkcji cytokin.

Na początku opisu wyników zawarto informacje dotyczące uczestników badania. Wyjaśniono, iż pacjenci otrzymywali ketaminę dożylną zgodnie z protokołem w dawce 0,5mg/kg mc. przez 40 minut oraz że nie obserwowano istotnych działań niepożądanych leku. U każdego pacjenta otrzymującego ketaminę zaobserwowano obniżenie wyniku w skali MADRS po zakończeniu całego badania w porównaniu z poziomem wyjściowym, choć jedynie u 5 pacjentów stwierdzono remisję zdefiniowaną jako całkowity wynik MADRS po zakończeniu wszystkich wlewów ketaminy ≤ 10 punktów, zaś 1 pacjent spełnił kryteria odpowiedzi na leczenie (spadek o ponad 50% punktacji wyjściowej w skali MADRS).

Opis wyników został podzielony na cztery podrozdziały. W pierwszych dwóch przedstawione zostały wyniki otrzymane bezpośrednio z krwi pobranej od pacjentów przed podaniem ketaminy i po otrzymaniu ketaminy. W kolejnym skupiono się na zależnościach pomiędzy odpowiedzią na leczenie a parametrami immunologicznymi. W ostatnim opisano wyniki otrzymane w badaniach in vitro.

Zaobserwowano, iż po 4 godzinach od podania ketaminy odsetek limfocytów T CD3+ istotnie wzrósł, a następnie po 24 godzinach istotnie zmalał. Podobne tendencje uwidoczono w przypadku limfocytów T CD4+, gdzie odnotowano statystycznie istotny spadek po 24 godzinach w porównaniu z 4 godzinami. Odwrotny efekt zaobserwowano w populacji limfocytów T CD8+, gdzie po 24 godzinach odsetek tych komórek istotnie wzrósł. Ostatecznie stosunek CD4+/CD8+ zmniejszył się istotnie po 24 godzinach w porównaniu z 4 godzinami od podania ketaminy. Po 4 godzinach od podania ketaminy wzrósł odsetek komórek CD4+CD28+, a następnie spadł po 24 godzinach. Po 4 godzinach zaobserwowano również wzrost odsetka subpopulacji CD4+CD25. Odsetek subpopulacji CD4+CD69+ oraz CD4+HLA-DR+ wykazał istotny spadek po 24 godzinach w porównaniu z punktem wyjściowym. Nie stwierdzono różnic statystycznych w odsetku limfocytów CD4+CD95+. Po 24 godzinach odnotowano istotne zmniejszenie odsetka limfocytów CD8+CD25+ oraz CD8+HLA-DR+ w porównaniu z 4 godzinami po podaży leku. Odsetek limfocytów CD8+HLA-DR+ był również niższy w porównaniu z punktem sprzed podania ketaminy. Nie stwierdzono różnic w odsetkach subpopulacji CD8+CD28+, CD8+CD69+ ani CD8+CD95+.

W przypadku cytokin, stężenie IL-10 w surowicy znacznie wzrosło po 24 godzinach w porównaniu do poziomu po 4 godzinach od podania ketaminy (Rycina 2). Stężenie IL-6 wykazało przejściowy, choć nieistotny wzrost po 4 godzinach, a następnie istotny spadek po 24 godzinach od podania ketaminy. Najciekawsze jednak wyniki dotyczyły zmian stężenia IL-8. W poprzedniej pracy zwróciliśmy uwagę na fakt, iż pacjenci z lekooporną depresją mieli wyższe stężenie IL-8 w surowicy niż osoby zdrowe, a poziomy wyjściowy IL-8 powyżej 19,55 pg/mL wiązały się z ponad 10-krotnie zwiększonym ryzykiem depresji lekoopornej. Początkowo obserwowane przed podaniem ketaminy bardzo wysokie stężenie IL-8 znacząco spadło po 4 godzinach, zaś po 24 godzinach spadek ten był jeszcze silniejszy. Co więcej, wykazaliśmy na przykładzie dziewięciu pacjentów, że zmiany w stężeniach IL-8 na przestrzeni 4 tygodni, podczas których otrzymywali oni kolejne dawki ketaminy, dalej utrzymują się na niskim poziomie.

Jeśli chodzi o korelacje pomiędzy parametrami immunologicznymi a skalą MADRS przed i po zakończeniu podaży wszystkich wlewów ketaminy, zaobserwowano głównie związek ze stężeniem IL-8 – najbardziej zauważalnym parametrem, który uległ zmianie. Osoby z wyższym stężeniem IL-8 przed podażą ketaminy oraz 24 godziny po jej podaniu miały też wyższy poziom punktacji w skali MADRS po zakończeniu terapii, a więc uzyskały niższą redukcję objawów depresyjnych na koniec badania.

Ostatni podrozdział zawiera wyniki z hodowli komórkowych, w których limfocyty pacjentów i osób zdrowych stymulowane były za pomocą monoklonalnego przeciwciała anty-CD3 w obecności dwóch stężeń ketaminy. Nie zaobserwowano istotnych statystycznie różnic w fenotypie stymulowanych limfocytów CD4⁺ i CD8⁺. Zaobserwowano jedynie wyższy odsetek proliferujących limfocytów CD4⁺, które wyizolowane zostały od pacjentów, w obecności wysokiego stężenia ketaminy (350 ng/ml). Limfocyty pacjentów stymulowane w obecności wyższego stężenia ketaminy produkowały wyższy poziom IL-8 w porównaniu do niższego poziomu ketaminy (185 ng/ml) już po 4 godzinach od stymulacji. Podobną tendencję zauważono w kontekście IL-6, ale dopiero po 72 godzinach stymulacji.

W rozdziale Dyskusja przeanalizowano otrzymane wyniki i porównano je z dotychczasowymi doniesieniami. Na podstawie naszych badań wysnuto wniosek, iż ketamina powoduje krótkoterminowe przesunięcie równowagi immunologicznej, z dwufazową odpowiedzią polegającą na początkowej aktywacji bądź mobilizacji limfocytów (czego przejawem był głównie wzrost odsetka pomocniczych limfocytów T CD4⁺ z ekspresją antygenu CD28 i CD25), a następnie zahamowaniem układu odpornościowego, czego odzwierciedleniem był spadek odsetka limfocytów CD4⁺

z ekspresją takich antygenów jak CD69 czy HLA-DR. Ponownie podkreślono wagę wyników w kontekście IL-8. Interleukina 8, znana również jako CXCL8, jest chemokiną odgrywającą kluczową rolę w rekrutacji i aktywacji neutrofili podczas reakcji zapalnych. Jej wysokie poziomy obserwowane są w różnych chorobach o podłożu autoimmunologicznym, ale także u osób z chorobą otyłościową. W literaturze naukowej istnieją doniesienia o podwyższonym poziomie tej interleukiny we krwi oraz płynie mózgowo-rdzeniowym w chorobach psychiatrycznych. Prezentowane w tym artykule wyniki potwierdzają wcześniejsze obserwacje. Co więcej, podwyższony poziom IL-8 w krwi obwodowej może stanowić nie tylko potencjalny biomarker depresji lekoopornej, ale też wskaźnik odpowiedzi na leczenie przeciwdepresyjne. Silna dodatnia korelacja między końcowymi wynikami w skali MADRS a poziomami IL-8 mierzonymi przed leczeniem oraz 24 godziny po pierwszym podaniu ketaminy sugeruje również możliwy związek między odpowiedzią zapalną a efektem klinicznym.

Rozbieżność między wynikami *ex vivo* a *in vitro* wskazuje, że obserwowane *ex vivo* działanie immunomodulujące ketaminy nie jest prawdopodobnie wynikiem wyłącznie bezpośredniego wpływu na limfocyty, lecz obejmuje znacznie bardziej złożone, systemowe i zależne od kontekstu mechanizmy. Różnica w poziomach IL-6 i IL-8 wskazuje dodatkowo, że w zależności od stężenia ketaminy, efekt immunomodulujący tego leku może być zupełnie różny.

W dalszej części opisano ograniczenia badania wskazując na potrzebę kontynuacji badań nad immunomodulacyjnym efektem ketaminy na większej grupie osób, aby wysnuć ostateczne wnioski, lepiej wyjaśnić mechanizm jej działania i pomóc w identyfikacji pacjentów, którzy odniosą największe korzyści z terapii.

XI. PODSUMOWANIE WYNIKÓW BADAŃ

I. Charakterystyka pacjentów z depresją lekooporną.

1. Pacjenci z depresją lekooporną w porównaniu do osób zdrowych prezentowali:
 - obniżony odsetek limfocytów CD4+CD25+ oraz CD8+CD95+,
 - obniżony poziom IL-12p70 oraz TNF- α ,
 - znacznie podwyższony poziom IL-8.
2. Stężenia IL-8 powyżej 19.55 pg/mL zwiększały ryzyko depresji lekoopornej o 10,26 razy.
3. Nie zaobserwowano korelacji między zmianami immunologicznymi a wynikiem w skali MADRS.
4. Im dłużej trwał epizod depresyjny, tym wyższy był odsetek limfocytów T CD4+, a niższy limfocytów T CD8+.
5. Im wyższy był odsetek limfocytów CD4+CD69+ oraz CD4+HLA-DR, tym wyższe były poziomy IL-8.

II. Zmiany w układzie immunologicznym u pacjentów z depresją lekooporną po podaniu ketaminy.

1. U każdego z pacjentów otrzymujących ketaminę zaobserwowano redukcję w skali MADRS po zakończeniu cyklu podaży ketaminy.
2. 4 godziny po podaży ketaminy ex vivo w porównaniu do stanu sprzed podania leku zaobserwowano:
 - wzrost odsetka limfocytów CD3+, CD4+CD28+ oraz CD4+CD25+,
 - znaczący spadek poziomu IL-8.
3. 24 godziny po podaży ketaminy ex vivo w porównaniu do stanu sprzed podania leku zaobserwowano:
 - wzrost odsetka limfocytów CD4+CD69+,
 - spadek odsetka limfocytów CD4+HLA-DR+ oraz CD8+HLA-DR+,
 - znaczący spadek poziomu IL-8.
4. 24 godziny po podaży ketaminy ex vivo w porównaniu do momentu po 4 godzinach podania leku zaobserwowano:
 - spadek odsetka limfocytów CD3+, CD4+ oraz stosunku CD4+/CD8+,

- wzrost odsetka limfocytów CD8+,
 - spadek odsetka limfocytów CD4+CD28+, CD8+CD25+ oraz CD8+HLA-DR+,
 - wzrost poziomu IL-10 oraz IL-6.
5. Im wyższe było stężenie IL-8 przed podaniem ketaminy oraz po 24h po podaży ketaminy, tym wyższe były wyniki w skali MADRS na zakończenie leczenia.
 6. Wyniki badań in vitro po zakończeniu hodowli komórkowych nie wykazały statystycznych różnic między osobami zdrowymi i chorymi w odsetkach subpopulacji limfocytów T, niezależnie od obecności ketaminy.
 7. Obecność ketaminy w hodowli komórkowej w dawce 350 ng/ml:
 - zwiększała odsetek proliferujących, stymulowanych komórek CD4+ pacjentów w porównaniu do stymulowanych komórek pacjentów bez obecności ketaminy,
 - powodowała zwiększoną produkcję IL-8 oraz IL-6 przez komórki stymulowane pacjentów w porównaniu do komórek stymulowanych pacjentów w obecności niższego stężenia ketaminy (185 ng/ml).

WNIOSKI KOŃCOWE

Uzyskane wyniki badań wskazują, że pacjenci z depresją lekooporną charakteryzują się zaburzeniami w układzie immunologicznym. Ketamina wykazuje znaczące działanie immunomodulacyjne, zarówno we krwi pacjentów w trakcie leczenia, jak i w warunkach hodowli in vitro. W szczególności na duże zainteresowanie zasługuje oznaczanie stężenia IL-8 w surowicy pacjentów, która nie tylko może stanowić potencjalny biomarker depresji lekoopornej, ale może być też wskaźnikiem odpowiedzi na leczenie przeciwdepresyjne. Silna dodatnia korelacja między końcowymi wynikami w skali MADRS a poziomami IL-8 mierzonymi przed leczeniem oraz w trakcie podaży ketaminy sugeruje również możliwy związek między odpowiedzią zapalną a efektem klinicznym.

XII. PIŚMIENICTWO

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XIII. PUBLIKACJE WCHODZĄCE W SKŁAD ROZPRAWY

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REVIEW



The Influence of Antidepressants on the Immune System

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Abstract

Depression is one of the most frequently diagnosed condition in psychiatry. Despite the availability of many preparations, over 30% of treated patients do not achieve remission. Recently the emphasis is put on the contribution of the body's inflammatory response as one of the causes of depression. The interactions between nervous and immune systems are the main issue addressed by psychoneuroimmunology. In patients suffering from depression changes in the plasma concentrations of cytokines and in the number and level of activation of immune cells has been found. Attention is paid to the high levels of pro-inflammatory cytokines, the prevalence of Th1 responses to Th2, weakening of NK cell cytotoxicity and changes in lymphocyte proliferation and apoptosis. A number of studies focus on influence of antidepressants and non-standard methods of depression treatment, such as ketamine infusion, on patients' immunology. Many of them seem to regulate the immune responses. The study results encourage to look for new ways to treat depression with immunomodulatory drugs. In this article authors present the current knowledge about immune system changes accompanying depression as well as the study results showing the influence of drugs on the immune system, especially in the context of reducing the symptoms of depression.

Keywords Depression · Antidepressants · Lymphocytes · Cytokines · Proliferation · Apoptosis

Introduction

Depression is a condition that occurs in the course of many psychiatric disorders. It can be caused by various factors and have varying severity and duration. Using the term "depression" we usually mean a major depressive episode during the recurrent depressive disorder or major depressive disorder. A depressive episode can be also a component of bipolar disease in which depressive episodes are intertwined with manic episodes. Typical symptoms of depression include depressive mood, anhedonia, sleep changes, psychomotor retardation, decreased appetite and libido, weight loss, feeling of worthlessness as well as suicidal thoughts and suicide attempts, although not all of them must be present for the diagnosis of depression. To meet the criteria of a depressive episode (both according to ICD-10 and DSM-V), the symptoms should last for a minimum of 2 weeks for most of the

day. These symptoms may be accompanied by anxiety and psychotic symptoms, such as delusions (most often of guilt or sin) and hallucinations (American Psychiatric Association 2013; World Health Organization 2016).

Depression as a syndrome is the body's response to a variety of genetic, psychological, environmental and biological factors (Murawiec and Wierzbński 2016). Nowadays attention is paid to the role of inflammation resulting from the activation of the immune system in the course of depression, especially in its drug-resistant form. Also, it has been proven that antidepressants modulate immune responses thus affecting the activation, proliferation and survival of leukocytes.

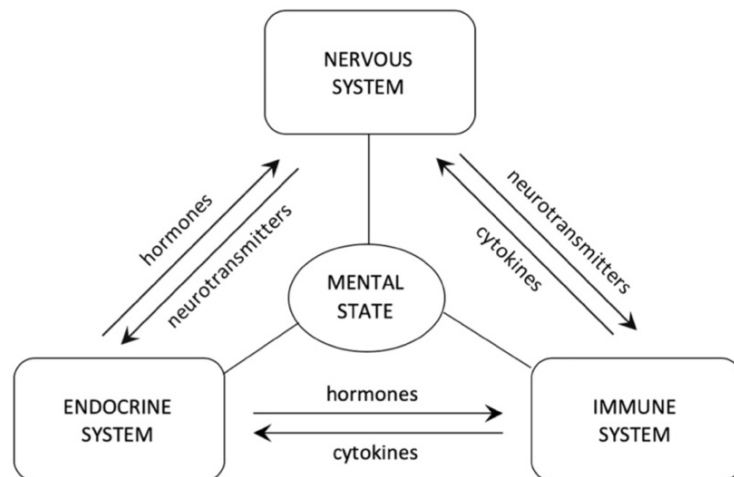
Psychoneuroimmunology is a field of study based on the concept of mutual interaction between nervous, endocrine and immune systems taking into account the influence of mental state on patient's immunity (Pariante 2015) (Fig. 1). It has been scientifically proven that the function of lymphatic organs and cells of the immune system can be regulated by the nervous system with the help of hormones, secreted mainly by the pituitary gland, and by neurotransmitters, among others, noradrenaline, acetylcholine released directly from the axons of cells of the autonomic nervous system (Talbot et al. 2016). Lymphocytes and macrophages

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Fig. 1 The interactions between nervous, endocrine and immune systems modulated by secreted hormones, neurotransmitters and cytokines



are also known to express on their surface a number of receptors for neurotransmitters like noradrenaline, acetylcholine, dopamine, serotonin, gamma-aminobutyric acid, endorphins and hormones such as corticotropin (CRH), allowing them to respond to signals sent by nervous system (Rybakowski 2002). In response to the stimulation of receptors with the aforementioned substances, a number of processes in immune cells are activated, including the production of cytokines secreted into the bloodstream allowing their systemic action, and hormones, mainly pituitary, such as corticotropin (ACTH), thyrotropin (TSH) or endorphins, mainly acting locally as auto- and paracrine hormones (Pállinger and Csaba 2008).

