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Research paper

Pro-inflammatory monocyte profile in patients with major depressive disorder and suicide behaviour and how ketamine induces anti-inflammatory M2 macrophages by NMDAR and mTOR

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ABSTRACT

Background: Depression is a highly prevalent disorder that is one of the leading causes of disability worldwide. Despite an unknown aetiology, evidence suggests that the innate and adaptive immune systems play a significant role in the development and maintenance of major depressive disorder (MDD). The non-competitive glutamatergic N-methyl-D-aspartate receptor (NMDAR) antagonist, (R,S)-ketamine (ketamine), has demonstrated rapid and robust efficacy as an antidepressant when administered at sub-anaesthetic doses.
Methods: Our goal was to characterize the pro-inflammatory profile of patients with MDD by measuring pro-inflammatory cytokines in plasma and circulating monocyte subsets and to understand how ketamine induces an anti-inflammatory program in monocyte and macrophages in vitro and vivo.
Finding: Our results show that patients with MDD without other comorbidities ($N = 33$) exhibited significantly higher levels of pro-inflammatory IL-12 and IL-6 in plasma and that these cytokines were associated with increased numbers of non-classical ($CD11b^{+}CD16^{bright}CD14^{neg}$) monocytes and increased activation state ($CD40^{+}CD86^{+}$) of classical monocytes in circulation. Remarkably, we have demonstrated that sub-anaesthetic doses of ketamine programs human monocytes into M2c-like macrophages by inducing high levels of CD163 and MERTK with intermediate levels of CD64 and stimulating mTOR-associated gene expression in vitro. The NMDAR antagonist MK-801, but not the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) antagonist, NBQX, also polarizes macrophages to an M2c-like phenotype, but this phenotype disappears upon mTOR pathway inhibition. Sub-anaesthetic doses (10 mg/kg) of ketamine administration in mice both promote reduction of circulating classical pro-inflammatory monocytes and increase of alternative M2 macrophage subtypes in the spleen and CNS.
Interpretation: Our results suggest an anti-inflammatory property of ketamine that can skew macrophages to an M2-like phenotype, highlighting potential therapeutic implications not only for patients with MDD but also other inflammatory-based diseases.
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Research in context

Evidence before this study

Major depressive disorder (MDD) is a prevalent and debilitating disorder in which treatment often results inadequate in approximately two-thirds of patients. The increasing involvement of

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inflammation in the pathophysiology of depression, during the last two to three decades, has provided new potential therapeutic avenues. MDD patients exhibited all cardinal features of inflammation, including increased circulating levels of inflammatory cytokines, chemokine, and activated immune cells. The discovery of the rapid-acting antidepressants effects of ketamine has opened a new opportunity to develop a novel class of safe and effective therapy for MDD. The mechanistic action of ketamine on CNS has extensively explored. However, it is not entirely understood how ketamine impacts on the activation and polarization program of macrophages. This is a critical aspect considering the inflammatory bias as an aetiological factor for MDD.

Added value of this study

Our results contribute to understanding better some cellular and cytokine players associated with MDD. We observed a clear correlation between the increased proportion of non-classical monocyte and pro-inflammatory cytokines (IL-6 and IL-12) levels. But interestingly not all patients have shown high cytokine levels or a pro-inflammatory cellular profile. Increased activation of CD86 and CD40 markers were also observed in the classical subset. Additionally, we have demonstrated how ketamine primes monocytes to initiate an anti-inflammatory program in vitro via NMDA receptor and mTOR signalling dependency. Finally, we also show that an in vivo administration of sub-anaesthetic doses of ketamine promotes an anti-inflammatory macrophage skewing in CNS and spleen.

Implications of all the available evidence

There still are controversial opinions in regard to inflammatory players, treatment and particularly to determine causality in human patients with MDD. So, measurements with several immune markers and longitudinal prospective study are needed to identify depression-specific biomarkers that will also allow better immunotherapeutic treatment strategies.

1. Introduction

Depression is a highly prevalent and disabling disease that comprises a set of pleomorphic syndromes with emotional, cognitive, visceral, and behavioural symptoms domains. According to the World Health Organization, over 350 million people worldwide suffer from depression [1], and the Global Burden of Disease Study revealed that Major Depressive Disorder (MDD) is one of the leading causes of the global burden of disease [2].

Despite the public impact of depression, its aetiology remains unknown. However, in recent years, a compelling body of evidence has emerged suggesting the role of the innate and adaptive immune systems in the development and maintenance of MDD. Patients with MDD exhibit cardinal features of an inflammatory response, such as increased levels of pro-inflammatory cytokines and their receptors along with increased release of acute-phase reactants, chemokine, and soluble adhesion molecules in peripheral blood and cerebrospinal fluid [3–5]. Additionally, higher levels of inflammation appear to increase the risk for the development of de novo depression [6]. Cytokines can induce depressive-like behaviours; in studies where healthy participants are given endotoxin infusions to trigger release of inflammatory cytokines, classical depressive symptoms emerge. Exogenous cytokine infusions also cause the classical phenotypic behavioural and cognitive features of depression [4,5,7].

Monocyte-macrophage lineage cells are multifunctional and found in nearly all tissues throughout the body. They orchestrate the initiation and resolution phases of both innate and adaptive immunity, significantly impacting protective immunity and immune-mediated pathological damage [8,9]. Circulating monocytes, the precursors of macrophages, migrate across the vascular endothelium to peripheral tissues where they mature into macrophages and adopt different activation states.

Previous studies by Carvalho et al. [10] and Grosse et al. [11] have highlighted the pro-inflammatory state of circulating sorted $CD14^+$ monocytes in patients with MDD. However, new gene expression studies in human monocyte biology emphasise the importance of defining the roles of monocytes populations in circulation based on the expression of CD14 and CD16 [12,13]. The $CD16^+$ subset can be further segregated into two subpopulations: the intermediate subset that expresses CD16 coupled with CD14 ($CD14^+$ $CD16^+$), and the non-classical subset that expresses high levels of CD16 with low levels of CD14 ($CD14^{neg}$ $CD16^{bright}$). The third subset that constitutes approximately 85–90% of circulating monocytes in human peripheral blood expresses high levels of CD14 with no CD16 expression, and is termed classical monocytes ($CD14^{bright}$ $CD16^{neg}$). It has been demonstrated that the three subsets of monocytes circulate in dynamic equilibrium, with 99% of classical monocytes leaving the circulation and the remaining 1% becoming intermediate monocytes, 100% of which then mature in the circulation to become non-classical monocytes under steady state conditions [14]. Moreover, non-classical monocytes have been proposed to act as custodians of vasculature by maintaining endothelial cells integrity [14,15]. Additionally, several studies have shown that circulating $CD16^+$ monocytes are found in large numbers in patients with inflammatory processes and infectious diseases [16–18].

Macrophages assume critical roles in almost every tissue and disease state through their ability to adopt distinct functional capacities in different microenvironments [19]. A major goal of the macrophage field is to link specific functions with specific cellular and molecular pathways associated with different macrophage activation profiles. Although macrophage polarization is not a fixed state due to the plasticity and ability of these cells to integrate multiple signals, two major groups are the classical pro-inflammatory M1 and the regulatory and tissue repair M2 macrophages that capture a snapshot of the inflammatory milieu at a specific point in time and space [9,20]. Pro-inflammatory M1s are characterized by strong induction of pro-inflammatory cytokines, such as TNFα, IL-6, IL-1β, and IL-12, high expression of CD64 and low levels of CD206 and CD163, all of which are necessary for pathogen clearance. High levels of CD206, CD163, and MERTK expression characterize the anti-inflammatory M2 repertoire of macrophages that are involved in tissue repair and regulatory function [9,20,21]. Additional details about the polarization phenotype markers used in this study can be found in **Table S1**.

The high prevalence and burden associated with MDD is partially explained by the lack of efficacy of available treatments. Studies published during the last decade showed that the magnitude of the effect of antidepressants has been overestimated for many years. One third of patients with MDD did not achieve remission despite multiple trials using different types of antidepressants [22]. Moreover, as a result of selective reporting, the effect size achieved with antidepressants is almost one third less than that reported in published data [23], which has led to the search of new therapeutic targets for the development of antidepressant drugs. This search identified the non-competitive glutamatergic N-methyl-D-aspartate receptor (NMDAR) antagonist, (R,S)-ketamine (ketamine), which has demonstrated rapid and robust efficacy as an antidepressant by improving core depressive symptoms including depressed mood, anhedonia, and suicidal thoughts in treatment-refractory unipolar and bipolar depressed patients when administered at sub-anaesthetic doses. Remarkably, these actions are observed within hours following a single administration and persisted for approximately 7 days with a single infusion [3,24–26].

The mechanism by which ketamine elicits a rapid decrease in depressive symptoms does not appear to be explained entirely by its antagonizing effect on neuronal NMDAR alone since its half-life is approximately three hours while the antidepressant effect lasts for

7 days, suggesting that it is not continual blockade of NMDAR that mediates the antidepressant response but rather synaptic plasticity mechanisms caused by ketamine that are involved in the longer term behavioural effects. While it has been suggested that ketamine has anti-inflammatory properties, results in previous studies conducted in humans and animals have been inconsistent [27–29]. However, a recent clinical study has shown a positive correlation between changes in cytokine levels after ketamine infusion and improvement in depressive symptoms with treatment-resistant depression [30].

We hypothesized that ketamine induces an anti-inflammatory program in macrophages that contribute to the rapid and sustained anti-depressant effect. In order to test this hypothesis, we evaluated the major cellular and cytokine inflammatory profile in patients with MDD and the effect of ketamine on macrophage programming during macrophage differentiation and polarization in vitro and in vivo.

