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Defective inflammatory pathways in never treated depressed patients is associated with poor treatment response

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Author contributions

WEC, BD, HM, and CBN designed the PReDICT study and collected human samples. EB and JAL performed cytokine and cell measurements. PJDRV, RK and DWD performed inflammasome measurements. SS and DAL performed all the statistical analyses. EB, SS, DAL, FD and CBN wrote the manuscript.

Declaration of Interests

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Summary

Inflammation has been involvement in the pathophysiology and treatment response of major depressive disorder (MDD). Plasma cytokine profiles of 171 treatment-naïve, MDD patients (none of the MDD patients received an adequate trial of antidepressants or evidence-based psychotherapy) and 64 healthy controls (HC) were obtained. MDD patients exhibited elevated concentrations of 18 anti- and proinflammatory markers and decreased concentrations of 6 cytokines. Increased inflammasome protein expression was observed in MDD patients, indicative of an activated inflammatory response. The plasma of MDD patients was immunosuppressive on healthy donor peripheral blood mononuclear cells inducing reduced activation of monocytes/dendritic cells and B cells and reduced T cell memory. Comparison between 33 non-responders and 71 responders at baseline and 12 weeks, revealed that after treatment, anti-inflammatory cytokine levels increase in both groups, whereas 5 pro-inflammatory cytokine levels were stabilized in responders, but continued to increase in non-responders. MDD patients exhibit remodeling of their inflammatory landscape.

ETOC paragraph

Treatment-naïve depressed patients have increased levels of pro- and anti-inflammatory markers, but overall the balance shifts towards immunosuppression of immune cells. Consistent with these findings, absence of response to antidepressant treatments has been associated with defective anti-inflammatory response.

Keywords

Major depressive disorder; treatment naïve; cytokines; PBMC; inflammasome

Introduction

Major depressive disorder (MDD) is a widespread and debilitating disorder with a lifetime prevalence rate in the United States of 21% in women and 11% in men (Kessler et al., 2005). It is characterized by disturbances in sleep, appetite, concentration, ability to experience pleasure, and psychomotor alterations [Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5)]. It is also associated with a markedly increased risk for suicide and a variety of comorbid medical disorders (stroke, myocardial infarction, diabetes and others) (Otte et al., 2016). Various approaches ranging from functional brain imaging of patients to animal models of depression have been proposed to discover biomarkers to identify at risk populations and/or to predict individual treatment responses. However, because of the current lack of validated biomarkers and heterogeneity in different MDD patient populations, these approaches with some exceptions (Raison et al., 2013; Schmidt et al., 2011; Sen et al., 2008) have provided only limited advances towards these goals. This current limitation is particularly evident in studies of the role of inflammation in depression (Maes et al., 2009). It is now well-established that psychological stress, a prominent risk factor for MDD, induces an inflammatory response, and in multiple meta-analyss, two cytokines, interleukin (IL)-6 and tumor necrosis factor (TNF), are reproducibly elevated in the blood and cerebrospinal fluid (CSF) of depressed patients (Dowlati et al., 2010; Howren et al., 2009; Köhler et al., 2017; Liu et al., 2012; Maes et al., 2009; Miller et al., 2009). Furthermore, elevation of these proinflammatory cytokines in healthy volunteers is associated with the development of depressive symptoms (Miller and Raison, 2015), suggesting that investigating proinflammatory cytokines represents a reasonable strategy to identify biomarkers for depression.

The immune system induces the expression of anti-inflammatory cytokines to diminish inflammation. Thus, it is particularly intriguing that the levels of antiinflammatory cytokines IL-2, IL-4, and IL-10 are also often elevated in depressed patients, raising the question of whether these anti-inflammatory cytokines play a role in the onset or recovery from depression (Dowlati et al., 2010). As discussed below, elevated anti-inflammatory cytokines may represent responses to antidepressant treatments or compensatory mechanisms related to the duration of depressive episodes; this emphasizes the need for a study with a relatively homogeneous population of patients with a minimal number of depressive episodes and free of antidepressant treatment exposure to interpret changes in the inflammatory system.

The role of inflammation in MDD is supported, in part, by the results of a meta-analysis that supported the efficacy of non-steroidal anti-inflammatory drugs (NSAIDs) compared with placebo in the treatment of depression (Köhler et al., 2014). It is, important to note, however, that although NSAIDs have broad anti-inflammatory actions, they do not block the effects of inflammatory cytokines. Indeed, in the SADHART study all patients received NSAIDs, but they continued to exhibit an elevation in inflammatory markers (Glassman et al., 2002). With the development of novel targeted therapeutics in other inflammatory diseases such as psoriasis and rheumatoid arthritis, FDA-approved monoclonal antibodies and other cytokine inhibitors have been used to block individual cytokines in depressed patients, and they have demonstrated significant antidepressant properties in some patient cohorts (Kappelmann et al., 2016). For example, TNF inhibitors such as adalimumab (Loftus et al., 2008; Menter et

al., 2010) or etanercept (Tyring et al., 2013; Tyring et al., 2006), IL-12/IL-23 antagonists (Langley et al., 2010), or IL-4Ra antagonists (Simpson et al., 2015) have been shown to be more efficacious than placebo in the treatment of MDD symptoms. Similar effects have been observed in non-randomized and/or non-placebo controlled trials that targeted TNF or IL-6 (Kappelmann et al., 2016), indicating an improvement of depressive symptoms with anti-cytokine treatments. Infliximab, a TNF neutralizing antibody, only benefited a sub-population of treatmentresistant MDD patients with elevated levels of inflammation (CRP>5 mg/l) (Miller and Raison, 2015; Raison et al., 2013). This suggests that anti-cytokine approaches might only provide benefit in depressed patients with prominent inflammation.

Response to antidepressant treatments has been reported to be impaired by proinflammatory cytokines, which may be overcome by co-administering antiinflammatory drugs (Köhler et al., 2014). Although antidepressants are generally thought to shift the balance towards anti-inflammatory response (Kubera et al., 2001; Lanquillon et al., 2000; Maes et al., 1999; Sluzewska et al., 1997), the overall net effect of antidepressants on cytokines remains unclear, as antidepressants have also been reported to promote proinflammatory cytokine production (Warner-Schmidt et al., 2011). Nonetheless, high levels of proinflammatory cytokines are often observed in treatment-resistant depressed patients, suggesting a negative correlation between pro-inflammatory cytokine levels and treatment response (Kubera et al., 2001; Lanquillon et al., 2000). Taken together, the data suggest that changing the balance between pro- and anti-inflammatory cytokines may promote antidepressant actions. However, no prior studies have focused on previously untreated patients and, furthermore, there are limited data on multiple inflammatory and anti-inflammatory cytokines.