Cytokines of immune cells are secreted both systemically and locally within the central nervous system (CNS). Some of them, such as interleukin (IL)-6, delivered by the bloodstream can pass through the blood–brain barrier (BBB) (Banks et al. 1994). They also affect indirectly the inflammatory process within the central nervous system by cerebral endothelium stimulation for the expression of adhesion molecules with co-stimulatory properties, such as intercellular adhesion molecule 1 and vascular cell adhesion protein 1, and the major histocompatibility complex class II with interferon (IFN)- γ , so that T lymphocytes specific for the brain antigens may be activated to cross BBB and stimulate the inflammation. Additionally, both neurons and glial cells also can produce and secrete cytokines locally (Stokłosa 2017).

Knowledge about mechanisms of anti-inflammatory and pro-inflammatory processes, activity of immune cells and their mutual relations with the nervous and endocrine systems may help to understand the role of immune system in affective disorders. The purpose of this article is to show that, in addition to the classical effects on the

neurotransmitter system in the brain, antidepressants can also modulate the immune responses in patients suffering from depressive disorders, and thus contribute to clinical state improvement in patients during anti-depressive treatment.

Immune System Dysregulation in Depression

Regulation of immune system activity and chronic inflammation seems to play an important role in the pathogenesis of depression. One of the basic and best-studied mechanisms of interaction between nervous, endocrine and immune systems is their mutual influence during chronic stress or chronic inflammation accompanying some of the patients presenting features of the depressive syndrome.

Stress induces the transmission of nerve signals from the cerebral cortex to the hypothalamus where it stimulates the secretion of CRH, then ACTH by the pituitary gland which results in the release of glucocorticoids from adrenal cortex. Glucocorticoids act in immunosuppressive manner on the immune cells through the inhibition of cytokines production essential for the development of the inflammation, e.g., IL-1, IL-6, tumor necrosis factor (TNF)- α , IL-2 or IFN- γ , simultaneously reducing the T lymphocytes activity (Oppong and Cato 2015). They also favor the recruitment of transcription factors to promoters of genes encoding proteins with anti-inflammatory properties, such as IL-10, I κ B, annexin 1 or α -2-microglobulin (Oppong and Cato 2015).

The CNS can also inhibit the immune responses during chronic inflammation; when locally activated immune cells secrete into the blood stream a number of pro-inflammatory

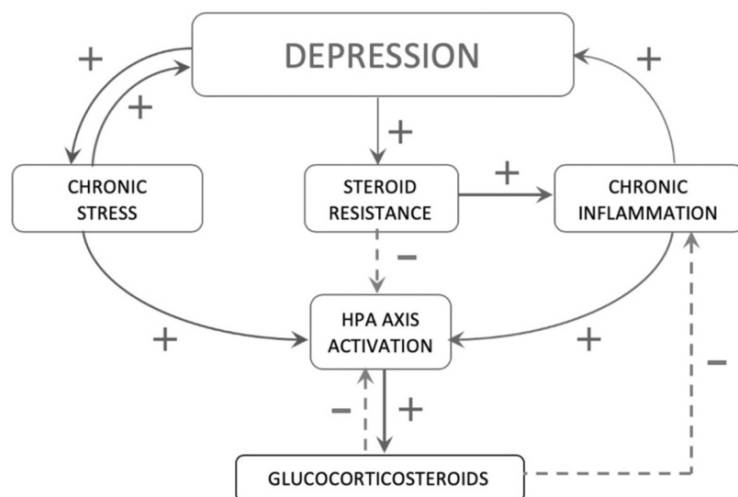
cytokines (IL-1, IL-6, TNF- α), the hypothalamic–pituitary–adrenal (HPA) axis is secondarily stimulated which consequently leads to the secretion of CRH and ACTH. Again, this results in suppression of the immune response through adrenocortical hormones (Hilal-Dandan and Brunton 2014). Unfortunately, in the situation of depressive episode, the immunosuppressive effect of the HPA axis seems to be insufficient to reduce inflammation associated with depression, which may be result of steroid resistance of immune cells or decrease in the threshold of hypothalamic sensitivity to pro-inflammatory cytokines secreted by these cells (Hilal-Dandan and Brunton 2014) (Fig. 2).

The inflammatory hypothesis of depression has been described and confirmed in a number of scientific studies. Elevated concentration of cortisol was observed in the blood of patients with the first major depressive episode diagnosed at an early age (Cubała and Landowski 2014, Cubała et al. 2016). It has been demonstrated that concentrations of pro-inflammatory cytokines are elevated in the serum of patients suffering from depressive disorders, mainly IL-1, IL-6, IFN- γ and TNF- α , which has been confirmed in several meta-analyses (Dowlati et al. 2010, Haapakoski et al. 2015, Schmidt et al. 2014a) (Fig. 3). The increase in the level of pro-inflammatory cytokines was accompanied by increased plasma concentrations of granulocyte–macrophage colony-stimulating factor (Schmidt et al. 2014a) and monocyte chemoattractant protein 1 (Király et al. 2017). Pro-inflammatory cytokines are mainly produced by type 1 T helper (Th1) cells which together with macrophages, dendritic cells and cytotoxic T cells are involved in a cell-mediated immunity essential for the elimination of the intracellular pathogens. Elevated concentrations of other cytokines, such as IL-5, IL-7, IL-8, IL-10, IL-12, IL-13 (Schmidt et al. 2014a) have

also been reported in some studies. These cytokines are involved in the development of humoral response against extracellular pathogens supported by Th2 cells. Increased activation of inflammasome NLRP3 (a multi-oligomer responsible for the activation of inflammatory responses) in peripheral blood mononuclear cells (PBMCs) of patients suffering from the depression has been described as well (Alcocer-Gómez et al. 2017). It is also known that IFN- α exogenously administered to treat chronic viral hepatitis C can cause transient symptoms of depression (Bonaccorso et al. 2002). In patients vaccinated against *Salmonella typhi* depressive symptoms occurred after vaccine administration and were positively correlated with elevated levels of IL-6 (Wright et al. 2005).

Changes of the immune system in patients suffering from depression are also noticeable in the number and ratio of leukocyte populations. An increase in the number of cells involved in the inborn immune responses, i.e., monocytes, macrophages and neutrophils (Demir et al. 2015), as well as increased production of reactive oxygen species (ROS) has been reported (Wei et al. 2015). In turn, a reduction in the number of NK cells, which are responsible for natural cytotoxicity was observed (Patas et al. 2018), accompanied by a simultaneous decrease in their activation compared to healthy people (Stokłosa 2017, Zorrilla et al. 2001). Some authors reported an increase in the ratio of CD4⁺ T (Th) cells to CD8⁺ cytotoxic (Tc) T lymphocytes (Di Rosso et al. 2016; Zorrilla et al. 2001). In addition, T lymphocytes of depressed patients presented significantly reduced expression of chemokine receptors CXCR3 and CCR6 (Patas et al. 2018), which play an important role in the recruitment of leukocytes to places where the inflammatory process is present. Additionally, an increase in the percentage of CD25⁺

Fig. 2 The participation of chronic stress and chronic inflammation in the development of depression and steroid resistance



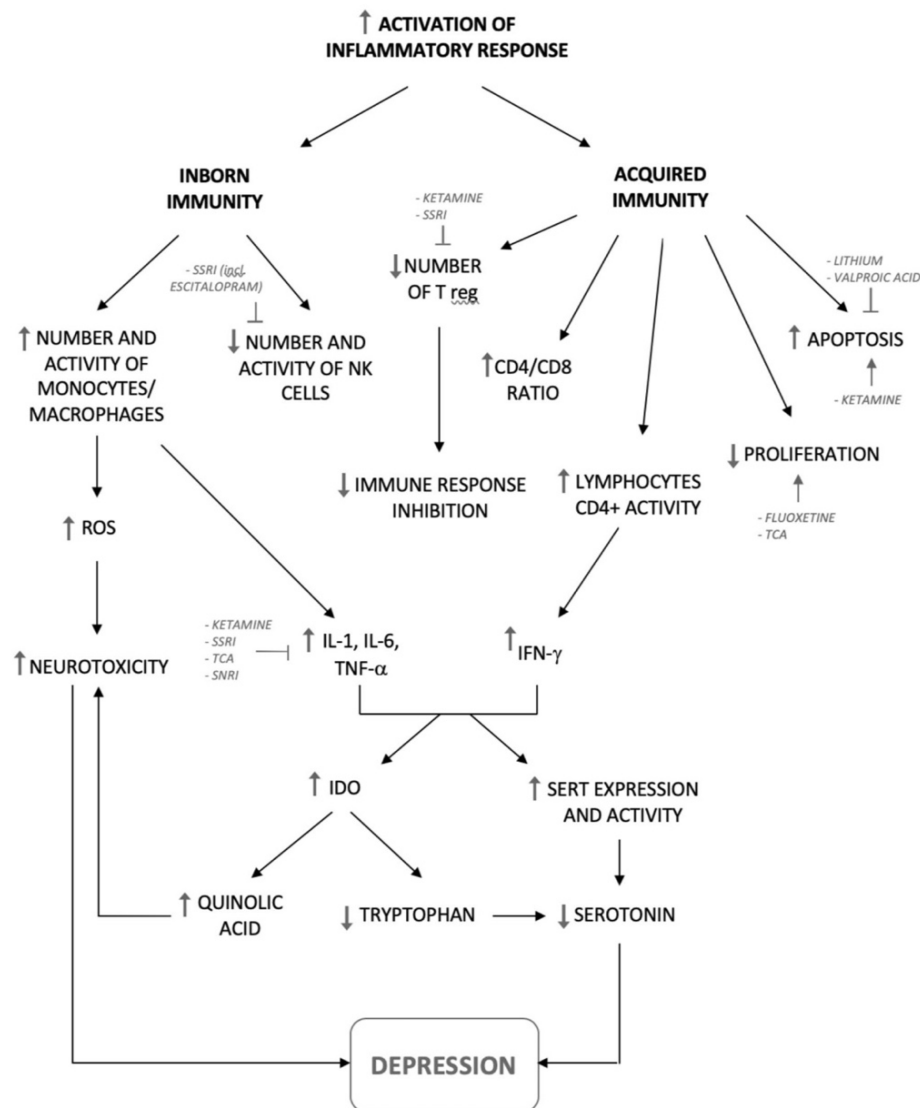


Fig. 3 Changes in the immune system in depressive patients with the identification of pathways modified by antidepressants. SERT: serotonin transporter

cells (activated T cells) (Müller et al. 1993; Patas et al. 2018) with the accompanying decrease in the total number of regulatory T lymphocytes (Treg) (Toben and Baune 2015), which are responsible for suppression of immune responses, within CD4+ T cell population have also been observed. Some authors demonstrated that lymphocytes of depressed patients were also characterized by decreased response to

mitogen stimulation in vitro (Darko et al. 1989; Kronfol et al. 1986; Toben and Baune 2015), while study of Schleifer et al. (1999) reported the opposite effect. Moreover, PBMCs of patients were also more prone to apoptosis (programmed cell death) compared to healthy people (Ivanova et al. 2007).

Even though there are animal and human studies suggesting that there can be stress-related B lymphocyte decrements

due to high levels of glucocorticoids, both the number of B cells and the level of antibodies in the serum of patients suffering from depression seem to be comparable to those observed in healthy persons (Dubois et al. 2016). Some authors suggest that autoantibodies, such as anti-ribosomal-P and anti-N-methyl-D-aspartate receptor antibodies, which are present in patients suffering from autoimmune diseases such as systemic lupus erythematosus might contribute to pathophysiology of depression in that group (Iseme et al. 2014; Postal and Appenzeller 2015). Recently, Ahmetshahic et al. (2018) demonstrated that frequencies of naive B cells as well as regulatory B cells are reduced in severely depressed patients as compared to healthy donors or mildly to moderately depressed patients, which suggests that the humoral response could play a role in the development of inflammation in the course of depression. However, there are still only a few articles about disorder of the humoral response in patients suffering from depression and this topic requires further research.

The changes in the immune system described above may contribute to the development of depression in various ways (Schmidt et al. 2014a). The pro-inflammatory cytokines such as IFN- γ , IL-1 or IL-6 affect the HPA axis by stimulating the secretion of CRH and increasing steroid resistance (Cubala and Landowski 2014), which secondarily stimulates the organism to produce more immunosuppressive glucocorticoids (Pariante and Lightman 2008). Also, cytokines induce production of inducible nitric oxide synthase, which leads to increased synthesis of ROS that cause protein and DNA damage that result in neurotoxicity and neurodegeneration (Inserra et al. 2018). Moreover, NO decreases norepinephrine production (Karolewicz et al. 2001) and inhibits the dopamine transporter, which decreases the availability of inter-synaptic dopamine (Sandor et al. 1995).

The most known neurobiological theory explaining mechanism of depression is monoamine theory, especially the serotonin hypothesis. The serotonin hypothesis of depression postulates that a reduction in serotonin levels may cause depression (Cowen and Browning 2015). Indeed, most of the widely used anti-depressive medications in the medical practice focus on increasing serotonin level in the synaptic cleft, mostly by inhibition of the serotonin reuptake. There are studies showing that immune system may interfere with serotonin system and contribute to the decrease of serotonin levels in CNS. It is postulated that pro-inflammatory cytokines, such as IFN- γ , IL-1 or IL-6, produced by activated immune cells are responsible for triggering pro-depressive effects through the induction of indoleamine 2,3-dioxygenase (IDO), an enzyme involved in the metabolism of tryptophan. IDO activates kynurenine pathway, thus causing depletion of tryptophan, which may be one of the important factors decreasing serotonin concentration in depressed patients. In addition to lower serotonin levels, there is also an increase in

neurotoxic metabolites of kynurenine—quinolic acids (Zou 2015). Moreover, pro-inflammatory cytokines also increase the expression and activity of the presynaptic serotonin transporter, which results in more excessive neurotransmitter reuptake from the synaptic space (Chou et al. 2016; Malynn et al. 2013). However, it is important to notice that only 50% of patients respond to the selective serotonin reuptake inhibitors (SSRI) and effective remission occurs less than 30% of the time (Trivedi et al. 2006). In the non-responders group, changing SSRI to other antidepressants that modulate also dopamine and/or norepinephrine levels causes remission in only two-thirds of patients (Stahl 2013). It shows that other mechanisms causing depression, not connected to synaptic serotonin or other monoamines levels, such as dopamine and norepinephrine, must exist.

Antidepressants and Their Effect on the Immune System

Currently, as mentioned above, the main drugs used for pharmacotherapy of depression are drugs that affect the level of neurotransmitters in the synaptic cleft, such as tricyclic antidepressants (TCA), selective serotonin/serotonin and noradrenaline reuptake inhibitors (SSRI and SNRI) as well as antiepileptic drugs (e.g., lamotrigine, valproic acid) and antipsychotics (including quetiapine, olanzapine, amisulpride). In addition to the known mechanism of action of these drugs on neurotransmitter transporters and pre- and post-synaptic receptors located on the neuronal membrane (serotonergic, dopaminergic, adrenergic), it is considered that they may have immunomodulatory properties which can contribute to achieving a therapeutic effect in patients with depression through this mechanism as well.

