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The role of monocyte phenotype and steroid-related gene
expression in major depressive disorder

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1. Abstract

Background: Inflammation has been associated with major depressive disorder (MDD). Meta-analytic evidence has shown increased levels of circulating pro-inflammatory cytokines in groups of patients with MDD. However, little is known about the cellular source of these inflammatory signals. Recent studies have suggested that innate and adaptive immunity may be differentially affected in MDD. Neuroendocrine pathways tightly regulate cellular inflammation via glucocorticoids, such as cortisol. While some studies have found neuroendocrine disturbances in MDD, it has remained unclear whether impairments in glucocorticoid signalling are cell-specific. Therefore, we interrogated phenotype and glucocorticoid signalling of key innate (monocytes) and adaptive (T cells) immune cell populations in patients with MDD and healthy controls (HC).

Methods: 35 well-characterized antidepressant-free patients with MDD and HC individually matched for age, sex, smoking status and body mass index were enrolled. All participants were free of immunomodulatory medications or significant medical illness and non-pregnant. Immunophenotyping was performed by flow cytometry following established guidelines. Cell-specific steroid signalling was determined by mRNA expression of pre-receptor regulation (11 β -hydroxysteroid dehydrogenase type 1 [*11 β -HSD1*]), receptor expression (glucocorticoid [*GR*] and mineralocorticoid receptor [*MR*]), and its main downstream target (glucocorticoid-induced leucine-zipper [*GILZ*]). Salivary cortisol (collected on two consecutive days at 8 a.m. and 10 p.m.) and serum levels of IL-6, IL-1 β and TNF- α were analysed with ELISA.

Paired-samples t-tests were used for continuous variables and McNemar's Test for dichotomous variables. Repeated-measures ANOVA was used for cortisol values. Effects sizes were calculated as Hedges' *g*.

Results: In patients with MDD, we observed a shift in monocyte repertoire towards elevated frequencies of non-classical monocytes ($p < 0.01$; Hedges' $g = 0.88$). In contrast, frequency and phenotype of major lymphocyte subsets were similar ($ps > 0.05$). Furthermore, monocytes but not T cells from patients with MDD showed lower expression of *GR* ($p = 0.018$; Hedges' $g = 0.21$) and *GILZ* ($p = 0.045$; Hedges' $g = 0.39$), indicative of steroid resistance. Finally, altered monocyte phenotype and steroid gene expression occurred against the backdrop of unchanged salivary cortisol ($p = 0.32$) and serum cytokine levels ($ps > 0.05$).

Conclusion: Our results suggest that in MDD regulatory mechanisms of inflammation are affected in a cell-specific manner. More specifically, we found a shift towards a pro-

inflammatory phenotype and gene expression consistent with steroid resistance that was restricted to monocytes and occurred without neuroendocrine alterations. Our results could outline avenues for tailored cell-specific treatments to target aberrant inflammation in MDD.

2. Abstract (German)

Hintergrund: Entzündliche Prozesse stehen mit der Pathophysiologie von Depression (MDD) in Verbindung. Meta-Analysen deuten auf erhöhte Spiegel von zirkulierenden pro-inflammatorischen Zytokinen in Patientengruppen hin. Bisher ist nur wenig über den zellulären Ursprung dieser entzündlichen Signale bekannt. Jüngere Untersuchungen konnten zeigen, dass adaptive und angeborene Immunität bei MDD differentiell beeinträchtigt sein könnte.

Glukokortikoide wie Cortisol sind potente neuroendokrine Regulatoren von Inflammation. Eine veränderte neuroendokrine Regulation ist bei Patienten mit MDD beschrieben worden, allerdings ist unklar, ob diese neuroendokrinen Veränderungen zell-spezifisch auftreten. Das Ziel dieser Arbeit ist es, Phänotyp sowie Steroidregulation von Monozyten und T-Zellen, den wichtigsten Zellpopulationen von angeborenem und erworbenem Immunsystem, bei Patienten mit MDD und gesunden Kontrollen (GK) zu untersuchen.

Methoden: 35 Antidepressiva-unmedizierte Patienten mit MDD und 35 GK (individuell parallelisiert nach Alter, Geschlecht, Raucherstatus und Body-Mass-Index) wurden für die Studie rekrutiert. Alle Probanden waren frei von immunmodulatorischer Medikation, ohne signifikante medizinische Begleiterkrankungen und nicht-schwanger.

Immunphänotypisierung wurde mithilfe von Durchflusszytometrie gemäß etablierter Standards durchgeführt. Zell-spezifische Steroidregulation wurde anhand der mRNA-Expression auf Ebene von prä-Rezeptor- (11β -hydroxysteroid dehydrogenase type 1 [11β -HSD1]), Rezeptor- (Glukokortikoid- [GR] und Mineralokortikoidrezeptor [MR]) sowie post-Rezeptor-Ebene (glucocorticoid-induced leucine-zipper [$GILZ$]) bestimmt. Speichel-Cortisol zweier konsekutiver Tage (8:00 und 22:00 Uhr) sowie IL-6, IL-1 β und TNF- α -Serum-Spiegel wurden mittels ELISA analysiert. Kontinuierliche Variablen wurden mittels des t-Tests für verbundene Stichproben, dichotome Variablen mit McNemar's Test und Speichel-Cortisol mit einer messwiederholten Varianzanalyse ausgewertet. Hedges' g wurde als Maß der Effektstärke berechnet.

Ergebnisse: Bei Patienten mit MDD zeigte sich eine signifikante Verschiebung des Monozyten-Repertoire hin zu einer erhöhten Frequenz von nicht-klassischen Monozyten ($p < 0.01$; Hedges' $g = 0.88$). Dem gegenüber gab es keine Gruppenunterschiede in Frequenz oder Phänotyp wichtiger Lymphozyten-Populationen ($ps > 0.05$). In Einklang hiermit zeigten nur Monozyten, nicht aber T-Zellen, von Patienten mit MDD geringere mRNA-Level von GR ($p = 0.018$; Hedges' $g = 0.21$) und $GILZ$ ($p = 0.045$; Hedges' $g = 0.39$) als Monozyten von Kontrollen, was

auf Steroidresistenz hindeutet. Diese Veränderungen waren unabhängig von Speichel-Cortisol- ($p = 0.32$) und Serum-Zytokin-Spiegeln ($ps > 0.05$).

Conclusio: Unsere Ergebnisse deuten darauf hin, dass die Regulation von Inflammation bei Patienten mit MDD auf zell-spezifische Weise beeinträchtigt ist. Es zeigte sich eine Verschiebung hin zu einem pro-inflammatorischen Phänotypen gekoppelt mit einer Monozyten-spezifischen Genexpression, die mit Steroidresistenz konsistent ist. Diese Veränderungen traten bei depressiven Patienten ohne Erhöhung der Speichel-Cortisol-Spiegel auf. Diese Ergebnisse könnten Hinweise für zukünftige zell-spezifische Behandlung von entzündlichen Prozessen im Rahmen von MDD liefern.

3. Major depressive disorder, the immune system and stress hormones

3.1 Background

Major depressive disorder (MDD) is a highly prevalent mental illness that is associated with severe mortality and morbidity (1). Several hypotheses about its pathobiology have been put forward, including alterations in (monoaminergic) neurotransmission (2,3), physiological stress systems such as the hypothalamus-pituitary-adrenal (HPA) axis (4) or neurotrophic factors (5). More recently, links between MDD and aberrant immune processes have been suggested (6). For example, observational studies found that infectious illnesses increase the risk of developing MDD (7). Reversely, an MDD diagnosis is a risk factor for major somatic illnesses and infections (8–10). Animal studies and preclinical work has lent further support to the role of immune deregulation in depression. Inflammatory stimuli, such as typhoid vaccines, reliably evoke symptoms reminiscent of MDD, including fatigue, anhedonia and sleep disturbances, in healthy volunteers (11). Termed “sickness behaviour”, these effects are likely mediated by the actions of pro-inflammatory molecules on central nervous system pathways that strongly overlap with MDD-associated networks (12).

Meta-analyses have strengthened earlier reports that levels of circulating pro-inflammatory cytokines, such as interleukin-6 (IL-6) or tumour necrosis factor α (TNF- α), are raised in a subgroup of patients with MDD (13,14). However, little is known about their specificity and cellular origin. In fact, a wide range of immune-competent cells secrete inflammatory cytokines, including T cells, monocytes, macrophages and natural killer (NK) cells. As it is now increasingly recognised that these cells may play different roles in the aetiology of MDD, their inflammatory signals are likely to underpin different biological functions. To illustrate this point further, MDD does not seem to be underlined by uniform immune (de)activation, but more subtle disturbances in adaptive and innate immunity (15,16). For example, evidence in support of innate immune activation in MDD has accrued, including upregulated expression of pro-inflammatory genes by monocytes (17,18), elevated levels of circulating pro-inflammatory cytokines (13,14) and a link with allelic variations for pro-inflammatory cytokine genes (19). Conversely, adaptive immunity and especially T cells function seems to be impaired (16,20–22). Thus, not all immune branches may be impaired equally. One pathway that could explain this specificity is the HPA axis. Glucocorticoids (GCs), such as cortisol, are potent regulators of inflammation. They exert anti-inflammatory effects primarily by binding to glucocorticoid receptors (GR) on leukocytes. When liganded, the GR translocates from the cytoplasm to the

nucleus of the target cell, where it regulates transcription of several inflammation-related genes. For example, GCs modulate T cell trafficking, apoptosis, proliferation and cytokine production (23) and favour differentiation of monocytes with anti-inflammatory features (24). Interestingly, there is some evidence of blunted responsiveness to GCs in T cells from patients with MDD (25–28). These deficits have mostly been interpreted as blunted GR functioning. Likewise, initial evidence suggests that monocytes and peripheral blood mononuclear cells (PBMCs, a cell population that contains lymphocytes, monocytes and a small fraction of dendritic cells) show similar alterations (17,18,29,30). Thus, cell-specific GC dysfunction provides an attractive framework that integrates deregulations in certain immune compartments and the HPA axis.

Previous studies have largely focused on GR function, disregarding other levels of regulations that individually or collectively may bear relevance to MDD. In fact, GC signals in lymphocytes and monocytes are transmitted along a cascade of pre-receptor, receptor and post-receptor regulation. For example, on a pre-receptor level, immunocompetent cells regulate the conversion of biologically inactive cortisone to cortisol via 11 β -hydroxysteroid dehydrogenase type 1 (11 β -HSD1) (31). Genetic ablation of 11 β -HSD1 was linked with an antidepressant-like phenotype in an animal model of depression (32), supporting its possible relevance. Cortisol's main biological signals are transmitted via stimulation of the GR and mineralocorticoid receptor (MR), two nuclear receptors that reside in cytoplasm. Upon ligand binding, they translocate to the cell nucleus and suppress or enhance the transcription of target genes. As mentioned above, liganded GR induces a breadth of anti-inflammatory effects via the suppression of pro-inflammatory (e.g., interleukin-1) (33) and the stimulation of anti-inflammatory gene expression (e.g., interleukin-10) (34). A host of preclinical and clinical studies also supports the immunomodulatory role of MR stimulation (35) and there is evidence to suggest that MR function may be impaired in MDD (36). Finally, most of the anti-inflammatory signals of GCs are mediated by the glucocorticoid-induced leucine zipper (GILZ), a protein induced upon GR stimulation (37). Interestingly, several observations (38,39) have independently linked GILZ to MDD, although not yet in a cell-specific manner.

In the context of MDD, GR function has mainly been assessed in single cell populations (mostly lymphocytes), lacking appropriate comparator populations. This means that whether differences in GR function occur in a cell-specific manner has not been conclusively investigated. Given accruing evidence that MDD is underpinned by subtler imbalances between immune compartments, however, this might have overlooked cell-specific differences. Finally, MDD is a highly heterogeneous illness that is associated with a width of comorbid diseases, unfavourable

demographical characteristics and behaviours (1). As many of these characteristics affect immune function, they need to be carefully considered in participant selection. As a case in point, smoking (40) and body mass index (41) affect a diffuse set of immune parameters and, on average, patients with MDD have a higher prevalence of smoking (42) and higher body mass indices (1) than the general population.

To sum up, several strains of evidence suggest that in MDD impaired regulation of inflammation may be limited to certain immune branches. This divergence could be mediated by cellular alterations in GC function. However, a rigorous examination of this hypothesis is still missing. The aim of this thesis was to characterise the phenotype and GC-related gene expression of monocytes and T cells, key mediators of innate and adaptive immunity.

3.2 Participants and methods

3.2.1 Participants and study assessment

Patients with MDD were recruited either from on-site psychiatrist referral, Charité Campus Benjamin Franklin Clinic for Psychiatry and Psychotherapy inpatient ward or via online advertisements. Healthy controls were exclusively recruited via online advertisements. Main exclusion criteria for both groups were significant medical illness (e.g., diabetes, autoimmune or infectious illnesses), current immunomodulatory treatment or pregnancy. MDD patients unanimously had a psychiatrist-confirmed MDD diagnosis, no antidepressant treatment for at least two weeks and no other psychiatric disorders except milder anxiety disorders (cf. Table 1). Healthy controls were free of any psychiatric illness (including subclinical depressive symptom) and were not permitted to have a first-degree relative with any affective disorder. A total of 35 patients with MDD and healthy controls satisfied all criteria and were enrolled in the study.

All participants completed several self-report questionnaires including Beck Anxiety Inventory (BAI) (43), Beck Depression Inventory II (BDI-II) (44) and Childhood Trauma Questionnaire (CTQ) (45). A psychiatric interview (Mini-International Neuropsychiatric Interview) (46) was then conducted before participants' depression severity was rated using the Montgomery Asberg Depression Rating Scale (MADRS) (47). These steps were all performed by a single trained rater (the author of this thesis). Sample characteristics including routine blood works performed by an affiliated diagnostic lab (Labor Berlin—Charité Vivantes GmbH, Germany) are elaborated in Table 1. An individual overview over all study participants is given on pages 58-60.

For biological analyses, patients with MDD and healthy controls were matched on age, BMI, sex and smoking status to create “twin pairs” that minimize the impact of confounding variables.

		MDD	HC	p
Demographic parameters	Age	31.7 ± 11.2 years	31.7 ± 10.2 years	> 0.99
	BMI	23.9 ± 3.6 kg/m ²	23.5 ± 3.2 kg/m ²	> 0.05
	Females	25 (71.4 %)	25 (71.4 %)	> 0.99
	Smokers	12 (34.3 %)	12 (34.3 %)	> 0.99
Clinical parameters	MADRS	24.9 ± 5.3	1.2 ± 1.8	< 0.01 [†]
	BDI-II	29.9 ± 6.5	2.9 ± 3.4	< 0.01 [†]
	BAI	20.7 ± 12.4	3.8 ± 2.9	< 0.01 [†]
	CTQ*	40.9 ± 15.9	32.9 ± 9.8	0.03 [†]
	Comorbid anxiety disorder	10 (28.6 %)	-	-
	Inpatients	18 (51.4 %)	-	-
	Recurrent disease course	28 (80 %)	-	-
	Previous episodes	3.8 ± 1.7	-	-
Blood count	Lymphocytes	1.9 ± 0.7 /nl	1.9 ± 0.5 /nl	0.56
	Monocytes	0.5 ± 0.2 /nl	0.5 ± 0.2 /nl	0.67
	Eosinophils	0.2 ± 0.2 /nl	0.2 ± 0.2 /nl	0.66
	Basophiles	0.04 ± 0.03 /nl	0.05 ± 0.03 /nl	0.14
	Neutrophils	3.3 ± 1.5 /nl	3.6 ± 1.8 /nl	0.42
	CRP	1.6 ± 1.7 mg/dl	1.4 ± 1.5 mg/dl	0.54

Table 1. Demographic, clinical and blood count parameters for patients with MDD (n=35) and healthy controls (n=35)

BAI: Beck Anxiety Inventory, BDI-II: Beck Depression Inventory II, BMI: body mass index, CTQ: Childhood Trauma Questionnaire, HC: healthy control, MADRS: Montgomery Asberg Depression Rating Scale, MDD: patients with major depressive disorder

Values represent mean ± standard deviation for continuous variables and n (%) for dichotomous variables. Blood count is shown in cells / nl or mg / dl. Paired-samples t-test was used for continuous and McNemar's test for dichotomous variables. *CTQ total score [†]significant at p < 0.05

3.2.2 Sample collection and handling

For biological analyses, all participants provided 70ml of venous blood that was collected in heparinized tubes for immune cell-specific analyses and serum separator tubes for cytokine assessment. A strict 12h fasting regiment prior to sampling was required for all participants and all samples were drawn between 8.00 a.m. and 9:30 a.m. to minimize circadian confounding. Participants also provided diurnal saliva samples from two days chosen to resemble typical weekdays (i.e., excluding with extraordinary events such as exam or stressful medical interventions). Handling and analysis of all biological material followed protocols optimized in our lab for the specific readouts of this study. Detailed explanation of all steps including cell isolation, sample handling and storage is provided on pages 48-50). Finally, all experimental and study parameters were equalized between groups to achieve maximum internal validity.