The Predictors of Remission in Depression to Individual and Combined Treatments (PReDICT) study was designed to identify predictors of the response to 3 well-established and effective interventions: escitalopram (10–20 mg/day), duloxetine (30–60 mg/day) and Cognitive Behavior Therapy (CBT:16 sessions) among MDD patients who had never previously received an evidence-based treatment for depression (Dunlop et al., 2012). This unique and relatively homogeneous population of treatment-naïve depressed patients represents an ideal paradigm to identify predictors of treatment response and to identify biomarkers by comparing MDD patients to healthy volunteers. The PReDICT study demonstrated that antidepressants and CBT treatment were similarly efficacious with remission rates in the 44–52% range (Dunlop et al., 2017a). Further, magnetic resonance imaging (MRI) using resting state functional connectivity distinguished between the likelihood of remitting or failing to benefit from CBT or antidepressant treatment in the PReDICT cohort (Dunlop et al., 2017b).

In the current investigation, we measured 27 cytokines, chemokines, and growth factors in the PReDICT cohort of treatment-naïve MDD patients and healthy volunteers. The majority of patients exhibited elevated levels of both proinflammatory and anti-inflammatory cytokines, pointing towards an increased inflammatory response. We also found that independent of treatment type, responders exhibited stabilized levels of proinflammatory cytokines whereas non-responders exhibited continued increases in proinflammatory cytokines. In contrast, anti-inflammatory cytokines, remained elevated in both responders and non-responders.

In addition, we found associated with the increased cytokine production, an increase in the inflammasome protein levels, pointing towards an upregulation of the cytokine production machinery in MDD patients compared to healthy controls. Examination of the potential effect of the plasma content of MDD patients on PBMC activation revealed that plasma of MDD patients exhibit immunosuppressive capacity consistent with previous literature (Maes, 1995). Nevertheless, response to antidepressant treatments was not associated with changes in the inflammasome proteins, nor PBMC activation suggesting that cytokine concentrations might represent better biomarkers to evaluate the effects of intervention in treatment-naïve MDD patients 12 weeks after initiating the treatment.

STAR methods

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-CD4 clone SK3	BD Horizon	Cat# 566104 Lot7068600
anti-CD11b clone CBRM1/5	BD Horizon	Cat# 566313 Lot6105692
anti-CD19 clone SJ25C1	BD Horizon	Cat# 566396 Lot7146702
anti-CD45RO clone UCHL1	BD Pharmingen	Cat# 555493 RRID:AB_395884 Lot6320585
anti-CD25 clone 2A3	BD Horizon	Cat# 564467 Lot7054527
anti-CD69 clone FN50	BD Horizon	Cat# 562617 Lot7158806
anti-CD8 clone RPA-T8	BD Horizon	Cat# 563823 RRID:AB_2687487 Lot7167933
anti-CD86 clone FUN-1	BD Horizon	Cat# 562999 Lot7144880
Critical Commercial Assays		
human 27Plex cytokine multiplex assay	Bio-rad	M500KCAF0Y
CART/Simple Plex Assay	Protein simple	SPCKA-PS-000786
Biological samples		
Human PBMCs	ALLCELLS	PB001 Lot A5962
Instruments		
MAGPIX	Luminex	Cat# MAGPIX
Celesta flow cytometer	BD Bioscience	Cat# 660343
Ella System	Protein Simple	
Software		
SPSS 24.0 software	IBM	RRID:SCR_002865
FACS DIVA 8.0 software	BD Bioscience	RRID:SCR_001456
FlowJo 9.9.5 software	FlowJo	RRID:SCR_008520

REAGENT or RESOURCE	SOURCE	IDENTIFIER
PRISM 5.0 software	Graph Pad	

CONTACT FOR REAGENT AND RESOURCE SHARING

For further information and requests for reagents may be directed to, and will be fulfilled by, the Lead Contact and Corresponding author, Charles Nemeroff **(**cnemeroff@med.miami.edu).

EXPERIMENTAL MODEL AND SUBJECTS DETAILS

Study overview—The rationale, methods and design of the PReDICT study has been published previously (Dunlop et al., 2012) (ClinicalTrials.gov ID NCT00360399). The study was conducted between January 2007 and May 2013 through the Emory University's Mood and Anxiety Disorders Program. The study was approved by the Emory Institutional Review Board and the Grady Hospital Research Oversight committee.

Patients—Male and female patients were recruited through clinical referrals and advertising. Eligible participants were adult outpatients 18–65 years of age who met the DSM-IV criteria for a primary diagnosis of MDD without psychotic features. The primary diagnosis and absence of exclusionary diagnoses were assessed through a study psychiatrists interview and administration of the Structured Interview for DSM-IV (SCID). Inclusion criteria included a 17-item Hamilton Depression Rating Scale (HDRS) (Hamilton, 1960) total score ≥15 at the randomization visit. Patients had to have never previously received an evidence-based treatment for a mood disorder, operationalized as lifetime exposure to 4 sessions of an evidence-based psychotherapy or 4 weeks of an antidepressant medication. Of the patients entered into the study, 15 (8.8%) had ever received an antidepressant medication. Of these 7 had received an inadequate trial as defined in the inclusion criterion. The mean period of time they were treated was 10.5 ± 2.4 days and this occurred 8.9± 3.1 years prior to study entry. The only exception was one patient who had been treated for "premenstrual tension" 2 years prior to study entry. These patients did not differ from the others in the study either in terms of inflammatory markers or treatment response.

Exclusion criteria: Lifetime criteria for bipolar disorder or a psychotic disorder, currently met criteria for OCD (past 12 months), substance abuse within 3 months prior to screen or substance dependence within 12 months, significant current suicidality or homicidality, pregnant or breast-feeding women, contraindication to MRI scanning. If present, comorbid current post traumatic stress disorder (PTSD) could be only mild in severity as assessed by the study psychiatrist and could not be the focus of treatment.

Interested patients were seen for a screening visit, which was comprised of a SCID interview, HDRS, medical history, routine laboratory testing (including urine drug screen), electrocardiogram, and physical exam.

Randomization and Treatments—Patients were randomized at a 1:1:1 ratio to receive 12 weeks of treatment with either escitalopram (10–20 mg/day), duloxetine (30–60 mg/day), or 16 sessions of CBT of 50 min, administered according to a standardized protocol (Beck, 1979). Escitalopram and duloxetine were initiated at 10 mg/day and 30 mg/day, respectively, with the option to increase the dose beginning at week three depending on tolerability and response. All patients who had not achieved remission by week six had the dose increased to 20 mg/day of escitalopram or 60 mg/day of duloxetine unless prevented by tolerability concerns.

Clinical outcomes—We defined positive response if HDRS scores at week 12 were < 50% of baseline.

Sample collection—Blood samples were collected from antecubital veins using standard techniques into EDTA tubes at baseline, and at week 12 between 8 am and 4 pm. Within 10 minutes of being obtained, the EDTA tubes were centrifuged at 4°C, the plasma aliquoted into 1 ml samples and frozen at −80°C. Healthy control (HC) volunteer plasma were also collected in EDTA tubes. We also selected healthy control volunteers who reported no medication (comprising psychiatric or other types of medications) at the time of blood collection.

Cytokine Measurements—Cytokines, chemokines and growth factors were measured in plasma samples isolated as above using a commercially available multiplex analysis human 27Plex cytokine multiplex assay (M500KCAF0Y, Bio-Rad) on a MAGPIX. Samples were run on 9 plates, blind to treatment. 4 samples were run on every plate to control for the inter-plate variability (%CV varied between 2.3–12%, supplementary Figure 1C). Assays were checked for quality control to fit the standard curves. A standard curve was run for each lot, and samples were normalized to the averaged standard curve values.

