# Peripheral blood cell-stratified subgroups of inflamed depression

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Table 1

**Abstract**

*Background* Depression has been associated with increased inflammatory proteins but changes in circulating immune cells are less well defined.

*Methods* We used multi-parametric flow cytometry to count 14 subsets of peripheral blood cells in 206 cases of depression and 77 age- and sex-matched controls (total N = 283). We used univariate and multivariate analyses to investigate the immunophenotypes associated with depression and depression severity.

*Results* Depressed cases, compared to controls, had significantly increased immune cell counts, especially neutrophils, $CD4^+$ T cells and monocytes, and increased inflammatory proteins (C-reactive protein, CRP, and interleukin-6, IL-6). Within-group analysis of cases demonstrated significant associations between the severity of depressive symptoms and increased myeloid and $CD4^+$ T cell counts. Depressed cases were partitioned into two subgroups by forced binary clustering of cell counts: the inflamed depression subgroup (N=81 out of 206; 39%) had increased monocyte, $CD4^+$ and neutrophil counts, increased CRP and IL-6, and was more depressed than the uninflamed majority of cases. Relaxing the presumption of a binary classification, data-driven analysis identified four subgroups of depressed cases: two of which (N=38 and N=100; 67% collectively) were associated with increased inflammatory proteins and more severe depression, but differed in terms of myeloid and lymphoid cell counts. Results were robust to potentially confounding effects of age, sex, body mass index, recent infection, and tobacco use.

*Conclusions* Peripheral immune cell counts were used to distinguish inflamed and uninflamed subgroups of depression and to indicate that there may be mechanistically distinct subgroups of inflamed depression.

## Introduction

There has been recent growth in the evidence for an association between depression and inflammation. Increased blood levels of inflammatory proteins (cytokines, like interleukin-6 (IL6), and C-reactive protein (CRP)) and increased expression of innate immune-related genes have been repeatedly reported in case-control studies of major depressive disorder (MDD) compared to non-depressed controls (1-6). There is experimental evidence that inflammation can *cause* depressive behaviours, both from animal studies showing that exposure to pro-inflammatory cytokines results in social withdrawal and anhedonia (7), and from human studies demonstrating that treatment (for hepatitis) with a pro-inflammatory cytokine (interferon) is followed by increased incidence of MDD (8). Convergently, meta-analytic reviews of clinical trial data have consistently demonstrated that anti-inflammatory drugs can significantly improve mood and fatigue symptoms, measured as secondary endpoints, in cases with major inflammatory disorder (9-11).

Thus inflammatory mechanisms could be plausible targets for repurposing or de novo development of anti-inflammatory drugs for anti-depressant efficacy in cases with “inflamed depression”, i.e., clinical symptoms of depression associated with clinical or biomarker evidence of inflammation. Inflamed depression hypothetically includes cases of “co-morbid” depression associated with major medical inflammatory disease; as well as a subgroup of MDD cases with low-grade inflammation detectable by blood or brain biomarkers. The concept of inflamed depression as a subgroup of MDD implies that there is an un-inflamed subgroup of cases who are depressed without any evidence for inflammation. This is an important distinction to be able to make in the design of clinical

trials for immunergic anti-depressant drugs, which should be precisely focused on the cases most likely to have a positive benefit:risk response to treatment.

Blood proteins – like cytokines and CRP – have been the focus of most immune biomarker research in psychiatry, to date; the potential utility of cellular immune markers has been relatively under-explored (12-18). Most case-control studies of leucocyte subsets have used small samples, limited immunophenotyping panels, and have generated somewhat inconsistent results. Depression has been reproducibly associated with leucocytosis, increased neutrophil to lymphocyte ratio, and increased ratio of $CD4^+$ to $CD8^+$ T cells (19-21). However, there are less consistent results concerning regulatory T cells, Th17 cells, natural killer (NK) cells, monocytes, and B cells (12-16, 18, 22). Other psychiatric disorders, including schizophrenia, bipolar disorder and autism have also been associated with altered cell counts (23-25). Notably, most prior studies have measured the relative frequency of each immune cell subset in proportion to the superset of peripheral blood mononuclear cells (PBMCs). Such relative cell counts are difficult to interpret since a decrease in the relative proportion of any given subset may reflect either an absolute decrease in their number or an absolute increase in the number of another PBMC subset.