2. Material and methods

2.1. Patients

The present exploratory analysis used data and blood samples obtained from patients enroled in a multicenter prospective cohort study conducted in Buenos Aires, Argentina. The cohort recruited patients who had been admitted to the emergency department of psychiatric hospitals for current suicidal ideation or a recent suicide attempt. Eligible study participants were aged 18–65 years, hospitalized for a suicide attempt within 48 h, and had a diagnosis of MDD. Exclusion criteria included an inability to respond autonomously (ie, due to sedative effects of medication or language limitations), met DSM-IV diagnostic criteria for substance abuse, or presence of a medical illness. Baseline characteristics along with clinical and psychopharmacology treatment of patients with MDD were collected and summarized in Table 1. Any additional clinical illnesses were reported at the screening interview, and no medications beyond the psychopharmacological treatments were allowed.

The control group ($N = 20$) consisted of gender and age-matched individuals (25% men & 75% women, with a median age of 34.5 years old and a SD of 5.3) who had not taken any medication.

Table 1
Demographics and clinical characteristics of patients with MDD and suicide behaviour ($n = 33$).

Characteristic	n (%)*
Age (yr), mean (SD)	36.36 (12.75)
Sex	
Men	8 (24.20)
Women	25 (75.80)
Marital status	
Partnership/cohabiting	27 (81.80)
Separated/divorced	6 (18.20)
Height (m), mean (SD)	1.64 (0.99)
Weight (kg), mean (SD)	66.83 (14.97)
BMI, mean (SD)	24.58 (3.27)
Education (yr), mean (SD)	11.33 (3.18)
Suicide behaviour	
SI	8 (24.2)
SA	25 (75.8)
Lethality index SA	
Very low	9 (36)
Low	9 (36)
High	7 (28)
Age at first SA (yr), mean (SD)	23.88 (13.75)
Previous SA	
No	11 (66.6)
Yes	22 (33.3)
Psychiatric treatment history	
No	14 (42)
Yes	19 (58)
Receiving medications	
No	14 (42)
Yes	19 (58)
Type of medications	
Antidepressants	13 (68)
Mood stabilizers	6 (32)
Antipsychotics	12 (63)
Benzodiazepines	18 (94)
Other	1 (5)

SD=standard deviation; m=metre; kg=kilogram; BMI=Body Mass Index; yr=year; SI=suicide ideation; SA=suicide attempt; lethality index SA=lethality of the index (ie, suicide attempt that resulted in hospitalization at study enrolment) suicide attempt.
* unless otherwise specified.

2.2. Ethics statement

All participants gave written informed consent to participate in the study. The study protocol was approved by the institutional review board at each participating hospital. The present analysis included 33 patients recruited between May 2015 and March 2017 who met DSM-IV diagnostic criteria for MDD. Patients were evaluated by specialized psychiatrists through semi-structured clinical psychiatric interviews, which included specific questions about clinical and demographic variables. The Mini International Neuropsychiatric Interview (MINI) [31] was used for diagnostic purposes. At the end of the assessment, blood samples were collected into EDTA-coated tubes (BD, Vacutainer) and transported to the laboratory.

2.3. Peripheral blood mononuclear cells (PBMCs) isolation and characterization of monocyte subsets by flow cytometry

Plasma and PBMCs from patients with MDD and healthy volunteer controls (HC) were obtained by centrifugation as previously described [32]. The plasma fraction was preserved at −80 °C for cytokine measurement. PBMCs were isolated by density gradient centrifugation employing Ficoll® Paque Plus (GE Healthcare Cat# 17144003), cryopreserved in 90% foetal bovine serum (FBS, Gibco Cat # 10091148) plus 10% dimethyl sulfoxide (Sigma Aldrich, St. Louis, MO, USA), and stored in liquid nitrogen until use. Monocyte subsets were analysed from cryopreserved PBMCs. Briefly, cells were thawed, washed, and blocked in 1x PBS + FBS 2% on ice for 30 min. Cells were then washed with 1x PBS + FBS 2% and incubated for 30 min on ice with appropriate antibody combinations against human CD11b-allophycocyanin/Cy7 (APC)/Cy7) (BioLegend Cat # 101225, RRID: AB_830,641), CD14-PE (BioLegend Cat # 325605, RRID: AB_830678) CD16-fluorescein isothiocyanate (FITC) (BioLegend Cat # 302005, RRID: AB_314205), CD40-PECy7 (BioLegend Cat # 334321, RRID: AB_10643414), and CD86 biotin (BioLegend Cat # 305404, RRID: AB_314524) plus DyLight™ 649-conjugated Streptavidin (BioLegend Cat # 405224). Live cells were distinguished using Fixable Viability Dye eFluor™ 450 (eBioscience Cat # 65-0863-14). After staining, cells were fixed with Cytofix/Cytoperm kit (BD Bioscience Cat# 554714) and acquired using a FACS Canto I (Becton Dickinson). All analysis was carried out with FlowJo software (Tree Star).

2.4. In vitro lipopolysaccharide (LPS) stimulation of PBMCs

2.5×10^5 PBMCs from patients with MDD or HC were seeded in 96-well plates with RPMI 1640 (Gibco Cat # 11875119) supplemented with 10% FBS and 1% penicillin-streptomycin (PS) (Sigma Aldrich). Cells were stimulated with *E. coli*-derived LPS (1–10 ng/mL; O111:B4, Sigma Aldrich Cat # L3024) and cultured in a humidified incubator at 37 °C with CO_2 (5%) for 24 h. Supernatants were collected at indicated time points and stored at −80 °C for cytokine measurements.

2.5. In vitro differentiation and stimulation of human macrophages

$CD14^+$ monocytes were isolated from PBMCs using the EasySep™ Human CD14 positive selection kit (StemCell Tech Cat # 18058)

following manufacturer's instructions. Macrophage differentiation was performed by plating 2.5×10^5 $CD14^+$ monocytes in 48-well plates containing 500 μL of RPMI 1640 supplemented with 10% FBS and
1% PS. On day 5 of culture, the following reagents were added for an additional 2 days of culture for macrophage polarization: LPS (1 ng/mL) plus IFNγ (50 ng/mL) for M1, IL-4 (40 ng/mL) for M2a, and dexamethasone (0.1 μM) for M2c. Recombinant cytokines were purchased from R&D Systems (Cat # 285-IF for IFNγ and Cat # 204-IL for IL-4) and dexamethasone from Sigma Aldrich (Cat # D4902).

Ketamine-derived macrophages were obtained by adding (R,S)-ketamine hydrochloride (Cost, Fada Pharma, Argentina) from day 0, at indicated concentrations, and analysed after 7 days by flow cytometry. Response to LPS was evaluated by challenging macrophages with 1 ng/mL of LPS for additional 24 h. NMDA selective receptor antagonist, (+)-MK-801 hydrogen maleate (Sigma Aldrich Cat # M107), AMPA selective receptor antagonist NBQX hydrate (Sigma Aldrich Cat # N171) or mTOR inhibitor rapamycin (Apexbio Technology Cat # A8167) were also added to the culture from day 0 at the indicated concentrations.

On day 7 of macrophage culture, supernatants were collected and stored at −80 °C for cytokine measurement, and macrophages were harvested by 15 min incubation with 1x PBS + FBS 2% + 1 mM ethylenediaminetetraacetic acid (EDTA) from Sigma Aldrich (St. Louis, MO, USA) on ice followed by gentle pipetting detachment from the bottom of the well. Macrophages were then washed twice with 1x PBS + FBS 2% to remove the EDTA.

The phenotype and activation of macrophages were characterized by cell surface staining employing the appropriate combination of directly conjugated antibodies against human CD11b-APC/Cy7 (BioLegend Cat # 101225, RRID: AB_830641), CD64-PE/Cy7 (BioLegend Cat # 305021, RRID: AB_2561583), CD163-PerCP/Cy5.5 (BioLegend Cat # 333625, RRID: AB_2,650629), CD206-AlexaFluor 488 (BioLegend Cat # 321113, RRID: AB_571874), CD14-PE (BioLegend Cat # 325605, RRID: AB_830678), HLA-DR-FITC (BioLegend Cat # 980402, RRID: AB_2616625), CD80-PE (BioLegend Cat # 305207, RRID: AB_314,503), and MERTK-APC (R&D Systems Cat # FAB8912A RRID:AB_357213) along with its control isotype IgG1-APC (R&D Systems, Cat # IC002A). Viability was assessed with Fixable Viability Dye eFluor 450 (e-Bioscience, Cat # 65-0863-14). After washing, cells were fixed with Cytofix/Cytoperm kit (BD Bioscience, Cat # 554714). Cells were acquired using a FACS Canto I cytometre (Becton Dickinson), and all analysis was carried out with FlowJo software (Tree Star). The fluorescence minus one (FMO) controls were used to set negative signal in each interested channel.

2.6. Enzyme linked immunosorbent assay (ELISA)

Plasma levels of IL-6 (e-Bioscience Cat # BMS213HS) and IL-12 (e-Bioscience Cat # BMS238HS) were detected with high sensitivity precoated ELISA kits while TNFα (Cat # 88-7346, RRID: AB_2575093), IL-6 (Cat # 88−7066, RRID: AB_2574993), and IL-10 (Cat# 88-7106; RRID: AB_2575001) levels in culture supernatants were assessed with ELISA Ready-SET-Go kits (e-Bioscience) according to manufacturer's protocol.

2.7. Intracellular cytokine measurement

2.5×10^5 PBMCs were seeded in 96-well plates with RPMI 1640 (Gibco Cat # 11875119) supplemented with 10% FBS and 1% PS (Sigma Aldrich) and incubated with monensin (Golgistop; BD Biosciences Cat # 554715) for 5 h. After that, PBMCs were stained with anti-CD11b APC/Cy7 (BioLegend Cat # 10,225, RRID: AB_830641) anti-CD14-PerCP (BioLegend Cat # 325631, RRID: AB_2563327), anti-CD16-FITC (BioLegend Cat # 302005, RRID: AB_314205). Live cells were distinguished using Fixable Viability Dye eFluor™ 450 (eBioscience Cat # 65-0863-14). After staining of surface markers, cells were fixed and made permeable according to the manufacturer's instructions BD Cytofix/Cytoperm Kit (BD Bioscience Cat # 554715). Then, the cells were stained with anti-human IL-12-biotin (ThermoFisher Cat # AHC7129, RRID: AB_2536290) plus PE-conjugated Streptavidin (BioLegend Cat #405203), and acquired using a FACS Canto I (Becton Dickinson). All analysis was carried out with FlowJo software (Tree Star).