A meta-analysis by Strawbridge based on 35 clinical trials performed in over 20 years has shown that in patients suffering from unipolar depression, who have responded to widely understood antidepressant treatment, serum level of TNF- α and IL-6 significantly decreased (Fig. 3) while no significant change in C-reactive peptide concentration before and after treatment was observed (Strawbridge et al. 2015). Other meta-analysis involving the examination of 22 studies showed that the level of IL-1 β in patients' serum significantly decreased after pharmacotherapy (SSRI, SNRI or TCA), IL-6 level decreased slightly in patients receiving SSRI, while TNF- α levels did not change significantly regardless of the taken medicine (Hannestad et al. 2011). In study of Dahl et al. (2014), which measured peripheral blood cytokine levels before and after 12-week antidepressant therapy in 50 patients, a significant reduction in the level of cytokines IL-6, IL-7, IL-8, IL-10, G-CSF, IFN- γ and IL-1 receptor antagonist was reported. Importantly, the drop in the levels of these cytokines was seen only in patients

who demonstrated clinical response to treatment (Dahl et al. 2014). In another randomized trial a decrease in the concentration of pro-inflammatory cytokines was also observed in 73 patients who received sertraline (some of the patient received also transcranial magnetic stimulation therapy). Unfortunately, in this study the decrease also occurred in people taking placebo and was not related to the antidepressant effect (Brunoni et al. 2014). In another study, in which the level of soluble TNF receptor (sTNFR) was measured before and after treatment with sertraline, showed no significant difference in the concentration sTNFR in plasma after sertraline administration (Brunoni et al. 2015).

Antidepressants can influence activity and numbers of immune cells as well but not only that. For example, fluoxetine and mirtazapine have been shown to increase the expression of the serotonin transporter on the surface of T lymphocytes (Peña et al. 2005), which may improve the reuptake of serotonin thus lowering its concentration in the environment of lymphocytes and, consequently, at the same time reduce their ability to proliferate (Ahern 2011). Immunomodulatory properties of fluoxetine have also been reported in animal models and human. In mice treated with fluoxetine a number of T lymphocytes in the peripheral blood was decreased but the CD4⁺/CD8⁺ ratio remained unchanged (Di Rosso et al. 2016). In vitro, a decrease in the proliferative activity of T lymphocytes in patients with depression, who were treated fluoxetine, was also observed (Fazzino et al. 2009). In another study, not only the decrease in the concentration of pro-inflammatory cytokines was observed in 16 patients, after 6 weeks of treatment but also an increased number of Treg cells was observed (Grosse et al. 2016; Himmerich et al. 2010). Schleifer et al. (1999), who demonstrated that in patients with depression T cell response to mitogenic stimulation is significantly increased compared to healthy people, also showed that in patients receiving TCA lymphocyte activity significantly decreases. Several studies showed that pharmacotherapy of depression can also increase the number and cytotoxic activity of NK cells (Mizuruchin et al. 1999; Park et al. 2015).

Alcocer-Gómez et al. (2017) examined the expression level of genes encoding elements of NLRP3 inflammasome in mononuclear cells taken from patients receiving antidepressants from the SSRI group (paroxetine), SNRI (venlafaxine, desvenlafaxine), TCA (imipramine, amitriptyline) and patients treated with agomelatine or mianserin, were measured. All of these drugs not only significantly decreased the expression of genes but also reduced inflammasome activity, which decreased of plasma concentrations of IL-1 β and IL-18 (Alcocer-Gómez et al. 2017). Changes in the concentration of brain-derived neurotrophic factor (BDNF), a factor responsible for the survival of neurons, stimulating the formation of new nerve cells and synapses, with a broadly defined anti-inflammatory properties, were also observed

(Papathanassoglou et al. 2015). Increase in patients' serum BDNF has been demonstrated as soon as 5 weeks of treatment with sertraline and 6 months when venlafaxine was administered (Matrisciano et al. 2009). Moreover, studies show that the measurement of BDNF in serum may correlate with the level of BDNF in the central nervous system (Klein et al. 2011).

In the recently published work of Pietruczuk et al. (2018) it was demonstrated that T lymphocytes of patients suffering from bipolar disorder treated with lithium or valproate are characterized by decreased proliferative activity and increased susceptibility to apoptosis. The authors also showed that in vitro both drugs have an anti-apoptotic effect but do not affect in any way the ability of lymphocytes to proliferate (Pietruczuk et al. 2018).

Ketamine, an intravenous anesthetic affecting glutamate neurotransmission, which has also been used in the treatment of drug-resistant depression, also modulates the immune response. Presently, there are no studies showing how ketamine can influence the immune system of patients suffering from affective disorders. However, its immunomodulatory effect has been shown in oncological patients and patients in the postoperative period. It seems that low doses of ketamine administered parenterally cause a temporary decrease in TNF- α and IL-6 concentration in the patient's blood in the postoperative period (Beilin et al. 2007). Studies performed on mononuclear peripheral blood cells collected from oncological patients showed that ketamine may increase the CD4⁺/CD8⁺ ratio as well as the percentage of Treg cells (Hou et al. 2016). Meanwhile, studies in healthy subjects have shown that ketamine can inhibit the differentiation of Th2 cells, which are responsible for regulating humoral responses (Gao et al. 2011). It has also been shown that ketamine may exhibit an immunosuppressive effect not only by reducing the pro-inflammatory cytokines synthesis or the ability of the leukocyte to adhesion and migration (Larsen et al. 1998; Kawasaki et al. 2001) but also by inducing apoptosis of T lymphocytes (Braun et al. 2010) and the inhibition of the maturation of dendritic cells responsible for the antigen presentation (Zeng et al. 2011).

Immunomodulatory Drugs as Antidepressive Treatment?

An additional argument that the treatment of depression may be effective due to the modification of the immune response, is the results of researches carried out with the use of non-steroidal anti-inflammatory drugs (NSAIDs), cytokine inhibitors, polyunsaturated fatty acids or curcumin (Menon and Sudheer 2007). For example, it has been shown that administration of NSAIDs can reduce the symptoms of depression. The cyclooxygenase-2 inhibitor celecoxib turned out to be

particularly effective (Köhler et al. 2014). Etanercept, a soluble TNF- α receptor, also was shown to reduce depressive symptoms (Schmidt et al. 2014b). Patients suffering from depression treated with infliximab, an anti-TNF antibody, for longer period of time, also benefited from the therapy in terms of improving their mental state (Raison et al. 2013). Curcumin, a nutrient present in plants of the ginger family, may also contribute to the reduction of depressive symptoms when supplemented with classic antidepressant treatment (Yu et al. 2015), most likely by inhibiting the production of inflammatory mediators, such as prostaglandins, leukotrienes or nitric oxide and increasing in the concentration of biogenic amines in the brain (Kulkarni et al. 2008).

Raison and colleagues who performed randomized clinical trial with above-mentioned infliximab concluded that TNF antagonism does not have generalized efficacy in treatment-resistant depression but may improve depressive symptoms in patients with high baseline inflammatory biomarkers (e.g., high sensitivity C-reactive protein) (Raison et al. 2013). The meta-analysis of randomized, placebo-controlled trials of omega-3 fatty acid treatment of major depressive disorder demonstrated no significant benefit of omega-3 fatty acid administration (Bloch and Hannestad 2012). Authors also concluded that nearly all of the anti-inflammatory treatment efficacy in major depressive disorder observed in the published literature may be attributable to publication bias caused by heterogeneity of the group including somatic or mental comorbidities and pro-inflammatory markers baseline. Lower methodological quality or trials of shorter duration can also affect the final results. Köhler et al. (2014) in their meta-analysis emphasized that identification of subgroups that could benefit from anti-inflammatory treatment is needed especially since NSAIDs treatment is often accompanied by well-known adverse effects.

Conclusions

According to the assumptions of psychoneuroimmunology, impaired regulation of immune system activity and chronic inflammation may play an important role in the pathogenesis of depression. Furthermore, the results of numerous studies presented in this article show that the mechanism of action of currently used antidepressants is more complex than only their influence on the concentration of neurotransmitters in the synaptic cleft. Unfortunately, the results presented here do not always coincide, which may be the result from many different factors, including the heterogeneity of a group of patients diagnosed with a depression, a various mechanism of action of widely understood antidepressants, different doses of drugs administered, treatment period, co-morbidity, polypharmacotherapy or patients' age. Nevertheless, it seems that modification of the immune system activity

may also improve the mental state of patients suffering from affective disorders, which makes the research on the interaction of the immune and nervous systems particularly interesting.

Compliance with Ethical Standards

Conflict of interest All co-authors reported no biomedical financial interests or potential conflicts of interest.

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Article

Changes in T-Cell Subpopulations and Cytokine Levels in Patients with Treatment-Resistant Depression—A Preliminary Study

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Abstract: Although there is some evidence for the involvement of cytokines and T cells in the pathophysiology of treatment-resistant depression (TRD), the nature of this relationship is not entirely clear. Therefore, we compared T-cell subpopulations and serum cytokine levels in TRD patients to find relationships between their immunological profiles, clinical presentation, and episode severity. Blood samples from TRD patients ($n = 20$) and healthy people ($n = 13$) were collected and analyzed by flow cytometry. We analyzed the percentages of helper and cytotoxic T cells according to the expression of selected activation markers, including CD28, CD69, CD25, CD95, and HLA-DR. The serum levels of inflammatory cytokines IL12p70, TNF- α , IL-10, IL-6, IL-1 β , and IL-8 were also determined. TRD patients had a lower percentage of CD3⁺CD4⁺CD25⁺ and CD3⁺CD8⁺CD95⁺ cells than healthy people. They also had lower serum levels of IL-12p70 and TNF- α , whereas IL-8 levels were significantly higher. Receiver operating characteristic (ROC) analysis demonstrated that serum IL-8 values above 19.55 pg/mL were associated with a 10.26 likelihood ratio of developing TRD. No connections were found between the MADRS score and immunological parameters. These results show that TRD patients have reduced percentages of T cells expressing activation antigens (CD25 and CD95) and higher serum concentrations of proinflammatory and chemotactic IL-8. These changes may indicate reduced activity of the immune system and the important role of IL-8 in maintaining chronic inflammation in the course of depression.

Keywords: treatment-resistant depression; T cells; IL-8; cytokines; flow cytometry



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1. Introduction

Depression is a severe mental condition that may occur as major depressive disorder (MDD) or bipolar disorder (BD). According to psychoneuroimmunology, mutual interactions between the nervous, endocrine, and immune systems could influence a patient's mental state [1]. Therefore, dysregulation of immune system activity and chronic inflammation seems to play an essential role in the pathogenesis of depression.

Several meta-analyses confirmed that MDD patients have increased serum levels of cytokines regulating inflammation, including interleukin (IL)-6, tumor necrosis factor-alpha (TNF- α) [2–4], IL-1 β , IL-8, IL-10, and IL-18 [5–7]. A few studies demonstrated that patients suffering from depression have a reduced percentage of blood lymphocytes [8,9] but a high concentration of serum IL-2, a cytokine essential for T-cell differentiation into effector cells [6,7,10]. Some authors suggest that the stimulating effect of IL-2 is suppressed by the soluble IL-2 receptor (sIL-2R) in the blood [3]. In some other studies, the percentage of CD3⁺ T cells, especially CD3⁺CD4⁺ (helper) T cells, was reported to be increased in MDD patients, whereas the percentage of CD3⁺CD8⁺ (cytotoxic) T cells was reported to be decreased [11,12]. In addition, in meta-analysis, Zorrilla et al. [13] reported an increase in the CD4⁺/CD8⁺ ratio in MDD patients. However, the most recent studies show no evidence that the percentage of T cells or their two central populations, CD4⁺ and CD8⁺ cells, changes during depression [10,14,15]. T cells of MDD patients have also been shown

to express some activation markers, including CD95, which is involved in the process of apoptosis [16,17]; CD69, an early activation marker [18,19]; and CD25, an alpha chain of the IL-2 receptor [18,20].

Several studies showed that MDD patients present with high concentrations of IL-12 [10,21], which is involved in promoting Th1-type immune responses and stimulating the production of high levels of interferon-gamma (IFN- γ) [10,22]. Th17 cell number, a subset of proinflammatory T-helper cells, also increases in depression [23,24], most likely due to higher serum IL-6 levels. MDD patients also are characterized by a decreased percentage of CD4⁺CD25⁺FoxP3⁺ cells, which are regulatory T cells (Tregs) responsible for maintaining tolerance against self-antigens, thus preventing autoimmune reactions [9,23,25].

In BD patients, several cytokines are elevated, indicating the hyperactivity of innate immunity. A meta-analysis by Modabbernia et al. [26] showed that serum IL-4, IL-10, TNF- α , sIL-2R, sIL-6R, and soluble TNF receptor 1 (sTNFR1) in BD patients are, in general, significantly increased compared to healthy people. A recent meta-analysis also showed that IL-8 levels in BD patients are significantly increased [27]. Few alterations in the lymphocyte profile in patients with BD have been demonstrated, especially in a subpopulation of T cells. For example, female euthymic patients are characterized by fewer Tregs and a higher number of cytotoxic CD8⁺CD28[−] T cells than healthy people [28]. Younger euthymic BD patients (younger than 40 years old) have a higher percentage of Tregs [29]. In another study, euthymic BD patients showed significantly increased numbers of Th17 and Tregs compared with healthy controls [30]. A reduced number of cytotoxic T cells and an increased number of activated (CD4⁺CD25⁺) helper T cells with fewer IL-10-expressing Tregs were also described [31].

Although several pharmacological and non-pharmacological therapeutic options are available, treatment resistance among MDD patients is high and can reach up to one-third of patients suffering from depression [32]. Treatment resistance is an inadequate response to two or more antidepressant trials of adequate doses and duration [33]. For bipolar depression, we define treatment-resistant depression (TRD) as a clinically unsatisfactory response following at least two trials of different medicinal treatments in presumably adequate doses and durations within a specific phase of bipolar disorder [34].

Several studies have shown that MDD patients with TRD have higher serum IL-6, IL-8, TNF- α [35], and sIL-6R [36,37] levels than non-TRD patients. However, the most recent study showed that MDD patients with TRD were characterized by lower elevation of IL-6 and TNF- α than non-TRD patients [38]. There are no data available on T-cell changes in this particular patient group. Another study showed that BD patients with TRD were characterized by higher levels of IL-6 and TNF- α and lower levels of sTNFR1 than MDD patients [39]. In BD patients, elevated IL-1 β was proposed to predict treatment resistance [40].

In this paper, we compare main T-cell subpopulations and serum cytokine levels in TRD patients to find relationships between patients' immunological profiles, clinical presentation, and episodic severity. Although there is some evidence for the involvement of cytokines and T cells in TRD pathophysiology, the nature of this relationship is not entirely clear. Therefore, we analyzed the percentages of helper and cytotoxic T cells according to the expression of the CD28 antigen due to its role in antigen presentation and selected activation markers, including CD69, CD25, CD95, and HLA-DR. The serum levels of inflammation-related cytokines IL12p70, TNF- α , IL-10, IL-6, IL-1 β , and IL-8 were also determined.

2. Results

2.1. Comparison of T-Cell Subpopulations and Serum Cytokine Levels between TRD Patients and Healthy People

The study groups consisted of 20 inpatients diagnosed with TRD in the course of MDD or BD without psychotic features and 13 healthy people (Table 1). There was no difference between TRD patients and healthy people in terms of age or BMI.

Table 1. Basic characteristics of study participants.

	HC (n = 13)	TRD (n = 20)	p Value
Sex (male/female)	7/6	9/11	0.728
Age (years)	51 ± 17.63	47.50 ± 19.03	0.509
BMI (kg/m ²)	24.47 ± 5.34	28.24 ± 6.27	0.115
Length of current episode (weeks)	n.a.	38 ± 6.08	n.a.
MADRS	n.a.	30.2 ± 1.49	n.a.
Medication *			
SSRIs	n.a.	5	n.a.
SNRIs	n.a.	7	n.a.
SARIs	n.a.	2	n.a.
NaSSAs	n.a.	6	n.a.
TCAs	n.a.	2	n.a.
Atypical antipsychotics	n.a.	10	n.a.
Antiepileptic drugs	n.a.	5	n.a.
Low-potency antipsychotics	n.a.	4	n.a.
Lithium	n.a.	6	n.a.

Data are shown as mean results with standard deviation according to unpaired *t*-test. HC—healthy control, TRD—treatment-resistant depression, BMI—body mass index, SSRIs—serotonin reuptake inhibitors, SNRIs—serotonin and norepinephrine reuptake inhibitors, SARIs—serotonin antagonist and reuptake inhibitors, NaSSAs—noradrenergic and specific serotonergic antidepressants, TCAs—tricyclic antidepressants. * Please note that some patients received more than one medication.

The percentage of CD3⁺ T cells and their main subpopulations, CD3⁺CD4⁺ cells (helper T cells) and CD3⁺CD8⁺ (cytotoxic T cells) cells, were analyzed in healthy people and TRD patients, then compared with each other. In addition, in each of the T-cell populations, we analyzed the percentage of CD28⁺ (costimulatory antigen), CD69⁺ (early-activation antigen), CD25⁺, HLA-DR⁺ (middle-activation antigens), and CD95⁺ (activation protein also involved in apoptosis) subpopulations. Figure 1 shows an exemplary cytometric analysis of T-cell subpopulations in TRD patients.