3.2.3 Flow cytometry

Samples were prepared for flow cytometry analyses using a pre-tested, optimized protocol that is described together with all antibody panels and clones on pages 49 and 57,

respectively. Identification of leukocyte subpopulations followed state-of-the-art recommendations for general immunophenotyping in humans (48). Briefly, we identified lymphocytes by forward and sideward scatter properties, followed by exclusion of doublets and dead cells. Next, we selected T cells based on CD3 positivity and non-T cells based on CD3 negativity. T cells were further subdivided into CD4⁺ T helper and CD8⁺ cytotoxic T cells. Finally, among CD4⁺ T cells, we used the expression of the CD25 and CD127 to define regulatory T cells as CD4⁺CD25⁺CD127 (49). Among non-T cells, B cells were identified using CD20, a common pan-B cell marker. Next, among CD20⁻ cells, pan-natural killers cells were defined as CD56 positive and CD14 negative. Lastly, among natural killer (NK) cells, we defined cytotoxic NK cells (NKc) as CD56⁺CD16⁺ and NK cells with a putatively regulatory phenotype (NKreg) as CD56⁺CD16^{dim/-} as suggested by (48).

While there is a certain consensus regarding gating strategies for lymphocytes, accurate monocyte subtype identification can be more challenging (50). Therefore, we used an optimized monocyte identification approach for reliable detection of classical, non-classical and intermediate monocytes (Figure 1).

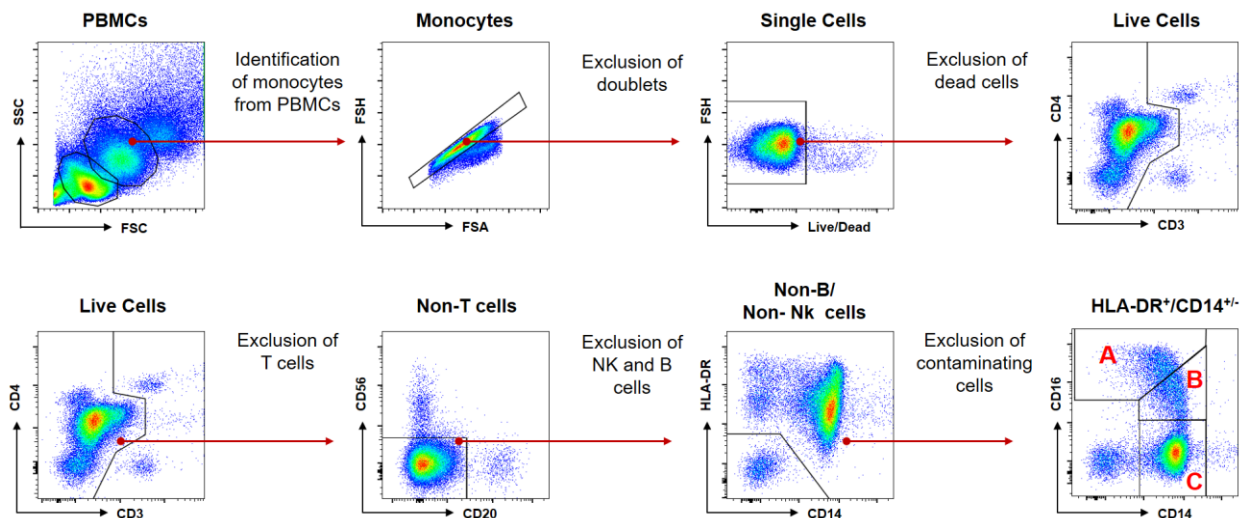


Figure 1. Identification of classical, intermediate and non-classical monocytes

Monocytes were identified by forward (FSC) and sideward scatter (SSC), followed by exclusion of doublets, dead cells, remaining T cells, B cells (CD20⁺) and natural killer (NK) cells (CD56⁺) as well as CD14⁻/HLA-DR⁻ cells (e.g., neutrophils). Monocytes were classified as either non-classical (A: CD14⁺CD16⁺⁺), intermediate (B: CD14⁺⁺CD16⁺) or classical (C: CD14⁺⁺CD16⁻) following established guidelines (51).

Manual gating is subject to significant inter-individual and inter-lab variability (48). To the author's knowledge, this thesis is among the first to employ automated analysis to flow cytometry data in the context of MDD. More specifically, CITRUS (cluster identification, characterization, and regression) offers an automated, data-driven method for highly reliable cell subsets identification in flow cytometry (52). Briefly, CITRUS first identifies clusters of cells

that are phenotypically similar in an unsupervised manner by using hierarchical clustering. Next, it extracts biologically relevant features of each cluster on a per-sample basis and feeds them into a regularized regression model for predictive or correlative purposes.

Visualization of t-distributed stochastic neighbor embedding (viSNE) is an algorithm that provides a two-dimensional categorized map representation of multi-parameter single cell data. There are several parameters that allow further fine-tuning of viSNE maps, including perplexity, theta and number of iterations. Perplexity refers to the degree of spatial separation of events on the viSNE map and was set to 50 to provide a distinct distribution of cell populations. Theta is a measure of balance between speed and accuracy in the viSNE map and was set to 0.5. Number of iterations was set to 5000 to provide adequate resolution of the cellular populations on the two-dimensional viSNE map. We used equal sampling with 10^4 events per sample. The input population (either CD3⁺ T cells and/or CD3⁻ non-T cells) as well as the selection of clustering channels depended on the flow cytometry panel that was analysed. For non-T cells, cell populations were pre-gated to exclude debris, doublets, dead cells as well as CD3⁺ T cells. CD14, CD16, HLA-DR, CD45RA and CD20 were selected as clustering channels.

Next, a cluster identification, characterization, and regression (CITRUS) analysis was conducted using the same input populations. In the case of non-T cells, minimum cluster-size threshold (MCST, i.e. the minimum frequency of total input events that the smallest clusters must contain) was set to 1.5% to ensure that all populations identified via manual gating were included in the analysis. The false discovery rate (FDR) was generally set to 1%. To determine stratifying features between the study groups, we decided to run a correlative association model (significance analysis of microarrays = SAM), which created a list of clusters whose abundance is associated with the experimental (MDD) group.

3.2.4 T Cell and monocyte purification and RT-qPCR

To study cell-specific gene expression, live T cells and monocytes were purified from PBMCs using magnetic activated cell sorting (MACS) with CD3 and CD14 MicroBeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Briefly, MicroBeads are antibody-coated magnetic nanoparticles that bind to specific cell surface markers that can be either expressed on the cell population of interest (positive selection) or unwanted cells (negative selection). After incubation with the MicroBeads, the cell suspension is applied to a separator column on a magnet, allowing the unlabelled cell populations to pass through while the

population of interest is retained in the column. With our protocol, we achieved a mean cell purity (\pm SD) of 96.5% (\pm 1.2%) for T cells and 92.3% (\pm 1.7%) for CD14⁺ monocytes (n=5).

RNA was isolated from purified T cells and monocytes using the Qiagen RNeasy Plus Mini Kit (Qiagen GmbH, Hilden, Germany) following manufacturer's instruction. In line with established guidelines, only samples with adequate purity were transcribed to complementary DNA (cDNA). cDNA was synthesised from RNA without intermittent freezing using random hexamer primers and the RevertAid H Minus First Strand cDNA Synthesis Kit (ThermoFisher Scientific, Germany). cDNA was then used as a template to study the expression of key genes along the GC signalling cascade with a TaqMan Gene Expression Assay (ThermoFisher Scientific, Darmstadt, Germany). Target genes included the glucocorticoid receptor (*GR*; Hs00353740_m1), mineralocorticoid receptor (*MR*; Hs01031809_m1), glucocorticoid-induced leucine zipper (*GILZ*; Hs00608272_m1) and 11 β -hydroxysteroid dehydrogenase type 1 (*11 β HSD-1*; Hs01547870_m1). Real-time reverse transcription-polymerase chain reaction (RT-qPCR) was used to quantify messenger (m)RNA levels. Gene expression was referenced to two internal controls, importin 8 (*IPO8*; Hs00183533_m1) and TATA Box Binding Protein (*TBP*; Hs00427620_m1), that are invariantly expressed in both T cells (53) and monocytes (54)s.

3.2.5 Enzyme-linked Immunosorbent Assay (ELISA)

ELISA was used to quantify saliva cortisol and serum levels of interleukin-6 (IL-6), interleukin-1 β (IL-1 β) and tumour necrosis factor alpha (TNF- α). A competitive enzyme-linked immunosorbent assay (IBL, Germany) was used for the analysis of saliva cortisol. Serum cytokines were analysed with high-sensitivity sandwich ELISA (R&D Systems Europe, UK). All ELISAs were conducted following manufacturer-optimized instructions with matched MDD/HC pairs on the same microplate. All mean inter- and intra-assay coefficients of variations were <10%. For detection limits and technical specifications, see page 50.

3.2.6 Statistical analyses

Cell populations identified via flow cytometry were expressed as percentage of a suitable reference population or, where possible, shown as absolute cell counts. SPSS version 21 (IBM Inc., USA) and GraphPad Prism version 7 (GraphPad Software Inc., USA) were used for statistical analyses. FlowJo version 10.1 (Treestar Inc., USA) and Cytobank analysis software (Cytobank Inc., USA) were used for the analysis of flow cytometry data.

Because of the close matching of patients with MDD and healthy controls (Table 1), paired-samples t-test was used for the analysis of continuous and McNemar's test for the analysis of

dichotomous variables statistics (55). A repeated-measures analysis of variance (ANOVA) was run to assess differences in cortisol between groups (two levels, MDD vs. HC) and time (four levels, day 1 8:00 am vs. day 1 10:00 pm vs. day 2 8:00 am vs. day 2 10:00 pm). Hedges' g was calculated a measure of effect size for t-tests and partial η^2 (η^2) for ANOVA, respectively. To assess linear relationships between variables of interest, we used Spearman's correlation coefficient to account for non-normal data distribution (for brevity, results from correlation analyses are depicted on pages 61-62).

3.3 Results and discussion – A role for monocytes in MDD?

3.3.1 Patients with MDD show a shift towards non-classical monocytes

Patients with MDD showed a significantly higher relative frequency of non-classical ($p < 0.0001$) and intermediate ($p = 0.02$) monocytes and a down-regulation of classical monocytes ($p < 0.0001$). This shift in monocyte subset composition was present in 82% of patient control pairs. When referenced to absolute monocyte counts, it became evident that this shift was driven by an expansion of non-classical ($p < 0.01$) and intermediate monocytes ($p = 0.02$). Reversely, there were no changes in major T cell or natural killer (NK) cell subpopulation (all p values > 0.05 ; Table 2). The same pattern was reproduced using an unsupervised clustering algorithm (CITRUS), which showed group differences in the abundance of only those clusters that were consistent with intermediate and non-classical monocytes expressing CD16 (Figure 2).

		MDD	HC	p*	g ⁺
Monocytes	Classical				
	<i>Relative frequency</i>	83.3 ± 7.9 %	88.8 ± 4.4 %	< 0.01 [†]	0.79
	<i>Absolute cell count</i>	0.42 ± 0.16 /nl	0.42 ± 0.14 /nl	0.89	0.03
	Intermediate				
	<i>Relative frequency</i>	5.8 ± 3.4 %	4.2 ± 1.7 %	0.02 [†]	0.58
	<i>Absolute cell count</i>	0.03 ± 0.02/nl	0.02 ± 0.01 /nl	0.02 [†]	0.66
Non-classical					
	<i>Relative frequency</i>	10.9 ± 6.4 %	7.0 ± 3.4 %	< 0.01 [†]	0.81
	<i>Absolute cell count</i>	0.05 ± 0.03 /nl	0.03 ± 0.02 /nl	< 0.01 [†]	0.87
Lymphocytes	CD4 ⁺ T cells	61.8 ± 10.5 %	63.7 ± 11.3 %	0.45	0.17
	CD8 ⁺ T cells	30 ± 7.9 %	29.3 ± 8.9 %	0.69	0.09
	TRegs	6.6 ± 1.7 %	6.7 ± 1.5 %	0.65	0.09
	NKc cells	13.9 ± 9.1 %	10.9 ± 5.5 %	0.13	0.4
	NKReg cells	0.8 ± 0.6 %	0.8 ± 0.3 %	0.84	0.05
	B cells	7.4 ± 2.9 %	6.9 ± 2.9 %	0.57	0.17

Table 2. Frequencies of monocyte and lymphocyte subpopulations (mean ± standard deviation)

HC: healthy controls; MDD: patients with major depressive disorder; NKc cells: cytotoxic natural killer cells; NKReg cells: regulatory natural killer cells; TRegs: Regulatory T cells. Relative frequency was determined using flow cytometry as described in 3.2.3. Absolute cell count was only available for monocytes.

* calculated using paired-samples t-test ⁺ Hedges' g [†] significant at $p < 0.05$

Human monocytes are generally short-lived and transition from classical ($CD14^{++}CD16^{-}$) via intermediate ($CD14^{++}CD16^{+}$) to mature non-classical ($CD14^{+}CD16^{++}$) monocytes. Monocyte subtypes are characterised by significant functional heterogeneity. While classical monocytes are mostly phagocytic (expressing high levels of the scavenger receptors CD36 and CD163) and devoid of inflammatory features, non-classical monocytes are highly pro-inflammatory (50). For example, they are potent producers of pro-inflammatory cytokines and underlie a wide range of conditions characterised by chronic inflammation, including obesity (56), cardiovascular illness (57,58), neuromyelitis optica spectrum disorder (59) and arteriosclerosis (60).