2.8. Quantitative PCR (qPCR)

For gene expression analysis, macrophages were differentiated for 5 days and different concentrations of ketamine (0.1, 1 and 10 μM) or validated polarizing stimuli were added for an additional 48 h. On day 7 of culture, cells were washed and then harvested with Trizol (Life Technologies Cat # 15596018) following manufacturer's instructions. Reverse transcription was performed using 100 ng of RNA in 20 μl of reaction volume by employing iScript cDNA synthesis kit (Bio-Rad Cat # 4106228). qPCR reactions were assessed using 1 μl of cDNA in 10 μl of reaction volume by employing Power SYBR Green PCR Master Mix kit (Applied Biosystems Cat # A25741) and Applied Biosystems 7500 equipment. Primers used in this study are listed in **Supplementary Table S2**. The reaction was normalized to housekeeping gene expression levels, the elongation factor eEF-1 alpha (EEF1A1), and the specificity of the amplified products was checked through analysis of dissociation curves.

2.9. Mice

Eight to nine-week old male C57BL/6 (B6) mice were provided by the animal facility of the CIBICI (Centro de Investigaciones en Bioquímica Clínica e Inmunología)-CONICET, Facultad de Ciencias Químicas de la Universidad Nacional de Córdoba, and maintained in a specific pathogen-free environment. Mouse experiments were performed according to National Institutes of Health guidelines with approval of the Institutional Animal Care and Use Committee.

2.10. In vivo ketamine administration and immune compartment analysis

Ketamine (10 mg/kg/mouse) or saline solution for control mice was administered via intra-peritoneal injection [33]. After 72 h, animals were euthanized and peripheral blood, bone marrow, spleens, and brains were collected.

Mouse peripheral blood was obtained by cardiac puncture while spleen cells and CNS leucocytes were isolated as previously described [34]. Red blood cells were lysed with ACK lysing buffer (Gibco Cat # A1049201); cells were then stained with appropriate antibody cocktails against Alexa Fluor 647-CD11b (BioLegend Cat # 101220, RRID: AB_493546), Ly-6G-APC/Cy7 (Biolegend Cat # 127623, RRID: AB_10645331) and Ly-6C-PE/Cy7 (BioLegend Cat # 128017, RRID: AB_1732093) for 30 min at room temperature and analysed by flow cytometry. Cells were acquired in a FACS Canto II (Becton Dickinson). All analysis was carried out with FlowJo software (Tree Star).

2.11. Murine blood leucocyte composition

Blood smears were prepared with one drop of blood obtained as aforementioned, stained with May-Grünwald stain for 3 min, and washed with water. The smear was then incubated with previously prepared Giemsa (1 vol Giemsa + 9 vol water) for 15 min. Cells were counted under optic microscope (Boeco, Germany).

2.12. Isolation of murine CNS immune cells

Briefly, mice were transcardially perfused with ice-cold 1x PBS, and brains were collected in DMEM supplemented with sodium pyruvate, penicillin, streptomycin, and glutamine cocktail (Gibco),

gently mechanically disaggregated and re-suspended in 1x PBS containing 3 mg/mL collagenase D plus 10 μg/mL DNAse (Sigma-Aldrich). Then, CNS homogenates were filtered through 40 μm pore size cell strainers (BD Falcon), centrifuged at 1800 rpm and resuspended in 38% Percoll® (GE Healthcare Cat # 17089101) before a 25 min centrifugation at 800 g without acceleration or brake. Myelin and debris were discarded. Pelleted cells were washed and then labelled with the following antibodies: CD45-APC/Cy7 (BioLegend Cat # 368515, RRID: AB_2566375), CD11b-PE/Cy5 (BioLegend Cat # 101209, RRID: AB_312792), and F4/80-PE (BioLegend Cat # 123109, RRID AB_893498). Fixable Viability Dye eFluor 450 (eBioscience Cat # 65-0863-14) was used according to manufacturer's instructions to distinguish live cells. After washing, cells were fixed with Cytofix/Cytoperm kit (BD Bioscience Cat # 554714) and then stained with Arginase-1-APC antibody (R&D Systems Cat # IC5868A).

2.13. Murine spleen cells flow cytometry

Spleen red blood cells were lysed with ACK lysing buffer (Gibco), and cell viability was determined by Trypan blue exclusion staining using a haemocytometre. Cells were then stained with CD11b-PE/Cy5 (BioLegend Cat # 101209, RRID: AB_312792) and F4/80-PE (BioLegend Cat # 123109, RRID: AB_893498). Fixable Viability Dye eFluor 450 (eBioscience Cat# 65-0863-14) was used according to manufacturer's instructions to distinguish live cells. After washing, cells were fixed with Cytofix/Cytoperm kit (BD Bioscience Cat # 554714) and then stained with Arginase-1-APC antibody (R&D Systems Cat # IC5868A). Cells were acquired using a FACS Canto II cytometre (Becton Dickinson), and all analysis was performed in FlowJo software (Tree Star).

2.14. Bone marrow derived macrophages (BMDM) differentiation and stimulation

BMDMs were obtained from control and ketamine-treated mice as previously described [35]. Briefly, cells were cultured in RPMI 1640 supplemented with 13% supernatant of the mouse L929 cell line (conditioned medium) for 7 days. Then, cells were stimulated with LPS (100 ng/mL) or maintained in media for 24 h. Cells were harvested and stained with CD86-APC/Cy7 (BioLegend Cat # 105,029, RRID: AB_2074993), CD206-PE/Cy7 (BioLegend Cat # 141719, RRID: AB_2562247), and CD36-PE (Biolegend Cat # 102605, RRID: AB_389348) antibodies before analysis by flow cytometry with the FACS Canto II cytometre (Becton Dickinson).

2.15. Statistical analysis

Data are expressed as mean ± SEM. Two-tailed unpaired Student's *t*-test or one-way ANOVA test were used accordingly to determine significance. When non-parametric Mann Whitney test was used, it is denoted in figure legends. Correlation was assessed by Spearman test. Statistical significance was set at $p < 0.05$. Analysis was performed using GraphPad Prism software.

3. Results

3.1. Elevated plasma levels of IL-12 strongly correlate with a higher percentage of pro-inflammatory non-classical monocytes and more activated classical monocytes in the blood of patients with MDD and suicide behaviour

Inflammation has been associated with MDD in recent years highlighting the critical role of the immune response in the pathophysiology of this disease [3]. In a cohort of 33 patients with MDD with no comorbidities, we found that 36% of patients (13/33) showed higher levels of IL-12 (> 0.1344 pg/mL) and 54% of patients (18/33) had higher levels of IL-6 (> 0.9345 pg/mL) when compared with a distribution of age- and gender-matched healthy controls (HC) (Fig. 1a and b). Furthermore, 27% of patients with MDD (9/33) demonstrated increased levels of both cytokines. The threshold for high cytokine level was set as the mean value plus 1 SD of control group.

Taking into account that both cytokines are major pro-inflammatory mediators secreted by activated monocytes, the three populations of circulating classical ($CD11b^{++}CD16^{neg}CD14^{bright}$), non-classical ($CD11b^{++}CD16^{bright}CD14^{neg}$), and the intermediate ($CD11b^{++}CD16^{+}CD14^{+}$) monocytes from HC and patients with MDD were analysed from PBMC samples. Gating strategy is depicted in Supplementary Fig. S1 and representative dot plots from HC and patients with MDD as well as colour gates to discriminate the three monocytes subsets, denoted as blue (classical), red (intermediate) and green (non-classical), are shown in Fig. 1c and d.

We observed a significant reduction in the frequency of classical monocytes with a concomitant increase of the non-classical and intermediate subsets in patients with MDD compared with HC (Fig. 1e–g). Interestingly, when patients with MDD were segregated based on cytokine levels, a higher level of IL-12 (alone or with IL-6) revealed a higher frequency of non-classical monocytes (Fig. 1e–g), and this association shows a strong positive correlation (Fig. 1h). Even though a significant positive correlation of IL-6 and increased non-classical monocytes was also observed (Fig. 1i), patients with only high IL-6 do not show a significant increase of this monocyte fraction, which may be explained by the co-presence of IL-12 in some samples of high IL-6. Patients with high levels of only IL-6 or low levels of both cytokines denoted a clear tendency of increased percentage of the intermediate monocyte fraction, indicating that another cytokine could be involved.

Notably, classical monocytes from patients with MDD demonstrate a higher activation state (measured as $CD86^{+}CD40^{+}$ cells) and associated high levels of IL-6 or IL-12 than those from HC (Fig. 1j and k). The increased activation state of classical monocytes positively correlated with an increased frequency of non-classical monocytes (Fig. 1l). In the same sense, a significantly increased level of intracellular IL-12 was observed in the $CD11b^{+}CD16^{neg}CD14^{bright}$ subset of MDD patients at basal condition (Fig. 1m,n) underscoring that more activated classical monocytes could be one of the sources of the increased IL-12 favouring the transition to non-classical monocytes. Additionally, higher production of IL-6 and IL-12 were found in the culture supernatant of PBMCs after 24 h of LPS treatment (Supplementary Fig. S2).

3.2. Ketamine induces high levels of CD163 and MERTK expression and intermediate levels of CD64 associated with M2c-like human macrophages

Human circulating monocytes are the main source of macrophages that infiltrate tissues under inflammatory conditions or stress. Here, we tested if sub-anaesthetic doses of ketamine affect $CD14^{+}$ monocyte differentiation and polarization into human macrophages in comparison with classical M1 (IFNγ plus LPS), M2a (IL-4) and M2c (dexamethasone) in-vitro polarization. Interestingly, we found that monocytes treated with ketamine from day 0 of culture acquired a M2c-like phenotype with increased levels of MERTK and CD163 expression together with intermediate levels of CD64, when compared with classical M1 and M2c profiles (Fig. 2a, b and d). We did not observe changes in CD206, a classical M2a marker (Fig. 2c). These results clearly demonstrate that ketamine promotes an M2c-like macrophage.