Inflammasome Protein Measurements—Inflammasome signalling protein levels (ASC, caspase-1 and IL-18) in plasma were analysed as described in (Brand et al., 2016), using the Ella System and analysed by the Simple Plex Explorer software (Protein System). Results correspond to the mean of each sample run in triplicates for each analyte.

Peripheral Blood Mononuclear Cells (PBMC) stimulation

PBMCs from a non-hispanic white female healthy donor (age 50, weight: 142 lbs, height 68 in) unmedicated for a week, were freshly isolated at ALLCELLS. No other information regarding this donor was available. When received in the lab, PBMCs were plated at 4×10^5 cells/well (86% viability) in 96-well plates, and the next day, PBMCs were stimulated with LPS (100 ng/mL) or not and/or with 10% of plasma of HC subjects or MDD patients for 24 h. All the samples were run in duplicate. The plasma of 27 HC subjects and 40 MDD patients (20 non-responders and 20 responders) were tested. The demographics of the patients were chosen so there was no difference between the 3 groups.

Cells were then stained for flow cytometry using BV480-conjugated anti-CD4, BV786-conjugated anti-CD8, BV421-conjugated anti-CD11b, BB700-conjugated antiCD19, PE-conjugated anti-CD45RO, BB515-conjugated anti-CD25, PE-CF594conjugated anti-CD69,

and BV605-conjugated anti-CD86 (BD Bioscience, supplementary material) and analysed on a Celesta, BD bioscience flow cytometer. Data were analysed using Flow Jo.

Data analysis—The data were analyzed using SPSS Version 24. For all analyses, any samples that were under the detection limit were included as the minimal detectable value of the assay. More than 50% of the values for IL-10, IL-5, IL-12, G-CSF and GM-CSF were under the minimal detection range in the HC group. Depending on the analytes, some samples demonstrated aberrant values and were excluded.

Statistical Analyses

Baseline demographic differences between HC and MDD groups were performed using one-way analysis of variance procedures and chi-square analyses for dichotomous variables. The criteria for statistical significance was $p<0.05$. In analyses comparing variables associated with an inflammatory response and the upregulation of the inflammasome pathway, we employed ANCOVA analyses to account for HC and MDD initial differences in age, gender, percentage of African-American participants and Hispanic participants. The Bonferroni correction was applied to adjust for family-wise Type 1 alpha error rates due to multiple comparisons. Following a statistically significant Bonferroni Test, additional nonparametric Mann-Whitney Tests were performed to insure that distributional characteristics of the data and heterogeneity of variance in a comparison of groups with unequal n's did not affect the obtained findings.

We tested if antidepressant treatments for 12 weeks altered the inflammatory molecule profile and if there were differences between responders and non-responders. Due to the modest number of subjects who completed treatment in each arm, we pooled all the treatments together as there were no differences in the cytokine levels and response outcome between the 3 treatments (Supplementary Table 5C). We hypothesized that MDD responders would have reduced proinflammatory cytokine levels compared with MDD non-responders after 12-weeks of treatment. To test this hypothesis, a 2 X 2 (Responder by Time) multivariate analyses of variance (MANOVA) with IL-6, IL-1β, TNF, IFNγ, and IL-17A serving as outcome measures. We focused on the Responder X Time interaction term since this would reveal any potential differences in patterns of change of pro-inflammatory cytokines in responders versus non-responders, overtime. Following a statistically significant omnibus multivariate test of significance, individual univariate analyses conducted for each pro-inflammatory cytokine. A similar MANCOVA approach was employed for anti-inflammatory markers.

For analyses comparing cytokine values at baseline of MDD patients in the PReDICT study versus healthy controls, a series of Levine tests were used and demonstrated significant heterogeneity of variance. As a result, Welch's F-Value for Unequal Variances was applied. We also completed ANCOVAs using baseline differences between groups with regards to age and gender, BMI as covariates. Since the underlying assumptions of ANCOVA may be violated in the presence of both heterogeneity of variance described above coupled with unequal numbers of subjects in MDD patients versus HC subjects, it was judged that a most robust test of group differences could be obtained using a series of Mann-Whitney U tests of

ranks that do not require parametric assumptions such as homogeneity of variances, normality and unequal numbers of subjects. Both parametric and non-parametric tests yielded similar results.

To examine changes in cytokines as a function of treatment condition among responders versus non-responders, we conducted a series of Diagnostic Group x responder x Time (Baseline versus 12 weeks) mixed model repeated measures MANOVA for both proinflammatory cytokines and anti-inflammatory cytokines. Given the equivalence of age, gender and body mass index in responders versus non-responders, no covariates were required in the model. Following a statistically significant multivariate effect, post-hoc univariate analyses (ANOVAs) were conducted for proinflammatory and anti-inflammatory cytokines respectively. Multiple comparison corrections were made and stated within each legend.

Results

Patient characteristics

171 patients meeting DSM-IV criteria for MDD and 64 healthy controls were included in the analyses. The sociodemographic and clinical characteristics of the patients are presented in Table 1, Supplemental Tables 1A, 3, and Supplemental Figures 3A, 4A.

Depressed patients exhibit an inflammatory response

Because the populations of HC subjects and MDD patients were significantly different in demographic characteristics (age, gender, ethnicity, BMI) that could significantly impact the dependent variables (Table 1), we carefully matched 62 HC subjects with 62 MDD patients on these demographic characteristics to avoid any bias in our conclusions (Supplementary Table 1A-B). Two HC participants could not be effectively matched. This matching strategy resulted in equivalent age, gender and ethnicity as well as BMI among the HC and MDD matched groups (Supplementary Table 1A). We then compared these results to statistical analysis performed with the full compliment of 64 HC subjects and 171 MDD patients. Because the results were identical for the cytokine data, we present the data for the total sample below.

Compared to HC subjects, the 171 MDD patients exhibited elevated levels for six of the seven measured proinflammatory cytokines, including IL-12, TNF, IL-6, IFNγ IL9, and IL-17A, with levels that were ~2- to 13-fold those in HC subjects, and elevations were evident in 66–100% of the MDD patients (Table 2, Supplementary Fig 1A). Of the 7 potential anti-inflammatory cytokines measured, among the 171 MDD patients 5 were elevated (IL-5, IL-15, IL-10, IL-2, IL-13) ranging from ~1.6- to 17-fold levels in HC subjects, and 2 were diminished (IL-1RA, IL-4) by ~25%. The effects found with the potential anti-inflammatory cytokines occurred in 79–100% of MDD patients (Table 2, Supplementary Fig 1B).