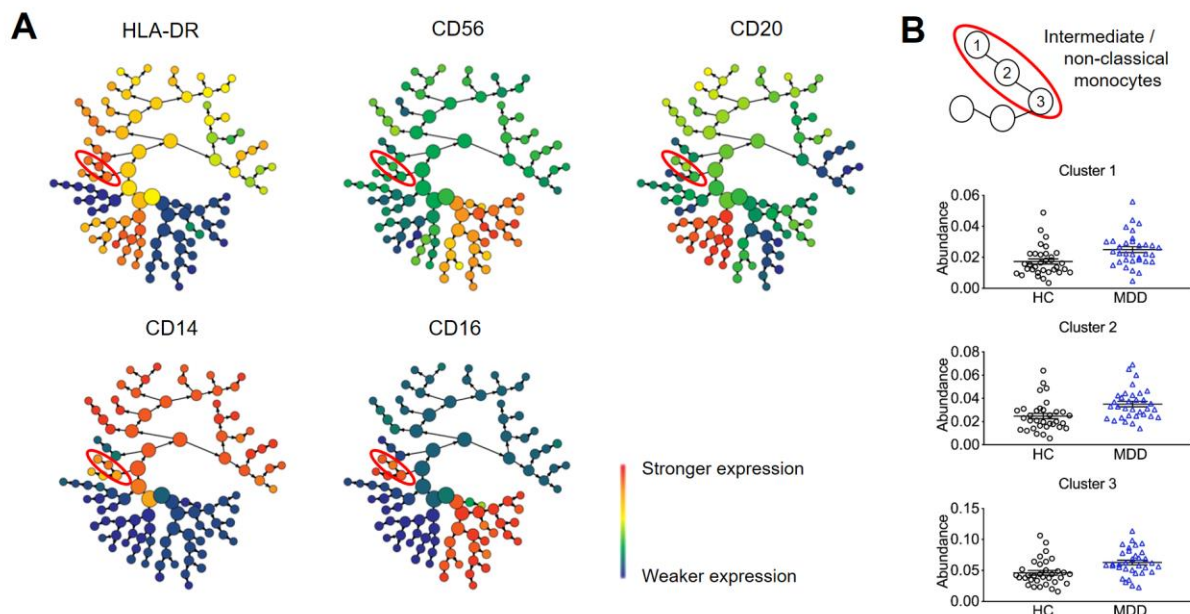


Figure 2. Identification of monocyte subtypes using CITRUS (A) and comparison of abundance of clusters representing monocytes expressing CD16 (B)

A: CITRUS hierarchical clustering of cellular populations based on phenotypical similarity. $CD3^{-}$ cells were used as input population (after removal of debris, doublets and dead cells) and HLA-DR, CD56, CD20, CD14 and CD16 were chosen as clustering channels to identify monocyte subpopulations (10^4 events sampled per file, minimum cluster size threshold of 1.5%). Clusters likely representing classical ($CD14^{++}/CD16^{-}$), intermediate ($CD14^{++}/CD16^{+}$) and non-classical monocytes ($CD14^{+}/CD16^{++}$) are highlighted in red.

B: Abundance of clusters 1, 2 and 3, previously identified as being consistent with monocytes expressing CD16 (A) per sample. Values shown as mean \pm standard error of the mean. Group differences were evaluated using paired-samples t-tests and were all significant at $p < 0.01$. No group difference in any other cluster reached statistical significance ($p < 0.05$: results not shown).

This study is the first to directly demonstrate a role of non-classical monocytes in the pathophysiology of MDD. This assumption receives support from several converging lines of research. First, convincing evidence suggests that they underlie conditions of persistent low-grade inflammation (see above) and a chronically inflamed milieu has also been observed in MDD patients (11). Interestingly, several of these conditions show a high comorbidity with MDD, suggesting inflammation may be a common disease pathway (1). Second, non-classical monocytes are the main source of monocytic $TNF-\alpha$, pro-inflammatory cytokine associated

with MDD (13), also independently of medical illness (61). Third, both MDD as well as pro-inflammatory monocytes have been associated with chronic stress (62,63). Fourth, a similar expansion in non-classical monocytes was recently observed in paediatric obsessive-compulsive disorder (64), a condition whose neurobiology strongly overlaps with MDD (65). What is more, among patients, those treated with an antidepressant had intermediate levels of non-classical monocytes between untreated patients and healthy controls. Fifth, non-classical monocytes have been associated with mood disturbances in women with HIV infection (66). Thus, there are good grounds to assume that monocyte subsets play a role in MDD.

This thesis is in contrast to earlier investigations (67–69). In one of the first studies, Schlatter and colleagues (67) found similar frequencies of CD14⁺ and CD16⁺ cells among individuals with MDD, dysthymia and healthy controls. Monocytes are highly heterogeneous and accurate monocyte subtype identification can be challenging as CD16 (the constituent marker of intermediate and non-classical monocytes) is also expressed on other cells, including neutrophils and Nk cells. CD14⁺ and CD16⁺ are thus insufficient for reliable characterisation of monocyte subtypes. What is more, the study by (67) had a relatively small sample size and was likely not matched on several important parameters of immune function, including BMI (70) and smoking (40).

There are good grounds for assuming that the significant clinical heterogeneity of MDD is mirrored in its underlying pathobiology. The results by Duggal and colleagues (68) are likely to provide an example of this. More specifically, they found that elderly hip fracture patients (minimum enrolment age was 60 years) who developed mild symptoms of depression following surgery did not have an altered monocyte subset distribution compared to controls. Besides the differences in age, it should be noted that the patients had generally low, sub-clinical levels of depression and had not received a formal diagnosis (in fact, lifetime diagnosis of MDD was exclusion criteria). For these reasons, a direct comparison to our findings is challenging.

Finally, Suzuki and colleagues (69) reported similar levels of classical and non-classical monocytes accompanied by changes in lymphocyte populations in patients with MDD and healthy controls. While their design was similar, several explanations could account for the differences to our results. First, our participants met stricter inclusion criteria and were free of significant medical illness, recent vaccinations or medications with known immunomodulatory effects (including non-steroidal anti-inflammatory drugs). Second, and in a similar vein, the patients with MDD studied by Suzuki and colleagues had a significantly higher BMI compared to the control group. In fact, with 29.6 kg/m² the average BMI in the MDD group was close to

the established obesity cut-off. This may be important because differences in BMI are associated with significant variation on immune parameters, including major lymphocyte (41) and monocyte populations (70). Given the complexity of the immune system, statistically controlling for these using linear procedures (e.g., analysis of covariance) may be inadequate. Finally, more than half of MDD patients in their study were either mildly depressed (n=11) or in partial remission (n=18). Conversely, the cohort studied in this thesis consisted of well-characterised individuals (mild episode: n=7; moderate episode: n=27; severe episode: n=1) who were largely inpatients (n=18) with a recurrent disease course (n=28).

Taken together, the results of this thesis are the first to suggest that non-classical and intermediate monocytes may be a prominent factor in MDD. While our results are among the first to support such a link, our rigid methodology and the high effect sizes strengthen the relevance of studying CD16 expressing monocytes in MDD. Considering the results by (69), they may be particularly relevant for more severe forms of MDD in otherwise relatively healthy individuals. In these cases, contributions from other immune cells, such as lymphocytes, may be secondary (see below). This is consistent with accumulating evidence that, in MDD, cellular immunity can be differentially affected and that this is likely to vary across patient characteristics (15).

Unlike monocytes, the distribution of major lymphocyte subsets (Nk cells, CD4⁺, CD8⁺ and regulatory T cells) was similar in patients with MDD and healthy controls. CD4⁺ T cells can be divided into regulatory T (Treg) cells and conventional T cells. Tregs are crucial in limiting adaptive immunity and maintaining self-tolerance (71). Decreased Treg percentages have been linked with subgrade chronic inflammation in stress-related conditions, such as post-traumatic stress disorder (72). Interestingly, the role of Tregs in MDD has remained poorly understood as both decreases (73,74) and increases (69) in Treg frequencies have been reported. Possibly important for subclinical forms of MDD, a study with older participants found that higher Treg frequency was associated with more depressive symptoms and lower mental health (75). What is more, antidepressant treatment has been shown to increase circulating Treg percentages, although it has remained unclear if this is important for mediating antidepressant effects (74,76).

Both increases and decreases in Tregs fit with various findings for MDD. For example, given their immunosuppressive role, a reduced Treg frequency would be consistent with the chronic low-grade inflammation observed in some patients with MDD (20). Conversely, because Treg percentages increase with age (77), excessive frequencies would also be consistent with accounts of accelerated cellular aging found in MDD (78). Conceptually integrating these findings is thus

challenging. Together with the results of this thesis, it could be argued that enumerative changes in Tregs may underlie only some patients with MDD. In fact, it has long been recognised that immune suppression, activation or a mix thereof is present in only a proportion of patients. Future studies stratifying for important clinical and demographical variables will need to investigate whether there is a homogenous patient group characterised by Treg alterations.

In line with similar Treg percentages, there were no differences in the frequencies of conventional ($CD4^+$ and $CD8^+$) T cells between patients with MDD and healthy controls. Little is known about the role of these cell subtypes in the pathophysiology of MDD. The results of this thesis are consistent with some (69,74,79), but not all previous studies (80). Interestingly, a higher $CD8^+$ T cells count was associated with non-response to antidepressants in one study (74) and several studies have linked $CD8^+$ T cells with comorbid depression among patients with medical illnesses, such as HIV (81), multiple sclerosis (82) or liver cirrhosis (83). Together, these findings suggest that $CD8^+$ T cells may be more closely associated with MDD in the context of underlying medical illnesses or antidepressant response which we did not study.

NK cells can be divided into cytotoxic (NKc; $CD56^+CD16^+$) and putatively immunoregulatory (NKReg; $CD56^+CD16^{dim/-}$) subtype. NKc cells are the main NK population and mediate early innate responses against a host of different pathogens. Impaired NK cytotoxicity has been advanced as one reason why patients with MDD show poorer prognosis for cancer and infectious diseases (8,84). Reversely, NKReg cells produce a wide range of immunoregulatory molecules to maintain cellular homeostasis (85,86), making them functionally similar to Tregs (87). From this perspective, unchanged regulatory NK cell frequencies in this thesis are consistent with the fact that patients with MDD and healthy controls showed comparable Treg percentages.

The results from this thesis do not support a universal role for NK cells in the pathobiology of MDD. Reduced NK cytotoxicity is one of the most commonly reported findings in MDD (88), although later studies have tended to be more heterogeneous (15). Possibly reconciling these inconsistencies, the NK system is closely linked with variations on demographical and lifestyle factors that tend to be overrepresented in patients with MDD (89), including female sex (90), larger BMI (91), smoking (92) and psychiatric comorbidity, such as alcohol dependence (93,94). The results of this thesis integrate previous inconsistencies by highlighting the role of moderating factors in the involvement of the lymphocyte system in MDD. Together with other research, this suggests that in “pure” forms of MDD (i.e., without significant medical illness or psychiatric comorbidity), enumerative and functional changes in the lymphocyte system might not be prominent disease pathways. This theory does not necessarily argue against alterations in

other aspects of immunity (in fact, decreased NK cytotoxicity can co-occur with increased inflammation in individuals with MDD (95)), but will need to be evaluated by future studies.

Finally, while patients with MDD showed higher level of (pro-inflammatory) non-classical monocytes, levels of serum cytokines commonly secreted (50) by those cells (i.e., IL-6, IL-1 β and TNF- α ; all p values > 0.05 ; page 53) were not elevated. This is not surprising considering that these cytokines are produced by several different cell populations. Considering that non-classical monocytes constitute $<10\%$ of monocytes (which make up 10-30% of human PBMCs), significantly larger samples would be needed to detect such an effect. Such a mechanism would be highly consistent with the fact that patients with MDD showed strikingly increased frequencies of non-classical monocytes, a monocyte subtype that secretes high levels of pro-inflammatory molecules when activated.

3.3.2 Altered gene expression in monocytes from patients with MDD

The second finding of this thesis is that altered gene expression indicative of GC resistance occurred in a cell-specific manner (Table 3). More specifically, monocytes from patients with MDD showed significantly lower mRNA levels of *GR* ($p = 0.018$) and *GILZ* ($p = 0.045$) compared to healthy controls. Consistent with the results obtained by flow cytometry, this was not found in T cells (all p values > 0.05). Importantly, this occurred against the backdrop of similar circadian cortisol profiles in both groups, a common index of HPA axis functioning (group x time: $F_{(3;84)}=1.19$, $p = 0.32$, $\eta p^2 = 0.04$; page 66).

	T cells (CD3 ⁺)				Monocytes (CD14 ⁺)			
	MDD	HC	p^*	g^+	MDD	HC	p^*	g^+
GR	3.7 \pm 1.6	3.6 \pm 1.3	0.43	0.05	4.1 \pm 1.9	4.6 \pm 2.1	0.02 [†]	0.21
MR	0.9 \pm 0.3	1.0 \pm 0.4	0.16	0.25	0.1 \pm 0.1	0.1 \pm 0.1	0.51	0.09
GILZ	13.9 \pm 8.2	15.5 \pm 8.4	0.29	0.19	12.5 \pm 5.7	14.8 \pm 5.9	0.04 [†]	0.39
11 β -HSD1	0.1 \pm 0.2	0.1 \pm 0.2	0.34	0.04	2.7 \pm 4.3	2.4 \pm 3.9	0.35	0.06

Table 3. Cell-specific expression of steroid-related genes in purified T cells and monocytes

11 β -HSD1: 11 β -hydroxysteroid dehydrogenase type 1; GILZ; glucocorticoid-induced leucine zipper; GR: glucocorticoid receptor; HC: healthy controls; MDD: patients with major depressive disorder; MR; mineralocorticoid receptor.

Gene expression (mRNA) is indicated as fold change relative to TBP and IPO8 (housekeeping genes). Values are shown as mean \pm standard deviation.

* calculated using paired-samples t-test [†] Hedges' g [†] significant at $p < 0.05$

Chronic stress (including stress caused by mental illness) has long been known to affect gene expression and behaviour of various immune cells, including monocytes (63,96,97). In depression, accumulating evidence has pointed to a reduction in monocyte GR expression as clinically relevant (17,18). The results of this thesis expand our understanding of the GC system

in monocytes from MDD patients in several significant ways. First, we included a suitable reference population (T cells) that allowed us to investigate whether impairments occurred in a cell-specific manner rather than systemically. This is particularly relevant considering that cell-mediated immunity does not seem to be uniformly affected in MDD (15). Second, mirroring the biological complexity of GC signalling, the scope of this thesis included key steps in the cellular steroid signalling cascade beyond receptor expression. This allowed us to consider reduced GR expression in a broader context. In this vein, probing HPA axis functioning further increased the specificity of our findings by discounting higher cortisol exposure as a possible explanation.

Taken together, this thesis suggests that rather than a consequence of MR dysfunction, altered conversion of cortisol (by 11 β -HSD1) or hyperactive HPA axis, impaired cell-specific GC regulation is likely attributable to GR dysfunction. This highlights pathways beyond classic HPA signalling, such as direct effects of inflammatory molecules, as contributing factors to cellular GR resistance. In fact, some pro-inflammatory cytokines have been shown to modulate GR phosphorylation, translocation, binding to promoter DNA, and stimulate expression of the inactive GR β isoform (98). Conceptually, these pathways together may bear relevance for a more complete understanding of monocyte behaviour and its contribution to MDD (see below).

Monocytes from patients with MDD showed reduced mRNA levels of *GILZ*, a GR-inducible protein that exerts most GR-mediated anti-inflammatory effects. Little is known about the role of *GILZ* in the pathophysiology of MDD. However, its relevance is supported by several independent strains of evidence. First, reduced expression of *GILZ* in whole blood was associated with smaller hippocampal volumes among patients with MDD in one study (38). This is consistent with links between GC signalling, immune deregulation and selective brain atrophy in MDD (99). Second, microglia from chronically stressed mice (an animal model of MDD) showed a link between down-regulated *GILZ* expression and up-regulation of inflammatory markers (39). Third, *GILZ* inhibits key inflammatory pathways, including NF-kB, p38 MAPK and JNK, that have been implicated in MDD (89,100–102). Fourth, reduced *GR* and *GILZ* mRNA levels have been found in PBMCs from fibromyalgia patients, a condition that has a high comorbidity of depression (103). Together with the results from this thesis, mounting evidence implicates *GILZ* in the pathophysiology of MDD. As discussed below (3.5), together with future mechanistic studies, this knowledge may prove relevant to treatment.

The results of this thesis suggest that, in MDD, GR function is more prominently affected in monocytes than T cells. This may shed new light on older reports of blunted GR sensitivity in lymphocytes from patients with MDD. For the most part, these relied on proliferation assays that

measured how GC administration affected cell proliferation by PBMCs or whole blood in response to mitogens, such as lipopolysaccharides (LPS). Rather than using purified T cells, it was assumed that since only T cells proliferate, the extent to which GCs modulated proliferation reflected their GR function. However, such an assumption ignores several key observations. For example, this thesis agrees with other investigations in showing that evidence for reduced GR expression in peripheral immune cells in MDD is heterogeneous (104). In addition, and consistent with our gene expression findings in monocytes, indirect effects from other immune cells contained in PBMCs or whole blood warrant consideration. T cell proliferation occurs in the context of other cells, including monocytes. As a case in point, upon stimulation by agents commonly used in proliferation assays (e.g., LPS), monocytes secrete a host of different cytokines that steer T cell responses (105), including proliferation (106). It therefore remains to be tested if functional GC insensitivity in patients with MDD can be replicated with purified T cells and whether there are contributing influences from molecular alterations beyond the major components of the steroid cascade (11 β -HSD-1, GR, MR, GILZ).