Furthermore, we also discovered that ketamine-induced macrophages exhibit lower percentages of $CD80^{+}$ and HLA-DR^{+} activation

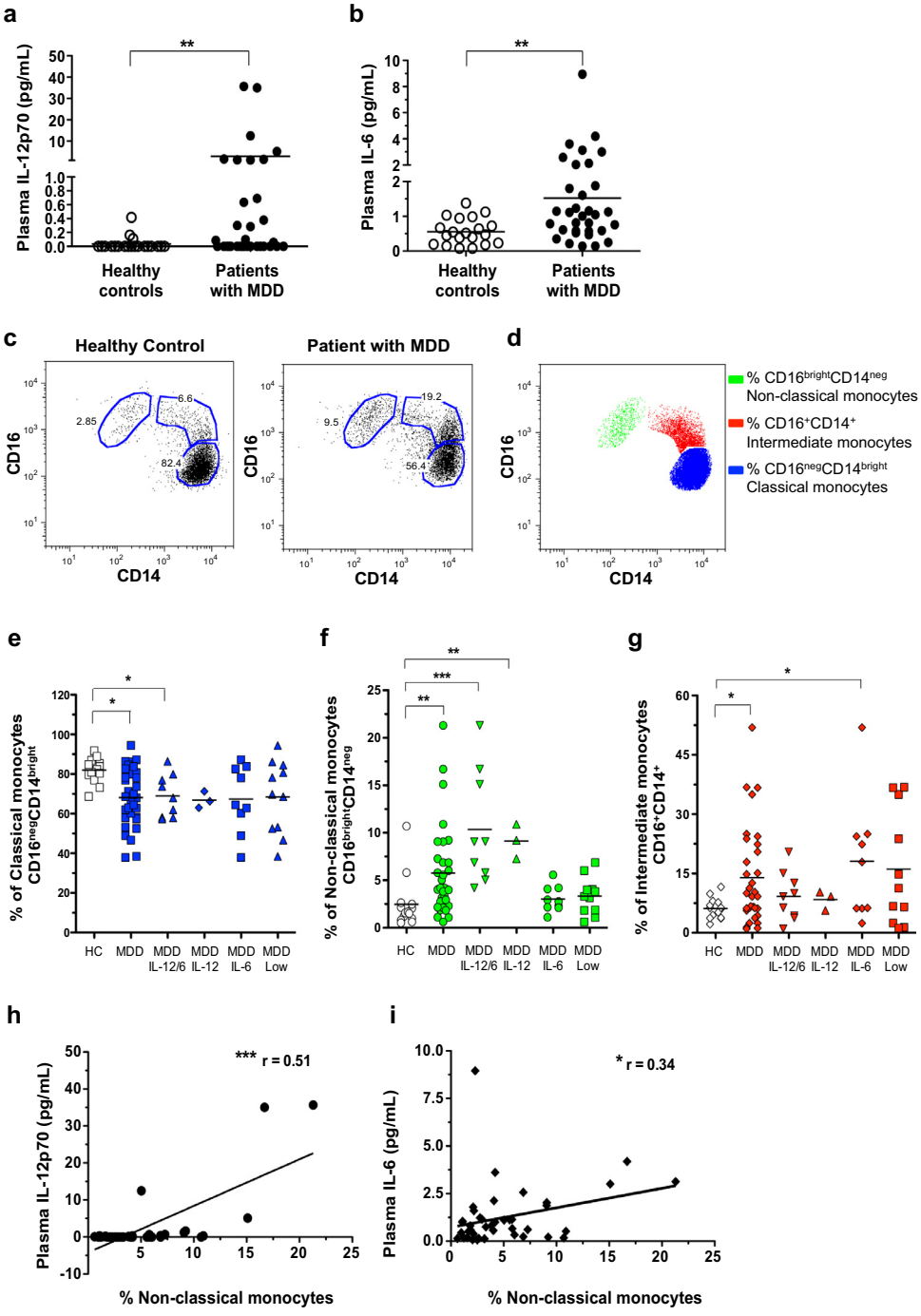
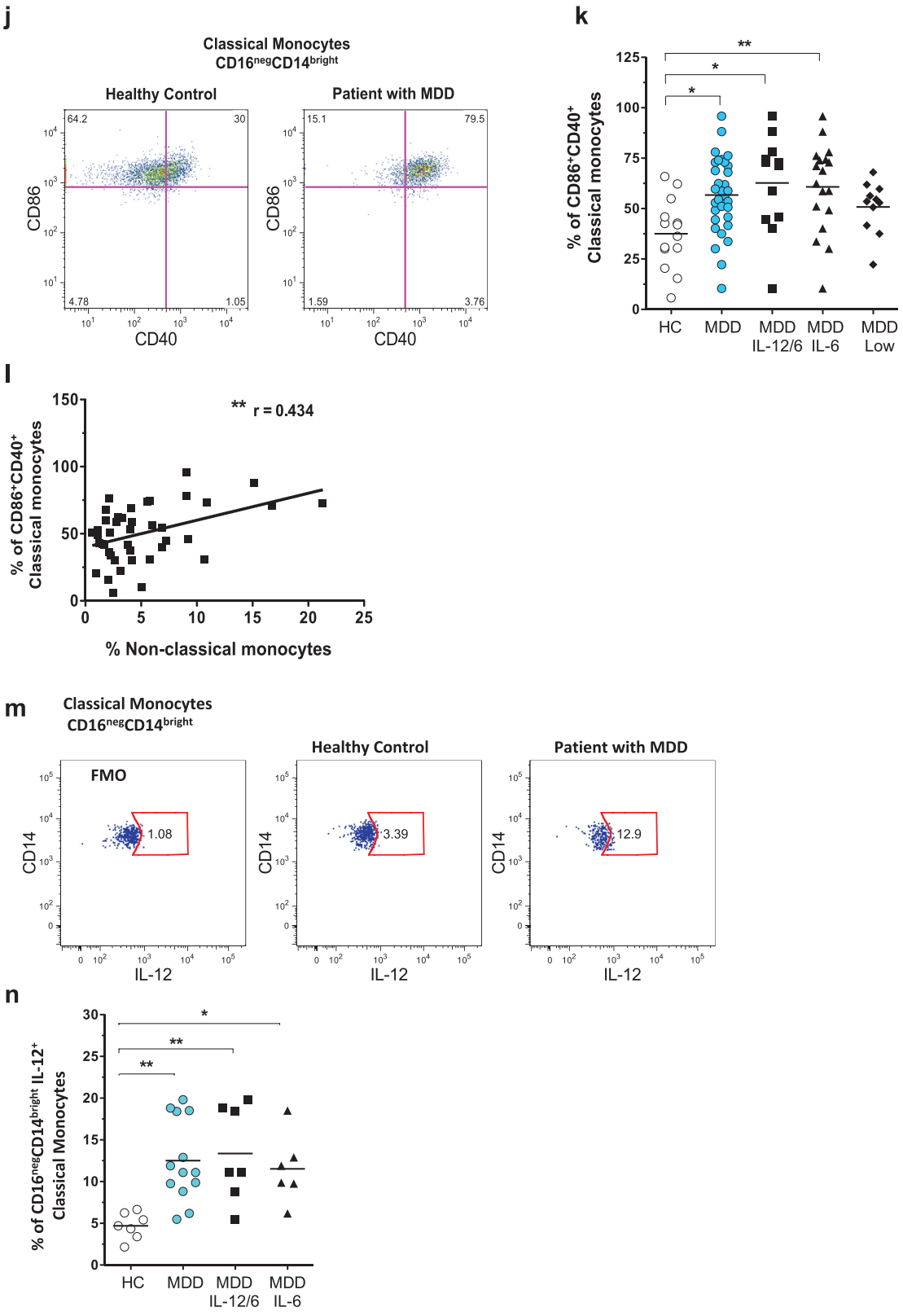


Fig. 1. Increased plasma levels of IL-12 and IL-6 correlate with increased non-classical CD16^{bright}CD14^{neg} monocytes as well as with increased activation of classical CD16^{neg}CD14^{bright} monocytes in patients with MDD and suicide behaviour. Plasma levels of (a) IL-12p70 and (b) IL-6 in patients with MDD (n=33) and healthy controls (n=20) as determined by ELISA. Significant differences between patients with MDD and healthy controls were calculated using a *2-tailed Mann-Whitney test* (**$p < 0.01$). (c) Representative dot plots, after gating in mononuclear CD11b^{+} cells, showing the frequency of the three monocyte subsets based on CD14 vs. CD16 expression level in healthy controls and patients with MDD. Detailed gaiting strategy is shown in Supplementary Fig. S1. (d) The three monocytes subset are depicted as classical CD16^{neg}CD14^{bright} (blue), non-classical CD16^{bright}CD14^{neg} (green) and intermediate CD16^{+}CD14^{+} (red) for further analysis. Independent data are graphed in (e-g) showing decreased percentages of classical CD16^{neg}CD14^{bright} monocytes alongside an increased percentage of non-classical CD16brightCD14neg monocytes in peripheral blood of patients with MDD. The frequency of the three subpopulations of monocytes from the cohort of patients with MDD was also segregated based on IL-6 and IL-12 plasma levels. The threshold for high cytokine level was set as the mean value plus 1 SD of control group. One-way ANOVA test was performed (**$p < 0.01$; ns $p > 0.05$). (h) and (i) Increased plasma levels of IL-12 and IL-6 were positively correlated with the percentage of non-classical monocytes in the peripheral blood of patients with MDD and healthy controls. Correlation was assessed by *Spearman test*. (j) Representative dot plots of CD86 and CD40 expression in classical monocyte subpopulations are shown. (k) Independent data showing percentage of CD86^{+}CD40^{+} classical monocytes in healthy controls and patients with MDD and, further segregated by IL-12 and IL-6 plasma levels. (l) Increased percentage of non-classical monocytes was positively correlated with an increased percentage of CD86^{+}CD40^{+} classical monocytes. *One-way ANOVA test* was performed (***$p < 0.001$). Correlation was assessed by *Spearman test*. (m) Representative dot plots showing the frequency of IL-12 positive cells after 5h of incubation with Golgi Stop and gating in CD11b^{+}CD16^{neg}CD14^{bright} subset. The Fluorescence Minus One (FMO) was used to set positive staining. (n) Independent data showing the percentage of Il-12 positive cells in a healthy control group (n=8) and patients with MDD (n=13) and, further segregated by IL-12 and IL-6 plasma levels. *One-way ANOVA test* was performed (**$p < 0.01$; *$p < 0.05$). Patients were segregated as follows: MDD IL-12/6, high levels of both cytokines; MDD IL-12, high levels of IL-12 only; MDD IL-6, patients high levels of IL-6 only; and MDD low, patients with similar levels of cytokines compared with healthy controls.