We found no significant difference in the central populations of T cells (CD3⁺, CD3⁺CD4⁺, and CD3⁺CD8⁺ cells) between healthy people and TRD patients (Table 2). There were differences in the percentage of CD3⁺CD4⁺CD25⁺ (Figure 1D,E) and CD3⁺CD8⁺CD95⁺ cells. TRD patients had a lower percentage of CD3⁺CD4⁺CD25⁺ cells (median value of 6.99 compared to 13.16 in HC) (Table 2, Figure 2A). They also had a lower percentage of CD3⁺CD8⁺CD95⁺ cells (median value of 18.2 compared to 30.3 in HC) (Table 2, Figure 2B). No significant changes in other examined subpopulations were found between groups (Table 2).

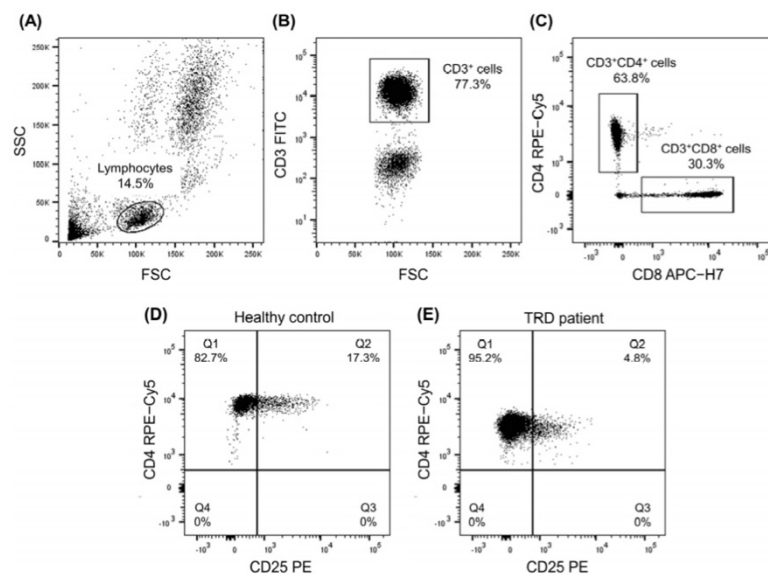
There were significant differences in serum cytokine levels between TRD patients and healthy people (Table 2). Healthy people had a higher level of serum IL-12p70 (median value of 2.65 pg/mL) compared to TRD patients, who, in most cases, had IL-12p70 contents below the level of detection (median value of 0 pg/mL) (Table 2, Figure 3A). They also had higher TNF-α level (median value of 1.2 pg/mL) compared to TRD patients (median value of 0 pg/mL) (Table 2, Figure 3B).

On the other hand, IL-8 levels were significantly increased in TRD patients (median value of 185.9 pg/mL) compared to healthy people (median value of 11.33 pg/mL) (Table 2, Figure 3C). Receiver operating characteristic (ROC) analysis demonstrated that serum IL-8 levels above 19.55 pg/mL were associated with a 10.26 likelihood ratio of developing TRD, with a sensitivity of 78.95% and a specificity of 92.31% (Figure 4).

Table 2. Comparison of T-cell subpopulations and cytokine levels between TRD patients and healthy people.

	HC	TRD	p Value
CD3 ⁺ (%)	75.66 (62.2; 80.82)	74.35 (43.3; 83.6)	0.407
CD3 ⁺ CD4 ⁺ cells (%)	61.4 (44.54; 76.88)	67 (48.8; 82.5)	0.286
CD3 ⁺ CD8 ⁺ cells (%)	32.8 (17.01; 48)	32.65 (13.2; 48.5)	0.630
CD4 ⁺ /CD8 ⁺ ratio	1.95 (1.08; 3.95)	2.05 (1.03; 6.25)	0.964
CD3 ⁺ CD4 ⁺ CD28 ⁺ cells (%)	59.02 (38.98; 69.81)	60 (36.7; 82.1)	0.434
CD3 ⁺ CD4 ⁺ CD25 ⁺ cells (%)	13.16 (5.08; 24.3)	6.99 (1.42; 43.6)	0.006
CD3 ⁺ CD4 ⁺ CD69 ⁺ cells (%)	0.63 (0.04; 12.5)	0.4 (0.03; 20)	0.242
CD3 ⁺ CD4 ⁺ CD95 ⁺ cells (%)	35.8 (18.3; 53.33)	29.1 (17; 45.8)	0.080
CD3 ⁺ CD4 ⁺ HLA-DR ⁺ cells (%)	1.68 (0.44; 7.18)	1.11 (0.09; 20.5)	0.715
CD3 ⁺ CD8 ⁺ CD28 ⁺ cells (%)	17.71 (9.59; 28.1)	18.95 (11.2; 35.3)	0.377
CD3 ⁺ CD8 ⁺ CD25 ⁺ cells (%)	0.64 (0.06; 4.84)	0.39 (0.06; 3.82)	0.191
CD3 ⁺ CD8 ⁺ CD69 ⁺ cells (%)	0.44 (0.07; 2.92)	0.45 (0.01; 2.44)	0.805
CD3 ⁺ CD8 ⁺ CD95 ⁺ cells (%)	30.3 (14.23; 48.56)	18.2 (9.11; 35.1)	0.024
CD3 ⁺ CD8 ⁺ HLA-DR ⁺ cells (%)	2.79 (0.92; 13.5)	8.8 (0.57; 15.9)	0.059
IL-12p70 (pg/mL)	2.65 (0; 12.74)	0 (0; 4.7)	0.001
TNF- α (pg/mL)	1.2 (0; 4.36)	0 (0; 62.8)	0.025
IL-10 (pg/mL)	2.7 (0; 6.15)	1.8 (0; 5.6)	0.153
IL-6 (pg/mL)	2.89 (0; 22.57)	2.8 (0; 110.4)	0.726
IL-1 β (pg/mL)	2.26 (0; 18.3)	0.7 (0.0; 12.6)	0.133
IL-8 (pg/mL)	11.33 (5.5; 20.2)	185.9 (8.1; 4660)	<0.0001

Data are presented as median with minimum and maximum results according to two-tailed Mann–Whitney U test. The results in bold are statistically significant at $p < 0.05$.

**Figure 1.** Cytometric analysis of T-cell subpopulations. Lymphocytes were selected based on forward- and side-scatter characteristics (A). Then, T cells were identified based on their positivity for the CD3 antigen (B). Next, helper T cells were identified based on the expression of the CD4 antigen and cytotoxic T cells based on CD8 expression (C). Finally, subpopulations expressing different activation antigens, e.g., the CD25 antigen, were identified. Exemplary dot plots with CD4⁺CD25⁺ cells from a healthy donor (D) and from a TRD patient (E).

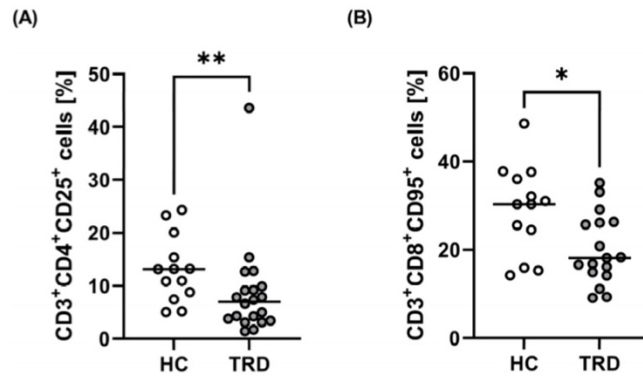


Figure 2. Comparison of percentage of CD4⁺CD25⁺ cells (A) and CD3⁺CD8⁺CD95⁺ cells (B) in TRD patients and healthy people. The line represents the median according to two-tailed Mann–Whitney U test; * $p < 0.05$, ** $p < 0.01$.

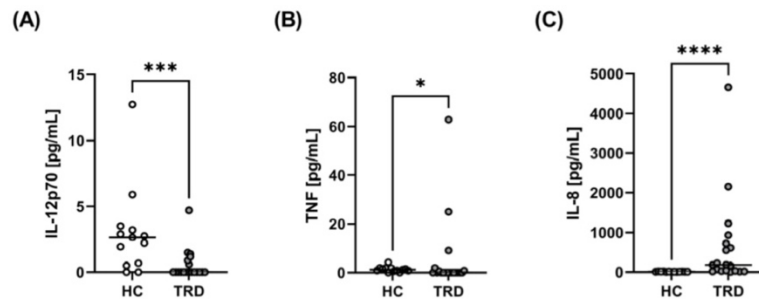


Figure 3. Comparison of serum concentrations of IL-12p70 (A), TNF- α (B), and IL-8 (C) between TRD patients and healthy people. The line represents the median according to two-tailed Mann–Whitney U test; * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

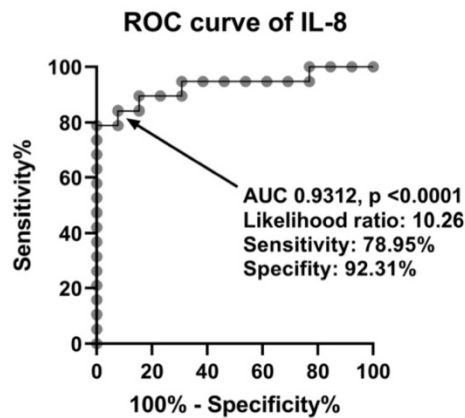


Figure 4. ROC curve for IL-8 in TRD patients.

2.2. Correlations between Immunological and Clinical Parameters

A psychiatrist assessed all patients with the MADRS scale. The median MADRS score in MDD patients was 32.5. We performed a correlation test of clinical characteristics and

immunological parameters in both patient groups. No correlation was observed between MADRS and any immunological parameter in TRD patients. MADRS was only negatively correlated with patient BMI ($r = -0.563$, $p = 0.010$). Patient age was also negatively correlated with serum IL-1 β ($r = -0.548$, $p = 0.015$) and IL-8 ($r = -0.502$, $p = 0.029$).

Additionally, a positive correlation was found between the length of the current episode and percentage of CD3⁺CD4⁺ cells ($r = 0.620$, $p = 0.004$), and a negative correlation was identified between episode length and percentage of CD3⁺CD8⁺ cells ($r = -0.599$, $p = 0.005$) (Table 3). Current episode length was also positively correlated with the CD4⁺/CD8⁺ ratio ($r = 0.604$, $p = 0.005$). After adjusting for potential confounders (sex, age, BMI, and smoking), there was a significant interaction between these variables.

Table 3. Correlations between current episode length and immune parameters.

	r	p Value
CD3 ⁺ (%)	−0.160	0.500
CD3 ⁺ CD4 ⁺ cells (%)	0.620	0.004
CD3 ⁺ CD8 ⁺ cells (%)	−0.599	0.005
CD4 ⁺ /CD8 ⁺ ratio	0.604	0.005
CD3 ⁺ CD4 ⁺ CD28 ⁺ cells (%)	0.364	0.115
CD3 ⁺ CD4 ⁺ CD25 ⁺ cells (%)	0.007	0.977
CD3 ⁺ CD4 ⁺ CD69 ⁺ cells (%)	0.303	0.222
CD3 ⁺ CD4 ⁺ CD95 ⁺ cells (%)	0.482	0.052
CD3 ⁺ CD4 ⁺ HLA-DR ⁺ cells (%)	0.230	0.359
CD3 ⁺ CD8 ⁺ CD28 ⁺ cells (%)	−0.358	0.121
CD3 ⁺ CD8 ⁺ CD25 ⁺ cells (%)	−0.052	0.826
CD3 ⁺ CD8 ⁺ CD69 ⁺ cells (%)	0.040	0.875
CD3 ⁺ CD8 ⁺ CD95 ⁺ cells (%)	−0.249	0.332
CD3 ⁺ CD8 ⁺ HLA-DR ⁺ cells (%)	−0.102	0.686
IL-12p70 (pg/mL)	0.208	0.394
TNF- α (pg/mL)	0.127	0.605
IL-10 (pg/mL)	−0.273	0.259
IL-6 (pg/mL)	0.142	0.562
IL-1 β (pg/mL)	0.058	0.815
IL-8 (pg/mL)	−0.031	0.901

Two-tailed Spearman rank correlation test. The results in bold are statistically significant at $p < 0.05$.

2.3. Correlations between Immunological Parameters

We have found some correlations between the percentages of T-cell subpopulations and serum cytokine levels in TRD patients (Table 4). There was a significant positive correlation between IL-10 and CD3⁺CD8⁺ ($r = 0.468$, $p = 0.043$) and CD3⁺CD8⁺CD95⁺ cells ($r = 0.579$, $p = 0.021$). There was a negative correlation between IL-10 and CD3⁺CD4⁺ cells ($r = -0.473$, $p = 0.041$) and between IL-10 and the CD4⁺/CD8⁺ ratio ($r = -0.470$, $p = 0.042$). There was a positive correlation between IL-6 and CD3⁺CD8⁺CD95⁺ cells ($r = 0.573$, $p = 0.022$). IL-1 β concentration was positively correlated with CD3⁺CD4⁺CD25⁺ ($r = 0.573$, $p = 0.01$) and CD3⁺CD4⁺CD69⁺ ($r = 0.592$, $p = 0.014$) cells. IL-8 levels correlated positively with CD3⁺CD4⁺CD69⁺ cells ($r = 0.564$, $p = 0.02$) and CD3⁺CD4⁺HLA-DR⁺ ($r = 0.544$, $p = 0.026$) cells.

Table 4. Correlations between immunological parameters in TRD patients.

	IL-12p70 (pg/mL)	TNF-α (pg/mL)	IL-10 (pg/mL)	IL-6 (pg/mL)	IL-1β (pg/mL)	IL-8 (pg/mL)
CD3 ⁺ (%)	−0.195	−0.233	0.218	−0.045	0.061	−0.168
CD3 ⁺ CD4 ⁺ cells (%)	−0.006	0.049	−0.473	0.091	0.072	0.226
CD3 ⁺ CD8 ⁺ cells (%)	0.040	−0.03	0.468	−0.087	−0.037	−0.242
CD4 ⁺ /CD8 ⁺ ratio	−0.023	0.039	−0.470	0.092	0.054	0.234
CD3 ⁺ CD4 ⁺ CD28 ⁺ cells (%)	0.168	−0.089	−0.312	0.133	0.214	0.135
CD3 ⁺ CD4 ⁺ CD25 ⁺ cells (%)	0.396	0.013	0.236	0.258	0.573	0.363
CD3 ⁺ CD4 ⁺ CD69 ⁺ cells (%)	0.401	0.470	0.046	0.395	0.592	0.564
CD3 ⁺ CD4 ⁺ CD95 ⁺ cells (%)	−0.064	−0.029	−0.304	0.272	0.139	−0.085

Table 4. Cont.

	IL-12p70 (pg/mL)	TNF- α (pg/mL)	IL-10 (pg/mL)	IL-6 (pg/mL)	IL-1 β (pg/mL)	IL-8 (pg/mL)
CD3 ⁺ CD4 ⁺ HLA-DR ⁺ cells (%)	−0.066	0.119	−0.378	0.219	0.416	0.544
CD3 ⁺ CD8 ⁺ CD28 ⁺ cells (%)	0.295	−0.056	0.190	−0.172	0.236	−0.188
CD3 ⁺ CD8 ⁺ CD25 ⁺ cells (%)	0.336	−0.018	0.242	0.236	0.301	−0.182
CD3 ⁺ CD8 ⁺ CD69 ⁺ cells (%)	−0.104	0.040	0.165	0.088	0.124	0.239
CD3 ⁺ CD8 ⁺ CD95 ⁺ cells (%)	0.224	0.132	0.579	0.573	0.327	0.003
CD3 ⁺ CD8 ⁺ HLA-DR ⁺ cells (%)	0.075	−0.072	−0.183	−0.022	0.248	0.088

Two-tailed Spearman rank correlation test. The results in bold are statistically significant at $p < 0.05$.

3. Discussion

Our results show several significant findings in the immune profiles of TRD patients. First, they had significantly higher serum IL-8 levels, with a median of 185.9 pg/mL, which is sixteen times higher compared to healthy people. Additionally, TRD patients were characterized by lower serum IL-12p70 and TNF- α levels than healthy people. Third, we found no statistically significant differences in the most often described proinflammatory cytokines, such as IL-1 β and IL-6. Finally, with respect to T-cell subpopulations, the median percentage values of CD3⁺CD4⁺CD25⁺ and CD3⁺CD8⁺CD95⁺ cells were twice as low as in healthy people, without any changes in other subpopulations.