3.3.3 Integration of results

GR signalling is an important regulator of cellular inflammation whose role in MDD has been the subject of intense study. This thesis has found that patients with MDD showed a shift towards a pro-inflammatory monocyte phenotype, reduced *GR* and *GILZ* gene expression specific to monocytes and no HPA axis hyperactivity or increases in serum levels of commonly measured pro-inflammatory cytokines (IL-6, IL-1 β , TNF- α). Together with other research, these findings imply that in MDD GCs such as cortisol exert impaired control over monocyte differentiation because of diminished GR availability. A key consequence of this is blunted transcription of pleiotropic anti-inflammatory factors, such as *GILZ*, removing the “brakes” on inflammation. This is likely to go hand in hand with an expansion of pro-inflammatory cells, such as non-classical monocytes (24,107), whose actions further blunt monocyte GR function (98). While a scarcity of samples prevented us from studying this possibility, it could be speculated that these cells show a particularly high degree of steroid resistance and pro-inflammatory activity in patients with MDD. Tantalisingly, this scenario receives preliminary support from animal and human studies on chronic stress (62,63,108). In the long term, these pathways are likely to become increasingly independent of physiological feedback mechanisms, such as the HPA axis. Ultimately, perturbed immune-neuroendocrine interactions might extend to other parts of the body, such as the central nervous system (CNS). Already there are studies

showing that (non-classical) monocytes can be recruited from the periphery to the CNS during stress (109), where they are likely to exacerbate neuroinflammatory signalling (110).

The aim of this study was to provide a first glimpse into cell-specific steroid gene expression in MDD and to inform future studies with a broadened research arsenal. As such, this thesis has highlighted, in the medium to long term, mechanistic targets for future interventions as well as preclinical and clinical research (see below).

3.4 Possible clinical and translational implications

A significant number of patients with MDD does not satisfactorily benefit from conventional pharmacological treatments (111,112). This underlines the need for a broader and possibly more individually tailored treatment arsenal. Mounting evidence has highlighted the relevance of neuroendocrine and immunological targets in this context (113). As a case in point, several attempts have been made to use available immunomodulatory agents, such as non-steroidal anti-inflammatory drugs or cytokine inhibitors, in the treatment of MDD (114). While initial results have been promising, there is significant demand for more customised mechanism-based approaches. This thesis highlights the role of cell-specific alterations in monocyte phenotype and GR pathways in MDD, reiterating earlier calls for studies into agents that boost monocyte GR function (62). Taking this one step further, this thesis outlines GILZ as a possible intermediate target for further investigation in the context of MDD.

GILZ mediates the majority of anti-inflammatory effects associated with GR stimulation (101) and was recently shown to be a feasible target for treating inflammatory illness. More specifically, Srinivasan and colleagues (115) synthesised a GILZ mimetic (GILZ-P) that binds the p65 subunit of NF- κ B and exerts anti-inflammatory effects *in vivo*. Excitingly, GILZ-P suppressed many of the clinical symptoms in experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis. Considering preliminary evidence for a role of GILZ hypo-expression in MDD (38,39,100,103), future studies should evaluate the potential of GILZ-P in animal models of depression, especially those involving inflammatory pathways.

Preclinical research has identified several other possibilities of modulating GILZ signalling, for example modifying transcription factors activity (116). In-depth analyses (e.g., using structure-function analyses) of interaction targets are likely to unravel further “druggable” mechanisms. As demonstrated by GILZ-P, this could broaden the clinical scope for mimetic agents (115). Promising targets in this vein are the NF- κ B and phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) signalling pathways that have both been implicated in MDD (117,118).

Other studies have examined compounds that modify GR function. Such agents include partial GR agonists and selective GR modulators, which have been developed to separate “beneficial” immunoregulatory effects of GCs from their unwanted side effects (119,120). What is more, a host of studies has linked cAMP/protein kinase A (PKA) pathways with GR function (reviewed by (113)). Interestingly, stimulation of PKA has been shown to inhibit NF- κ B signalling by binding to the p65 subunit in a manner similar to GILZ-P (121). This means that c-AMP/PKA pathways converge on simultaneous boosting of GR function and inhibition of inflammation, making them particularly attractive pharmacological options (122). However, until confirmed by future trials, their therapeutic utility remains theoretical.

Via inhibition of major inflammatory pathways such as NF- κ B, GILZ modulates monocyte differentiation and phenotype (123,124). This suggests that GILZ could help to normalise the phenotypical shift among monocytes in patients with MDD that were observed in this thesis. This is supported by preliminary evidence that GCs induce an anti-inflammatory monocyte phenotype (24) *in vitro* and may even selectively deplete non-classical monocytes *in vivo* (107,125). Although not specifically tested, considering its inhibition of major pathways of inflammation, it seems plausible that a substantial part of this effect is mediated by GILZ (101).

In this context, it would be exciting to study if antidepressant treatment reverses the monocyte-specific alterations described in this thesis. In fact, several classes of antidepressants including the commonly prescribed selective serotonin reuptake inhibitors modulate immune (126,127) and GR function (128). To the author’s knowledge, the extent to which their effects are mediated by monocytes is not known. Interestingly, a study with paediatric obsessive-compulsive disorder (OCD) patients found that antidepressant treatment was associated with a monocyte subset distribution more similar to healthy controls than unmedicated OCD patients (i.e., lower frequency of non-classical and intermediate monocytes) (64). In this context, the robust biological signatures that we found underline the potential for biomarker research (30,129). This may be conducive to the identification of more homogenous patient groups and, ultimately, treatment choice (74,130). Given that a significant share of non-responders to conventional treatments show immune disturbances (131,132), such improved biomarkers are direly needed.

Finally, the question of how to specifically modulate GR function in monocytes is likely complex and will require substantial additional research. Interestingly, while the anti-inflammatory features of vitamin D are well-known (133), recent evidence suggests that a key mechanism behind this may be boosted monocytic GR function (134). Highlighting its potential clinical relevance, this effect was also found in patients with steroid insensitivity (135). Some

studies have found lower vitamin D levels in patients with psychiatric disorders, including MDD (136), and given the low costs and risks associated with adjuvant vitamin D treatment, it would be an interesting first step to explore. Ideally, this would be linked with assessment of monocyte subtype distribution and GR function.

An improved understanding of the molecular pathways underlying impaired regulation of inflammation and neuroendocrine stress regulation in patients with MDD will propel future research and, ultimately, personalised treatments. With GILZ, this thesis has highlighted one possible mechanism-informed approach that might prove relevant to future investigations. Preclinical studies and clinical trials will need to establish how the theoretical attractiveness of GILZ-related pathways can translate into practical benefits for patients with MDD.

3.5 Future research and outlook

This thesis has examined major monocyte and lymphocyte populations based on strong *a-priori* hypotheses. However, a plethora of other immune-competent cells also bear relevance for MDD. High-throughput experimental techniques (e.g., mass cytometry) allow a more filigree immunophenotyping and will broaden our understanding of the biological heterogeneity behind MDD. Such investigations should also reach beyond traditional markers of functionality (i.e., cytokine production) and examine cell-specific expression of markers related to exhaustion (e.g., PD-1), senescence (e.g., CD57) or antigen-specific responses (e.g., to EBV) (137). Together with fully automated data analysis techniques, such as CITRUS, mass-scale immunophenotyping is set to induce a long overdue “knowledge spill-over” into other determinants of immune cell function (e.g., T receptor function). Together, this will help us unravel the myriad links between MDD and immunity likely integrate other findings into a coherent picture.

Peripheral inflammatory signals can reach the brain in several ways where they modulate diffuse mood circuitries (113), suggesting that resolving peripheral inflammation could also ameliorate CNS pathology. Monocytes can be recruited from the periphery to the CNS where they augment inflammatory signals (109) during periods of stress. Microglia are CNS-resident cells that share many characteristics with monocytes and whose role in MDD is just starting to be understood (138,139). Based on the findings of this thesis, excessive infiltration of pro-inflammatory peripheral monocytes may be one reason for the particularly pronounced CNS inflammation and adverse microglia behaviour seen in neuropsychiatric illnesses such as MDD (110). This view is tentatively supported by an animal study (140) that found that microglia from mice suffering repeated social defeat actively recruited pro-inflammatory monocytes to the brain. There, they showed enhanced production of IL-1 β (a cytokine also prominently produced by human non-

classical monocytes (50)) which has been linked with inducing anxiety. Such links between peripheral monocyte subtype and CNS cells provides an interesting perspective that will need to be evaluated in future studies.

This thesis has examined key steps in cellular steroid signalling – yet a plethora of other factors modulate the translation of cortisol signals and remain to be fully elucidated. Next steps may include cell-specific investigation of chaperones and co-chaperones of the cytosolic GR multiprotein complex (e.g., FKBP5), transcriptional regulation via factors such as AP-1 and post-translational modifications, such as altered GR phosphorylation or impaired ability of the GR complex to interact with its transcriptional targets (such as the GILZ promoter).

Interestingly, evidence (62,63,96,97) suggests that chronic stress leaves a transcriptional signature on human monocytes that is consistent with acquired GR insensitivity. Given that early adversity is a risk factor for MDD, similar patterns seem probable. Such hypotheses could be investigated using in-depth interrogation of cell-specific transcription signatures, for example RNA sequencing (141). In a similar vein, cell-specific epigenetic alterations, such as methylation in the *GR* gene (*NR3C1*), are likely to provide further clues on the molecular link between life adversity, inflammation and the development of depression (142,143). In fact, similar alterations were recently described in T cells from patients with post-traumatic stress disorder (144).

It has been recognised that metabolism is a crucial determinant of immune cell phenotype and function (145,146). For example, T cells and monocytes show a pronounced switch from oxidative phosphorylation to glycolysis when activated. Several lines of evidence have converged on the relevance of metabolic changes in MDD. For example, metabolic illness is highly prevalent in this population (1), earlier theories have linked mitochondrial dysfunction to MDD (147) and unfavourable peripheral lipid ratios in MDD patients have been reported (148). Finally, impaired energy metabolism has been found in PBMCs (149) and thrombocytes (150) from depressed individuals. These studies can now be extended with sophisticated instruments such (e.g., Seahorse XF⁹⁶) that allow measurement of metabolic changes in live cells.

3.6 Conclusion

This thesis highlights the importance of cell-specific immune deregulations in MDD. More specifically, we showed that changes in phenotype and gene expression were restricted to monocytes and did not occur in lymphocytes. As discussed above, these findings could provide the starting point for further research into cell-specific therapeutics and, ultimately, will bring us one step closer to the goal of individually tailored treatments.

3.7 References

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4. Eidesstattliche Versicherung

„Ich, Helge Hasselmann, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema „The role of monocyte phenotype and steroid-related gene expression in major depressive disorder“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) werden von mir verantwortet.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Betreuer/in, angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.org) zur Autorenschaft eingehalten. Ich erkläre ferner, dass mir die Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis bekannt ist und ich mich zur Einhaltung dieser Satzung verpflichte.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

Datum

Unterschrift

5. Detailed Declaration of Contribution

Publication: **Helge Hasselmann**, Stefanie Gamradt, Aline Tänzer, Jan Nowacki, Rami Zain, Kostas Patas, Caren Ramien, Friedemann Paul, Katja Wingefeld, Dominique Piber, Stefan M. Gold, Christian Otte; Pro-inflammatory monocyte phenotype and cell-specific steroid signaling alterations in unmedicated patients with major depressive disorder. *Front. Immunol.* 2018. doi: 10.3389/fimmu.2018.02693

Detailed Contribution

Mr. Hasselmann's contribution to this scientific publication is tantamount to 90%. The tasks he completed were conducted with the utmost care, independence and scientific integrity. It deserves mentioning that Mr. Hasselmann has a background in psychology, but nonetheless decided to pursue a doctorate in experimental laboratory work, which he successfully and independently completed.

Study preparation

Mr. Hasselmann adapted the study materials (clinical rating scales, patient-reported measures, clinical documentation forms) based on thorough examination of existing studies and finalized the material together with his supervisors. More specifically, he created the case report forms (CRFs) based on the questionnaires and materials that matched his research question. This also included translation of some materials from English to German to use state-of-the-art diagnostic criteria (depression subtypes according to DSM5). Besides CRF preparation, Mr. Hasselmann was also integral in setting up the required laboratory infrastructure and other study logistics.

Recruitment of study participants and conduction of study visits

Mr. Hasselmann screened all patients with depression and healthy controls for suitability and if applicable enrolled them in the study. All study visits were conducted by Mr. Hasselmann autonomously, including conducting structured psychiatric interviews, depression rating and saliva sample collection. He also provided logistical support for blood draws by the study physicians. He showed particular diligence in participant selection and data acquisition, which is

demonstrated by the well-matched groups (Table 1).

Experimental part

Mr. Hasselmann's share in the experimental analysis of all collected samples is 90%. More specifically, he individually completed the following experimental steps:

- Establishing experimental procedures for analyses
- Contributing to sample processing (e.g. isolation of peripheral blood mononuclear cells (PBMCs) by gradient centrifugation and cryopreservation)
- Preparing, staining, and running samples on a flow cytometer and analyzing cell phenotype by manual-gating based software (Figures 1a, S1-S3)
- Purification of T cells and monocytes from peripheral mononuclear cells using magnetic beads and quality control using flow cytometry
- Isolation of RNA from purified T cells and monocytes
- Synthesis of complementary DNA
- Quantitative analysis of gene expression using real-time reverse transcription-polymerase chain reaction (Figure 2)
- Analysis of saliva cortisol using ELISA (Figure S4)

Data analysis and statistics

All data processing and analyses pertinent to the abovementioned publication except serum cytokines and CITRUS analyses were conducted by Mr. Hasselmann independently and contained the following tasks:

- Analysis of flow cytometry data using FlowJo
- Analysis of gene expression using Microsoft Excel
- Pre-Processing of all experimental data in Microsoft Excel
- Statistical analysis of all experimental in IBM SPSS and GraphPad Prism using adequate statistical procedures
- Creation of graphs and figures for the abovementioned publication in GraphPad Prism

Manuscript composition

- Conceptually planning the manuscript together with his mentors
- Independent draft generation, including abstract, introduction, methods, results and discussion
- Composition of the final manuscript version according to co-author feedback

Publication process

- Drafting of the cover letter accompanying the manuscript
- Drafting of manuscript revision including a point-by-point response to reviewer feedback
- Composition of final revised manuscript together with supervisors and co-authors

Unterschrift, Datum und Stempel des betreuenden Hochschullehrers/der betreuenden Hochschullehrerin

Unterschrift des Doktoranden/der Doktorandin

6. Journal Summary List (ISI Web of Knowledge)

Journal Data Filtered By: **Selected JCR Year: 2017** Selected Editions: SCIE,SSCI
 Selected Categories: **"Immunology"** Selected Category Scheme: WoS
Gesamtanzahl: 155 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	NATURE REVIEWS IMMUNOLOGY	39,215	41.982	0.085360
2	Annual Review of Immunology	17,086	22.714	0.028800
3	NATURE IMMUNOLOGY	41,410	21.809	0.102290
4	IMMUNITY	46,541	19.734	0.136360
5	TRENDS IN IMMUNOLOGY	11,204	14.188	0.026850
6	JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY	49,229	13.258	0.083800
7	Lancet HIV	1,476	11.355	0.007950
8	JOURNAL OF EXPERIMENTAL MEDICINE	62,537	10.790	0.078310
9	IMMUNOLOGICAL REVIEWS	14,555	9.217	0.028540
10	Cancer Immunology Research	4,361	9.188	0.021180
11	CLINICAL INFECTIOUS DISEASES	61,618	9.117	0.120010
12	AUTOIMMUNITY REVIEWS	8,956	8.745	0.020990
13	Journal for ImmunoTherapy of Cancer	1,675	8.374	0.007130
14	CURRENT OPINION IN IMMUNOLOGY	9,275	7.932	0.020120
15	JOURNAL OF AUTOIMMUNITY	6,410	7.607	0.015490
16	Cellular & Molecular Immunology	3,633	7.551	0.008300
17	EMERGING INFECTIOUS DISEASES	29,657	7.422	0.057980
18	Mucosal Immunology	6,105	7.360	0.021860
19	SEMINARS IN IMMUNOLOGY	4,552	7.206	0.010950
20	EXERCISE IMMUNOLOGY REVIEW	740	7.105	0.001110
21	Journal of Allergy and Clinical Immunology-In Practice	2,802	6.966	0.009670
22	CLINICAL REVIEWS IN ALLERGY & IMMUNOLOGY	2,741	6.442	0.005880
23	Seminars in Immunopathology	2,967	6.437	0.009290
24	BRAIN BEHAVIOR AND IMMUNITY	12,583	6.306	0.026850
25	ALLERGY	16,476	6.048	0.025790
26	Emerging Microbes & Infections	1,318	6.032	0.005910
27	Advances in Immunology	2,423	5.935	0.004250
28	Current Topics in Microbiology and Immunology	5,633	5.829	0.011740
29	World Allergy Organization Journal	1,352	5.676	0.003800
30	Frontiers in Immunology	16,999	5.511	0.067470

Retrieved January 12 2019.