We measured absolute numbers of 14 immune cell subsets from peripheral blood samples in 206 cases of depression and 77 healthy controls. We used multiple univariate and multivariate methods to identify cell counts that were significantly different between all cases and controls, and to explore the correlations between immune cells, inflammatory proteins and clinical variables. We tested the hypothesis

that a subgroup of depressed cases would have peripheral inflammation (26, 27) by a "top-down" analysis, dividing the cases into two subgroups based on their immune cell profiles, then testing for significant differences between them in terms of inflammatory proteins and clinical variables. We also used a more "bottom-up" or data-driven analysis to identify a theoretically unconstrained number of immune cell-stratified subgroups of cases and then tested for immunological and clinical differences between subgroups.

## Materials and Methods

### Study design

This was a case-control study of peripheral blood cell counts in depression cases and healthy controls.

Depression cases were ascertained as those participants who screened positive for current or past depressive symptoms on the SCID screening questionnaire (28), completed the Hamilton Rating Scale for Depression (HAM-D), and screened negative for bipolar disorder or non-affective psychosis. 114 cases (55%) had moderate-severe depressive symptoms (HAM-D ≥ 17), of whom 61% were currently taking anti-depressant medication; 50 cases (24%) had mild depressive symptoms (HAM-D 8-16) of whom 90% were currently medicated; and 42 cases (20%) had minimal depressive symptoms (HAM-D ≤ 7) of whom 100% were medicated. By design, this was a clinically heterogeneous sample inclusive of depressed cases across a spectrum of symptom severity and antidepressant medication exposure.

Matched healthy controls were recruited from the general population by advertisement and defined as participants with no personal history of depression, no previous antidepressant treatment for any indication, no history of any major psychiatric disorder as defined by SCID screening questionnaire, and by current HAM-D total score <7.

All participants satisfied inclusion criteria including age 25-50 years, and exclusion criteria including major medical disorder and immune-modulating drug treatment; see SI Methods for full list of eligibility criteria.

All study assessments were completed at one of five UK centers as part of the Biomarkers in Depression (BIODEP) study (4), which was approved by an independent research ethics committee (National Research Ethics Service East of England, Cambridge Central, UK; 15/EE/0092). All participants gave informed consent in writing and received £100 compensation.

*Assessments*

Participants completed the following clinical assessments and self-report questionnaires: Hamilton Depression Rating Scale (29); Beck Depression Inventory v2.0 (30); Chalder Fatigue Scale (31); Snaith-Hamilton Pleasure Scale (32); State-Trait Anxiety Inventory (33); Childhood Trauma Questionnaire (34); and Life Events Questionnaire (35). Height and weight were measured to calculate body mass index (mass / height$^2$). For 269 of the 283 participants, the HAM-D, CRP, absolute cell counts and flow cytometry were measured in the same month; and, for all participants, these assessments were completed within 80 days.

Fasting venous blood samples were taken between 8am and 10.30am for measurement of absolute blood cell counts (neutrophils, eosinophils, basophils, lymphocytes, monocytes, red cells and platelets); flow cytometry ($CD4^+$ T cells, $CD8^+$ T cells, B cells, classical monocytes, non-classical monocytes, intermediate monocytes, $CD16^{hi}$ NK cells, $CD56^{hi}$ NK cells and NKT cells); high-sensitivity C-reactive protein; lipid profile; and plasma interleukin-6 (IL6).