As mentioned in the Introduction, some studies have shown that TRD patients have higher serum levels of IL-6 and TNF- α than non-TRD patients [35,39]. However, other studies show that TRD patients are characterized by a lower elevation of IL-6 and TNF- α compared to non-TRD patients [38]. Kiraly et al. [41] recently demonstrated that TRD patients have only high serum IL-6 compared with healthy people, without any changes in IL-1 β or TNF- α . In our study, TRD patients had lower TNF- α levels, without changes in IL-1 β or IL-6. TNF- α is a proinflammatory cytokine produced by several cells, primarily macrophages, in response to bacterial products and IL-1 signaling [18]. TNF- α , IL-1 β , and IL-6 are mainly involved in acute inflammation, leading to healing, trigger removal, tissue repair, and some systemic autoimmune diseases such as rheumatoid arthritis. Chronic inflammation accompanying some diseases, including depression, is characterized by a lack of canonical biomarkers [42]. The diagnostic value of IL-1 β or TNF- α is also somewhat limited due to their half-life of 5–10 min [43]. In our study, the serum level of IL-1 β was slightly decreased in TRD patients, which could explain the lower TNF- α level observed in patient sera. Lower (sometimes even undetectable) cytokine levels in TRD patients could indicate reduced macrophage activation due to prolonged, chronic inflammation.

IL-12p70, which is mainly produced by macrophages and dendritic cells, induces activation and proliferation of Th1 cells, which are responsible for cell-mediated response to pathogens [44]. According to some authors, MDD patients present with high concentrations of IL-12 [10,21]. In our study, TRD patients were characterized by lower serum IL-12p70 levels than healthy people, which may indicate a shift in immune response from Th1 (cellular) to Th2 (humoral) response. Similar results were reported by Cosma et al. [45]. However, in vitro studies demonstrated increased activation of M1 macrophages, which are responsible for activating Th1 responses. This difference may occur because in circulation, cells are influenced by hormones and neurotransmitters, including glucocorticoids, whereas in vitro, this influence is limited.

We also observed very high IL-8 levels in TRD patients, with a maximum of 2156 pg/mL, compared to a maximum level of 20.2 pg/mL in healthy people. Similar results were reported by Strawbridge et al. [35], who also suggested that elevated IL-8 is indicative of treatment-resistant MDD. Using ROC analysis, we were able to confirm that high serum IL-8 levels are associated with a 10.26 likelihood ratio of developing TRD. IL-8 is a crucial mediator that is mainly produced by monocytes, macrophages, and neutrophils and mainly responsible for neutrophil migration. However, its role in depression is currently unclear. Some previous findings indicated that human microglia can synthesize IL-8 in response to proinflammatory stimuli and that anti-inflammatory cytokines downregulate the production of this chemokine [46]. Furthermore, in a study by Taub et al. [47], a marked

T-cell infiltrate was observed in the IL-8 skin injection site, confirming that IL-8 is also a chemoattractant for lymphocytes.

We failed to demonstrate any correlation between cytokines and MADRS score. The latest study by Kofod et al. [48] showed no correlation between any measured inflammatory markers, including IL-1 β , IL-6, TNF- α , or IL-8, and MADRS in patients with moderate–severe depression. In our study, the MADRS score was negatively correlated only with patient BMI. Obesity can be characterized by a chronic low-grade inflammatory state that originates primarily from adipocytes capable of producing proinflammatory cytokines. In a study by Huet et al. [49], severely obese MDD patients (BMI > 40 kg/m²) had higher MADRS scores compared to lean people and people with overweight or moderate obesity. However, in another study, no relationship was between MADRS and the severity of obesity measured by BMI, although women with low BMI (≤ 18.5 kg/m²) achieved significantly higher MADRS scores than other women [50]. Differences in body fat distribution among TRD patients could explain disparities in these results. Whereas BMI is the most common measure of obesity, it is not fully representative of body fat distribution, especially central adiposity, because, compared to subcutaneous adipose tissue, visceral adipose tissue is considered to be associated with inflammation [51].

We found no difference in the percentage of T cells or their two central populations, CD3⁺CD4⁺ (helper) and CD3⁺CD8⁺ (cytotoxic) cells, which is in line with reports by other authors [9,14,15]. However, we observed correlations between current episode length and main T-cell subpopulations: a positive correlation with the percentage of CD3⁺CD4⁺ and the CD4⁺/CD8⁺ ratio and negative correlation with CD3⁺CD8⁺ cells. The results obtained in this study suggest that the longer the current episode, the higher the percentages of helper T cells. However, because no similar results have been reported in the literature, the meaning of this correlation remains unclear. We also observed correlations between percentages of several T-cell subpopulations and serum levels of IL-10, IL-6, IL-1 β , and IL-8, confirming that the lymphocyte profile in TRD patients is related to inflammation-related cytokines. Proinflammatory IL-1 β and IL-6 were mainly correlated with T cells expressing activation antigens (CD25, CD69, HLA-DR, or CD95). Moreover, anti-inflammatory IL-10 was negatively correlated with CD3⁺CD4⁺ cells, possibly as a response to chronic inflammation.

The median percentage of CD3⁺CD4⁺CD25⁺ cells in TRD patients was nearly twice as low as that in healthy people. This finding is consistent with previous studies on lymphocyte profiles in MDD patients [2,12,22], where CD25-positive cells were identified as Tregs. Unfortunately, the lack of a Treg marker, i.e., the FoxP3 transcription factor, does not allow us to indisputably conclude that we are dealing with a Treg deficiency. The CD25 antigen is also an activation marker in CD4⁺ and CD8⁺ T cells. Therefore, CD25-positive cell deficiency may be associated with an inadequate T-cell response as expressed by changes in serum IL-12p70 or TNF- α in TRD patients.

Previous studies reported an association between increased expression of CD95 on T cells with increased cell susceptibility to apoptosis [16,48]. In our study, TRD patients were characterized by lower percentages of CD3⁺CD8⁺CD95⁺ cells. On the other hand, no change was found in the percentage of CD3⁺CD4⁺CD95⁺ cells. Unfortunately, the lack of newer studies makes it impossible to compare the obtained results. However, because CD95 is also associated with the activation of T cells, its deficiency, together with the deficiency of CD25-positive cells, could be associated with disorders of T-cell activation in TRD patients.

We did not observe differences in other antigens involved in T-cell activation, CD69, or HLA-DR. The CD69 antigen is a classical early activation marker that appears an hour after T-cell contact with the antigen and rapidly decreases within 4–6 h. In chronic conditions, CD69 is expressed on infiltrated leukocytes at inflammatory sites rather than in blood [52], which could explain why we did not observe changes in its expression in T cells from blood in TRD patients. HLA-DR, on the other hand, is a molecule typically expressed by antigen-presenting cells and is associated with antigen presentation. Therefore, this antigen is also expressed in T cells during their activation. According to some authors, reduced

HLA-DR expression is a mechanism that adapts to an overwhelming burst of inflammatory stimuli; a high frequency of HLA-DR⁺ T cells strongly correlates with severe lymphopenia, systemic inflammation, and cytokine storm rather than with chronic conditions [53].

The present study is subject to potential limitations. First, the included group samples are relatively small. Hence, we were not able to separate several subgroups based on the factors that might have influenced the immune system, such as specific psychiatric medications. The limited number of patients is particularly problematic in studying the T-cell phenotype in TRD. Second, bipolar depression may have been misdiagnosed as MDD because depressive episodes during BD might be indistinguishable from unipolar depression. Third, we did not compare TRD patients with non-TRD patients. Furthermore, our observations and those of other authors show that serum cytokine levels in depressed patients vary, which may be related to the type of treatment and the response to this treatment. Fifth, our study included only Caucasian individuals in each of the study groups. Therefore, our results might not be fully applicable to different ethnic groups.

4. Materials and Methods

4.1. Study Groups

The study groups consisted of 20 inpatients diagnosed with depression in the course of MDD or BD without psychotic features (Table 1). Subjects were examined by clinicians using the Mini International Neuropsychiatric Interview (MINI) to verify the diagnosis using the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) criteria, as well as with the Montgomery–Asberg Depression Rating Scale (MADRS). In addition, all participants were described as treatment-resistance during the current episode, with MDD patients assessed by the Massachusetts General Hospital Antidepressant Treatment Response Questionnaire. A total of 13 healthy people of similar age were also included in the study.

The exclusion criteria for the present study were uncontrolled arterial hypertension; unstable coronary artery disease; increased intracranial pressure; acute and chronic infectious diseases; inflammatory, autoimmune, and metabolic diseases; and neoplastic diseases. An additional inclusion criterion for the control group was an absence of mental disorders. Only medically stable adult inpatients who were able to communicate and provide consent were enrolled in the study. All subjects provided written informed consent to participate in the study. For each participant, written consent was obtained after the procedures had been fully explained. The Independent Bioethics Committee for Scientific Research approved the study (consent nos. NKBBN/172/2017 and NKBBN/172-674/2019). A flow chart of the study methodology is presented in

The tested material was 5 mL of peripheral venous blood collected in EDTA tubes to determine lymphocyte subpopulations. In addition, 5 mL of blood was collected in anticoagulant-free tubes to collect serum to assess concentrations of cytokines. We stored serum samples at 80 °C.

4.2. Ex Vivo Determination of T-Cell Subpopulations

Samples of 100 µL per tube blood were transferred for staining with monoclonal antibodies and red blood cell (RBC) lysis. RBCs were lysed with buffer containing 0.8% NH₄Cl and 0.1% KHCO₃. Cells were then washed with PBS (phosphate-buffered saline) and stained with FITC-conjugated anti-CD3 or anti-CD95, RPE-Cy5-conjugated anti-CD4 (BD Pharmingen, San Diego, CA, USA), APC-H7-conjugated anti-CD8, PE-conjugated anti-CD28, anti-CD25, anti-CD69, or anti-HLA-DR (BD Pharmingen, USA) for 30 min at 4 °C in the dark. Then, cells were washed with PBS and suspended in 200 mL of suitable buffer for flow cytometric analysis using a FACSVerse instrument (Becton Dickinson, Franklin Lakes, NJ, USA). Figure 5.

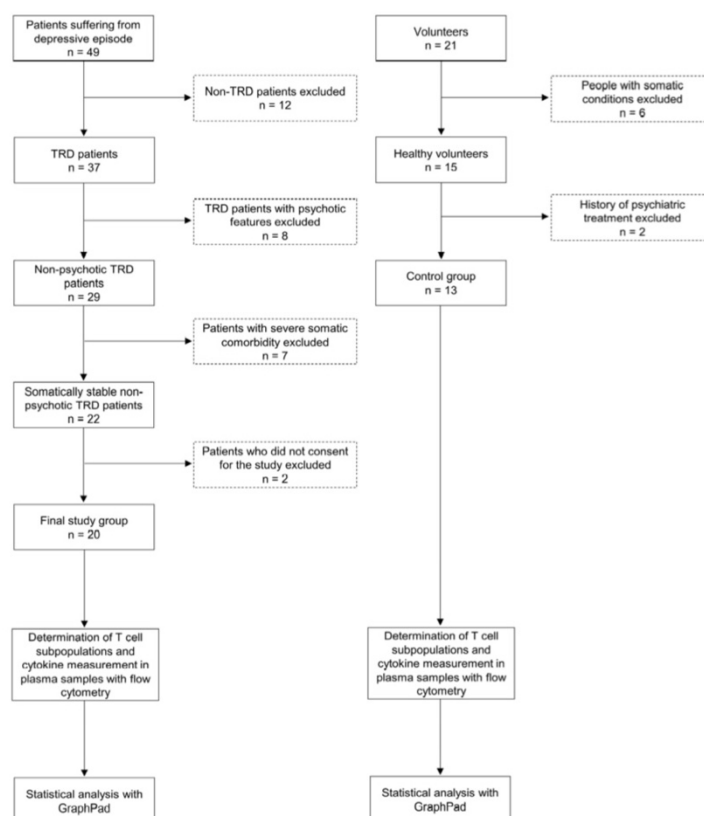


Figure 5. Flow chart of the study methodology.

4.3. Cytokine Measurement in Plasma Samples

A cytometric bead array (CBA) human inflammatory cytokine kit (BD Biosciences, USA) was used according to the manufacturer's protocol to determine the levels of IL12p70, TNF- α , IL-10, IL-6, IL-1 β , and IL-8 in serum samples from healthy people and both groups of patients. Quantitative cytometric fluorescence analysis was performed using a FACScan cytometer (Becton Dickinson, USA). The detection range for all measured cytokines was between 20 and 5000 pg/mL. The kit performance was optimized to analyze physiologically relevant concentrations (pg/mL levels) of specific cytokine proteins in tissue culture supernatants and serum samples. The limit of detection range for IL-8 was 3.6 pg/mL, 7.2 pg/mL for IL-1 β , 2.5 pg/mL for IL-6, 3.3 pg/mL for IL-10, 3.7 pg/mL for TNF- α , and 1.9 pg/mL for IL-12p70.

4.4. Analysis and Statistics

Twenty thousand events corresponding to the light-scatter characteristics of viable lymphocytes were acquired from each sample to analyze T-cell subpopulations. First, lymphocytes were selected based on forward- and side-scatter characteristics (Figure 1A). Then, T cells were identified based on their positivity for the CD3 antigen (Figure 1B). Next, helper T cells were identified based on the expression of the CD4 antigen, and cytotoxic T cells were identified based on CD8 expression (Figure 1C). Finally, subpopulations expressing different activation antigens, e.g., the CD25 antigen (Figure 1D,E), were identified. Cytometric data were analyzed with FlowJo 10 software (Beckton Dickinson, Franklin Lakes, NJ, USA).

Statistical data analysis was conducted using GraphPad 9 (version 9) statistical software. First, the distribution of the examined variables was checked with Kolmogorov–Smirnov and Shapiro–Wilk normality tests. Then, depending on data distribution, quantitative variables were analyzed with a two-tailed unpaired *t*-test, Mann–Whitney U test, or Spearman rank correlation test. A significance level of $p < 0.05$ was set for all analyses.

5. Conclusions

Although there is some evidence for the involvement of cytokines and T cells in TRD pathophysiology, the nature of this relationship is not entirely clear. Our results show that TRD patients have reduced percentages of T cells expressing activation antigens (CD25 and CD95) and higher serum levels of proinflammatory and chemotactic IL-8. These changes may indicate reduced activity of the immune system and the important role of IL-8 in maintaining chronic inflammation in the course of depression. Analyzing immunological parameters in these groups of patients requires larger sample sizes. The human immune system is susceptible to environmental factors and has many connections with the endocrine and nervous systems. Large study groups or prospective studies may answer the problem of differential observations in these groups of patients, making it possible to study the complicated relationships between TRD and response to treatment or correlation with disease activity.

Author Contributions: Conceptualization, Ł.P.S. and K.A.L.; methodology, K.A.L.; validation, K.A.L.; formal analysis, Ł.P.S.; resources, Ł.P.S. and W.J.C.; writing—original draft preparation, Ł.P.S.; writing—review and editing, K.A.L. and W.J.C.; visualization, Ł.P.S.; supervision, K.A.L.; funding acquisition, Ł.P.S. and W.J.C. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study was conducted according to the Declaration of Helsinki and approved by the Independent Bioethics Committee for Scientific Research at the Medical University of Gdańsk.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available upon request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

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Article

The Effect of Ketamine on the Immune System in Patients with Treatment-Resistant Depression

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Abstract

Treatment-resistant depression (TRD) is associated with immune dysregulation. Ketamine, a rapid-acting antidepressant, may exert effects via immunomodulation. The aim was to examine ketamine's impact on immune markers in TRD, including T-cell subsets, cytokines, and in vitro T-cell responses. Eighteen TRD inpatients received 0.5 mg/kg iv ketamine. Blood was sampled at baseline, 4 h, and 24 h to analyze T-cell phenotypes (CD28, CD69, CD25, CD95, HLA-DR) and serum cytokines (IL-6, IL-8, IL-10, TNF- α , IL-1 β , IL-12p70). In vitro, PBMCs from TRD patients and controls were exposed to low (185 ng/mL) and high (300 ng/mL) ketamine doses. Ketamine induced a transient increase in total T cells and CD4⁺CD25⁺ and CD4⁺CD28⁺ subsets at 4 h, followed by a reduction in CD4⁺ and an increase in CD8⁺ T cells at 24 h, decreasing the CD4⁺/CD8⁺ ratio. Activation markers (CD4⁺CD69⁺, CD4⁺HLA-DR⁺, CD8⁺CD25⁺, CD8⁺HLA-DR⁺) declined at 24 h. Serum IL-10 increased, IL-6 decreased, and IL-8 levels—initially elevated—showed a sustained reduction. In vitro, high-dose ketamine enhanced the proliferation of TRD CD4⁺ T cells and dose-dependent IL-8 and IL-6 secretion from activated cells. Ketamine induces rapid, transient immune changes in TRD, including reduced T-cell activation and cytokine modulation. A sustained IL-8 decrease suggests anti-inflammatory effects and potential as a treatment-response biomarker.