Chemokine level elevations in the 171 MDD patients compared to HC subjects were evident in 2 (MIP1α/CCL3, RANTES/CCL5; 1.7-fold and 6.8-fold of HC levels, respectively) out of 7 measured, and 4 were lower by 66–17% in the 171 MDD patients (IP10/CXCL10,

MCP1/CCL2, IL-8, MIP1β/CCL4) (Table 2, Supplementary Fig 2A). The 171 MDD patients demonstrated elevated levels on 4 of the 6 measured growth factors, including G-CSF, PDGF, FGF, IL-7, which were elevated to 1.2–11fold levels in HC subjects (Table 2, Supplementary Figure 2B). It is important to note, however, there were 3 chemokines and growth factors that were differentially regulated between the matched and whole sample analyses. RANTES/CCL5 and FGF were increased in the whole sample analysis in the MDD patients, but did not change or decreased, respectively, in the matched sample analysis in MDD patients compared to HC subjects. Similarly, GM-CSF did not change in the whole sample analysis in MDD patients, but significantly decreased in the matched sample analysis in MDD patients when compared to HC subjects. Although IL-6 concentration was increased in both the matched and whole sample analyses, the fold increase was ~3 fold lower in the 62 matched MDD patients. This suggests that these factors might be sensitive to age, gender, ethnicity and BMI variation.

Taken together, these results indicate that MDD patients commonly demonstrated elevated levels of many inflammatory molecules, along with several that had lower levels; this is in contrast to the notion that only a subset of MDD patients experience inflammatory dysregulation (Raison et al., 2013). In addition, both pro- and anti-inflammatory cytokines were increased in MDD patients, suggesting an overall increased inflammatory response in treatment-naïve MDD subjects.

Depressed patients exhibit an upregulation of the inflammasome pathway

We also examined whether MDD patients exhibited alterations of the cytokineproducing inflammasome pathway, which can be reliably analysed in plasma samples. Inflammasomes are intracellular multiprotein complexes that function as sensors of danger-associated molecular patterns (DAMPS) or pathogen-associated molecular patterns (PAMP), which leads to the activation of proinflammatory caspases and the cleavage and release of proinflammatory cytokines. Inflammasomes generally comprise three proteins: 1) an NLR (nucleotide binding domain, leucine-rich repeat family member); 2) the adaptor protein apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and 3) the pro-inflammatory cysteine aspartase, caspase-1 (Guo et al., 2015). The inflammasome regulates caspase-1 activity and, consequently, regulates the activation of the pro-inflammatory cytokines IL-1β and IL-18 (Fantuzzi and Dinarello, 1999). To date, there is only one report showing that caspase-1, NLRP3 mRNA expression, and NLRP3 protein levels are increased in PBMCs of MDD patients compared to HC subjects (Alcocer-Gómez et al., 2014).

Results here demonstrate that IL-18, caspase-1 and ASC-1, were significantly elevated in MDD patients compared to HC subjects (Table 3 and Supplementary Figure 3) suggesting that the upstream pathway responsible for the production of IL18 or IL-1β was elevated in MDD patients. However, IL-1β (Table 2) was not significantly different between the MDD and HC groups. These findings substantiate the idea that MDD patients exhibit elevated inflammatory responses.

Plasma of MDD patients displays impaired activation of PBMCs

Because plasma from MDD patients exhibited increased levels of both pro- and anti-inflammatory cytokines, we tested the overall effects of the plasma from MDD patients on freshly isolated PBMCs from a healthy volunteer donor. Both pro- and anti-inflammatory cytokines have been shown to modulate PBMC activation (Sancho et al., 2005; Subauste et al., 1998; Wieland and Shipkova, 2016). Compared with plasma from HC subjects, addition of plasma from MDD patients on donor PBMCs led to a lower population of the $CD86^{+}CD11b^{+}$ cells ($p=0.005$) (Table 4 and Supplementary Fig 4B) in control PBMCs, indicating a reduced activation of monocytes/dendritic cells, and a reduced proportion of $CD69^{+}CD19^{+}$ ($p=0.001$) cell population (Table 4 and Supplementary Fig 4B), indicative of reduced activated B cells. There was no change in the overall populations of $CD4^{+}$ ($p=0.292$), $CD8^{+}$ ($p=0.531$), $CD19^{+}$ ($p=0.913$) and $CD11b^{+}$ ($p=0.351$) cells from the donor PBMCs exposed to the plasma of MDD patients or HC subjects (Supplementary Table 2A). Furthermore, the percent of activated donor PBMCs exposed to plasma from MDD patients or HC subjects were similar after treatment with lipopolysaccharide (LPS) (supplementary Table 2B), suggesting that the reduced activation of PBMCs in the presence of the MDD patient's plasma was not the results of an incapacity of the PBMCs to become activated. The amount of lymphocyte activation marker CD69 expression on $CD8^{+}$ cells, Tregs ($CD4^{+}CD25^{+}$), and B cells was also significantly reduced after LPS treatment confirming there was less activation of the donor PBMCs by LPS in the presence of plasma from MDD patients compared to the plasma of HC subjects (Supplementary Table 2C). We also excluded any potential activating effect of the HC plasma on PBMCs, as PBMCs that did not receive any plasma, have similar PBMC activation level as PBMCs exposed to plasma from HC subjects (Supplementary Table 2D). We excluded any change in cell viability as the counts and frequency data were similar (data not shown). $CD4^{+}CD45RO^{+}CD69^{-}$ ($p<0.001$) memory cells from the healthy donor were also reduced after exposure to MDD patient's plasma compared to plasma from HC subjects (Table 4), suggesting that plasma of MDD patients exhibit properties that also inhibit cellular memory formation.

Taken together, these data suggest that MDD patients experienced a relatively major remodelling of the cytokine landscape, accompanied by an overall immunosuppressive phenotype at the cellular level.

Antidepressant treatment effects on the inflammatory response

We tested if antidepressant treatments for 12 weeks altered the inflammatory molecule profile and whether there were differences between responders and non-responders. Due to the modest number of subjects who completed treatment in each arm (33 non-responders and 71 responders), we pooled all the treatments together as there were no differences in the cytokine levels and response outcome among the 3 treatments (Supplementary Table 5) or on PBMC counts (Supplementary Table 7). We hypothesized that MDD responders would have reduced proinflammatory cytokine levels compared with MDD non-responders after 12-weeks of treatment. To test this hypothesis, we conducted a 2 X 2 (Responder by Time) multivariate analyses of variance (MANOVA) with IL-6, IL-1β, TNF, IFNγ, and IL-17A serving as outcome measures. No covariates were entered into the model because Responders and Non-Responders did not differ on demographic factors such as age, gender,

or BMI (Supplementary Table 3). We focused on the Responder X Time interaction term because this would reveal any potential differences in patterns of change of proinflammatory cytokines in responders versus non-responders, over time. Following a statistically significant multivariate test of significance, individual univariate analyses were conducted for each pro-inflammatory cytokine. As predicted, there was a Responder X Time effect [Wilks' lambda F(1,99)= 5.84; p=0.017] but there was no main effect for Time [Wilks' lambda [F(1,99)=0.46; p=0.5] or Responder [Wilks' lambda F(1,99)=0.74; p=0.392]. Univariate analyses on the interaction terms for each proinflammatory cytokines revealed that each of the five proinflammatory cytokines reached statistical significance (IFNγ, p=0.027; IL-1β, p=0.026; IL-6, p=0.041, IL-17A, p=0.035 and TNF, p=0.017) (Table 5A and Supplementary Fig 5A); furthermore, the pro-inflammatory markers tended to rise in non-responders during treatment while they were relatively lower or stabilized in treatment responders (Figure 1B).