With rank 30 out of 155, Frontiers in Immunology is in the top 20% of immunology journals ranked by impact.



Pro-inflammatory Monocyte Phenotype and Cell-Specific Steroid Signaling Alterations in Unmedicated Patients With Major Depressive Disorder

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Several lines of evidence have strongly implicated inflammatory processes in the pathobiology of major depressive disorder (MDD). However, the cellular origin of inflammatory signals and their specificity remain unclear. We examined the phenotype and glucocorticoid signaling in key cell populations of the innate immune system (monocytes) vs. adaptive immunity (T cells) in a sample of 35 well-characterized, antidepressant-free patients with MDD and 35 healthy controls individually matched for age, sex, smoking status and body mass index. Monocyte and T cell phenotype was assessed by flow cytometry. Cell-specific steroid signaling was determined by mRNA expression of pre-receptor regulation (11 β -hydroxysteroid dehydrogenase type 1; 11 β -HSD1), steroid receptor expression [glucocorticoid receptor (*GR*) and mineralocorticoid receptor (*MR*)], and the downstream target glucocorticoid-induced leucine-zipper (*GILZ*). We also collected salivary cortisol samples (8:00 a.m. and 10:00 p.m.) on two consecutive days. Patients showed a shift toward a pro-inflammatory phenotype characterized by higher frequency and higher absolute numbers of non-classical monocytes. No group differences were observed in major T cell subset frequencies and phenotype. Correspondingly, gene expression indicative of steroid resistance (i.e., lower expression of *GR* and *GILZ*) in patients with MDD was specific to monocytes and not observed in T cells. Monocyte phenotype and steroid receptor expression was not related to cortisol levels or serum levels of IL-6, IL-1 β , or TNF- α . Our results thus suggest that in MDD, cells of the innate and adaptive immune system are differentially affected with shifts in monocyte subsets and lower expression of steroid signaling related genes.

Keywords: depression, monocytes, inflammation, steroid hormones, innate immunity

INTRODUCTION

Several independent lines of evidence have implicated the immune system in the pathobiology of mood disorders, particularly major depressive disorder (MDD) (1–3). Numerous studies and several meta-analyses have demonstrated higher levels of circulating cytokines, such as interleukin 6 (IL-6) and tumor necrosis factor α (TNF- α), in MDD (4, 5). However, serum levels of cytokines reveal little about the specific cause of immune dysfunction and the cellular source of inflammation in MDD remains poorly understood.

Importantly, it is now becoming increasingly clear that MDD is not simply a state of general immune activation but that innate and adaptive immune responses might be differentially affected (6). Considerable evidence points toward activation of monocytes in MDD (7, 8). In contrast, recent studies have suggested that adaptive immunity, specifically T cell function, might be impaired in MDD (9–11).

This implies that immune activation and impaired regulation of inflammation in MDD might be limited to certain components of the immune system. Glucocorticoids (GCs) are among the most potent endogenous regulators of inflammation, and cell-specific alterations in steroid signaling are thus promising candidates in this respect. Several studies have suggested that leukocyte responsiveness to GCs is blunted in patients with MDD (7, 8, 12–15). If regulatory pathways of inflammation (such as glucocorticoid signaling) were indeed affected in a cell-specific fashion in MDD, this might have implications for developing tailored pharmacological approaches in the future.

In the current study, we therefore aimed to explore the interplay between inflammation and stress hormone signaling by contrasting the phenotype and glucocorticoid signaling of key cell populations in the innate immune system (monocytes) vs. adaptive immunity (T cells) in a sample of well-characterized, antidepressant-free patients with MDD and closely matched healthy controls (HC). In addition, we explored the association of these putative immune signatures with serum cytokines, clinical characteristics of MDD and important risk factors such as childhood trauma.

MATERIALS AND METHODS

Participants and Clinical Assessments

The study was approved by the local ethics committee (EA1/096/15). The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. All participants provided written informed consent and received financial reimbursement for their time and effort.

Patients with MDD between 18 and 60 years of age were recruited from our inpatient wards, via onsite psychiatrist referral, or online advertisements. Healthy controls were recruited from online advertisements. Patients and controls were matched pairwise on sex, smoking status, age, and body mass index (BMI) prior to running any biological analyses.

All participants were free of significant medical illness (e.g., diabetes, autoimmune or infectious illnesses), immunomodulatory treatment [e.g., non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids or antibiotics], pregnancy, and recent (<3 months) vaccinations. Inclusion criteria specific to MDD patients were a clinician-confirmed diagnosis of MDD, a minimum antidepressant-free period of 2 weeks and absence of comorbid psychiatric disorders (e.g., substance abuse in the past 12 months) except for mild-to-moderate anxiety disorders. Inclusion criteria specific to healthy controls were absence of any form of psychiatric illness, a Montgomery Asberg Depression Rating Scale (MADRS) score <7 and no clinically confirmed diagnosis of any affective disorder in a first-degree relative.

Diagnosis of MDD was confirmed by experienced psychiatrists (DP, CO). In addition, during the study visit, the Mini-International Neuropsychiatric Interview (16) and the MADRS (17) was conducted by a trained clinical rater (HH). Self-report questionnaires were obtained to quantify levels of anxiety (Beck Anxiety Inventory, BAI) (18), depression severity (Beck Depression Inventory II, BDI-II) (19) and adverse childhood experience (Childhood Trauma Questionnaire, CTQ) (20).

Blood and Saliva Collection

A sample of 70 ml of venous blood was collected in heparinized tubes (BD, Germany). To control for circadian rhythms and other potential confounds, samples were obtained between 8.00 a.m. and 9:30 a.m. after 12 h overnight fasting. Peripheral blood mononuclear cells (PBMCs) were immediately isolated and cryopreserved until assayed (see below for details).

Serum was collected in serum separator tubes (BD, Germany) and allowed to clot for 30 min at room temperature in the dark. Next, samples were centrifuged for 5 min, after which serum was aliquoted and stored at -20°C until analysis. Saliva samples were collected on 2 consecutive days at 8:00 a.m. and 10:00 p.m. using Sarstedt salivettes (Sarstedt, Germany) at home by the participants (within a week of their clinical visit) and shipped back to the lab in pre-stamped envelopes provided by the study team. All samples arrived within 7 days and were immediately processed and stored until assayed (see below for details).

Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

All blood samples were processed within 1 h of collection. PBMCs were isolated from heparin venous blood samples via density gradient centrifugation. In brief, samples were carefully layered on top of density medium (Biocoll, Biochrome, Germany). After centrifugation, PBMCs were harvested from the interphase, washed twice in phosphate-buffered saline (PBS) and taken up in RPMI-1640 + GlutaMax medium (Gibco, ThermoFisher Scientific, Germany) supplemented with 25% heat-inactivated fetal calf serum (FCS) (Biochrome, Germany) and 10% dimethylsulfoxide (Applichem GmbH, Germany) for cryopreservation. Cells were frozen at a concentration of 10^7

cells/ml at -80°C in a pre-cooled freezing container. After 24–48 h, cells were transferred to liquid nitrogen and stored at -196°C until analysis.

For thawing, the cryo vials were transferred to a water bath pre-warmed to 37°C . After 5 min, 10^7 cells were transferred into 10 ml of thawing medium (RPMI-1640 + GlutaMax containing 10% FCS at 37°C). Cells were then washed in medium, counted and prepared for phenotyping by flow cytometry or magnetic-activated cell separation (MACS Microbead Technology, Miltenyi Biotec, Germany) as described below.

Flow Cytometry

Antibody panels used for this study are presented in **Table S1** (all Biologend, UK). All monoclonal antibodies were pretested and titrated to optimal concentrations using PBMCs from healthy donors. All steps were conducted at room temperature unless otherwise specified.

First, PBMCs were incubated with a live/dead marker (Zombie NIR Fixable Viability Kit, BioLegend, UK) and the CCR7 antibody in PBS for 15 min. Next, antibody premixes were added in staining buffer (PBS + 0.5% bovine serum albumin Miltenyi Biotec, Germany + 0.02% sodium azide Sigma-Aldrich, Germany) and incubated for an additional 15 min. Cells were then washed and resuspended in staining buffer and immediately analyzed on a FACSCanto II (BD, Germany). Matched HC/MDD pairs were analyzed in the same run on the same day to avoid any systematic variation due to technical variability.

The gating strategies to identify PBMC subpopulations are depicted in detail in **Figures S1, S2**. Briefly, leukocyte identification followed recommendations for general immunophenotyping in humans (21, 22).

Results from manual analysis of flow cytometric data were further validated by means of an unsupervised clustering algorithm (CITRUS, as implemented in the cloud-based Cytobank software, Cytobank Inc. USA). This approach accounts for the continuous nature of immune subsets (e.g., monocyte subpopulations) and has been shown to better reflect pathophysiologic conditions than gating-based, threshold-driven manual analysis of distinct subtypes (23). Briefly, CITRUS performs hierarchical clustering of cellular populations based on phenotypical similarity and automatically identifies stratifying features between groups (24). In our analysis, we defined CD3^- non-T cells as input population after removing debris, doublets and dead cells. CITRUS was run with CD14, CD16, CD20, CD56, and HLA-DR as clustering channels on 10^4 events sampled per file with a minimum cluster size threshold of 1.5%. The correlative model SAM was used to detect associations between the relative cluster abundance of each sample and the study groups (HC or MDD). Using a false discovery rate of 1%, significant clusters were exported as FCS files, concatenated for the whole study cohort and projected on viSNE maps of the total non-T cell population. The viSNE algorithm reduces multi-dimensional flow cytometry data to two dimensions (tSNE1 and $\text{tSNE2} = \text{t-Distributed}$

Stochastic Neighbor Embedding) while retaining the single-cell representation of the data (25).

Cell Sorting, RNA Isolation, cDNA Synthesis, and Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-qPCR)

For analysis of cell-specific gene expression, T cells and CD14^+ monocytes after thawing were purified from PBMCs following manufacturer's instructions using magnetic-activated cell sorting (CD3 and CD14 MicroBeads, Miltenyi Biotec, Germany). Briefly, 10^7 were resuspended in 80 μL MACS buffer (PBS, 0.5% BSA, and 2 mM EDTA) and 20 μL of CD3 or CD14 MicroBeads, respectively, and incubated for 15 min at 4°C in the dark. After washing with 2 mL MACS buffer per 10^7 cells, cells were resuspended in 500 μL MACS buffer before proceeding to magnetic separation on MACS LS columns. Cell purity was checked using flow cytometry. In our hands, this yields a purity of $96.5 \pm 1.2\%$ for T cells and $92.3 \pm 1.7\%$ for CD14^+ monocytes. RNA was isolated from purified cells using Qiagen RNeasy Plus Mini Kit (Qiagen, Germany) following manufacturer's instruction. Purity and concentration were determined using a NanoDrop spectrophotometer (NanoDrop 2000c, ThermoFisher Scientific, Germany). Average RNA yield was similar across groups for T cells [MDD: 581.1 ± 480 ng; HC: 631.9 ± 383 ng; $T_{(34)} = 0.61$; $p = 0.55$] and monocytes [MDD: 815 ± 1358 ng; HC: 685 ± 524.6 ng; $T_{(34)} = 0.67$; $p = 0.51$] All steps were conducted at room temperature. Isolated RNA was directly transcribed to complementary DNA (cDNA) without intermittent freezing using the RevertAid H Minus First Strand cDNA Synthesis Kit (ThermoFisher Scientific, Germany) according to manufacturer's instructions and stored at -80°C until analysis. cDNA was amplified on a StepOne Real-Time PCR system (Applied Bioscience, Germany) using TaqMan Gene Expression Assays (ThermoFisher Scientific, Germany) for *GR* (Hs00353740_m1), *MR* (Hs01031809_m1), *GILZ* (Hs00608272_m1), *11 β -HSD1* (Hs01547870_m1). Gene expression was normalized using two housekeeping genes: Importin 8 (*IPO8*; Hs00183533_m1) and TATA Box Binding Protein (*TBP*; Hs00427620_m1). All RT-qPCRs reactions were performed in triplicates with a patient and matching control sample on the same plate. Gene transcript levels were assessed relative to *IPO8* and *TBP* using the $\Delta\Delta\text{CT}$ method.

Analysis of Salivary Cortisol

Circadian peak and nadir measures of hypothalamus-pituitary-adrenal (HPA) axis activity were estimated by salivary cortisol levels at 8:00 a.m. and 10:00 p.m. collected on 2 consecutive days. After collection, saliva tubes were centrifuged for 5 min and aliquots stored at -20°C until analysis. Samples were analyzed in duplicates using an enzyme-linked immunosorbent assay (ELISA) (IBL, Germany) following manufacturer's instructions. Matched HC/MDD pairs were measured on the same microplate. Standard curves were fitted using 4-parameter logistics. This method has a detection sensitivity of 0.135 nmol/L and intra- and inter-assay coefficients of variation $<10\%$.

Analysis of Serum Cytokines

Serum interleukin-6 (IL-6), interleukin 1 beta (IL-1 β) and tumor necrosis factor alpha (TNF- α) levels were analyzed in duplicates using commercially available high sensitivity ELISA kits (R&D Systems Europe, UK) following manufacturer's instructions. Matched HC/MDD pairs were measured on the same microplate. Optical density was determined on a CLARIOStar microplate reader (BMG Labtech, Germany). Standard curves were fitted using 4-parameter logistics. Calculated cytokine concentrations < 0.5 x limit of quantification (LOQ, i.e. lowest standard concentration) were set to 0.5 x LOQ. The mean limit of detection for IL-6, IL-1 β , and TNF- α as provided by the manufacturer is 0.031, 0.033, and 0.022 pg/mL, respectively. Intra- and inter-assay coefficients of variation were <10%.

Routine Blood Tests

Serum CRP analysis by particle-enhanced turbidimetric immunoassay (PETIA) and a differential blood cell count to enumerate circulating leukocyte subsets were conducted by a clinically licensed diagnostic lab (Labor Berlin—Charité Vivantes GmbH, Germany).