*Immuno-phenotyping*

Flow cytometry was performed on fresh PBMCs using live-dead stain and antibodies against CD3, CD4, CD8, CD19, CD56, CD14 and CD16 (**Table S1**). Data were manually gated, blind to case/control status of each participant, according to the strategy in **Figure S2**. Flow cytometry counts were recorded as percentages relative to larger cell subsets, then multiplied by the relevant absolute cell counts to calculate absolute cell counts used in this study. For example, the absolute count of classical monocytes was calculated as the proportion of total monocytes that were classical monocytes (measured by cytometry) multiplied by the absolute number of monocytes (from the absolute cell count). This allowed calculation of absolute cell counts for $CD4^+$ and $CD8^+$ T cells; classical, intermediate and non-classical monocytes; $CD16^{hi}$ and $CD56^{hi}$ NK cells; NKT cells, and B cells (see **Table S2** for all calculations).

**Statistical analysis**

All analyses were performed in [R] version 3.5.1 (R Core Team, 2018): see **Data availability** for code. *P*-values were corrected for multiple comparisons using the Benjamini-Hochberg procedure to control the false discovery rate (FDR). Group or subgroup effects on continuous variables were tested using Wilcoxon-Mann-Whitney or Kruskal Wallis tests, with FDR-corrected Conover non-parametric tests for post-hoc comparisons. (Sub)group effects on categorical variables were tested by $\chi^2$ tests with FDR-corrected $\chi^2$ tests for post-hoc pairwise comparisons; see SI Methods for details.

We used multivariate methods to deal with the high-dimensional, correlated data available on each participant. Principal components analysis (PCA) was used to identify the major dimensions of variation and co-variation over all 14 immune cell counts, using

data from all participants; partial least square discriminant analysis (PLS-DA) was used to identify the weighted function of immune cell counts most predictive of case/control status; partial least squares regression (PLS-R) was used to identify the weighted function of all 14 immune cell counts most strongly associated with multiple (four) clinical measures of depression severity within the cases, viz, HAM-D, BDI, Chalder Fatigue and SHAPS scores. For both PLS discriminant analysis and regression, *P*-values for the predictive ability of the models were estimated by permuting group labels or clinical score sets (5000 permutations). Significant weights were defined as those with an absolute bootstrapped *Z*-score >3 (24).

We used Gaussian finite multivariate mixture modeling, and consensus clustering, to identify subgroups of cases (and healthy controls) that shared an immune cell profile in common with each other and in contrast to the immune cells profile of cases in other subgroups (36, 37); see SI Methods for details.

## Results

*Sample characteristics*

Quality-controlled absolute counts of 14 cell types were available on a sample of 283 participants comprising 206 depressed cases (143 female, 66 male) and 77 healthy controls (52 female, 25 male). Case and control groups did not significantly differ in terms of mean age, sex, or current use of tobacco or cannabis. As expected, the cases were significantly more depressed, anxious and fatigued, and reported significantly more current stress, childhood trauma, alcohol use and unemployment, than controls (**Table 1**). By design, the cases were clinically heterogeneous, and enriched for moderate-severe depressive symptom scores despite current or past treatment with monoaminergic anti-depressant medication (**Figure S1**).

*Case-control differences in peripheral blood cell counts and inflammatory proteins*

We first estimated case-control differences in peripheral blood cell counts and inflammatory proteins using multiple univariate comparisons. Serum CRP (Mann-Whitney U: $P = 0.003$, effect size = 0.18) and plasma IL-6 concentrations ($P = 0.04$, effect size = 0.14), as well as absolute counts of neutrophils ($P = 0.01$, effect size = 0.15), intermediate monocytes ($P = 0.02$; effect size = 0.14) and $CD4^+$ (helper) T cells ($P = 0.003$, effect size = 0.18), were significantly increased in the depressed group (**Figure 1A, Table S3**). When case-control comparisons were corrected for the 16 biomarkers tested (FDR < 0.05), CRP and $CD4^+$ T cells remained significantly different between the groups.

*Correlational and principal components analysis of cellular, protein and clinical variables*

We estimated correlations between all immunological, clinical and demographic variables in the whole sample (N=283) (**Figure 1B**). Immune cell counts and inflammatory protein concentrations were positively correlated with each other, as were questionnaire measures of symptom severity and stress. The strongest pair-wise correlations between cell counts and clinical variables were between neutrophil count and HAM-D score (Spearman's $\rho$ = +0.27, FDR $P$ = 0.00003), and neutrophil count and BDI score ($\rho$ = +0.25, FDR $P$ = 0.0002). The correlation matrix estimated from data on cases only (N=206) was very similar to the whole sample matrix (**Figure S3**).