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markers (Fig. 2e and f) as well as reduced TNFα production (Fig. 2g) when they were challenged with 1 ng/mL of LPS. Intriguingly, ketamine-induced macrophages already showed lower levels of IL-6 and IL-10 at baseline compared with untreated monocyte-derived macrophages (Fig. 2h and i), supporting the phenotypic bias of ketamine-derived macrophages. After LPS stimulation, ketamine did not modify IL-6 levels yet significantly reduced IL-10 level under the same conditions (Fig. 2h and i).

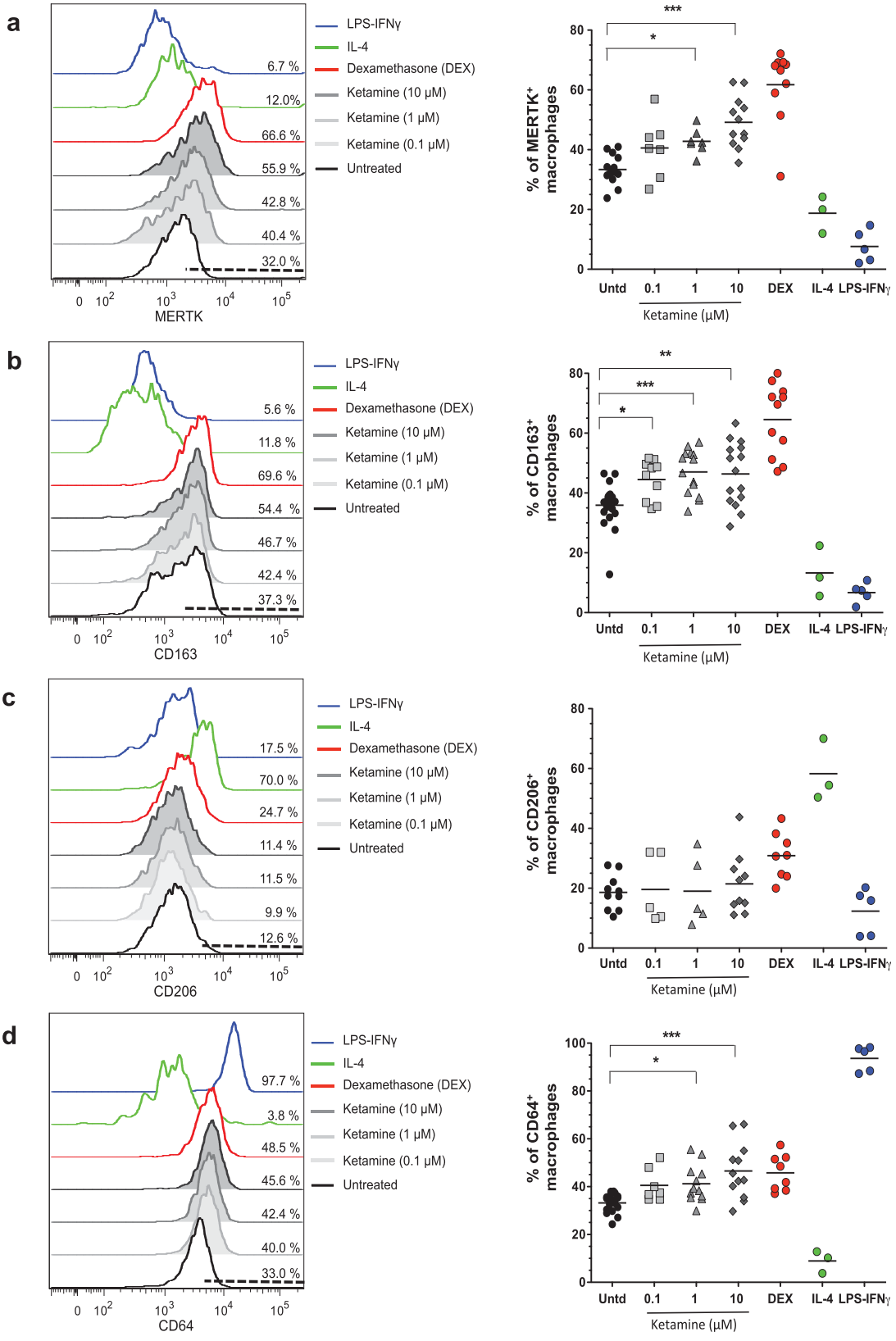

Fig. 2. Ketamine induces a M2c-like phenotype in monocyte-derived macrophages with increased levels of MERTK, CD163, and intermediate levels of CD64 while reducing the response to LPS. Monocyte-derived macrophages were differentiated for 7 days in the presence or absence of ketamine (0.1, 1 and 10 μM), and the percentage of (a) MERTK, (b) CD163, (c) CD206 and (d) CD64 positive CD11b$^+$ macrophages was analysed by flow cytometry. Macrophage polarization controls were performed using dexamethasone (0.1 μM) for M2c, IL-4 (40 ng/mL) for M2a, and LPS (1 ng/mL) plus IFN-γ (50 ng/mL) for M1. Representative and independent data are shown. (e-i) To analyse the response to an inflammatory stimulus, ketamine-induced macrophages were stimulated for 24h with 1 ng/mL of LPS. The activation markers (e) CD80 and (f) HLADR were evaluated by flow cytometry and (g) TNF-α, (h) IL-6 and (i) IL-10 production was assessed by ELISA. Each dot represents an independent donor and pooled data were graphed. *One-way ANOVA test* was performed and statistical significance is denoted as $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Untreated condition: Untd; dexamethasone: DEX.

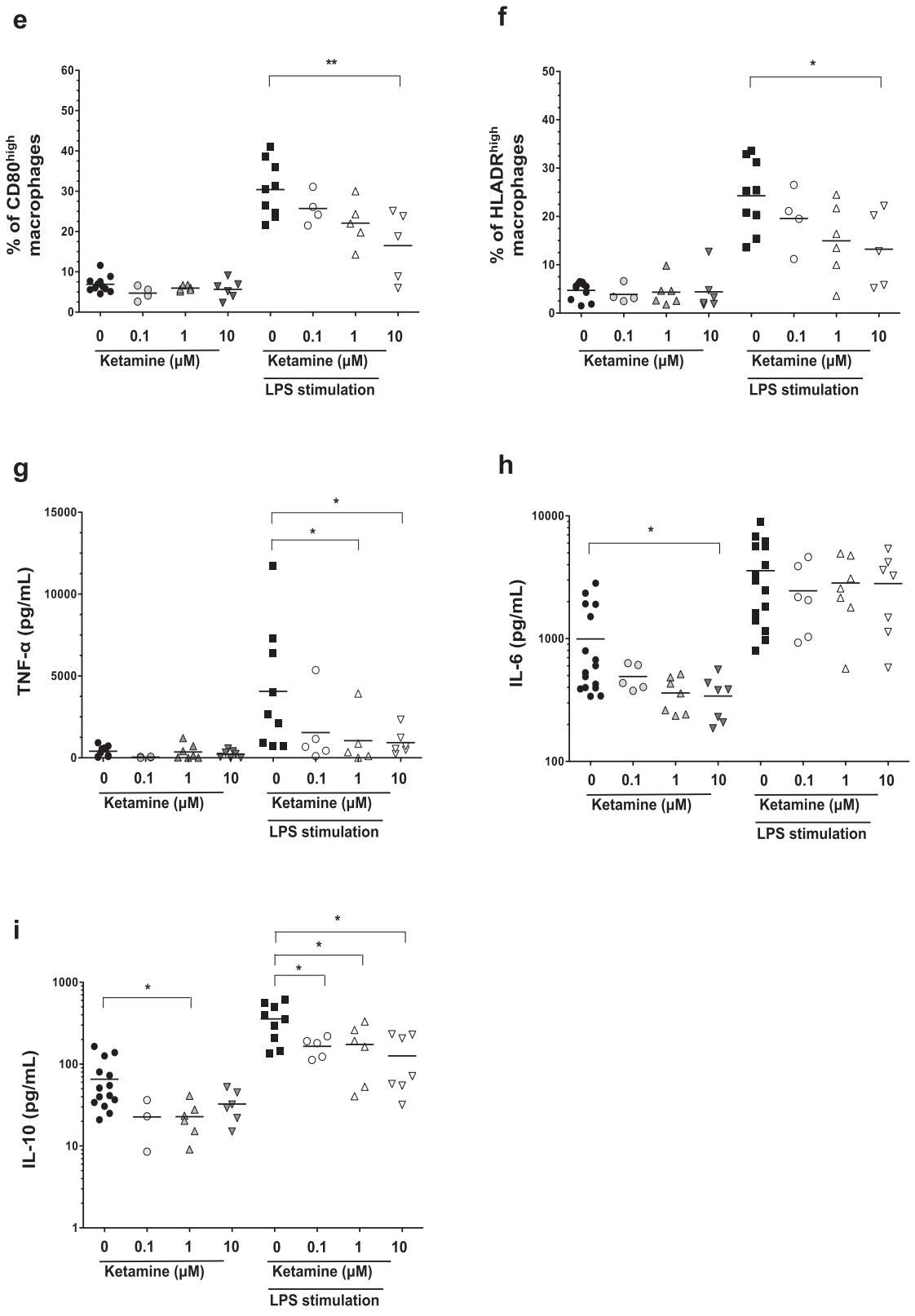

Fig. 2 Continued.