Statistics

Cell population specific parameters were expressed as either absolute cell counts or percentages normalized to suitable reference populations. Continuous variables were analyzed with paired-sample *t*-tests due to the close matching based on four variables (age, sex, smoking, BMI, see Table S2) between patients and healthy controls (26). Dichotomous variables were analyzed with McNemar's test. Associations with clinical variables and immune markers were explored using Spearman's correlation coefficients. A two (group = MDD vs. HC) \times four (time = day 1 morning vs. day 1 evening vs. day 2 morning vs. day 2 evening) repeated-measures analysis of variance (ANOVA) was run to investigate group differences in saliva cortisol levels. Effect sizes were calculated as Hedges' *g* for *t*-tests and partial eta² (η_p^2) for ANOVAs (27). Statistical analyses were conducted in SPSS version 21 (IBM Inc., USA) and GraphPad Prism version 7 (GraphPad Software Inc., USA). Flow cytometry data were analyzed using FlowJo version 10.1 (Treestar Inc., USA) and Cytobank analysis software (Cytobank Inc., USA).

RESULTS

Demographic and clinical characteristics of patients and controls are displayed in Table 1. The majority of MDD patients were inpatients ($n = 18$) and had a recurrent disease course ($n = 28$) with a mean of 3.75 previous episodes (range: 1–7, standard deviation 1.7 episodes). Mean MADRS scores indicated moderate depression severity.

As expected, the MDD group showed higher levels of anxiety and childhood trauma. There were no differences in any of the measured demographic or lifestyle variables (Table S2).

Immune Phenotype

When examining the phenotype of cell subsets in the innate and adaptive immune system, we observed a significantly reduced relative frequency of classical monocytes [$T_{(34)} = 4.81$; $p <$

TABLE 1 | Sample characteristics.

	MDD ($n = 35$)	HC ($n = 35$)	Test statistic	<i>p</i> -value*
Age, years	31.7 (11.2)	31.7 (10.2)	$T_{(df=34)} = 0$	> 0.99
BMI, kg/m ²	23.9 (3.6)	23.5 (3.2)	$T_{(df=34)} = 1.27$	0.21
% Females (<i>n</i>)	71.4 (25)	71.4 (25)	$\chi^2_{(df=1)} = 0$	> 0.99
% Current smokers (<i>n</i>)	34.3 (12)	34.3 (12)	$\chi^2_{(df=1)} = 0$	> 0.99
MADRS	24.9 (5.3)	1.2 (1.8)	$T_{(df=34)} = 26.31$	< 0.01
BDI-II	29.9 (6.5)	2.9 (3.4)	$T_{(df=34)} = 21.65$	< 0.01
BAI	20.7 (12.4)	3.8 (2.9)	$T_{(df=34)} = 7.41$	< 0.01
CTQ Total Score	40.9 (15.9)	32.9 (9.8)	$T_{(df=34)} = 2.32$	0.03
% Comorbid anxiety disorder (<i>n</i>)	28.6 (10)	–	–	–
% MDD subtype (<i>n</i>)	60 (21)	–	–	–
% Melancholic (<i>n</i>)	54.3 (19)	–	–	–
% Atypical (<i>n</i>)	5.7 (2)	–	–	–

BAI, Beck Anxiety Inventory; BDI-II, Beck Depression Inventory II; BMI, body mass index; CTQ, Childhood Trauma Questionnaire; HC, healthy control; MADRS, Montgomery Asberg Depression Rating Scale; MDD, major depressive disorder.

Unless specified otherwise, values represent mean (standard deviation).

*Paired-samples *t*-test for continuous and McNemar's test for dichotomous variables.

0.0001; Hedges' *g* = 0.88] and, conversely, elevated levels of "non-classical" monocytes [$T_{(34)} = 4.33$; $p = 0.0001$; Hedges' *g* = 0.80] in MDD patients compared to controls (Figure S3B). When expressed as absolute cell counts, significantly higher numbers in MDD were only detected for non-classical and intermediate monocytes (Figure 1A). There were no statistically significant differences in circulating numbers of leukocyte subsets (Figure S3A) or relative frequencies of major lymphocyte populations (Figures S3C,D) between the groups.

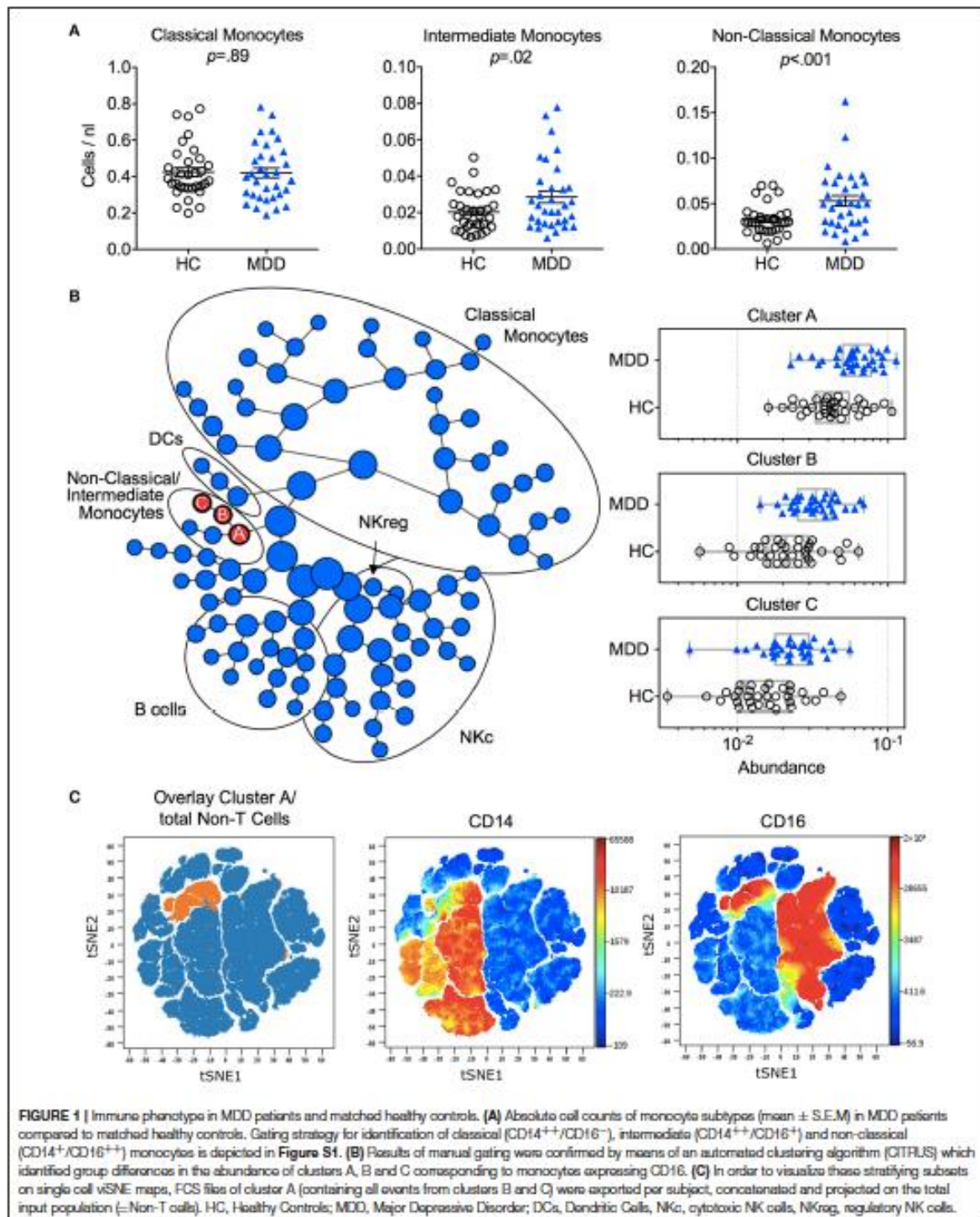
To validate monocyte subset results, we applied an unsupervised clustering algorithm (CITRUS), which automatically identifies differentially abundant cell clusters between groups. This analysis yielded one major cluster with two subclusters (A, B, C) corresponding to the non-classical and intermediate monocyte cell populations (Figures 1B,C). Further confirming the results from the manual gating, no additional group differences were detected by this algorithm (Figure 1B).

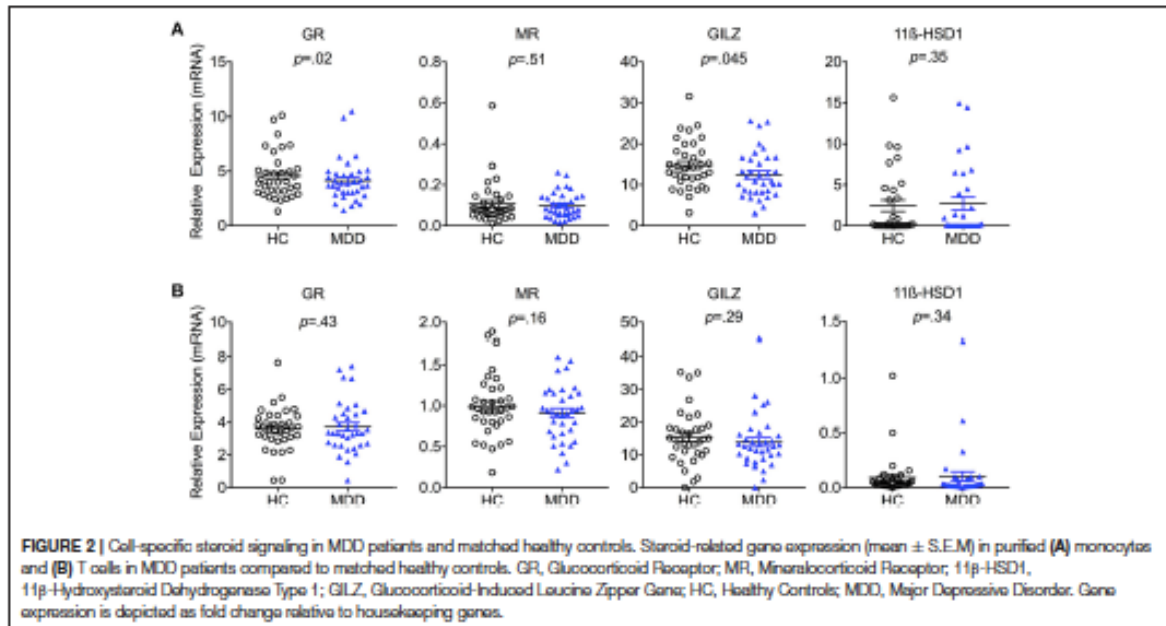
Cell-Specific Expression of Steroid-Signaling-Related Genes

In a next step, we explored cell-specific alterations in glucocorticoid signaling in monocytes and T cells. Purified CD14⁺ monocytes from MDD patients expressed significantly lower mRNA levels of *GR* [$T_{(34)} = 2.49$; $p = 0.018$; Hedges' *g* = 0.21] as well as its downstream target *GILZ* [$T_{(34)} = 2.08$; $p = 0.045$; Hedges' *g* = 0.39] (Figure 2A). In contrast, no group differences in monocyte expression of *MR* or *11 β -HSD-1* were observed (all *p*-values > 0.05). There were also no group differences in T cell expression levels of any of the genes examined (*GR*, *GILZ*, *MR*, or *11 β -HSD-1*) (Figure 2B).

HPA Axis Activity

Saliva samples were available from $n = 30$ patient/control pairs. Analysis of cortisol levels revealed no group \times time interaction or main effect of group [group \times time: $F_{(3;84)} = 1.19$, $p = 0.32$, $\eta_p^2 = 0.04$; group: $F_{(1;28)} < 0.01$, $p = 0.93$, $\eta_p^2 < 0.01$]. As expected,





there was a main effect of time [$F_{(3,84)} = 36.8, p < 0.01; \eta_p^2 = 0.57$] across groups, showing the typical circadian rhythm of HPA axis activity with higher cortisol levels after awakening compared to evening levels in both groups (Figure S4).

Serum Immune Markers

No significant group differences were observed for high sensitivity CRP levels (MDD: 1.67 ± 1.7 mg/L; HC: 1.46 ± 1.5 mg/L). Moreover, MDD patients and controls did not differ in serum levels of the cytokines IL-6, IL-1 β , or TNF- α (Figure 3). There were also no significant associations between serum cytokine levels and monocyte subset counts (see Figure 3).

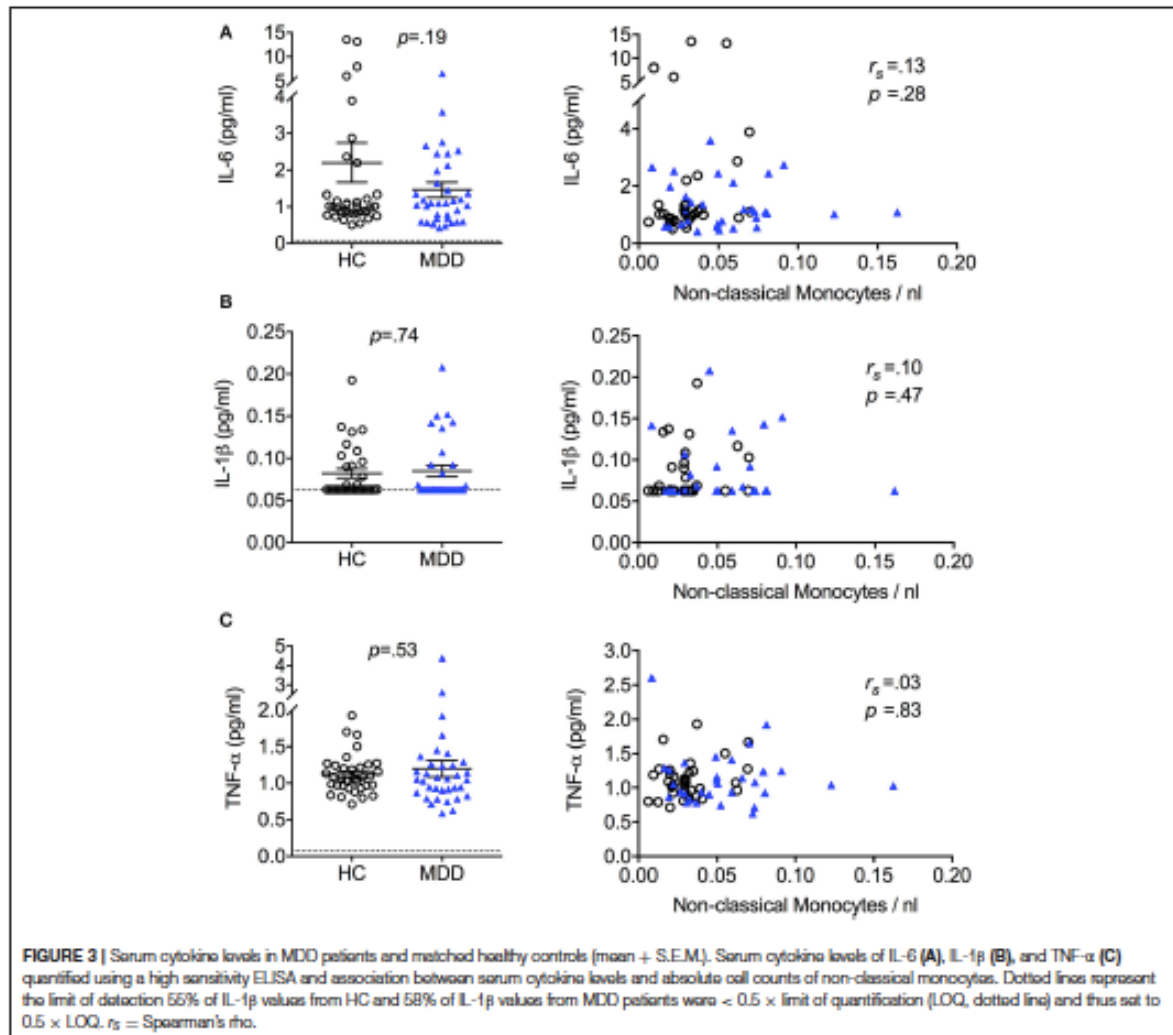
Clinical Correlates

To examine the relationship of steroid-related gene expression and immune phenotype with clinical variables, we ran correlation analyses. Out of 64 correlation coefficients computed, only two reached statistical significance, which is well within the range of chance findings (all p -values > 0.05 ; Table S3).