We used principal component analysis to summarise the correlated data on 14 blood cell counts in terms of the first 2 principal components, which together accounted for 29% of the total variance-covariance. The first principal component (PC1; 19% total (co)variance) was a weighted average of all cell counts, most strongly weighted on myeloid cells (neutrophils, basophils and classical monocytes) and $CD4^+$ T cells (**Figure 2A**). The second principal component (PC2; 10% total (co)variance) was most strongly weighted on classical and non-classical monocytes and $CD16^{hi}$ NK cells (**Figure S4A**). Similar results were obtained when PCA was repeated for sex-specific subgroups of cases (**Figure S4**).

PC1 scores were positively correlated with serum CRP ($\rho$ = 0.26, FDR $P$ = 0.00004), and IL-6 ($\rho$ = 0.34, FDR $P$ = 0.000004) concentrations. The depressed cases had higher mean PC1 scores than controls (Mann Whitney U: $P$ = 0.006, standard effect

size = 0.16, **Figure 2B**) and PC1 scores were positively correlated with multiple measures of symptom severity including observer-rated depressive symptoms (HAM-D, $\rho$ = 0.26, FDR *P* = 0.00004), self-reported depressive symptoms (BDI, $\rho$ = 0.24, FDR *P* = 0.0002) and anhedonia (SHAPS, $\rho$ = 0.23, FDR *P* = 0.0004), as well as BMI ($\rho$ = 0.24, FDR *P* = 0.00004) (**Figure 2B**). A scatterplot of each participant's scores on both PCs (**Figure 2A**) indicated that the majority of depressed cases had blood cell profiles overlapping those of healthy controls, but there was a subgroup of depressed cases with highly positive PC1 scores, indicating distinctively increased numbers of myeloid and $CD4^+$ T cells.

*Discriminant analysis of immune cell counts most predictive of case/control status*

We used partial least squares (PLS) discriminant analysis (**Figure 3A, S4E**) to find the weighted function of the 14 immune cell counts that most accurately discriminated between cases and controls. This discriminant function accounted for a small but significant proportion (6.3%) of the variability in diagnostic status (*P* = 0.002, permutation test). Absolute cell counts for $CD4^+$ T cells, neutrophils and eosinophils were significantly weighted on the discriminant function, indicating that a combination of these cell counts was most predictive of case/control status.

*Association of immune cell counts with severity of depression in cases*

We used PLS regression to test the hypothesis that a weighted function of immune cell counts predicted variability of depressive symptom severity among the cases. We found that a single PLS-R component accounted for a small (7.3%) but significant proportion of the variance in depressive symptom scores measured on multiple clinical

questionnaires (HAM-D, BDI, Chalder Fatigue and SHAPS) ($P$ = 0.001, permutation test). The cell counts significantly weighted on the PLS-R component were neutrophils, NKT cells and B cells, indicating that a combination of these three cell counts was most strongly related to symptom severity, especially as self-reported by the BDI (**Figure 3B**).

*"Top-down" analysis of two subgroups of depressed cases*

To make a binary partition of depressed cases into two subgroups based only on their immune cell count data, we used Gaussian finite multivariate mixture modeling under the constraint that the number of distributions in the mixture must be two. This analysis identified one subgroup of N=81 cases (39%) that had increased absolute counts of several immune cells (monocytes, granulocytes, $CD16^{hi}$ NK cells, NKT cells, B cells, T cells and platelets) compared to a second subgroup of cases (N=125, 61%) (**Figure 4A,B**).