We conducted a similar repeated measures MANOVA on anti-inflammatory cytokines (IL-4, IL-5; IL-10). IL-2 and IL-15 were not included because of significant missing data. There was no Responder X Time effect [Wilks' lambda F(1,102)=2.56; p=0.087], but there was a main effect for Time [Wilks' lambda [F(1,102)=12.11; p<0.001]. There was no Responder effect [Wilks' lambda F(1,102)=0.78; p=0.378]. Post-hoc tests on the main effect for time revealed the 3 cytokines (IL-4, p=0.001; IL5, p=0.002; and IL-10, p=0.027) generally thought to be anti-inflammatory cytokines were elevated in response to antidepressant treatments (Table 5B; Figure 1A and supplementary Fig 5B). There were only 31 responders and 21 non-responders who had a detectable level of IL-2 and a similar number of participants who had a detectable level of IL-15. The results of ANCOVA analyses revealed an identical main effect as observed on the MANOVA analyses of other anti-inflammatory markers. For IL-2 and IL-15, there was a statistically significant time effect [F(1,47)= 5.97; p=0.018] and F(1,47)=6.6; p=0.013], respectively. No Responder or Responder X Time Interaction effects were observed. Taken together, these results indicate that depressed patients undergoing 12 weeks of treatment have increases in anti-inflammatory markers independent of treatment response.

The pattern of results suggested by these data indicate that antidepressant treatments in general, promote anti-inflammatory cytokine production and inhibition of some pro-inflammatory cytokines (Supplementary Table 4 and Supplementary Fig 5 and 6).

We conducted exploratory analyses and did not find any difference between responders and non-responders in the activation of the inflammasome pathway (Supplementary Table 6), or in the proportion of activated CD4, CD8, B cells and CD11b cells (Supplementary Fig 7), suggesting that antidepressant treatments might target pathways downstream of cytokines rather than their production.

However, memory T cells ($CD4^+CD45RO^+CD69^-$) were significantly lower in responders compared to non-responders (Table 6). Associated with the reduced memory T cells, we found no change in the frequency of cells expressing the activation marker CD69 (Table 6), but reduced expression of CD69 at the cell level, on $CD8^+$ cells and B cells in responders compared to non-responders (Table 7). In contrast, CD69 expression on Tregs, though

significantly lower in responders compared to non-responders, did not survive the Bonferroni correction, suggesting that lower activation of T and B cells might be associated with lower memory.

Discussion

In this comprehensive assessment of inflammatory markers in treatment-naïve MDD patients, who had never received an adequate trial of antidepressant medication or evidence-based psychotherapy for the treatment of depression, we observed elevated levels of 18 pro- and anti-inflammatory cytokines, chemokines, and growth factors, together with lower levels of 6 cytokines and chemokines compared to HC subjects. These widespread differences indicate that a major remodelling of the inflammatory landscape occurred in MDD patients. Associated with these changes in cytokines was an upregulation of the inflammasome proteins IL-18, caspase-1 and ASC in MDD patients. There was a decrease of the activation and cellular memory of healthy donor PBMCs exposed to plasma of MDD patients compared to the plasma of HC subjects, suggestive of cellular immunosuppressive properties of the plasma of MDD patients. Overall, these findings demonstrated that treatment-naïve MDD patients exhibited features of a significant remodelling of their cytokine profiles.

Although the idea that depressed patients have increased pro-inflammatory and anti-inflammatory cytokines is now well-accepted (Dowlati et al., 2010), the novelty of the present study is based on the measurement of 27 cytokines in a unique population of treatment-naïve MDD patients. Most studies measuring cytokines in MDD patients have only measured a limited number of cytokines per patient. We found that levels of most cytokines (23/27) were different in MDD patients and HC subjects, which contrasts with a recent study that reported that treatment resistant depression (TRD) patients have only 5 cytokines differentially expressed compared to HC subjects (Kiraly et al., 2017). The discrepancy between our study and others' findings likely originates from the populations of MDD patients studied, and the fact that most previous studies focused on MDD patients that were previously exposed to multiple trials of antidepressant medications. Our population is unique because they are treatment-naïve and many were diagnosed for the first time, thereby providing a unique group for interrogation the role of the inflammatory response in MDD patients. Surprisingly, and in contrast to recent findings proposing that only a subgroup of MDD patients exhibit increased inflammatory markers (Raison et al., 2013), we found that the majority of MDD patients have elevated inflammatory markers above the mean of the HC subjects. Moreover, this increase in the cytokine levels occurred early in the disease as 5 cytokine (IL-2, IL-10, IL-12, IL15 and IL-17A), 1 chemokine (MIP1a/CCL3) and 2 growth factor (GM-CSF, FGF) levels were elevated in MDD patients within their first episode of the disease compared to MDD patients who experienced several episodes of the disease (data not shown).

Consistent with an increased production of cytokines in MDD patients (Dowlati et al., 2010; Howren et al., 2009; Köhler et al., 2017; Liu et al., 2012; Maes et al., 2009; Miller et al., 2009), the inflammasome pathway was also induced in MDD patients compared to healthy controls. There were differences in IL-18, ASC-1 and caspase-1 levels, but not in IL-1β levels in MDD patients compared to HC subjects. Caspase-1 and ASC-1 might possibly be

the limiting factors in the regulation of inflammasome activation, whereas the IL-18 and IL-1β are produced in excess (Marshall et 1999, Puren et al 1998) or encounter other regulatory interactions downstream of ASC-1 and capase-1, such as potassium efflux or cathepsin release (Guo et al 2015). This suggests that the cytokine production machinery is turned on in MDD patients, since the inflammasome is primed by an NF-$_K$B dependent stimulus and most cytokines that are increased in MDD patients do not depend on the inflammasome pathway, but on the NF-$_K$B pathway since NF-$_K$B is considered the main transcription factor controlling cytokine synthesis (Martinon et al., 2002). Importantly, inflammasome activation appears to bridge the gap between immune activation and metabolic danger signals or stress exposure, which are key factors in the pathogenesis of MDD (Dantzer et al., 2008b; Miller et al., 2009). Our study suggests that activation of the inflammasome is associated with the subsequent proteolysis and release of the pro-inflammatory cytokine IL-18 in MDD patients.

To test the overall effect of the cytokine profile remodelling on immune cells, we analysed the activation of healthy donor PBMCs in the presence of plasma of MDD patients or HC subjects. We found that the overall phenotype of plasma from MDD patients was immunosuppressive, as the PBMCs incubated with the plasma of MDD patients were less frequently activated than the PBMC cultured with plasma of HC subjects. This suggests that MDD patients experience an upregulation of the production of cytokines, whereas the overall effect of MDD plasma on immune cells are immunosupressive. It remains to be explored whether these immunosuppressive properties are due to the cytokine milieu favoring an anti-inflammatory response or other molecules present in the plasma providing immunosuppression. In addition, the fact that both pro- and anti-inflammatory cytokines are elevated in MDD patients but that the overall effect on healthy donor PBMCs is immunosuppressive, suggests that the anti-inflammatory response in MDD patients might be failing to terminate the inflammatory responses; this is consistent with the presence of chronic inflammation in MDD patients (Dantzer et al., 2008a).