DISCUSSION

Our study has three main results. First, patients with MDD showed a shift toward non-classical monocytes with no group differences in major T cell subset frequencies and phenotype, B cells or NK cells. Second, expression of key steroid-signaling genes *GR* and *GILZ* was lower in monocytes obtained from MDD patients with no group differences observed in T cells. Third, monocyte phenotype and steroid receptor expression was not related to circulating levels of cortisol or circulating levels of the cytokines IL-6, IL-1 β , or TNF- α .

Human monocytes can be divided into classical, intermediate and non-classical subsets that are functionally heterogeneous. Increasing evidence suggests that a higher frequency of the non-classical monocyte subtype is conducive to chronic inflammation, as seen in various illnesses (28–30). Here, we observed a significant shift among monocytes in patients with MDD. Intriguingly, non-classical monocytes are also associated with several somatic conditions that commonly co-occur in MDD patients (31), including coronary artery disease (32). Since patients in our MDD cohort were free of overt comorbid cardiovascular or metabolic disorders, our data suggest that monocyte subset shifts in MDD are not necessarily the consequence of comorbid somatic disorders but might occur independently or at least prior to such comorbidities in MDD. In contrast to our results, earlier studies in depressed patients did not find a link between depression and monocyte frequencies or phenotype (33) (34), although direct comparisons are hampered by differences in methodology (e.g., insufficient monocyte characterization) and study populations (e.g., elderly patients). Using an approach similar to ours, Suzuki et al. (35) recently reported no group differences in classical and non-classical monocytes between patients with MDD and healthy controls. In the study by Suzuki et al. (35) patients with MDD had a significantly higher BMI (close to obesity) compared to the control group. This is important because BMI can have a profound effect on immune responses, including major lymphocyte (36) and monocyte populations (37). Moreover, more than half of MDD patients in the study by Suzuki were only mildly depressed or in partial remission, while our sample consisted of patients with a well-described episode of at least moderate severity and many of our patients were currently hospitalized due to MDD. Thus, the exact relationship



between comorbidities, demographic variables, clinical severity, and monocyte subsets should be explored in detail in the future to determine the dynamics of immune alterations over the course of MDD.

Our second main result was that lower expression of steroid signaling related genes (i.e., *GR* and *GILZ*) was restricted to monocytes and not observed in T cells. Thus, our findings both replicate and expand previous studies (7, 8). More specifically, they suggest that monocytes in MDD are characterized by a reduction of *GR* expression (and more downstream, *GILZ*) rather than changes in *MR* or cortisol bioconversion.

Previous findings of functional steroid resistance as obtained by mitogen-stimulated proliferation assays (13) have often been attributed to reduced *GR* expression, however, evidence for this in peripheral immune cells is mixed (38). To our knowledge, no study to date has assessed *GR* expression or other GC-related genes in T cells specifically. Studies examining functional steroid

resistance have typically used proliferation assays or cytokine production in whole blood (13, 39) or PBMC culture systems (38, 40, 41). Our findings of unaltered *GR* and *GILZ* expression in T cells would suggest that functional steroid resistance in T cells as indicated by proliferation assays is unlikely to be explained by reduced steroid receptor expression in T cells but may either be due to alterations in other components of the signaling cascade or mediated indirectly via other cell populations such as monocytes.

Finally, healthy controls and the MDD group showed similar circadian HPA axis activity, CRP levels, and circulating levels of IL-6, IL-1 β , or TNF- α , which did not correlate with monocyte phenotype. A similar pattern of reduced *GR* expression and sensitivity in PBMCs without HPA axis hyperactivity was found in coronary heart disease patients with depression (42). Interestingly, evidence from studies with chronically stressed caregivers also suggests that blunted steroid signaling and altered inflammatory gene expression in monocytes can occur

independently of HPA axis output (43, 44). Taken together, this suggests that MDD-associated changes in monocyte phenotype and steroid signaling gene expression do not require the presence of detectable differences in cortisol or cytokines such as IL-6, IL-1 β , or TNF- α .

Strengths of our study include careful matching of patients and controls, in-depth manual as well as algorithm-based characterization of lymphocytes and monocytes and cell-specific investigation of steroid-related gene expression. Yet, several limitations need to be acknowledged. For example, we did not include cell-specific functional readouts, such as cytokine production. Our study adds relevant new information by providing direct evidence for specificity of steroid-signaling gene expression within the immune system of MDD patients. However, we acknowledge that our analysis of gene expression in pan-monocytes (CD14⁺) and pan-T cells (CD3⁺) still includes heterogeneous cell populations within each lineage. Some limitations also concern the clinical characteristics of our sample. Our main concern here was to control for somatic comorbidities, antidepressant medication, age, sex and BMI. This is important to limit the impact of such confounds on the variables of interest in case-control studies. However, this approach also limits generalizability to the MDD population at large. Similarly, we were likely underpowered in the exploratory analyses of clinical correlates and the non-significant results in this area should be interpreted with caution. Lastly, we did not measure several lifestyle factors such as physical activity, diet, sleep (45), that may affect cell-mediated immunity. However, given the close matching for BMI, at least major metabolic effects due to group differences in physical activity or diet appear unlikely.

In summary, our results suggest that in MDD, shifts in monocyte phenotype and altered expression of genes involved in steroid signaling, can occur in the absence of HPA axis hyperactivity or elevated levels of circulating cytokines such as IL-6, IL-1 β , or TNF- α . This provides evidence for a possible

divergence in steroid signaling-related gene expression between monocytes and T cells in MDD and could provide a starting point for further research into the role of monocyte subsets in major depression.

AUTHOR CONTRIBUTIONS

SMG and CO: conception and design. HH and SG: execution of experiments. HH, AT, JN, RZ, and DP: acquisition of data. HH, SG, and KP: analysis of data. HH, SG, SMG, and CO: interpretation of data. SMG and CO: obtained funding. HH, SG, and SMG: drafting of the manuscript. AT, JN, KP, CR, FP, KW, DP, and CO: revision of the manuscript for important intellectual content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2018.02693/full#supplementary-material>

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Material

“Pro-inflammatory monocyte phenotype and cell-specific steroid signaling alterations in unmedicated patients with major depressive disorder”

by Hasselmann *et al.*

TABLE S1 | Antibody Panels.

TABLE S2 | List of patients with MDD and healthy controls matched for age, sex, body mass index, and current smoking status.

TABLE S3 | Correlations between steroid signaling-related gene expression, PBMC phenotype and clinical variables in patients with MDD.

FIGURE S1 | Flow cytometry gating strategy for identification of lymphocyte phenotype.

FIGURE S2 | Flow cytometry gating strategy for identification of classical (CD14⁺⁺ CD16⁻), intermediate (CD14⁺⁺CD16⁺) and non-classical (CD14⁺CD16⁺⁺) monocytes and exemplary staining for a patient-control pair.

FIGURE S3 | Routine laboratory blood cell counts of circulating leukocytes and proportions of monocyte and T cells subsets, as well as B cells and Nk cells as measured by flow cytometry.

FIGURE S4 | Salivary cortisol levels in patients with MDD and matched healthy controls.

TABLE S1 | Antibody Panels.

T cell phenotype and activation	Treg	Non-T cells
HLA-DR (LN3)	HLA-DR	HLA-DR
CCR7 (G043H7)	CD25 (M-A251)	CD56 (HCD56)
CD4 (RPA-T4)	CD4	CD4
CD45RA (HI100)	CD45-RA	CD20 (2H7)
CD38 (HB-7)	CD127 (A019D5)	CD16 (3G8)
Zombie NIR	Zombie NIR	Zombie NIR
CD3 (OKT3)	-	CD14 (HCD14)
CD8 (SK1)	CD3	CD3

Three different antibody panels were applied to each sample to analyze PBMC subsets: Naive, Memory and Effector T cells as well as activated T cells (T cell phenotype and activation panel), regulatory T cells (Treg) or non-T cell peripheral blood mononuclear cells including monocytes, natural killer cells and B cells (Non-T cells). Antibody clones are indicated in brackets.

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TABLE S2 | List of patients with MDD and healthy controls matched for age, sex, body mass index and current smoking status. Depression severity (MADRS), psychiatric comorbidity as well as depression subtype according to DSM5 are given where applicable.

	Age	Sex	BMI	Currently Smoking	MADRS Score	Psychiatric Comorbidity	Subtype (DSM5)
MDD01	47	F	19.8	No	23	SP	-
HC01	42	F	19.0	No	0		
MDD02	36	M	27.3	No	24	-	-
HC02	31	M	27.5	No	0		
MDD03	22	F	21.2	No	27	SP	-
HC03	25	F	19.5	No	2		
MDD04	30	F	27.7	No	28	PD	
HC04	25	F	23.7	No	2		
MDD05	33	F	24.4	No	25	-	melancholic
HC05	28	F	22.4	No	0		
MDD06	20	F	26.6	No	30	-	melancholic
HC06	24	F	22.2	No	0		
MDD07	26	M	22.2	No	21	-	atypical
HC07	28	M	22.2	No	0		
MDD08	30	M	26.5	Yes	28	SP	melancholic
HC08	30	M	23.1	Yes	1		
MDD09	33	F	19.7	No	29	-	melancholic
HC09	33	F	23.1	No	0		
MDD10	32	F	27.7	No	26	PD	melancholic
HC10	28	F	23.5	No	1		
MDD11	24	F	26.1	Yes	25	-	melancholic
HC11	30	F	25.8	Yes	1		
MDD12	32	M	24.3	No	19	PD	melancholic
HC12	28	M	25.0	No	0		

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TABLE S2 | continued

	Age	Sex	BMI	Currently Smoking	MADRS Score	Psychiatric Comorbidity	Subtype (DSM5)
MDD13	35	F	19.7	Yes	22	-	melancholic
HC13	30	F	19.1	Yes	0		
MDD14	26	M	25.0	Yes	17	-	-
HC14	24	M	21.7	Yes	0		
MDD15	53	F	26.9	No	28	-	-
HC15	55	F	25.4	No	1		
MDD16	59	M	25.4	No	25	-	melancholic
HC16	57	M	21.6	No	1		
MDD17	32	M	27.6	Yes	17	-	-
HC17	27	M	25.3	Yes	0		
MDD18	39	F	20.3	No	28	-	-
HC18	41	F	23.1	No	0		
MDD19	50	F	26.7	No	27	-	melancholic
HC19	44	F	28.5	No	0		
MDD20	19	F	21.6	Yes	19	-	-
HC20	22	F	19.7	Yes	4		
MDD21	45	F	21.9	Yes	25	-	melancholic
HC21	52	F	22.5	Yes	1		
MDD22	21	F	23.2	Yes	25	-	-
HC22	19	F	21.6	Yes	0		
MDD23	31	M	20.5	No	29	-	melancholic
HC23	30	M	22.4	No	6		
MDD24	18	M	16.8	Yes	23	-	melancholic
HC24	23	M	17.7	Yes	5		
MDD25	47	F	19.5	No	27	GAD	melancholic
HC25	49	F	22.2	No	6		

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TABLE S2 | continued

	Age	Sex	BMI	Currently Smoking	MADRS Score	Psychiatric Comorbidity	Subtype (DSM5)
MDD26	26	M	18.3	Yes	32	-	melancholic
HC26	24	M	19.4	Yes	1		
MDD27	24	F	28.7	No	25	-	melancholic
HC27	28	F	26.6	No	0		
MDD28	18	F	30.4	No	19	SP	-
HC28	21	F	32.1	No	2		
MDD29	24	M	25.3	No	12	-	-
HC29	30	M	23.0	No	0		
MDD30	29	F	21.9	No	35	-	melancholic
HC30	26	F	24.8	No	5		
MDD31	25	F	21.0	No	31	PTSD	atypical
HC31	30	F	24.1	No	0		
MDD32	55	F	32.2	No	24	-	melancholic
HC32	46	F	31.9	No	0		
MDD33	27	F	25.3	Yes	31	SP & GAD	-
HC33	36	F	24.6	Yes	1		
MDD34	20	F	23.7	No	13	-	-
HC34	21	F	25.1	No	2		
MDD35	21	F	22.9	No	32	-	melancholic
HC35	22	F	21.9	No	1		

GAD: generalized anxiety disorder, PD: panic disorder, PTSD: post-traumatic stress disorder, SP: social phobia

TABLE S3 | Correlations between steroid signaling-related gene expression, PBMC phenotype and clinical variables in patients with MDD.

	BAI	BDI-II	MADRS	CTQ
Steroid-related Gene Expression				
T cells				
<i>GR</i>	-0.10	-0.297	-0.18	-0.154
	0.96	0.08	0.3	0.38
<i>MR</i>	0.012	-0.1	0.088	-0.224
	0.95	0.57	0.62	0.19
<i>GILZ</i>	0.322	0.025	0.089	-0.316
	0.06	0.89	0.61	0.06
<i>11β-HSD1</i>	0.137	-0.313	-0.14	-0.041
	0.44	0.07	0.43	0.82
Monocytes				
<i>GR</i>	-0.186	-0.232	-0.283	-0.007
	0.28	0.18	0.1	0.97
<i>MR</i>	0.129	0.128	0.236	-0.207
	0.46	0.47	0.17	0.23
<i>GILZ</i>	0.228	0.307	0.163	-0.169
	0.19	0.07	0.35	0.33
<i>11β-HSD1</i>	0.137	0.168	0.219	-0.278
	0.46	0.37	0.24	0.13
PBMC Phenotype				
Monocytes				
<i>Classical Monocytes %</i>	-0.047	-0.018	-0.014	-0.205
	0.79	0.92	0.94	0.25
<i>Intermediate Monocytes %</i>	0.221	0.033	-0.133	0.258
	0.22	0.86	0.46	0.15
<i>Non-classical Monocytes %</i>	-0.046	0.061	0.023	0.166
	0.96	0.74	0.9	0.36

TABLE S3 | continued

	BAI	BDI-II	MADRS	CTQ
Lymphocytes				
<i>CD4⁺ T Cells</i>	-0.009	0.203	0.035	-0.286
	0.96	0.26	0.85	0.11
<i>CD8⁺ T Cells</i>	0.043	-0.161	-0.112	0.383
	0.81	0.37	0.54	0.03
<i>Regulatory T Cells</i>	-0.083	-0.228	-0.332	-0.13
	0.67	0.24	0.08	0.5
<i>Cytotoxic Natural Killer Cells</i>	0.215	-0.102	-0.023	-0.034
	0.23	0.57	0.9	0.85
<i>Regulatory Natural Killer Cells</i>	-0.075	0.02	-0.371	0.324
	0.68	0.91	0.03	0.07
<i>B cells</i>	-0.147	-0.282	-0.212	-0.03
	0.41	0.11	0.24	0.86

BAI: Beck Anxiety Inventory, BDI-II: Beck Depression Inventory II, CTQ: Childhood Trauma Questionnaire, MADRS: Montgomery Asberg Depression Rating Scale. All correlation coefficients denote Spearman's rho with respective p values (two-tailed) indicated below.