The subgroup of cases with increased immune cell counts also had significantly increased inflammatory protein concentrations (CRP, $P$ = 0.03, standard effect size = 0.16; and IL6, $P$ = 0.02, standard effect size 0.19; **Figure 4C, Table S4**), compared to the second subgroup with decreased immune cell counts, and hence it was referred to as the inflamed depression subgroup. Cases of inflamed depression had significantly higher severity of observer-rated depressive symptoms (HAM-D, $P$ = 0.0002, effect size = 0.26) and self-reported depressive symptoms (BDI, $P$ = 0.01, effect size = 0.18), compared to the uninflamed depression cases. Inflamed vs uninflamed cases had twice the rate of unemployment (33% vs.17%, $P$ = 0.008), were slightly older (median age 38 years vs. 34 years, $P$ = 0.01), and more likely to be smokers (19% vs. 7%, $P$ = 0.01).

However, the two subgroups did not differ significantly on sex, study center, current antidepressant use, alcohol or cannabis use, reported recent infection or minor inflammatory disease, or BMI (**Figure S5A**,**B**, **Table S4**).

*Sensitivity analysis of immune cell binarization of un/inflamed depression*

To test the robustness of this key result – that binarization of all depression cases on cell counts identifies immunologically and clinically distinct subgroups of inflamed and uninflamed depression – we conducted two sensitivity analyses and a benchmarking study (detailed in Supplemental Material and summarized briefly here): (i) robustness to diagnostic eligibility criteria: we included only the subset of depression cases with a SCID diagnosis of major depressive disorder (MDD; N=139); (ii) robustness to potential confounds in case-control data: we used linear regression to mitigate the effects of age, sex, BMI, recent infection history and tobacco use before case-control analysis of residualised counts of the 14 immune cell subsets (**Figures 4, S5, S6**). In both these sensitivity analyses, we replicated identification of a subgroup of inflamed cases with more severe depressive symptoms. The first principal component of the residual cell counts was very similar to PC1 for the absolute counts (**Figure 4E**, **S5A**). Recapitulating the results for the absolute cell counts, PLS-R of residual cell counts identified a single component weighted on neutrophil and NKT cell counts as most predictive of depression severity (permutation test, $P = 0.01$). Binarization of depressed cases using residual immune cell counts again identified an inflamed subgroup with higher counts across all 14 cell types and increased HAM-D and BDI scores compared to an uninflamed subgroup (**Figure 4E, 4F, S6**). For benchmarking, the results of immune cell binarization of cases were compared to the results of binarization of controls (N=87).

Data-driven clustering identified a subgroup of controls (N=20) with very low cell counts and a subgroup with slightly higher cell counts (N=57) (**Figure S8A**). The marginally more inflamed subgroup was associated with higher BMI, higher IL-6, and male gender; but had significantly lower lymphoid and myeloid counts than the inflamed MDD cluster (**Figure S8B**).

*"Bottom-up" analysis of immune-cell stratified subgroups of cases*

We used Gaussian finite multivariate mixture modeling and consensus clustering, without prior constraint on the number of distributions in the mixture, to identify 4 subgroups of cases (**Figure 5, S7, Table S5**), each characterized by a distinct profile of absolute immune cell counts (**Figure 5B, S7A**). One subgroup comprised 58 cases (28%) with low counts for all cells and low CRP and IL6 levels and was designated uninflamed (S0). Subgroups 2 and 3 had significantly increased inflammatory proteins, and significantly increased depressive symptom severity scores, compared to S0; but they also differed from each other in terms of their immune cell profiles. Subgroup 3 had a stronger myeloid bias compared to subgroup 2, with significantly higher numbers of classical monocytes, intermediate monocytes, non-classical monocytes and neutrophils (**Figure S7A**). Subgroup 2 had a lymphoid bias with significantly higher numbers of adaptive immune cells ($CD4^+$ T cells, $CD8^+$ T cells and B cells) compared to the uninflamed subgroup (**Figure 5B, S7A**). This four-way, bottom-up stratification of cases was not simply nested within the top-down binarization (**Table S6**). There were no significant differences between these 4 immune cell-stratified subgroups in terms of multiple, potentially confounding demographic and clinical factors (**Figure S7B**, **Table S5**).