Although CD69 is considered to be an activation marker of T and B cells, CD69 has ambiguous immunoregulatory functions (Sancho et al., 2005). In humans, its expression is associated with an ongoing immune response and tissue damage. Our data are consistent with a proinflammatory role of CD69 in MDD patients, though the role of CD69 in MDD patients will need to be confirmed in PBMCs isolated directly from MDD patients. Cytokines have been shown to modulate CD69 expression (Sancho et al., 2005), with anti-inflammatory cytokines such as IL-10 that reduces CD69 expression (Mocellin et al., 2003). Reduced levels of CD69 have been shown to be associated with reduced memory cell formation, which might explain why memory cells are reduced in the responders compared to non-responders (Shinoda et al., 2012). Nevertheless, a deeper characterization of the memory immune cells is needed to follow-up this finding.

Because MDD patients have a significant remodelling of their immune response, we also analysed the effect of antidepressant treatments on the immune response, as antidepressants have been shown to modulate both cytokine production (Kenis and Maes, 2002) and immune cells (Miller and Raison, 2016). Because there were no differential effects in pro-inflammatory or anti-inflammatory cytokines in the different types of depression treatments,

we were able to model the profiles of MDD responders versus non-responders over the 12-week treatment period. The current results indicated that anti-inflammatory cytokines were increased in both MDD responders and non-responders, whereas only proinflammatory cytokines were stabilized in responders while they continued to increase in non-responders. This suggests that the antidepressant response may be associated with the ability of anti-inflammatory cytokines to block increasing levels of proinflammatory cytokines (e.g. IL-1β, IL-6, and TNF), while non-responders may have a defect in the response to anti-inflammatory cytokines. However, the mechanism of defective anti-inflammatory response remains to be determined.

It is unlikely that the difference in the production of cytokines between responders and non-responders is due to a reduction in the production of cytokines as upstream inflammasome activation is similar between responders and non-responders; thus, it is most likely due to an absence of the response to inflammatory termination signals. Consistent with this conclusion, the percent of $CD86^{+}CD11b^{+}$ cells, indicative of activated monocytes/dendritic cells cells, which represent activated cells of the innate immune response which in part regulate the inflammasome pathway (Guo et al., 2015), were similar between non-responders and responders. Furthermore, CD69, which is present on adaptive immune cells, was expressed similarly between non-responders and HC but it decreased in responders. Altogether, these findings suggest that the plasma of responders has anti-inflammatory and anti-activation properties that are absent in the non-responder group and are likely mediated by different cell types.

It is interesting to note that antidepressant actions, although likely expected to be different between escitalopram, duloxetine (Frampton and Plosker, 2007), and CBT (Hofmann et al., 2012), have similar effects on the immune response. Better mood outcomes were associated with a healthier immune response. It is possible that a direct central nervous system effect of the antidepressant medication and CBT influences the immune system response, perhaps by modulating HPA axis activity or the production of growth factors (McKay and Zakzanis, 2010). Because escitalopram and duloxetine are present in plasma, it is also possible that the medications directly affect the PBMCs (Greeson et al., 2016). However, because we found no difference between duloxetine, escitalopram and CBT in the activation levels of CD69, or cytokine levels, it is unlikely that the observed effects are due to a direct effect of the medication on PBMC activation, but rather results from downstream effects of the treatments leading to cytokine changes. In addition, it is possible that the absence of effects on the PBMC activation between responders and non-responders at the frequency level are due to the small changes of cytokine concentrations observed between these two groups. The differentiation of T cells, which is known to be regulated by cytokines (Zhu et al., 2009), might also be an important component to measure as T cells even though exhibiting the same level of activation might differentiate towards pro or anti-inflammatory subsets. It is particularly interesting that the regulatory T cells ($CD4^{+}CD25^{+}$), known to be anti-inflammatory cells, or CD8 cells or B cells expressing CD69 were reduced in responders compared to non-responders. This suggests that T and B cell activation markers might help to discriminate between patient's response to antidepressant treatments, consistent with a recent study of (Grosse et al., 2016).

Taken together, the results of this study show a broad effect of depression on the immune system, although unknown confounds may contribute to some of the differences reported here and that causality was not directly tested. Interestingly, effective treatments for depression modulate the inflammatory response, although these effects might be the results of epiphenomena e.g. sleeplessness, stress, weight loss associated with depression. Effective treatments seem to target both the innate and adaptive immune system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Treatment-naive depressed patients have elevated levels of inflammatory markers.
- Overall, plasma of treatment-naïve depressed patients is immunosuppressive.
- Defective anti-inflammatory response occurs in non-responders.