Keywords: ketamine; treatment-resistant depression; T cells; cytokines; IL-8; inflammation



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1. Introduction

Depression is a severe mental condition that may be classified as major depressive disorder (MDD) or depressive episode due to bipolar disorder (BD). Around 30% of patients with MDD do not achieve remission, even after trying several antidepressants and augmentation strategies [1]. Those patients are often classified as treatment-resistant. Treatment resistance is defined as an inadequate response to 2 or more antidepressant trials of adequate doses and duration [2]. Despite many available treatment methods, treatment-resistant depression (TRD) is a considerable challenge for mental health professionals. Discovering new pathomechanisms of depression and seeking novel anti-depressive agents with different mechanisms of action is necessary to help patients suffering from TRD.

Several reasons, including misdiagnosis, comorbidity, inadequate treatment, psychosocial factors, or lack of compliance, might cause treatment resistance. However, genetic,

neurobiological, and immunological factors play an important role in the pathomechanism of TRD [3]. Stress-induced excessive cortisol release induces dysregulation of the HPA (hypothalamic-pituitary-adrenal) axis. Cortisol activates the transcription of proinflammatory cytokines in microglia [4]. It has been shown that in TRD, pro-inflammatory cytokines induce an increase in the reuptake and impairment of the synthesis and release of monoamine neurotransmitters [5]. Moreover, pro-inflammatory cytokines interfere with the synthesis of monoamine neurotransmitters through the alteration of kynurenine and tetrahydrobiopterin (BH4) enzymatic pathways, resulting in reduced BDNF synthesis [4]. All of those processes contribute to depression and impaired treatment responsiveness.

Ketamine, a non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist, is a well-known, widely used anesthetic and analgesic drug. Esketamine, an S(+) enantiomer in the intranasal form, was approved by the Food and Drug Administration (FDA) in 2019 as a new-generation, rapidly acting antidepressant [6]. Ketamine is a safe, well-tolerated, and effective medication in the treatment of both unipolar and bipolar depression [7]. The rapid and robust antidepressant effects of ketamine, both in its intravenous form [8] and intranasal administration [9], have been extensively documented and investigated worldwide. Unlike traditional monoaminergic antidepressants, ketamine primarily modulates glutamatergic transmission. By inhibiting NMDA receptors on GABAergic interneurons, ketamine induces a surge in glutamate release, activating α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. This triggers intracellular signaling cascades involving brain-derived neurotrophic factor (BDNF) and the mechanistic target of rapamycin (mTOR), which promote synaptogenesis and restore synaptic connectivity in prefrontal and limbic regions implicated in depressive pathology [10,11]. Additionally, ketamine may upregulate serotonin 5-HT_{1B} receptor density in key brain areas, potentially enhancing serotonergic signaling [12]. It exhibits anti-inflammatory properties by reducing pro-inflammatory cytokines and modulating the kynurenine pathway, mechanisms increasingly recognized in the etiology of depression [13,14]. Moreover, some studies have suggested a potential role of the complement system in mediating ketamine's antidepressant effect [15]. However, it is still not fully understood by which mechanisms ketamine reduces depressive symptoms in TRD patients.

The immunomodulatory effect of ketamine has been previously reported in several studies, highlighting its potential role in modulating immune system activity in the context of depression [15,16]. It has been proven that ketamine modulates cytokine levels in patients with depression [17]. Kiraly et al. [18] performed a study in which TRD patients, after 4 h since receiving intravenous (iv) ketamine, presented decreased serum levels of interleukin 6 (IL-6) and granulocyte colony-stimulating factor (G-CSF), along with IL-1 α and interferon gamma-induced protein 10 (IP-10). Another randomized, double-blind control study showed a decrease in IL-6 and tumor necrosis factor- α (TNF- α) levels just after 40 min and was correlated with a lower Montgomery-Asberg Depression Rating Scale (MADRS) score [19]. This is the first clinical study to support a positive correlation between changes in serum cytokine levels after ketamine infusion and improvements in depressive symptoms in patients suffering from TRD.

We previously demonstrated that TRD patients differ immunologically from healthy individuals. They were characterized by, among other things, a lower percentage of CD4⁺CD25⁺ cells and high serum IL-8 levels [20]. This time, we hypothesize that ketamine infusion can modulate different parameters of the immune system of patients suffering from TRD, especially the activity of T cells and their ability to produce pro- and anti-inflammatory cytokines, which can reduce inflammation and thus affect the mental state of patients. Our study takes a broad approach to the immunomodulatory effect of ketamine, not only by measuring serum cytokine levels at one time point, but simultaneously studying

T-cell responses, phenotype, and cytokine levels in the blood over time after ketamine administration. Moreover, we included in vitro experiments to compare ex vivo results with a more exclusive examination of the ketamine immunomodulatory effect on T-cell activity.

We designed a study to compare how patients' selected immune parameters change 4 and 24 h after ketamine infusions. We analyzed the percentages of helper (CD4-positive) and cytotoxic (CD8-positive) T cells expressing CD28, CD69, CD25, CD95, and HLA-DR antigens essential for T-cell functioning. We also determined the serum levels of inflammatory cytokines: IL-12p70, TNF, IL-10, IL-6, IL-1 β , and IL-8. Additionally, we performed in vitro experiments to investigate how lymphocytes of TRD patients react to different concentrations of ketamine. We analyzed how ketamine influences their surface phenotype, proliferation capacity, and ability to produce cytokines. Our results were compared with samples from healthy volunteers.

2. Results

The mean of MADRS before ketamine administration was 28.9 ± 12 (min. 14; max. 42) points, while at the end of the series of ketamine administration (after 4 weeks) MADRS mean was 19.3 ± 8.6 (min. 1; max. 37) points (Table 1). In every patient receiving ketamine, we observed a reduction in the MADRS scale compared to the base level, although only 5 patients achieved remission (remission was defined as the total MADRS score arriving at ≤ 10 points [21]), and 1 patient met the criteria for response.

Table 1. Characteristics of TRD patients receiving ketamine, participating in the ex vivo part of the study.

	TRD (n = 18)
Sex (male/female)	5/13
Age (years) ¹	44.94 ± 19.72
BMI (kg/m ²) ¹	28.24 ± 6.27
Current episode length (weeks) ¹	41.1 ± 33.86
Bipolar depression (male/female)	7 (3/4)
Unipolar depression (male/female)	11 (2/9)
MADRS before ¹	28.9 ± 8.5
MADRS after ¹	$19.3 \pm 12^*$
Delta MADRS ¹	9.6 ± 8.6
Treatment:	
SSRIs	5
SNRIs	7
SARIs	1
NaSSAs	6
TCAs	1
Atypical antipsychotics	11
Antiepileptic drugs	7
Low-potency antipsychotics	3
Lithium	5

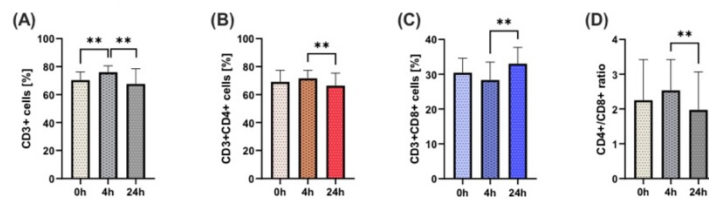
TRD—treatment-resistant depression, BMI—body mass index, SSRIs—serotonin reuptake inhibitors, SNRIs—serotonin and norepinephrine reuptake inhibitors, SARIs—serotonin antagonist and reuptake inhibitors, NaSSAs—noradrenergic and specific serotonergic antidepressants, TCAs—tricyclic antidepressants. * $p < 0.0001$ compared to MADRS before, the Wilcoxon test. Please note that some patients received more than one medication. ¹ Data are presented as mean $\pm$ SD.

Patients were administered a ketamine dose of 0.5 mg/kg body weight [22]. During ketamine administration, we did not observe any serious side effects that would lead to discontinuation of the treatment. Some patients experienced dizziness, drowsiness, and dissociative symptoms, which did not persist for more than a few minutes after the infusion was stopped. In a few patients, a transient, mild increase in blood pressure during ketamine infusion was observed [23].

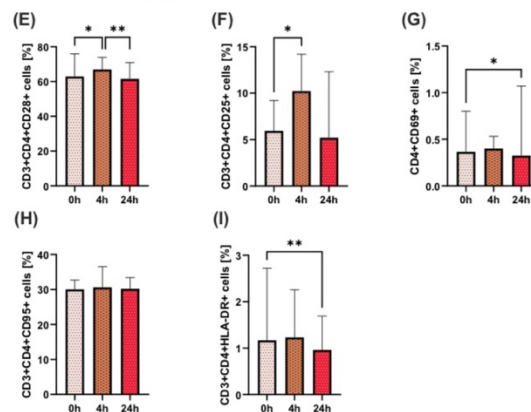
2.1. T-Cell Subpopulations and Serum Cytokine Levels Before and After Ketamine Administration Ex Vivo

We found a significant difference in the populations of CD3⁺ T cells between 4 h and 24 h after ketamine administration; at 4 h after ketamine administration, the percentage of CD3⁺ cells significantly rose, followed by a statistically significant drop after 24 h (Figure 1A). Similar tendencies were obtained for CD4⁺ T cells identified within the CD3 population, with a statistically significant fall after 24 h compared to 4 h (Figure 1B). On the other hand, a reversed effect was observed in the CD8⁺ T cells, where, after 24 h, the percentage of those cells increased significantly (Figure 1C). Finally, the CD4⁺/CD8⁺ ratio significantly decreased 24 h after vs. 4 h after ketamine administration (Figure 1D).

Basic T-cell populations



CD4⁺ T-cell subpopulations



CD8⁺ T-cell subpopulations

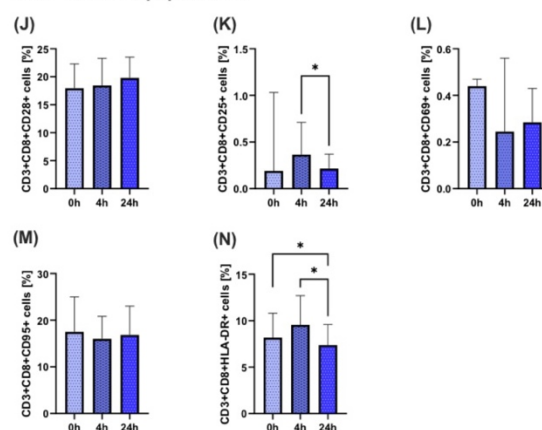


Figure 1. Comparison of percentage of CD3⁺ cells (A), CD3⁺CD4⁺ cells (B), CD3⁺CD8⁺ cells (C), CD4⁺/CD8⁺ ratio (D), CD3⁺CD4⁺CD28⁺ cells (E), CD3⁺CD4⁺CD25⁺ cells (F), CD3⁺CD4⁺CD69⁺

cells (G), CD3⁺CD4⁺CD95⁺ cells (H) CD3⁺CD4⁺HLA-DR cells (I), CD3⁺CD8⁺CD28⁺ cells (J), CD3⁺CD8⁺CD69⁺ cells (K), CD3⁺CD8⁺CD69⁺ cells (L), CD3⁺CD8⁺CD95⁺ cells (M), and CD3⁺CD8⁺HLA-DR⁺ cells (N) in TRD patients before 0 h, 4 h and 24 h after iv ketamine administration. The column bar graphs show the median value with confidence interval (CI). Wilcoxon test; * $p < 0.05$, ** $p < 0.01$.

Differences between 4 h and 24 h after ketamine administration were also observed in the percentages of CD4⁺CD28⁺ cells (Figure 1E). After 4 h, the rate of cells increased, then significantly decreased after 24 h of ketamine administration. A rise in the percentage of CD4⁺CD25⁺ subpopulation was observed after 4 h as well (Figure 1F). The percentage of CD4⁺CD69⁺ (Figure 1G) and CD4⁺HLA-DR⁺ (Figure 1I) showed a significant decrease after 24 h compared to the starting point. No difference was seen in the percentage of CD4⁺CD95⁺ cells (Figure 1H). A significant reduction after 24 h was identified in the percentage of CD8⁺CD25⁺ (Figure 1K) and CD8⁺HLA-DR⁺ cells (Figure 1N) compared with 4 h. The percentage of CD8⁺HLA-DR⁺ cells was also lower compared with 0 h (Figure 1N). No difference was seen in the percentage of CD⁺CD28⁺ (Figure 1J), CD8⁺CD69⁺ (Figure 1L), or CD8⁺CD95⁺ cells (Figure 1M).

In the case of cytokines, serum IL-10 concentrations after 24 h compared to 4 h levels after ketamine significantly increased (Figure 2A). There were significant differences in serum IL-6 levels, in a similar manner of transient, although non-significant elevation after 4 h and following, a statistically significant decrease in level 24 h vs. 4 h after ketamine administration (Figure 2B). The levels of IL-1 β , TNF, and IL-12p70 were close to 0 throughout the entire therapy, and therefore, were not included in Figure 2.

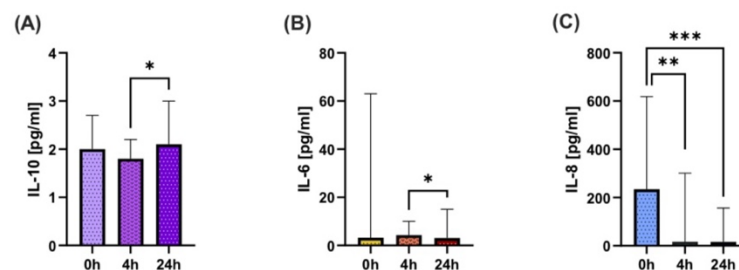


Figure 2. Comparison of serum concentration of IL-10 (A), IL-6 (B), and IL-8 (C) in TRD patients before 0 h, 4 h and 24 h after iv ketamine administration. The column bar graphs show the median value with CI. Wilcoxon test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The most interesting findings were changes in IL-8 levels. Recently, we have shown that the TRD patients, who later received ketamine in vitro and were mostly included in this study, had higher serum IL-8 than healthy people—serum IL-8 levels above 19.55 pg/mL were associated with a 10.26 likelihood ratio of developing TRD [18]. Initially, very high levels of IL-8 before ketamine administration were observed, and rapidly and significantly dropped 4 h after ketamine administration, and with even stronger statistical significance 24 h after (Figure 2C).

2.2. IL-8 Levels After Following Ketamine Administrations

Encouraged by the evident differences in serum IL-8 levels observed at 4 and 24 h after the first ketamine administration, we aimed to investigate whether this trend would remain over the following weeks of the study. Due to organizational constraints, we were able to collect blood samples at later stages of the study from only a limited number of participants. Blood samples were collected 24 h after the 3rd, 5th, and 7th (odd-numbered)

ketamine infusions, at weekly intervals. Although the limited sample size precluded a reliable statistical analysis, reduced IL-8 levels persisted during the following weeks of treatment (Figure 3).

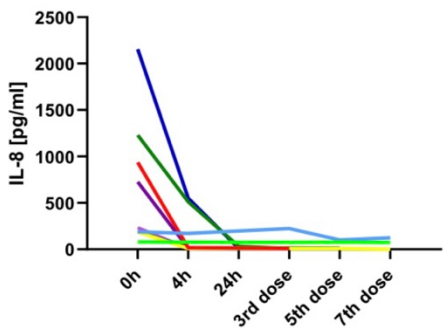


Figure 3. Serum IL-8 concentration during ketamine treatment in 9 TRD patients. Each colored line represents one of the patients.

2.3. Correlations Between Immunological and Clinical Parameters

Spearman’s rank-order correlation analysis revealed several statistically significant associations (Figure 4). First, a strong positive correlation was observed between baseline and endpoint MADRS scores ($r = 0.72, p = 0.001$). Furthermore, a statistically significant positive correlation was found between endpoint MADRS scores and IL-8 concentrations measured before treatment ($r = 0.52, p = 0.033$) and 24 h ($r = 0.69, p = 0.003$) after the first ketamine administration.