## Discussion

*Immune cell systems and depression*

We confirmed case-control mean differences in CRP and IL6, as well as increased absolute counts of neutrophils, intermediate monocytes, and $CD4^+$ T cells in depressed cases, by the conventional approach of multiple univariate testing (**Figure 1A**) (1, 4, 5, 19). We also observed that the immunological variables were correlated with each other, and with measures of depressive symptom severity, prompting further investigation with multivariate methods. The first principal component of the cellular data represented a weighted sum of all cell counts, especially myeloid and $CD4^+$ T cells, and was positively correlated with both inflammatory protein concentrations and depressive symptom scores. Partial least squares (PLS) identified the weighted functions of immune cell counts, especially neutrophil cell counts, that optimally discriminated between cases or controls, or were most predictive of variation in depressive symptom severity.

These results, in the context of the prior literature, tell us that peripheral blood cell counts are plausible as candidate biomarkers of “inflamed depression”, and the most informative cellular biomarkers are likely to summarise the status of a system of functionally or developmentally related cells, rather than a solitary “smoking gun”. Myeloid cells, especially neutrophils, were strongly implicated in these data. Absolute neutrophil numbers were increased in depressed cases, positively correlated with depressive symptom scores (**Figure 1B**), and strongly weighted on the PLS functions that optimally discriminated cases from controls or predicted symptom severity (**Figure 3**). These findings are compatible with prior emphasis on the role of the innate immune

system in depression and, more specifically, with reports of case-control differences in total leucocyte count, neutrophil count, or neutrophil/lymphocyte ratio (19-21, 38). The hypercortisolemia observed in some depressed cases (39) may thus relate to the neutrophilia observed in these data and other studies (40, 41). Neutrophils can traffic to the brain and neutrophil depletion has been shown to mitigate the effects of inflammation on behavior in animal models (42).

However, it would be simplistic at this stage to assert that myeloid cells are the only immune cells relevant to depression. For example, $CD4^+$ helper T cells were correlated with myeloid cell counts, increased in depressed cases, and strongly weighted on the PLS discriminant function. Helper T cells are known to facilitate cytokine production and other inflammatory responses by myeloid cells (43); and myeloid antigen presenting cells are important for activating and polarizing $CD4^+$ T cells towards a terminally differentiated state (44). In short, there are two-way interactions between myeloid and lymphoid cells that may underlie the observed pattern of depression-related change in multiple cell types. A role for adaptive as well as innate immunity in depression is also compatible with transcriptional results (6) that indicate coupled changes in peripheral whole blood expression of genes specialized for innate and adaptive immune functions.

*Immune cell stratified subgroups of depression*

These results also tell us that not all cases of depression are equally likely to be associated with abnormal immune cell counts, which is compatible with prior expectations of a subgroup of cases with “inflamed depression”. We tested this prediction more explicitly using mixture modeling to decompose the multivariate

distribution of immune cell counts in the depressed group into two or more component distributions or subgroups. Initially, we specified this analysis to identify two subgroups, which we found were indeed significantly different from each other immunologically and clinically. About 40% of depressed cases had increased immune cell counts, increased inflammatory proteins, and increased symptom severity scores, compared to the remaining 60% of uninflamed cases. These results are consistent with prior observations that approximately a third of MDD cases have CRP levels greater than the upper limit of the normal range (3 mg/L) and that depression is symptomatically more severe when associated with inflammation (4).

However, there is no prior reason to assume that there should be only one subgroup of inflamed depression. When the multivariate mixture analysis was repeated, without constraining the algorithm to find a binary solution, we found 4 immune cell-stratified subgroups, of which two were associated with equivalently-increased inflammatory proteins and depressive symptom scores compared to the uninflamed subgroup. These two inflamed subgroups together accounted for about two thirds of cases, suggesting that the proportion of depression cases associated with inflammation may be underestimated by the conventional cut-off of CRP > 3 mg/L. Intriguingly, the existence of two inflamed subgroups, differentiated by their distinctively myeloid- vs lymphoid-biased immune cell profiles, suggests that there may be more than one mechanistic pathway to the same syndrome of high depressive symptoms and increased inflammatory proteins. For example, some cases of inflamed depression may be caused primarily by proliferation or activation of myeloid cells, innately responding by pattern recognition receptors to acute stress or infection, whereas other cases may be driven by