Additionally, ketamine was able to dampen the macrophage activation markers (CD80 and HLA-DR) when these cells were pre-treated for 30 min before LPS stimulation (**Supplementary Fig. S3),** suggesting a rapid anti-inflammatory action of ketamine. Of note, inherent donor variability was observed in some experiments either during basal conditions or under treatment.

3.3. Ketamine up-regulates M2 gene expression profile including mTOR pathway associated genes

Considering that ketamine induced an M2c-like phenotype in macrophages, we compared the gene expression program primed by ketamine versus the classical M1 (IFNγ plus LPS), M2a (IL-4) and M2c

(dexamethasone) stimuli on human monocyte-derived macrophages. We observed that ketamine induced a particular gene profile, with higher levels of CCL22 and TGM2, two classical M2 genes (Fig. 3a and b). We did not observe induction of IRF4 or the classical M1 genes IRF1 and CXCL10 (Fig. 3c–e). On the other hand, we found that classical mTOR pathway associated genes (SGK1, EIF4B, and FOXO1) were specifically up regulated after ketamine treatment (Fig. 3f–h). Of note, the SGK1 gene product is specifically involved downstream of mTOR2 signalling and its expression is strictly transcriptionally and post-transcriptionally regulated.

3.4. Ketamine induces an M2 macrophage program through NMDAR and mTOR pathways

In order to better understand if NMDAR and/or AMPAR are involved in the M2c-like phenotype skewing by ketamine, we challenged monocytes with the NMDAR antagonist, MK801 (1–10 μM) and the AMPAR antagonist NBQX (1–10 μM) from day 0 of culture. We found that MK-801 induced a similar macrophage polarization program as ketamine treatment by augmenting MERTK but without modification in CD206 expression after 7 days of culture (Fig. 4a and b). On the other hand, NBQX (1–10 μM) did not induce MERTK but significantly increased CD206 expression (Fig. 4a and b). Considering that NMDAR triggers the mTOR pathway in CNS [33] and macrophage polarization depends on mTOR complex activation [36], we blocked this complex during ketamine-induced macrophage differentiation and polarization. Monocyte-derived macrophages differentiation in the presence of rapamycin (0.1–1 nM) significantly reduced the percentage of MERTK$^+$, CD64$^+$, and CD163$^+$ cells but increased the percentage of CD206 positive cells (Fig. 4c). Therefore, the opposing effect of mTOR inhibition on MERTK and CD206 expression clearly suggests a dichotomy on the pathway for M2a and M2c polarization.

3.5. Injection of sub-anaesthetic doses of ketamine modulates the circulating myeloid compartment and promotes differentiation of anti-inflammatory M2 macrophages in vivo

It has been demonstrated that injection of sub-anaesthetic doses of ketamine (10 mg/kg) improves the performance of mice stressed by swimming test by activating mTOR signalling in the CNS [33]. Nonetheless, how ketamine affects or modulates the immune compartment in vivo has not been clarified. Here, we demonstrated that intraperitoneal injection of sub-anaesthetic doses of ketamine in control mice significantly reduced the percentage of circulating pro-inflammatory monocytes (CD11b$^+$Ly6C^{high}Ly6G$^-$) with a concomitant increase in anti-inflammatory CD11b$^+$Ly6C^{low} Ly6G$^-$ monocytes after 72 h (Fig. 5a and b). Ketamine-treated mice did not show a significant difference in total blood leucocyte numbers or percentage of cell types, except for a significant increase of the eosinophil fraction (Supplementary Fig. S4). However, the bone marrow and spleen of ketamine-treated mice showed increased cell numbers (Fig. 5c and d). On the other hand, we observed a significant reduction of non-resident macrophages (CD45^{high}CD11b$^+$F4/80$^+$) in the CNS without changes in the frequency of microglia (CD45^{low}CD11b$^+$) (Fig. 5e and f). Strikingly, these CD45^{high}CD11b$^+$F4/80$^+$macrophages showed a significant increase of the M2 effector enzyme (Arginase-1$^+$) after 72 h of ketamine treatment (Fig. 5g). Similar results were observed in the spleens of ketamine-treated mice in comparison with saline-treated mice (Fig. 5h).

These results prompted us to evaluate if ketamine is capable of reprogramming macrophages differentiation and polarization in vitro. Remarkably, bone marrow progenitors from ketamine-treated mice demonstrated clear skewing to M2 macrophage polarization after 7 days of in vitro differentiation characterized by a lower percentage of CD86$^+$CD206$^-$ cells and lower levels of CD36$^+$ cells as well as increased expression of CD206$^+$CD86$^-$ fraction when stimulated with LPS (Fig. 5i–k).

4. Discussion

During the past two decades, an increasing body of evidence indicates that the immune-inflammatory pathways and activation of cell-mediated immunity are involved in the pathophysiology of MDD. Maes et al. were the first to report that psychological stress and stress-induced anxiety are related to a Th1-like response with increased TNF-α, IL-6, IL-1 receptor antagonist, and IFNγ production in humans [37]. Classical depressive symptoms emerged when healthy volunteers received endotoxin infusions to trigger the release of inflammatory cytokines [7]. Furthermore, activation of peripheral and/or central induced oxidative and nitrosative stress pathways may explain co-occurrence of depression with a variety of inflammatory disorders [38]. Even though increasing evidence shows that different types of psychosocial stressors may stimulate the pro-inflammatory network, the source of inflammation remains unclear [5]. The inflammation theory of depression involve a complex interaction of innate and adaptive immune cells, inflammatory cytokines, endocrine signalling molecules, and neuromodulatory processes in the periphery and the central nervous system that may have a profound effect on behavioural alterations and the development of depression [39,40]. An array of evidence using murine models has implicated monocyte activation and pro-inflammatory cytokine secretion in the increased traffic and infiltration to the brain, therefore contributing to the pathophysiology [41–43]. Nowadays, the opinions regarding the inflammatory players, causality, and treatment for patients with MDD remain controversial. Measurements with several immune markers are needed to identify depression-specific biomarkers that will also allow better immunotherapeutic treatment strategies.

Our results demonstrate that patients with MDD presented elevated plasma levels of inflammatory cytokines such as IL-6 and IL-12. The increased IL-6 levels in our cohort is consistent with a recently published meta-analysis compiling 42 studies in which patients with MDD had higher IL-6 concentrations compared with HC. In the same meta-analysis, only 4 studies evaluated the levels of IL-12 in patients with MDD, and though levels of IL-12 were increased, these results were not conclusive due to a high degree of heterogeneity [44]. However, in agreement with our results, Kim et al. (not included in meta-analysis) also found a significant increase of IL-12 levels in 34 patients with MDD compared with HC [45]. Interestingly, increased levels of IL-12 have also been detected in other inflammatory pathologies of the CNS such as multiple sclerosis (MS) [46], and it has recently been reported that patients with psoriasis undergoing treatment with anti-IL-23 (guselkumab) showed marked improvement in their levels of depression and anxiety as a secondary analysis of the VOYAGE trial [47], leading to an exploration of new treatments for MDD.

IL-12 is primarily produced by monocytes and macrophages and plays a central role in promoting the Th1 response, activating NK cells to produce IFNγ, and driving cell-mediated immunity [48]. Accordingly, we have focused our investigations not only on cytokines but also on important cellular sources of these cytokines, ie monocytes and macrophages. The three subsets of monocytes, classical (CD14^{bright}CD16$^-$), intermediate (CD14$^+$CD16$^+$), and non-classical (CD14^{neg}CD16^{bright}), circulate in dynamic equilibrium under steady state conditions, and only 1% of the classical subset become intermediate monocytes to mature later into non-classical monocytes [12]. It has been proposed that the type of pathogenic stimuli influences the differentiation of each subset, increasing classical monocytes in bacterial infections and non-classical fractions in viral infections or chronic inflammatory and autoimmune conditions such as MS [16,48,49]. Our results indicated that the frequency of non-classical monocytes was increased in patients with higher concentration of IL-