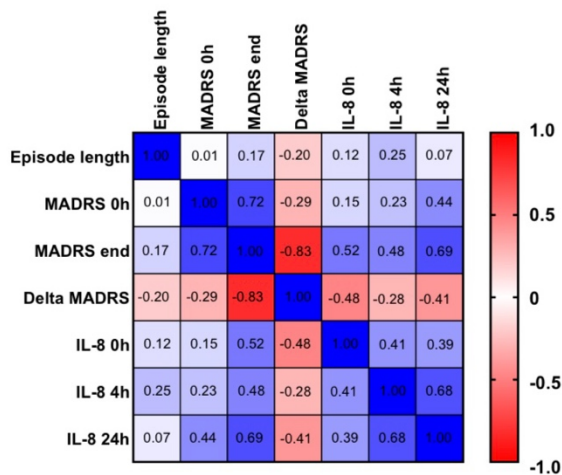


Figure 4. Heat map illustrating Spearman’s rank-order correlation (r) between episode length, MADRS scores at baseline and study endpoint, and IL-8 concentrations at various time points following ketamine administration. The color gradient reflects the strength and direction of correlations, with warm colors indicating positive correlations and cool colors representing negative correlations. The given r values are significant.

2.4. T-Cell Activity and Cytokine Levels After Cell Activation in the Presence of Different Concentrations of Ketamine in Vitro

We have found no difference in the percentages of CD4⁺ or CD8⁺ cells with expression of CD25, CD69, CD95, or HLA-DR antigens, regardless of the dose of ketamine

(Figure 5A–H). The only statistically significant difference was observed in the percentage of proliferating CD4⁺ cells in TRD patients in the presence of HD of ketamine compared to stimulated cells without ketamine (Figure 6B). No other significant alterations in lymphocyte proliferation were observed (Figure 6A,C,D). Comparisons across varying ketamine concentrations (in both sub-groups of stimulated and non-stimulated cells) and between patient and healthy control groups showed no statistically significant differences.

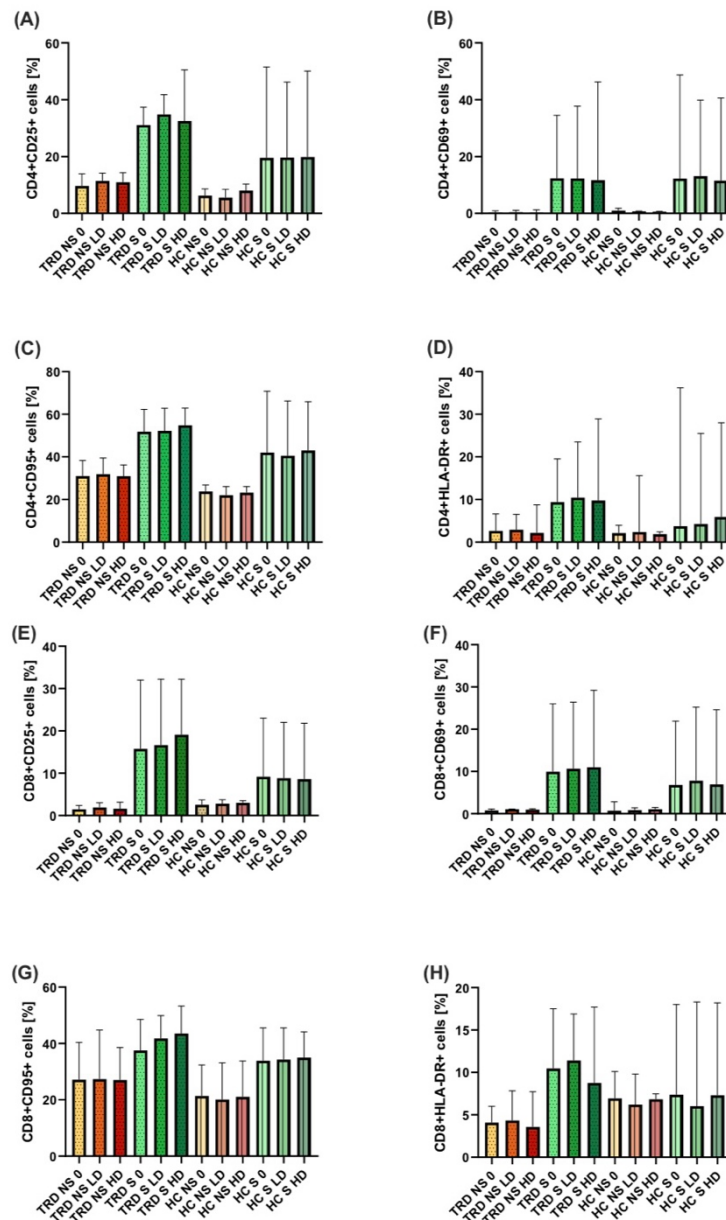


Figure 5. Comparison of the percentage of proliferating CD4⁺CD25⁺ cells (A), CD4⁺CD69⁺ cells (B), CD4⁺CD95⁺ cells (C), CD4⁺HLA-DR⁺ cells (D), as well as CD8⁺CD25⁺ cells (E), CD8⁺CD69⁺ cells (F),

CD8⁺CD95⁺ cells (G), and CD8⁺HLA-DR⁺ cells (H). Cells of healthy individuals (HC) and TRD patients were stimulated with anti-CD3 (S) in the presence of 185 ng/mL (low doses—LD) or 350 ng/mL (HD—high dose) ketamine or without it (0) for 72 h. Non-stimulated (NS) cells were also incubated with different doses of ketamine. The column bar graphs show the median value with CI.

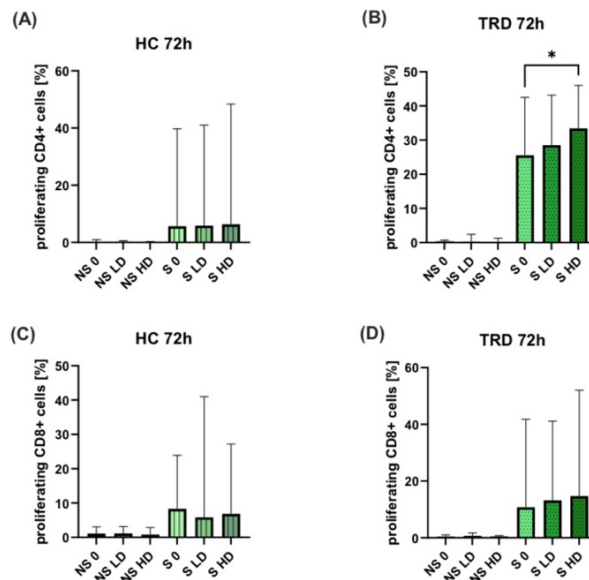


Figure 6. Comparison of the percentage of proliferating CD4⁺ and CD8⁺ cells from healthy controls (A,C) and TRD patients (B,D). TRD—patients, HC—healthy control, NS—non-stimulated, S—stimulated, 0—without ketamine, LD—low dose of ketamine, HD—high dose of ketamine. The column bar graphs show the median value with CI. ANOVA Friedman with Dunn's multiple comparisons test, * $p < 0.05$.

As to cytokines, changes were observed only in IL-8 (Figure 7A) and IL-6 (Figure 7B). Levels of IL-8 were lower in the presence of 185 ng/mL (LD) ketamine compared to 350 ng/mL (HD). Similarly, levels of IL-6 were lower in the presence of LD ketamine than HD ketamine.

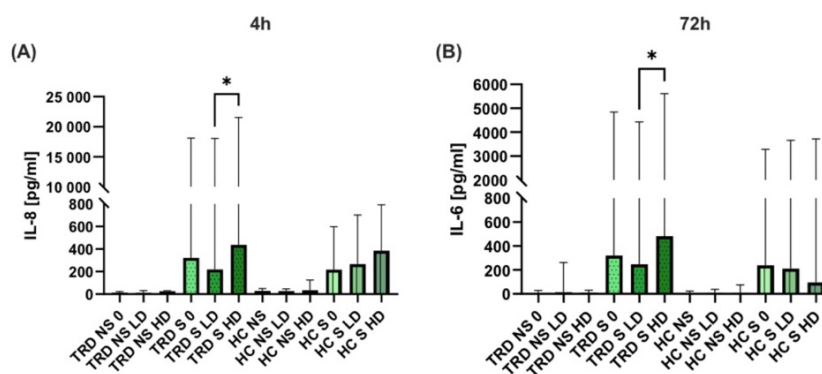


Figure 7. Supernatant concentrations of IL-8 (A) and IL-6 (B) after incubation with ketamine. TRD—patients, HC—healthy control, NS—non-stimulated, S—stimulated, 0—without ketamine, LD—low dose of ketamine, HD—high dose of ketamine. The column bar graphs show the median value with CI. ANOVA Friedman with Dunn's multiple comparisons test, * $p < 0.05$.

3. Discussion

Our study investigated immunological changes following ketamine administration in patients with treatment-resistant depression (TRD), using both ex vivo and in vitro models. In our previous study, we compared the immunological profile of TRD patients, who later received ketamine, to healthy volunteers. We found that patients with TRD exhibited a reduced percentage of CD4⁺CD25⁺, CD8⁺CD95⁺ cells; additionally, they showed lower serum levels of IL-12p70 and TNF- α , and highly elevated IL-8 levels compared to healthy controls [20]. Now, we observed dynamic alterations in T-cell subpopulations and cytokine profiles in the early period after a single ketamine infusion. These findings provide further evidence that the immunomodulatory effects of ketamine may contribute to its rapid antidepressant properties [24]. However, discussion of the effects of ketamine on the immune system is difficult. While there are isolated reports of its effects on serum levels of various cytokines [18,19], little is known about its effects on immune cells.

A transient activation of T lymphocytes was observed shortly after ketamine administration. Specifically, the percentage of total CD3⁺ T cells significantly increased 4 h after infusion, followed by a notable decrease 24 h post-administration. A similar pattern was found in CD4⁺ helper cells that play a central role in coordinating immune responses [25], while CD8⁺ cytotoxic T cells exhibited a reverse trend, with a delayed increase observed 24 h post-infusion. This divergence between CD4⁺ and CD8⁺ cells resulted in a decreased CD4⁺/CD8⁺ ratio at 24 h. The ratio is commonly used as an indicator of immune system homeostasis that is sensitive to stress and immunosuppressive agents [26]. Our results suggest a short-term shift in immune balance, with a biphasic response of initial activation followed by suppression of the immune system, possibly reflecting a state of temporary immune reorganization following ketamine administration.

Additional analyses of activation markers on CD4⁺ and CD8⁺ subsets supported this transient activation hypothesis. The percentages of CD4⁺CD28⁺ and CD4⁺CD25⁺ populations increased at 4 h, consistent with early T-cell activation, followed by a return to the initial level after 24 h. A similar observation was made for CD8⁺HLA-DR⁺ cells. Early activation marker CD69, a key T-cell activation marker, also showed a significant reduction at 24 h in CD4⁺ cells. These changes indicate that ketamine may initially trigger activation of helper and cytotoxic T cells, followed by a down-regulation of activation markers, potentially indicating an anti-inflammatory rebound or restoration of homeostasis.

The cytokine profile reinforces this interpretation. IL-6, a pro-inflammatory cytokine commonly elevated in depression, showed a significant reduction 24 h post-ketamine administration after a transient increase 4 h after ketamine. In parallel, levels of the anti-inflammatory cytokine IL-10 decreased after 4 h and then significantly increased 24 h later, supporting a transient shift toward a regulatory or resolving immune response. No changes were observed in other cytokines associated with inflammation, including TNF or IL-1 β .

What is striking is that most of these ex vivo parameters, both blood T-cell subpopulations and serum cytokines, are transient shortly after ketamine administration. Park et al. [27], who also observed transient changes in serum IL-6 in MDD and BD patients, suggested that this could be a non-specific stress response since similar observations are seen after saline administration in patients who were undergoing coronary artery bypass graft surgery [28]. However, if this were true, this change could apply to all parameters, not just selected ones. It is therefore possible that, as suggested above, ketamine administration leads to the mobilization or transient activation of immune cells.

The only variable that not only changed shortly after ketamine administration but also remained at the achieved level is IL-8. TRD patients showed markedly elevated baseline IL-8 before ketamine administration, which significantly decreased both 4 and 24 h post-infusion. Moreover, continuous monitoring of IL-8 levels in several patients throughout

the therapy showed that this decrease was maintained. Interleukin-8 (IL-8), also known as CXCL8, is a chemokine that plays a crucial role in the recruitment and activation of neutrophils during inflammatory responses. Elevated levels of IL-8 have been observed in various chronic inflammatory conditions, including major depressive disorder (MDD) [29]. The role of interleukin-8 as a marker of inflammation-related depression and predictor of treatment outcome has been previously described [30]. In our previous study, we found that in TRD patients, high serum IL-8 levels were associated with a 10.26 likelihood ratio of developing TRD [20]. The profound suppression of IL-8 may indicate a strong anti-inflammatory effect of ketamine, particularly on the innate immune system.

A strong positive correlation between endpoint MADRS scores and IL-8 levels measured before treatment and 24 h after the initial ketamine administration suggests a potential link between the inflammatory response and the clinical outcome. Specifically, higher IL-8 levels at 24 h were associated with greater residual depressive symptoms. None of the other immunological parameters showed such associations with treatment outcomes. Our findings strengthen the potential of IL-8 as a biomarker of inflammation-related depression and predictor of ketamine treatment response.

Our *in vitro* findings from cell cultures investigating the direct effects of ketamine on lymphocyte function were notably different and more limited in scope. In the cell culture model, ketamine did not significantly alter the expression of key activation markers (CD25, CD69, CD95, HLA-DR) on CD4⁺ or CD8⁺ T cells, regardless of the concentration used. This contrasts with our *ex vivo* data. The only statistically significant *in vitro* findings were an increase in CD4⁺ cell proliferation and increased levels of IL-6 and IL-8 in TRD patients' stimulated cells with high ketamine concentrations compared to lower concentrations, suggesting a potential activating effect on T cells under strong exposure to the medication.

Studies investigating the effects of ketamine on human lymphocytes *in vitro* remain limited. In one study focused on a topic, although performed in a population of patients with gastric cancer, their PBMCs were incubated for 24 h with different concentrations of ketamine (25, 50, and 100 μ M, which corresponds to 6.9, 13.7, and 27.4 ng/mL). The ratio of CD4⁺/CD8⁺ cells, as well as the percentage of Tregs, was significantly increased in the presence of rising concentrations of ketamine [31]. Their *in vitro* results somewhat overlap with our *ex vivo* results—in TRD patients, a transient increase in CD4⁺/CD8⁺ was observed 4 h after ketamine administration. Another study investigating the effects of ketamine on the proliferation of neural progenitor cells coming from healthy human-derived cells demonstrated that these cells exhibited a statistically significant increase in proliferation after 24 h of incubation. This effect was most pronounced at a ketamine concentration of 0.5 mM, which corresponds to approximately 137 ng/mL, and was inversely proportional to increasing ketamine concentrations [32]. In our study, proliferation of CD4⁺ cells was increased only in TRD patients and only in high doses of ketamine (350 ng/mL). These results show that the effects of ketamine are highly dependent on the dose, the type of cells stimulated, and even the cell donor.

Findings concerning cytokine production in the presence of ketamine partially align with the results of Kawasaki et al. [33]. In that study, lipopolysaccharide (LPS)-stimulated healthy human peripheral blood cells were incubated in the presence of ketamine. At a concentration of 100 ng/mL, ketamine was shown to directly inhibit the synthesis of TNF- α , IL-6, and IL-8 [33]. In our study, the production of IL-6 and IL-8 by lymphocytes from TRD patients stimulated in the presence of low-dose (185 ng/mL) ketamine revealed a downward trend. However, a high dose of ketamine significantly increased levels of those cytokines compared to the low-dose. Once again, these results show that the effect of ketamine is dose-dependent and cell donor-dependent.

The divergence between *ex vivo* and *in vitro* results indicates that the immunomodulatory effects of ketamine observed *in vivo* are likely not mediated only through direct action on lymphocytes but rather involve much more complex systemic and context-dependent mechanisms. These may include modulation of neuroimmune signaling via the HPA axis, autonomic nervous system, brain-derived neurotrophic factors, indirect effects through other immune cells such as monocytes or microglia, and cytokines and other substances secreted by them, changes in circulating metabolites such as kynurenines, as well as the permeability of the blood-brain barrier.