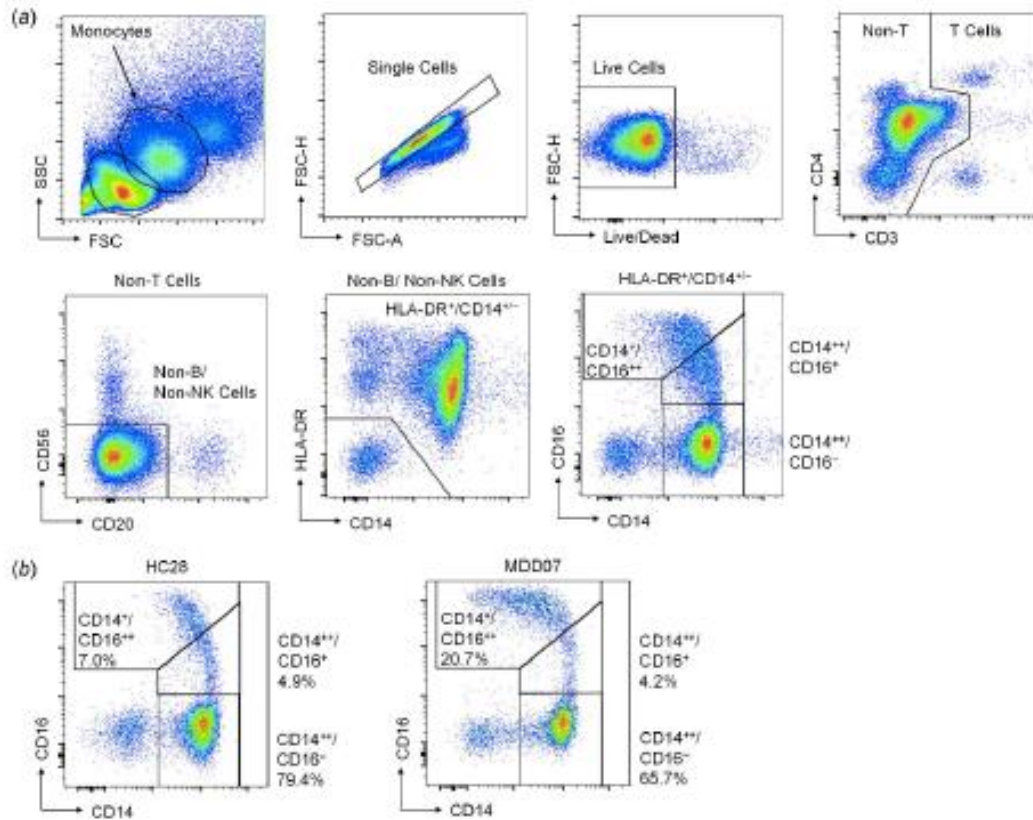


FIGURE S1 | Flow cytometry gating strategy for identification of classical (CD14⁺CD16⁻), intermediate (CD14⁺CD16⁺) and non-classical (CD14⁻CD16⁺) monocytes and exemplary staining for a patient-control pair. **(a)** Monocytes were identified by forward (FSC) and sideward scatter (SSC) properties. Next, doublets and dead cells were excluded as were remaining CD3⁺ T cells, B cells (CD20⁺), natural killer cells (CD56⁺) and CD14⁻/HLA-DR⁻ cells. Classification of monocytes based on surface expression of CD14 and CD16 followed established guidelines (21). **(b)** Exemplary monocyte staining in a representative patient-control pair.

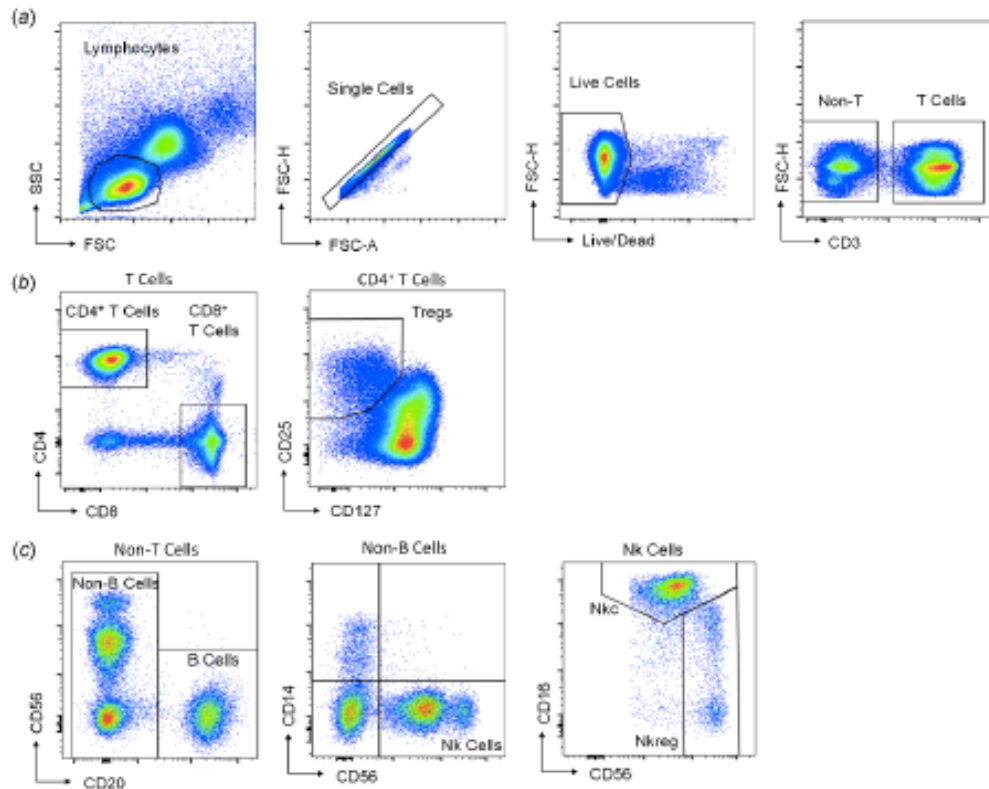


FIGURE S2 | Flow cytometry gating strategy for identification of lymphocyte phenotype. **(a)** First, lymphocytes were identified by forward (FSC) and sideward scatter (SSC) properties. Next, doublets and dead cells were excluded. Next, cells were gated for CD3 positivity (T cells) or negativity (Non-T cells). **(b)** T cells were further divided into CD4⁺ helper and CD8⁺ cytotoxic T cells. Among CD4⁺ T cells, regulatory T cells were identified as CD25⁺CD127⁻. **(c)** Among non-T cells, CD20⁺ B cells were identified. Next, among CD20⁻ cells NK cells were selected for CD56 positivity and CD14 negativity. Lastly, cytotoxic NK cells (Nkc) were defined as CD56⁺CD16⁺ and NK cells with a putatively regulatory phenotype (Nkreg) as CD56⁺CD16^{dim/-} as suggested by Maecker *et al.* 2012.

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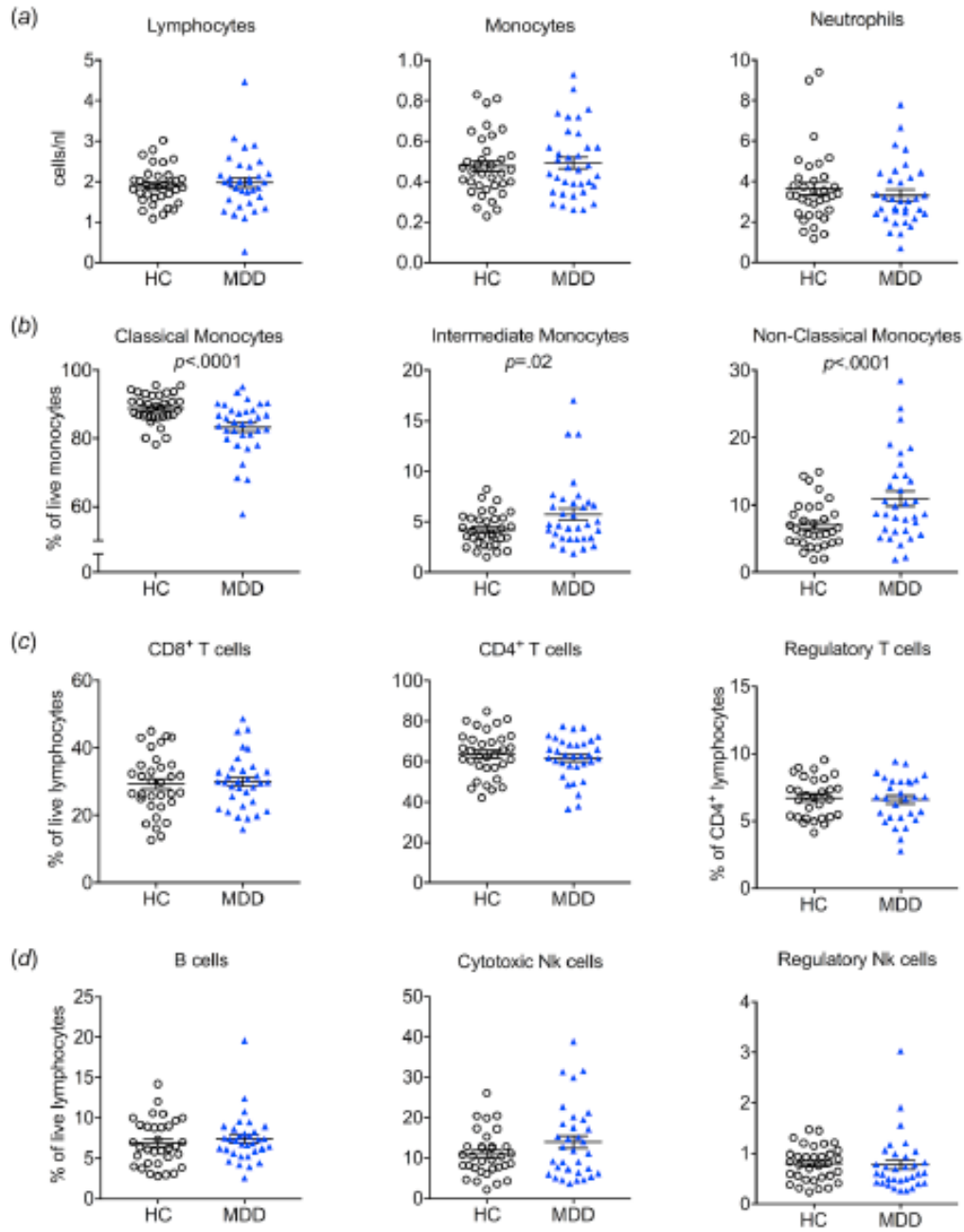


FIGURE S3 | (a) Routine laboratory blood cell counts of circulating leukocytes and proportions of (b) monocyte and (c) T cells subsets, as well as (d) B cells and Nk cells as measured by flow cytometry (mean \pm S.E.M.). If not depicted, all p -values > 0.1 .

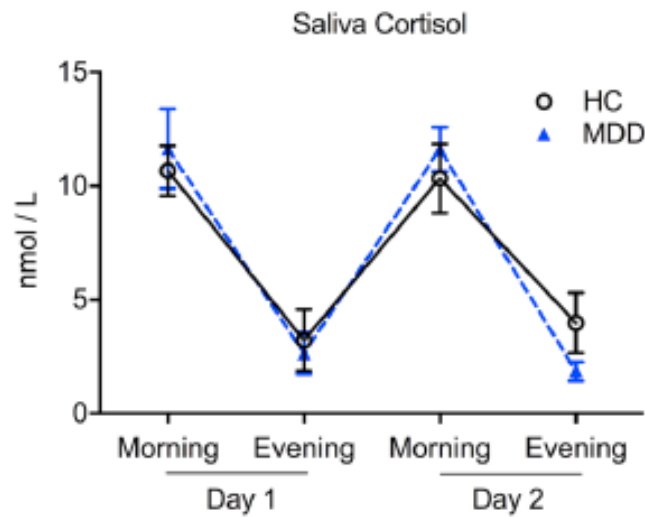


FIGURE S4 | Salivary cortisol levels in patients with MDD and matched healthy controls (mean \pm S.E.M.). Circadian HPA axis activity was estimated by salivary cortisol measures (8:00 a.m. and 22:00 p.m.) collected on two consecutive days (day 1 n = 28, day 2 n = 29).

8. Curriculum vitae

„Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.“

9. Publications and scientific contributions

9.1. Publications

Hasselmann H, Gamradt S, Taenzer A, Nowacki J, Zain R, Patas K, Ramien C, Paul F, Wingenfeld K, Piber D, Gold SM, Otte C (2018). Pro-inflammatory monocyte phenotype and cell-specific steroid signaling alterations in unmedicated patients with major depressive disorder. *Frontiers Immunology*, 9(2693). IF: 5.511

Baquet L, Hasselmann H, Patra S, Ketels G, Stellmann JP, Vettorazzi E, Engel AK, Rosenkranz SC, Poettgen J, Gold SM, Schulz KH, Heesen C (2018). Short-term aerobic training does not improve memory functioning in relapsing remitting multiple sclerosis – a randomized controlled trial. *PeerJ*. IF: 2.218

Gold SM, Enck P, Hasselmann H, Friede T, Hegerl U, Mohr D, Otte C (2017). Control conditions for randomised trials of behavioural interventions in psychiatry: a decision framework. *Lancet Psychiatry*, 4(9), 725-732. IF: 11.588

Veauthier C, Hasselmann H, Gold SM, Paul F (2016). The Berlin Treatment Algorithm. Recommendations for tailored innovative therapeutic strategies for multiple sclerosis-related fatigue. *EPMA* 7(25). IF: 0.49

de Kloet ER, Otte C, Kumsta R, Kok L, Hillegers MH, Hasselmann H, Kliegel D, Joels M (2016). Stress and Depression – a crucial role of the mineralocorticoid receptor. *J Neuroendocrinol* 28(8). IF: 3.470

Hasselmann H, Bellmann-Strobl J, Ricken R, Oberwahrenbrock T, Rose M, Otte C, Adli M, Paul F, Brandt AU, Finke C, Gold SM (2016). Characterizing the phenotype of multiple sclerosis-associated depression in comparison to idiopathic major depression. *Mult Scler*, 22(11), 1476-1481. IF: 4.840

Hasselmann H (2016). Interaction of ketamine and cannabis in bipolar disorder. *Biological Psychiatry*, 79(9). IF: 11.412

Hasselmann H (2015). Antidepressant and abuse potential of ketamine. In “The Neuropathology of Drug Addiction and Substance Misuse Volume 2” (Ed. Victor R. Preedy). Academic Press: London.

Godlewska, Hasselmann H, Igoumenou A, Norbury R, Cowen PJ (2014). Short-term escitalopram treatment and hippocampal volume. *Psychopharmacology*, 231(23), 4579-81. IF:3.308

Hasselmann H (2014). Scopolamine and Depression: A role for muscarinic antagonism? *CNS & Neurol Disord Drug Targets*, 13(4), 673-683. IF: 2.506

Hasselmann H (2014). Ketamine as antidepressant? Current state and future perspectives. *Curr Neuropsychopharmacol*, 12(1), 57-70. IF: 3.365

9.2. Posters

Hasselmann H, Gamradt S, Taenzer A, Nowacki J, Zain R, Patas K, Ramien C, Paul F, Wingenfeld K, Piber D, Gold SM, Otte C (2017). Cell-specific immune regulation and metabolic dysfunction in major depressive disorder. European Congress of Neuropsychopharmacology (ECNP) 30th Annual Congress, Paris. *Poster Award in the Category "Mood Disorders and Treatment"*

Hasselmann H, Bellmann-Strobl J, Ricken R, Oberwahrenbrock T, Rose M, Otte C, Adli M, Paul F, Brandt AU, Finke C, Gold SM (2015) Characterizing the phenotype of multiple sclerosis-associated depression in comparison to idiopathic major depression. European Committee of Treatment and Research in Multiple Sclerosis (ECTRIMS), 31st Congress, Barcelona.

Quested D, Hasselmann H, De Crescenzo F. (2015). Phase shift and bipolar disorder – casting light on a sometimes overlooked variable? British Association for Psychopharmacology Summer Meeting, Bristol.

9.3. Talks

Deutscher Kongress für Psychosomatische Medizin und Psychotherapie 2016: „Biologische Stress-Reaktionssysteme und Krankheitsaktivität bei MS“

Hanse-Wissenschaftskolleg Delmenhorst 2015: “Relevanz von Fatigue und vegetativen Symptomen im Kontext MS-assoziierter Depression“

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