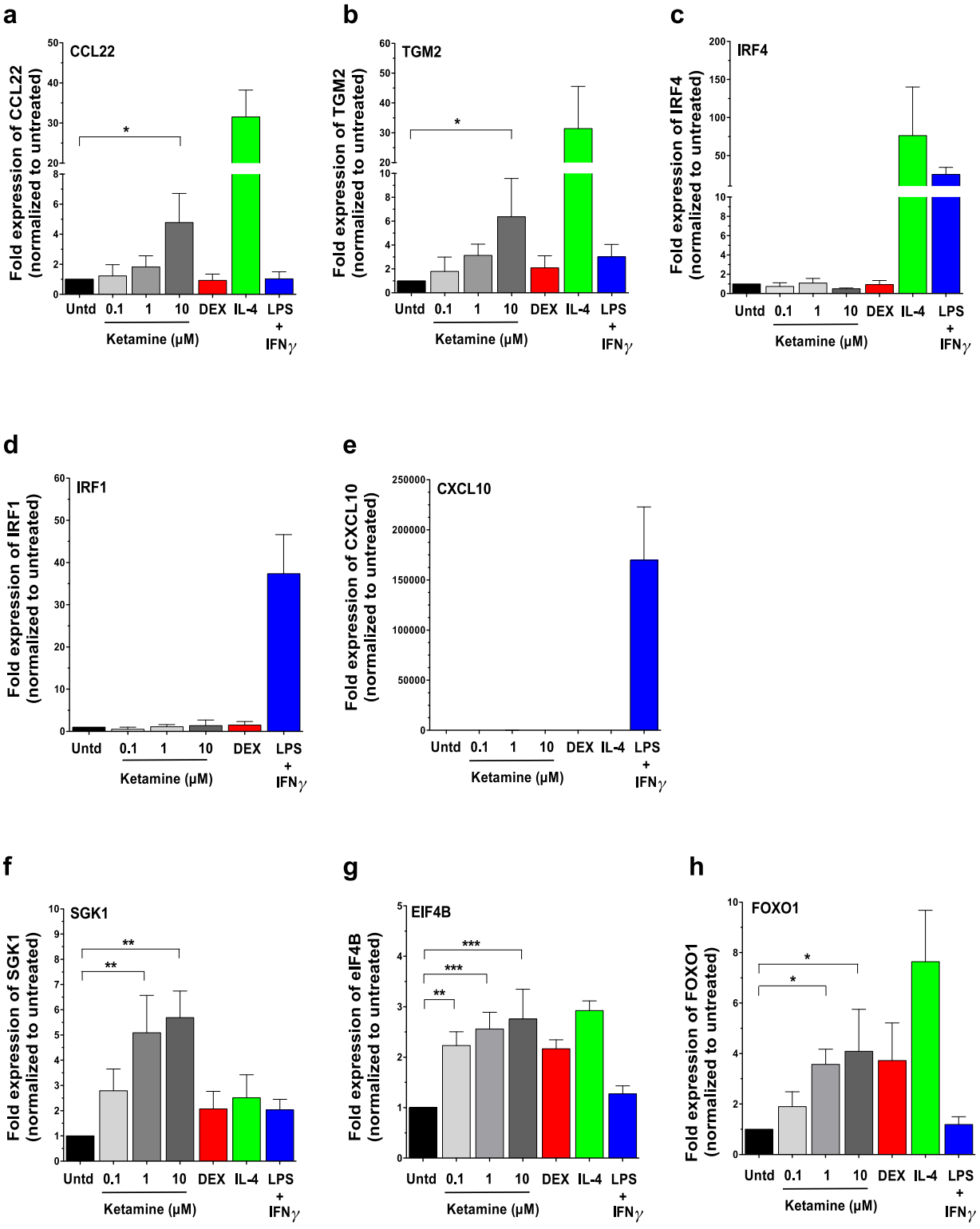

Fig. 3. Ketamine up-regulates M2 gene profile including mTOR pathway related genes. Relative quantification of (a) CCL22, (b) TGM2, (c) IRF4, (d) IRF1, (e) CXCL10, (f) SGK1, (g) E1F4B and (h) FOXO1 was performed by qPCR. EEF1A1 housekeeping gene was used as reference and fold change was normalized to untreated macrophages. The ketamine-induced gene profile was compared with untreated macrophages while dexamethasone (0.1 μM), IL-4 (40 ng/mL), and LPS (1 ng/mL plus IFN-γ [50 ng/mL]) were used as macrophage polarization controls. Each condition was performed with cells from at least 5 independent donors. *One-way ANOVA test* was performed to compare ketamine conditions to untreated macrophages and statistical significance is denoted as $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Untreated condition: Untd; dexamethasone: DEX.

12 or IL-12 plus IL-6 but not IL-6 alone. Moreover a stronger positive correlation was detected with the increased plasma level of IL-12, suggesting a potential link between IL-12 and non-classical monocytes as was suggested by Chuluundorj et al [16].

The exacerbated inflammatory profile of patients with MDD was also shown by the higher levels of CD86 and CD40 expression on $CD16^{neg}CD14^{bright}$ classical monocytes when compared with HCs. Increased pro-inflammatory cytokines (IL-6 and IL-12) were

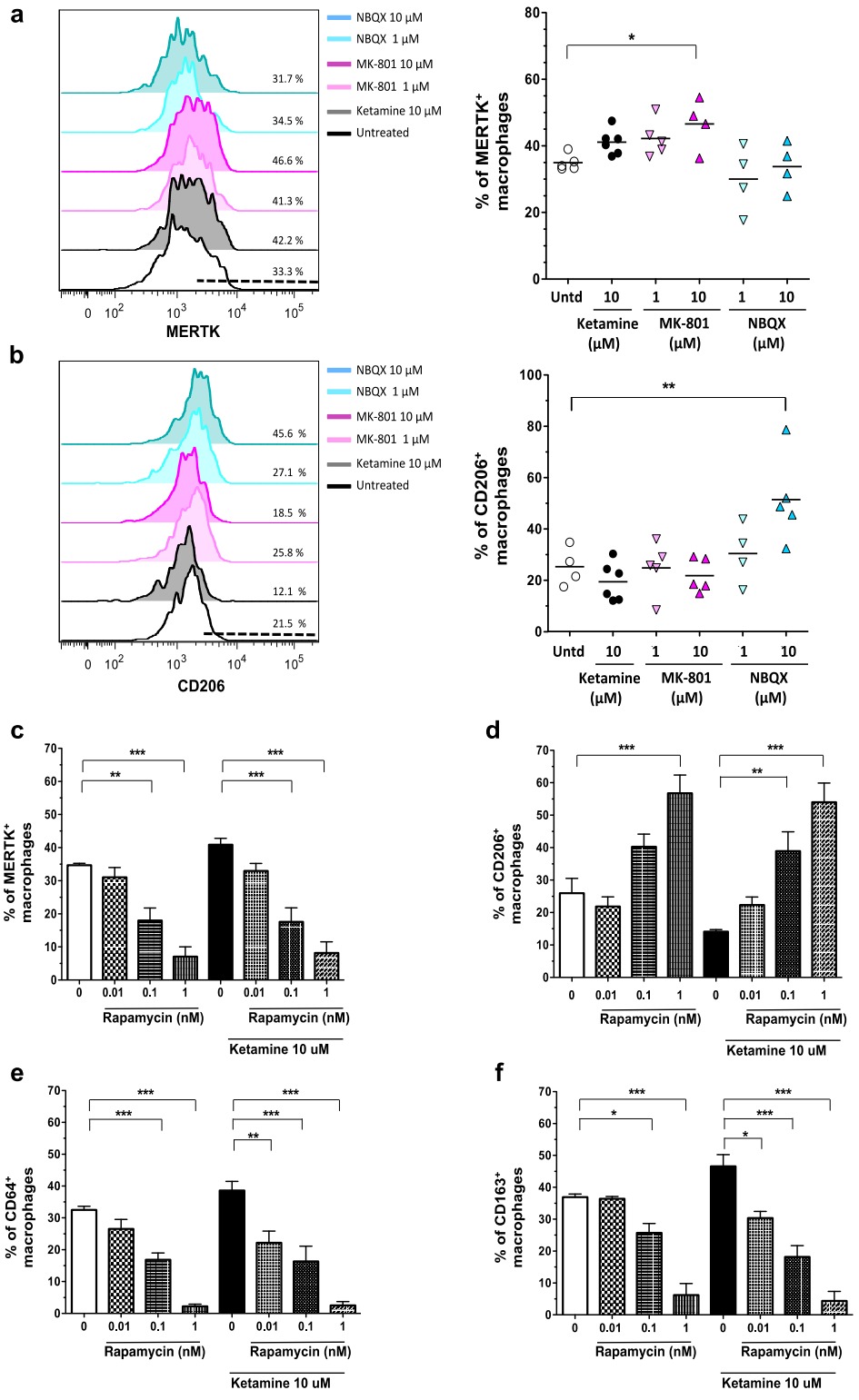

Fig. 4. NMDAR antagonist MK-801, but not the AMPAR antagonist NBQX, induces a similar M2 profile as ketamine, and this phenotype is completely abolished by the inhibition of the mTOR pathway. Monocyte-derived macrophages were differentiated for 7 days in the presence or absence of the NMDAR antagonist MK-801 (1 and 10 µM) or AMPAR antagonist NBQX (1 and 10 µM), and the percentage of (a) MERTK and (b) CD206 was analysed for M2 polarization by flow cytometry. Representative histograms and independent data are shown. Rapamycin (0.01–1 nM), added from day 0, was used to evaluate the role of the mTOR pathway in macrophage polarization after 7 days of culture. Viable $CD11b^+$ cells were analysed for the expression of (c) MERTK, (d) CD206, (e) CD64, and (f) CD163. Each experimental condition includes at least 4 independent donors. Pooled data were graphed and *one-way ANOVA test* was performed accordingly. Statistical significance is denoted as $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

observed in total mononuclear cells of patients with MDD compared with HC when stimulated with LPS. Additionally, the increased level of intracellular IL-12 in classical monocytes of patients with MDD reinforces the hypothesis that classical monocytes are becoming more activated promoting the transition to intermediate and finally to non-classical monocytes. These results clearly demonstrate an increased pro-inflammatory profile in patients with MDD.