Nonetheless, some alignment was found at the cytokine level. *In vitro*, high-dose ketamine led to reduced IL-8 secretion after 4 h of incubation in stimulated lymphocyte cultures from TRD patients, echoing the robust and rapid drop in serum IL-8 levels seen *ex vivo*. This supports the theory that IL-8 suppression may represent a direct immunological target of ketamine, particularly in immune-activated states. Additionally, *in vitro* reductions in IL-6 at lower ketamine concentrations (observed only in TRD-derived cells) also parallel the *ex vivo* decrease in circulating IL-6 levels 24 h post-infusion.

Our study has several limitations. Due to the inability to administer ketamine to healthy individuals, we were not able to compare the *ex vivo* effects of ketamine in healthy subjects versus those suffering from TRD. We also could not examine the pure effect of ketamine, as all patients continued their baseline oral medications, which varied between individuals. Furthermore, strict regulations surrounding ketamine administration limited the size of the study group. As a result, we were unable to divide patients into subgroups based on sex, diagnosis (MDD vs. BD), or comorbidities such as anxiety disorders, to obtain statistically meaningful results in those groups.

Further research in this area—particularly studies examining the immunological profile of patients over 4 weeks of ketamine treatment and correlating it with psychometric data—would significantly strengthen conclusions regarding ketamine’s immunomodulatory effects as a potential antidepressant mechanism of action. Understanding how ketamine’s effects on the immune system contribute to clinical improvement in patients with treatment-resistant depression—and identifying the immunological profiles of those most likely to benefit—could enhance the ability to match patients with this treatment, ultimately increasing the likelihood of therapeutic response across a broader population.

4. Materials and Methods

4.1. Study Group

The study groups consisted of 18 inpatients diagnosed with depression in the course of MDD or BD without psychotic features (Table 1). The study population includes subjects enrolled in a naturalistic observational registry protocol for intravenous ketamine treatment in TRD: A Naturalistic Study of Ketamine for Treatment-Resistant Mood Disorders (GDKet) (NCT04226963). Clinicians examined subjects using the Mini-International Neuropsychiatric Interview (MINI) to verify the diagnosis using the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) criteria and with the Montgomery–Asberg Depression Rating Scale (MADRS).

Only patients meeting the criteria for a TRD diagnosis were eligible for the study. A patient was defined responder at the end of the study if the improvement from the baseline total MADRS score was at least a 50% reduction of points and was not qualified as remitted. Remission was defined as the total MADRS score arriving at ≤ 10 points [21]. All participants were described as having treatment resistance for the current episode, assessed by the Massachusetts General Hospital Antidepressant Treatment Response Questionnaire (ATRQ).

The exclusion criteria from the research were uncontrolled arterial hypertension, unstable coronary artery disease, increased intracranial pressure, acute and chronic infectious diseases, inflammatory, autoimmune, and metabolic diseases, and neoplastic diseases. In addition, mental disorders could not be present in the control group. Only medically stable, able to communicate, and provide consent adult inpatients were enrolled in the study.

All subjects gave written informed consent to participate in the study. After the procedures had been fully explained, written consent was obtained from each participant. The Independent Bioethics Committee for Scientific Research approved the study (consent No. NKBBN/398/2017 received 12 October 2017). We performed all the experiments following the relevant guidelines and regulations.

The in vitro experiments were performed on 4 TRD patients from 18 who were qualified for this study and 4 healthy people, 2 women and 2 men in each group.

The tested material was up to 20 mL of peripheral venous blood collected into EDTA (ethylenediaminetetraacetic acid) tubes. In addition, 5 mL of blood was collected into anticoagulant-free tubes to collect serum to assess concentrations of cytokines. We stored serum samples at -80°C .

4.2. Study Design

The study followed an observational design; all patients continued their baseline psychotropic treatment (Figure 8). The therapeutic intervention included intravenous ketamine infusion, administered at a dose of 0.5 mg/kg based on the actual body weight of the patient and given over 40 min. The preparation used for infusions was Ketalar 50 (ketamine hydrochloride) 50 mg/mL; one vial contained 10 mL. The managing psychiatrist monitored safety before, during, and after the infusion every 15 min to 1.5 h, including periodic assessment of vital signs (heart rate, body temperature, respiration rate, blood pressure, and oxygen saturation). Safety monitoring also included the Brief Psychiatric Rating Scale (BPRS) and Clinician-Administered Dissociative States Scale (CADSS) at baseline and one hour after the infusion.

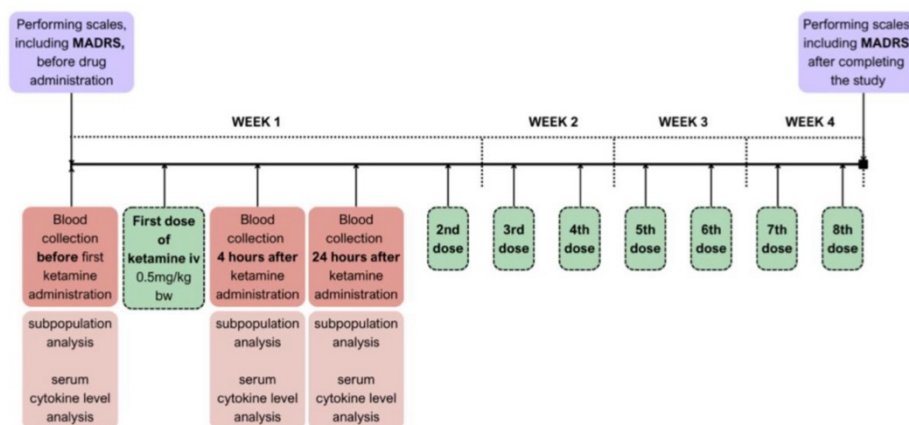


Figure 8. Timeline of the study procedure showing time points of intravenous ketamine administration, blood sample collection, and MADRS assessments.

Peripheral venous blood was collected three times from each patient, before ketamine infusion, 4 and 24 h after ketamine administration.

4.3. Determination of T Cell Subpopulations Ex Vivo

A total of 100 mL of blood samples were transferred for staining with monoclonal antibodies and red blood cell (RBC) lysis. RBCs were lysed with buffer containing 0.8% NH_4Cl and 0.1% KHCO_3 . Cells were then washed with PBS (phosphate-buffered saline) buffer and stained with FITC-conjugated anti-CD3 or anti-CD95, PE-Cy5-conjugated anti-CD4, PE-conjugated anti-CD28, anti-CD25, anti-CD69, or anti-HLA-DR, APC-H7-conjugated anti-CD8 (BD Pharmingen, San Diego, CA, USA) for 30 min at 4 °C in the dark. After this time, cells were washed with PBS and suspended in 200 mL of suitable buffer for flow cytometric analysis using the FACSVerse instrument (Becton Dickinson, Franklin Lakes, NJ, USA).

4.4. Cytokine Measurement in Serum and Plasma Samples

Cytometric Bead Array (CBA) Human Inflammatory Cytokines Kit (BD Biosciences, San Jose, CA, USA) was used according to the manufacturer's protocol to determine the level of IL12p70, TNF, IL-10, IL-6, IL-1 β , and IL-8 in the serum samples from TRD patients and culture supernatants. Then, quantitative cytometric fluorescence analysis was performed. The detection range for all measured cytokines was between 20 and 5000 pg/mL. The concentrations were analyzed using $X = \log(X)$ transformation and non-linear regression, with least squares regression fitting using GraphPad Prism version 9 (GraphPad Software, Boston, MA, USA).

4.5. PBMC Isolation and Stimulation

A total of 15 mL of peripheral venous blood was collected from 4 healthy volunteers and 4 patients with depression (2 diagnosed with BD and 2 with MDD) in tubes containing EDTA. Then, peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on Histopaque™ gradient (Sigma Aldrich Inc., Saint Louis, MO, USA). PBMCs were stained with Violet Proliferation Dye 450 (VPD450) (Becton Dickinson, Franklin Lakes, NJ, USA) for 12 min in the dark at 37 °C according to the manufacturer's protocol. Cells were then washed in phosphate-buffered saline (PBS) (EURx, Gdańsk, Poland) and resuspended in a complete culture medium (RPMI 1640 supplemented with 10% fetal bovine serum, 2 mm L-glutamine, 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin) at a concentration of 1 million cells per 1 mL medium. Cells were divided into three different subgroups according to the solution of ketamine in which they were incubated: 0 mmol/L (control), 185 ng/mL (low doses—LD), and 350 ng/mL (HD—high dose). The concentration of ketamine in the culture was selected based on reports of blood ketamine levels in individuals receiving the drug at a dose of 0.5 mg/kg body weight [22].

The cells were incubated with an immobilized (tissue-culture plate-bound) monoclonal anti-CD3 antibody (BD Pharmingen, San Diego, CA, USA) with the addition of ketamine solutions in standard culture conditions (5% CO_2 , 100% humidity at 37 °C) for three days.

Stimulated cells were collected after 4 h, 24 h, and 72 h and stained with the following antibodies: FITC-conjugated anti-CD95 and anti-CD25, PE-Cy5-conjugated anti-CD4, PE-conjugated anti-CD28, anti-CD69, and anti-HLA-DR, and APC-H7-conjugated anti-CD8 (BD Pharmingen, San Diego, CA, USA). Cells were also stained with PE-conjugated annexin V or 7-aminoactinomycin D (7-AAD), according to the manufacturer's protocol (BD Pharmingen, San Diego, CA, USA), and analyzed with flow cytometry.

4.6. Analysis and Statistics

Twenty thousand events corresponding to light scatter characteristics of viable lymphocytes were acquired from each sample to analyze lymphocyte subpopulations. First, lymphocytes were selected based on forward and side scatter characteristics (Figure 9A);

only single cells were included in the analysis (Figure 9B). Then, T cells were identified based on their positivity for the CD3 antigen (Figure 9C). Next, helper T cells were recognized based on the expression of CD4 antigen, and cytotoxic T cells based on CD4 and CD8 expression. Finally, subpopulations expressing different activation antigens, e.g., CD25 antigen, were identified (Figure 9D). The cytometric data were analyzed using the FlowJo version 10 software (Beckton Dickinson, Franklin Lakes, NJ, USA).

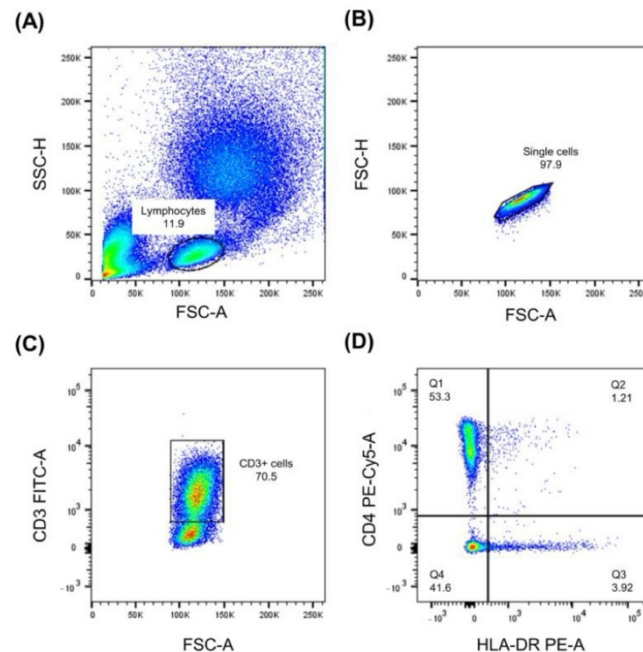


Figure 9. Analysis of lymphocytes ex vivo. Lymphocytes were selected based on forward and side scatter characteristics (A); only single cells were included in the analysis (B). T cells were identified based on their positivity for the CD3 antigen (C). Helper T cells were identified based on the expression of CD4 antigen, and finally, activation antigens, e.g., CD25 antigen, were identified (D). The figures are presented as density plots that show cell distribution with areas of high cell concentration appearing as denser, red colored.

To analyze lymphocyte proliferation patterns and cell phenotype after stimulation, thirty thousand events corresponding to lymphocytes were acquired from each sample. The expression of activation antigens, e.g., CD25, was identified on non-stimulated (Figure 10A) and stimulated (Figure 10C) lymphocytes. We used the dividing cell tracking (DCT) method to track cell proliferation using VPD450 (Figure 10B,D). After cleavage by cellular esterases within viable cells, the dye became fluorescent and covalently bound to proteins within the cells. As viable cells divided, the VPD450 dye was distributed uniformly between daughter cells, so each daughter cell kept approximately half of the VPD450 fluorescence intensity of its parent cell.

Statistical data analysis was conducted using GraphPad version 9 statistical software. The distribution of the examined variables was examined with Kolmogorov-Smirnov and Shapiro-Wilk normality tests. A significance level of $p < 0.05$ was set for all analyses.

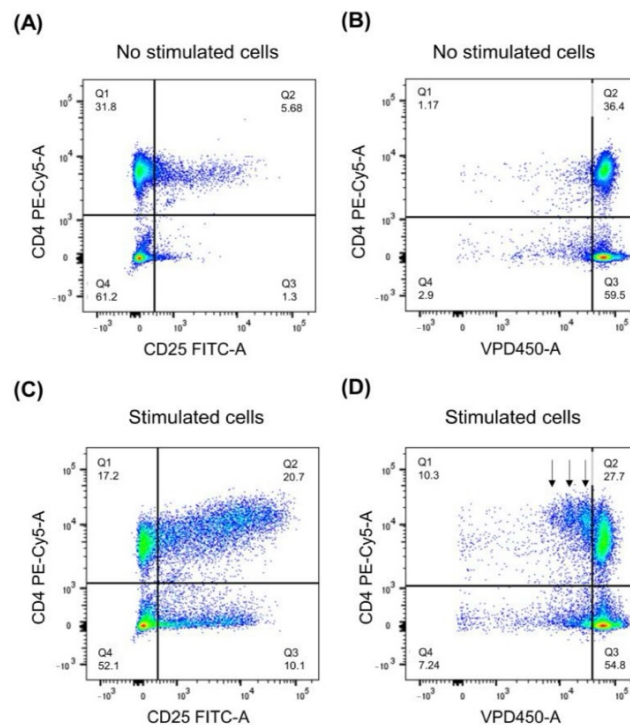


Figure 10. Analysis of phenotype and proliferation of lymphocytes in vitro. Non-stimulated (A,B) and stimulated (C,D) lymphocytes were identified based on the expression of CD4 antigen. Expression of activation antigens, e.g., CD25, was analyzed (A,C). VPD450 dye was used to analyze proliferating cells (B,D), which are marked with arrows (D). The figures are presented as density plots that show cell distribution with areas of high cell concentration appearing as denser, red colored.

5. Conclusions

Our findings demonstrate that intravenous infusion of ketamine induces rapid but transient immunological changes in patients with treatment-resistant depression *ex vivo*. A single infusion led to transient activation of T cells and modulation of key cytokines, including a sustained reduction in IL-8, an inflammatory marker previously associated with TRD. These results might support the hypothesis that ketamine's antidepressant effects could be mediated by immunomodulatory mechanisms. However, to accurately determine the direct relationship between ketamine's immunomodulatory effects and the final clinical response, further and more detailed studies—ideally including measurements throughout the entire 4-week period—should be conducted.

Notably, while *ex vivo* data showed time-dependent shifts in T-cell activation and cytokine profiles, *in vitro* effects were limited and dependent on ketamine dose and cellular context. The divergence between *ex vivo* and *in vitro* findings suggests that ketamine's immunomodulating properties likely involve complex systemic pathways rather than direct lymphocyte interaction alone.

The consistent suppression of IL-8 across models highlights its potential as a biomarker for inflammation-related subtypes of depression and response to ketamine therapy. Future research should focus on integrative approaches to better understand ketamine's neuroimmune mechanisms and identify immunological predictors of treatment response.

Author Contributions: Conceptualization, Ł.P.S. and K.A.L.; methodology, A.D. and K.A.L.; validation, A.D. and K.A.L.; formal analysis, Ł.P.S. and K.C.-F.; resources, Ł.P.S. and W.J.C.; writing—original draft preparation, Ł.P.S.; writing—review and editing, K.A.L.; visualization, Ł.P.S. and K.A.L.; supervision, K.A.L.; funding acquisition, Ł.P.S. and W.J.C. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study was conducted according to the Declaration of Helsinki and approved by the Independent Bioethics Committee for Scientific Research at the Medical University of Gdańsk (consent No. NKBBN/398/2017 received 12 October 2017).

Informed Consent Statement: Written informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available upon request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

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