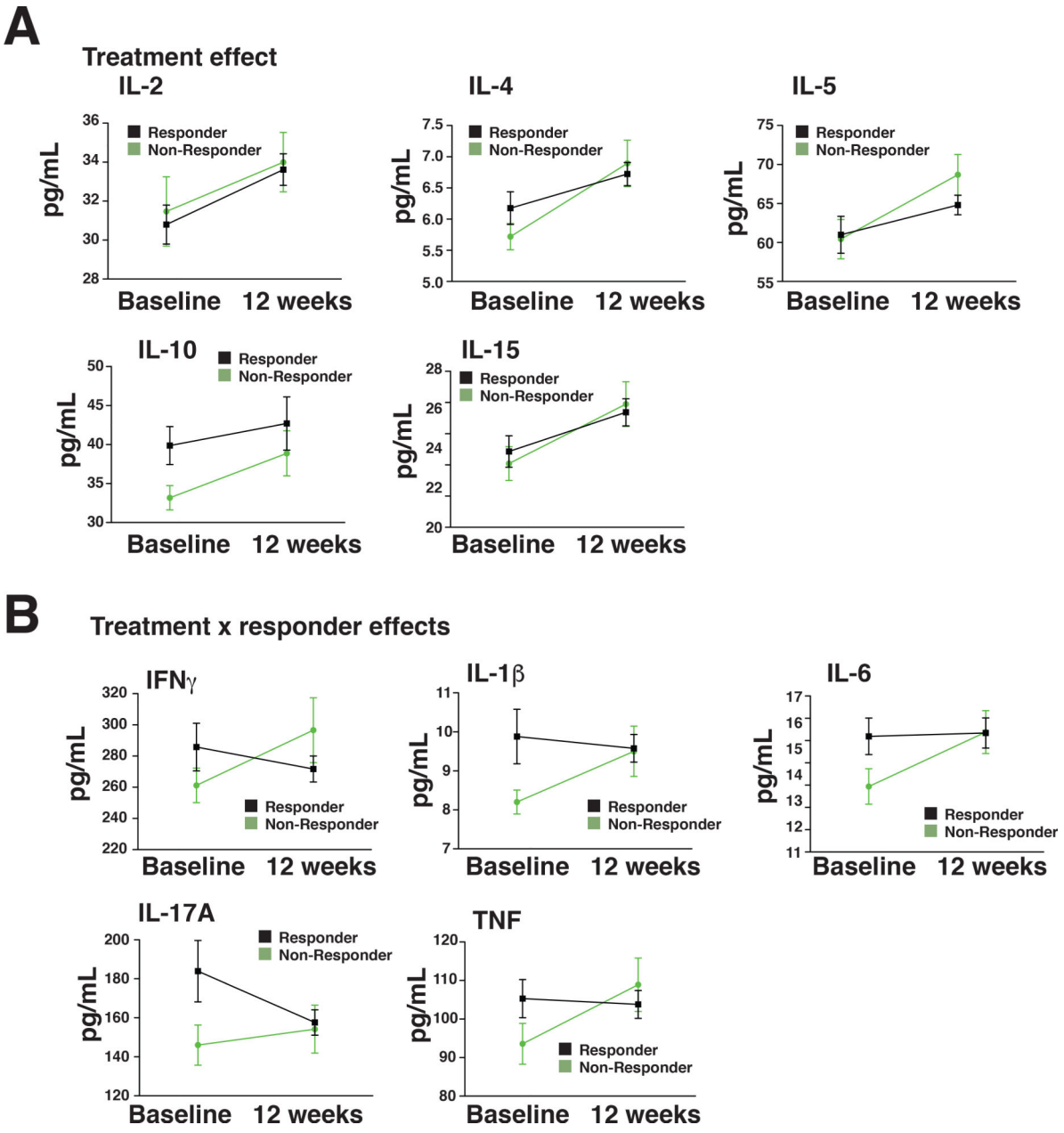

Figure 1: Anti-inflammatory cytokines increase in both responders and non-responders whereas proinflammatory cytokines are stabilized in responders but increased in non-responders.

Anti-inflammatory cytokines (A) and proinflammatory cytokines (B) were measured in the plasma of responders and non-responders, and the means were presented at baseline and 12 weeks after treatment.

Table 1.

Demographic characteristics of HC subjects and MDD patients

ANCOVA, mean ±SD, HSRD: Hamilton Depression Rating Scale, BDI: Beck depression Inventory, Major Dep ep: Major depressive episode, Fam hx MDD: Family history of MDD, BMI: body mass index

	HC n=64	**MDD n=171**	**p-*value***
Participants	64	171	-
Gender (F/M)	30/34	113/58	p=0.007 **
Age	45±11.8	39.4±11.8	p=0.001 **
BMI	29.2±7.2	28.8±6.18	p=0.728
Race, n (%)			p=0.562
Caucasian	8 (13%)	52 (30%)	
African-American	41 (64%)	41 (24%)	
Other	15 (23%)	78 (46%)	
Ethnicity			
-Hispanic	15 (23%)	72 (42%)	p=0.002 **
-Non-Hispanic	49 (77%)	99 (58%)	
Depression History			
HSRD-Baseline	-	20.0±3.73	
HSRD-Week12	-	7.78±3.73	
BDI-Baseline	-	23.3±6.78	
BDI-Week12	-	7.23±7.54	
# Major dep ep	-	5.15±16.7	
Fam hx MDD	-	57/171	

** p<0.01

Table 2:

Levels of cytokines, chemokines and growth factors in HC subjects and MDD patients

Due to differences between the HC subjects and the MDD patients, and to adjust for various covariates (age, gender, race), we performed a 1:1 match HC subjects and MDD patients and excluded any effects of the various covariates on the production of cytokines, chemokines and growth factors except for RANTES/CCL5, FGF and GM-CSF (Supplementary Figure 1). In addition, because there were a number of occasions where there was heterogeneity of variance, questionable distributional normality and an unequal n, a Non-Parametric Mann-Whitney U Test of Ranks test was also used, mean ±SD, CCL: chemokine ligand, FGF: Fibroblast Growth Factor, G-CSF: granulocyte colony-stimulating factor, GM-CSF: granulocyte-macrophage colony-stimulating factor, IFNγ: interferonγ, IL: interleukin, CXCL: chemokine (C-X-C motif) ligand, MCP-1: monocyte chemoattractant protein-1, MIP: macrophage inflammatory protein, PDGF: platelet derived growth factor, RANTES: regulated on activation, normal T cell expressed and secreted, TNF: tumor necrosis factor, VEGF: vascular endothelial growth factor

	HC Mean (pg/mL) n=64	**MDD Mean (pg/mL) n=171**	**MDD/ HC (%)**	**p-*value***
Proinflammatory cytokines				
Increased				
IL-12	4.6±6.2	60.3±36.0	1311	**p<0.001** **
TNF	27.4±14.7	101±47.9	369	**p<0.001** **
IL-6	11.6±13.0	41.2±16.5	355	**p=0.001** **
IFNγ	97.5±30.8	279±117	286	**p<0.001** **
IL-9	24.0±3.5	60.7±2.1	253	**p<0.001** **
IL-17A	93.4±65.8	182±173	195	**p<0.001** **
No change				
IL-1β	9.1±6.8	9.2±4.9	101	p=0.089
Anti-inflammatory cytokines				
Increased				
IL-10	13.7±73.2	37.9±21.0	277	**p<0.001** **
Decreased				
IL-1RA	394±280	290±170	74	**p=0.001** **
Other cytokines				
Increased				
IL-5	3.6±3.9	60.5±21.3	1681	**p<0.001** **
IL-15	2.1±0.0	23.0±7.8	1095	**p<0.001** **
IL-2	17.6±33.3	32.1±12.8	182	**p<0.001** **
IL-13	18.7±17.5	30.0±21.3	160	**p<0.001** **
Decreased				
IL-4	8.0±4.1	6.0±2.1	75	**p<0.001** **

	HC Mean (pg/mL) n=64	MDD Mean (pg/mL) n=171	MDD/ HC (%)	p-*value*
Chemokines				
Increased				
MIP1α/CCL3	2.0±0.6	13.6±1.6	680	**p<0.001** **
RANTES/CCL5	7310±856	12200±2270	167	**p<0.001** **
No change				
Eotaxin/CCL11	81.6±7.1	71.2±1.7	87	p=0.617
Decreased				
MIP1α/CCL4	61.4±3.06	50.7±1.9	83	**p<0.001** **
IL-8	20.7±9.5	16.3±6.0	79	**p<0.001** **
MCP-1/CCL2	116±9.3	69.1±4.8	60	**p<0.001** **
IP-10/CXCL10	1530±129	525±30.8	34	**p<0.001** **
Growth factors				
Increased				
G-CSF	10.0±2.5	106±3.4	1060	**p<0.001** **
PDGF	574±73.1	1290±126	225	**p<0.001** **
FGF	52.0± 2.9	104.3 ±3.9	201	**p<0.001** **
IL-7	31.8±11.4	38.7±13.4	122	**p<0.001** **
No change				
GM-CSF	62.8±1	62.2±2.8	99	**p=0.008** *
VEGF	81.6±12.4	64.4±5.3	79	p=0.320

* GM-CSF did not survive the Bonferroni correction

** p≤0.001

Table 3:

Inflammasome protein levels in HC subjects and MDD patients

Non-Parametric Mann-Whitney U Test of Ranks, mean ±SD, All measures survived Bonferroni correction.