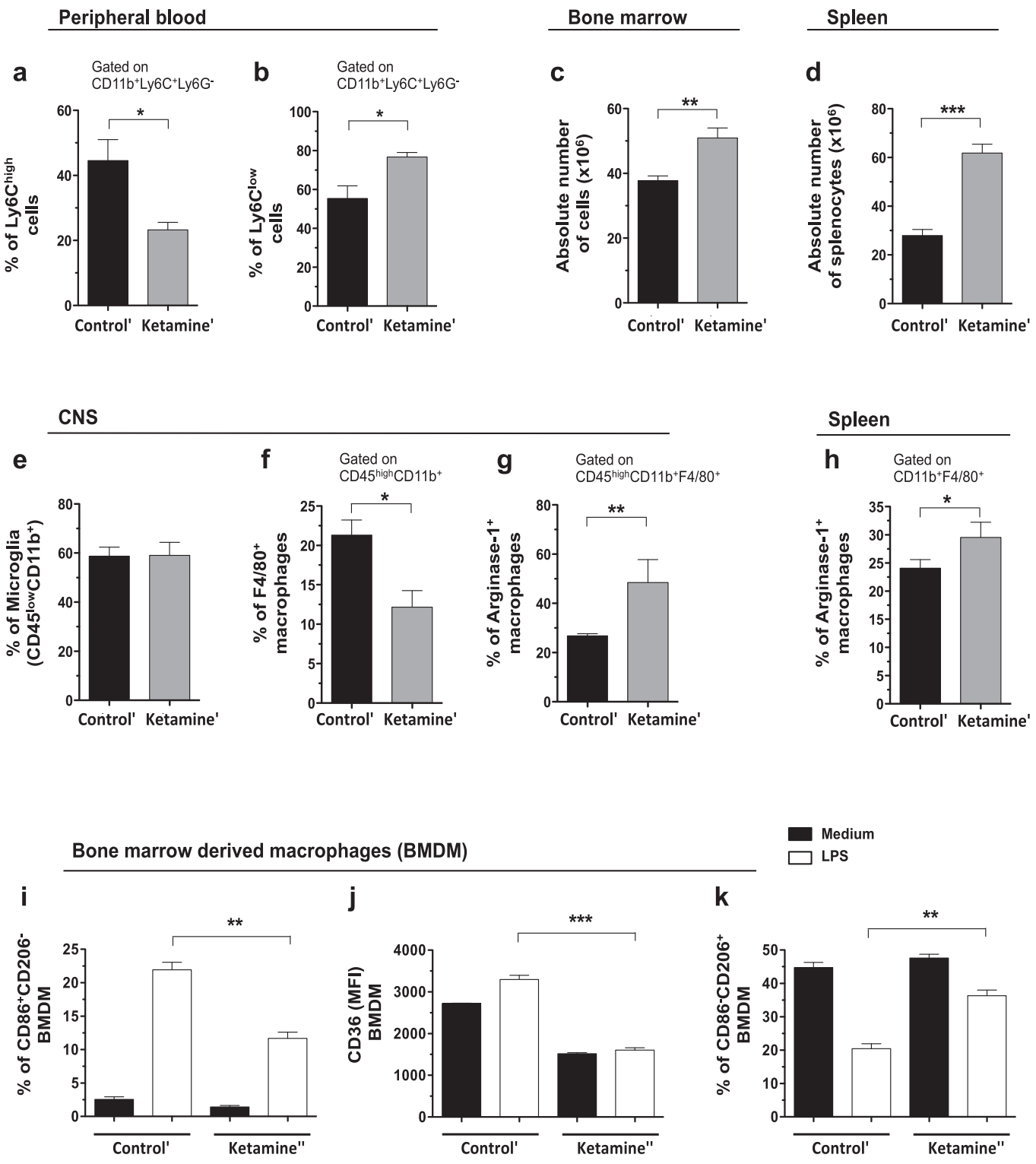

Fig. 5. Injection of sub-anaesthetic doses of ketamine modulates circulating myeloid compartment and promotes the differentiation of anti-inflammatory M2 macrophages in vivo. 72 hours after the intraperitoneal injection of sub-anaesthetic doses of ketamine (10 mg/kg) or saline solution, peripheral blood cells, bone marrow, spleen and total leukocytes from CNS were analysed. Peripheral circulating monocytes were analysed by gating on $CD11b^{+}Ly6C^{+}Ly6G$ by flow cytometry. Ketamine administration significantly reduces (a) the inflammatory $Ly6C^{high}$ monocytes and increases (b) the $Ly6C^{low}$ monocyte fraction. (c) The absolute number of bone marrow cells as well as (d) the total number of splenocytes was significantly increased 72 hours after the administration of sub-anaesthetic doses of ketamine compared with control mice. (e) The percentage of tissue resident $CD45^{low}CD11b^{+}$ cells in the CNS was similar between groups, but (f) the percentage of hematopoietic $CD45^{high}CD11b^{+}F4/80^{+}$macrophages was significantly reduced in CNS tissue from ketamine-treated mice. Furthermore, (g) the percentage of Arginase-1^{+} macrophages ($CD45^{high}CD11b^{+}F4/80^{+}$) was significantly increased in the CNS and (h) spleens of ketamine-treated mice. (i) and (j) BMDM from ketamine-treated or control mice, were differentiated for 7 days in the presence of M-CSF. These BMDMs were harvested and then challenged with 100 ng/ml of LPS for 24 hours. The percentage of (i) $CD86^{+}CD206$ cells and (j) the mean fluorescence intensity (MFI) of $CD36^{+}$ pro-inflammatory markers were significantly reduced in BMDM obtained from ketamine-treated mice. (k) The percentage of $CD206^{+}CD86$ macrophages was significantly increased in ketamine-treated compared with untreated mice. In-vivo experiments were performed with at least 4–5 mice per each condition, and repeated twice. Pooled data were graphed and statistical significance is denoted as $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Student's t test or *one-way ANOVA test* were performed accordingly.

Intriguingly, although all patients included in the present study were hospitalized for suicide behaviour with a severe MDD score, not all of them had high cytokine levels or a pro-inflammatory cellular profile. Two possibilities exist: Either inflammation is associated with only a fraction of these patients, or alternatively, inflammation indicates a particular stage of the disease. A limitation of our study is that cytokines and monocyte subset proportion could not be controlled by age, gender, BMI, or medication due to the number of samples; in order to gain insight into the two possibilities, a longitudinal prospective study is necessary. In this sense, several studies have demonstrated that peripheral immune deregulation may represent an important pathway for inducing functional and structural brain changes, specifically in the prefrontal cortex (PFC) and hippocampus that underpin the pathophysiology of MDD [50,51].

In the last few years, ketamine has emerged as prototype of a group of rapid-acting antidepressants (RAAD) with a clinical antidepressant response starting at 2 h, reaching maximal effect at 24 h, and lasting up to 7 days after a single infusion, giving ketamine a very different profile than traditional antidepressants and spurring an exciting field of research [3,26,52]. The mechanism that leads to the antidepressant action of ketamine is not completely understood. To date, two ketamine-induced glutamate neurotransmission changes appear to be critical to its RAAD effect: (a) A "transient" increase in post-synaptic glutamatergic stimulation in the PFC and (b) a sustained increase in PFC synaptic connectivity [53]. Mechanistically, ketamine leads to activity-dependant release of brain-derived neurotrophic factor, activation of mTOR pathway in the CNS, and increased protein synthesis and synaptic strength [33,53,54].

Ketamine is a racemic mixture comprising equal parts of (R) and (S) enantiomers, of which (S)-ketamine has been regarded as the active isomer because of its higher affinity for the NMDA receptor (3–4-fold), which pharmacologically explains the 4-fold greater anaesthetic potency and greater undesirable psychotomimetic side effects [55]. However, recent studies have shown that (R)-ketamine has a rapid-onset and sustained antidepressant action without psychotomimetic side effects [56–58]. Additionally, it was reported that its antidepressant effect was independant of mTOR pathway but involved extracellular signal-regulated kinase (ERK) signalling [59]. These previous findings suggest that mTOR signalling plays a role in the antidepressant effects of (S)-ketamine meanwhile ERK signalling plays a role in the antidepressant effects of (R)-ketamine. Nevertheless, further detailed studies are needed to better understand the mechanism of the antidepressant actions of racemic ketamine and the clinical implication of their enantiomers.

Until now, most research about ketamine focused on neuronal cells, but the effect of ketamine on innate immune response has just begun, with a recent clinical study showing a positive correlation between changes in cytokine levels after ketamine infusion and improvement in depressive symptoms in treatment-resistant depression [30].

Taking into account the predominant role of the monocyte-macrophage lineage in cytokine production as well as tissue infiltration and homoeostasis, we explored the role of ketamine in reprogramming the innate immune response and macrophage polarization. Here, we demonstrated that ketamine skewed monocytes into M2c-like macrophages by augmenting CD163 and MERTK expression, with intermediate levels of CD64 comparable with the regulatory phenotype driven by dexamethasone challenge. Similarly, ketamine was able to reduce macrophage activation markers (CD80 and HLA-DR) and dampen cytokine production induced by LPS, highlighting its anti-inflammatory properties. The central goal of macrophage biology is to define macrophage polarization on a molecular basis along with the specific pathways involved in physiological or pathological processes.

It has been described that in the CNS, ketamine activates NMDAR and mTOR pathway, inducing an increase of protein synthesis and synaptic strength [33], but how ketamine programs monocyte and macrophages to become an anti-inflammatory responder was not clearly explored. Our results showed that ketamine acts on monocytes by inducing a M2c-like phenotype via the NMDAR and mTOR pathway but also by promoting a particular gene expression profile that supports an independant way to induce the M2 program. In agreements with our results, murine models have also demonstrated the critical role of the mTOR complex for orchestrating macrophage polarization [36,60,61]. Remarkably, in vivo administration of ketamine at sub-anaesthetic doses promotes reduction of circulating classical pro-inflammatory monocytes and increase of the arginase-1^{+} M2 macrophage subset in the spleens and CNS of mice. Of note, to our knowledge no previous studies in murine models have described the effects of ketamine alone on spleen size or cellularity, yet our result showed that sub-anaesthetic doses of ketamine increased total cellularity in spleen and bone marrow. We speculate that the increased haematopoiesis may also impact splenic cellularity. However, ketamine-induced increase in splenocytes must be better studied in order to understand causality.

In summary, our data suggests that systemic ketamine administration biases the immune profile toward an anti-inflammatory response. This result potentially explains the rapid and sustained beneficial effect of ketamine in patients with MDD since ketamine can not only target the neurological system but can also skew the immune response toward an anti-inflammatory state. Our results have significant biological implications due to the historical antidepressant function of ketamine and its novel role as an anti-inflammatory mediator in other inflammatory-based diseases.

Declaration of Competing Interest

The authors have declared no potential conflicts of interest

CRediT authorship contribution statement

Wanda Nowak: Performed in-vitro experiments, Formal analysis, review & editing. **Leandro Nicolás Grendas:** Recruitment and follow up of patients, sample colection. **Liliana María Sanmarco:** Designed and perfomed in-vivo murine experiments, Formal analysis, review & editing. **Ivana Gisele Estecho:** Performed in vitro experiments. **Ángeles Romina Arena:** Sample processing of patients. **Natalia Eberhardt:** Performed in vitro experiments. **Demián Emanuel Rodante:** Recruitment and follow up of patients, sample colection. **María Pilar Aoki:** Designed and performed in-vivo murine experiments, Formal analysis, review & editing. **Federico Manuel Daray:** Conceived and designed the study, recruitment and follow up of patients, Writing - review & editing. **Eugenio Antonio Carrera Silva:** Conceived and designed the study, Formal analysis, Writing - review & editing. **Andrea Emilse Errasti:** Conceived and designed the study, Formal analysis, Writing - review & editing.

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Supplementary material

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2019.10.063.

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