ASC apoptosis associated speck-like protein containing a caspase recruitment domain, IL: interleukin

	HC Mean (pg/mL) n=24	MDD Mean (pg/mL) n=24	p-value
ASC1	233.9±85.7	443.9±164.7	**p<0.001***
Caspase-1	0.7± 0.6	2.7 ±1.6	**p<0.001***
IL-18	178.4±82.2	258.0±119.7	**p=0.013***

* p<0.05

Table 4:

Frequency of activated PBMC cells after stimulation with plasma of HC subjects and of MDD patients Non-Parametric Mann-Whitney U Test of Ranks, mean ±SD, All statistical significant results survived the Bonferroni correction. CD69 and CD86 are activation markers of the adaptive and the innate immune systems, respectively. $CD4^+CD25^+$ are T regulatory cells. $CD4^+CD45^+CD69^-$ are memory cells, and $CD4^+CD69^+CD45RO^-$ are effector T cells.

	HC Mean (%) n=27	**MDD Mean (%) n=40**	**p-value**
$CD4^+CD25^+CD69^+$	28.9±8.5	24.4±7.3	p=0.242
$CD4^+CD45RO^+CD69^-$	34.4±5.0	30.1±4.5	**p<0.001***
$CD4^+CD69^+CD45RO^-$	3.0±4.2	2.2±1.6	p=0.682
$CD8^+CD69^+$	25.3±8.0	20.9±7.7	p=0.182
$CD19^+CD69^+$	25.4±5.3	21.4±3.7	**p=0.001***
$CD11b^+CD86^+$	0.7±0.3	0.5±0.18	**p=0.005***

*p < 0.05

Table 5:

Pro- and anti-inflammatory cytokine levels in responders and non-responders

A: Proinflammatory cytokine levels in responders and non-responders: Repeated measure MANOVA, mean ±SD, *p<0.05, IFNγ: interferonγ, IL: interleukin, TNF: tumor necrosis factor. B: Anti-inflammatory cytokine levels in responders and non-responders Repeated measure MANOVA, mean ±SD

	Baseline Responder Mean (pg/mL) (n = 71)	**Baseline Non-Responder Mean (pg/mL) (n = 33)**	**12 week Responder Mean (pg/mL) (n = 71)**	**12 week Non-Responder Mean (pg/mL) (n = 33)**	**Effect Time F *p-value***	**Effect Responder F *p-value***	**Effect Time x Responder F *p-value***
A IFNγ	287.7±142.3	248.0±67.5	271.6±70.4	296.5±119.5	1.28 p=0.261	0.178 p=0.674	**5.05 p=0.027***
IL1β	10.2±6.6	7.9±1.9	9.6±3.0	9.5±3.7	1.092 p=0.299	2.060 p=0.154	**5.096 p=0.026***
IL-6	15.5±7.7	12.5±5.3	15.4±5.6	15.4±5.3	3.41 p=0.068	1.85 p=0.177	**4.3 p=0.041***
IL-17A	189.9±147.3	134.0±56.7	157.2±54.0	154.9±73.9	0.221 p=0.639	2.93 p=0.09	**4.56 p=0.035***
TNF	108.2±45.5	91.4±33.1	103.7±30.4	108.8±39.7	2.07 p=0.154	0.779 p=0.379	**5.93 p=0.017***
B IL2†	30.0±8.9	30.8±10.5	33.1±5.9	33.5±6.5	**5.97 p=0.018***	0.202 p=0.655	0 p=0.99
IL-4	6.2±2.4	5.5±1.3	6.7±1.5	6.8±2.1	**11.6 p=0.001***	0.884 p=0.35	3.05 p=0.084
IL-5	61.6±21.9	58.1±15.6	64.8±10.5	68.6±14.9	**10.5 p=0.002***	0.004 p=0.951	3.09 p=0.083
IL-10	40.1±22.3	32.5±9.7	42.7±28.7	38.9±16.6	**5.05 p=0.027***	1.74 p=0.19	0.913 p=0.342
IL-15††	23.4±5.4	21.6±5.4	25.2±5.7	25.1±5.7	**6.64 p=0.013***	0.541 p=0.466	0.573 p=0.453

*p<0.05, IL: interleukin

†31 responders-21 non-responders

††28 responders-21 non-responders

Table 6:

Frequency of activated PBMC cells after receiving plasma of responders and non-responders
Repeated measure ANCOVA, after Bonferroni correction, mean ±SD.

	Responder Baseline N=20	Non-Responder Baseline N=20	Responder 12 weeks N=20	Non-Responder 12 weeks N=20	Time effect F p-value	Responder effect F p-value	Time x responder effect F p-value
$CD4^+CD25^+CD69^+$	23.6±11.1	26.9±12.7	24.8±10.5	24.8±13.4	0.02 p=0.8	0.49 p=0.49	0.30 p=0.56
$CD4^+CD45RO^+CD69^-$	27.1±4.8	33.3±3.4	26.6±3.9	31.9±3.3	2.3 p=0.14	**20 p<0.001***	0.57 p=0.45
$CD4^+CD69^+CD45RO^-$	3.9±2.3	4.6±2.6	4.1±3.1	4.3±2.7	0.11 p=0.73	0.07 p=0.78	0.22 p=0.64
$CD8^+CD69^+$	19.2±8.7	23.2±11.5	20.5±8.9	22.6±9.7	0.035 p=0.85	1.5 p=0.21	0.21 p=0.65
$CD19^+CD69^+$	20.5±3.3	22.2±3.9	20.7±4.3	20.7±3.9	0.67 p=0.42	0.77 p=0.39	1.1 p=0.3
$CD11b^+CD86^+$	0.5±0.1	0.5±0.1	0.6±0.1	0.6±0.2	1.9 p=0.18	0.23 p=0.63	0.33 p=0.57

* p<0.005

Table 7:

CD69 expression (MFI) in PBMC cells receiving plasma from HC or of depressed patients
Repeated measure ANCOVA

	Responder Baseline N=20	**Non-Responder Baseline N=20**	**Responder 12 weeks N=20**	**Non-Responder 12 weeks N=20**	**Time effect F p-value**	**Responder effect F p-value**	**Time x responder effect F p-value**
$CD4^+CD25^+CD69^+$	296±127	313±142	270±121	318±165	0.5 p=0.82	**4.8 p=0.03***	0.01 p=0.93
$CD4^+CD45RO^+CD69^-$	677±107	627±116	579±80	614±139	2.5 p=0.12	1.7 p=0.20	0.6 p=0.44
$CD8^+CD69^+$	973±255	1171±473	952±308	1043±358	0.001 p=0.997	**7.1 p=0.01***	0.54 p=0.47
$CD19^+CD69^+$	1636±174	1816±248	1619±184	1704±217	**6.3 p=0.014**	**12.6 p=0.001***	3.5 p=0.065

* $p<0.05$