T helper cells or B cells with a longer term memory of past exposure to stress, infection or other antecedent immune challenges. This concept of multiple species of inflamed depression, rather than a monolithic subgroup, could have important implications for the design of immunological interventions targeting more fundamentally causal mechanisms, rather than downstream biomarkers such as CRP or IL6.

*Methodological issues*

Case-control designs are vulnerable to the effects of uncontrolled confounding variables and there are many demographic, clinical, and lifestyle factors that could have effects on peripheral immune biomarkers. This sample of cases was designed to encompasses considerable clinical heterogeneity, which is useful for the within-group analysis, but is not epidemiologically representative. The sample is large (N=283; 206 cases), and the number of cell subsets counted is large (p=14), by comparison to prior immune cell studies of MDD; the order of magnitude difference between N and p is desirable for multivariate analysis. However, it will require an order of magnitude increase in sample size to fully explore and exploit the cellular resolution of contemporary immuno-phenotyping for stratification of inflamed depression.

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E.B., P.C., N.H., W.C.D., J.C., D.J.; Project Administration, J.B., L.P., E.B., D.J.; Supervision, E.B. and M.R.C; Funding Acquisition, E.B., M.E.L., M.R.C., C.P., J.C., V.M., P.C., N.H., W.C.D, D.J.

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**Data and materials availability**: Further information and requests for resources and reagents should be directed to Mary-Ellen Lynall (mel41@cam.ac.uk). The sharing of data used in this study is restricted by the informed consent process. Our data cannot be made available on public repositories but will be shared with other scientifically accredited research groups on request (Apollo Repository https://doi.org/10.17863/CAM.40364). [R] code to support the analyses and figure generation is available on Github (maryellenlynall/2019_depression_flow_cytometry).

# Figure legends

**Figure 1: Peripheral immunophenotypes in MDD and control participants**

**(A)** Comparison of 14 absolute cell counts, high sensitivity CRP and plasma IL-6 in major depressive disorder (MDD, n=206) and matched controls (n=77). Boxplots show median and interquartile range, with the outer violin shape showing the full distribution of data. Color indicates statistical significance by Mann Whitney U test (purple $P < 0.05$; red FDR $P < 0.05$). FDR p-values are corrected for 16 multiple comparisons. See **Table S3** for underlying data and effects sizes.

**(B)** Spearman correlations between immunological, clinical and demographic variables. Only those correlations significant at FDR $P < 0.05$ are shown. FDR p-values are corrected for 325 multiple comparisons. Color indicates the correlation coefficient (Spearman's $\rho$); dark blue outlines group together similar variables.

**Figure 2: Principal components analysis of cellular immunophenotypes**

**(A)** Principal components analysis (PCA) for the 14 absolute cell counts across all participants (n=283). On the left panel, each point (MDD in blue; controls in grey) represents one participant's scores on the first two principal components (PC1 and PC2). Red arrows show the loadings of each cell count on the first two principal components. Ellipses show the 95% confidence ellipse for each group. Right hand panel shows the PCA eigenvector for PC1. See **Figure S4** for PC2 and PCA excluding cases with minor inflammatory conditions, and for each sex separately.

**(B)** Spearman correlations between the first principal component of the cellular immunophenotype (PC1), clinical features, demographic features, and peripheral proteins (n=283 participants). Only correlations significant at FDR $P < 0.05$ are shown. FDR p-values are corrected for 33 multiple comparisons. Color indicates the correlation coefficient (Spearman's $\rho$). The right-hand side boxplot shows the PC1 scores for MDD cases and controls (Mann-Whitney test, estimate=0.54, effect size=0.16, $P = 0.006$). Boxplots show median and interquartile range, outer violin shape shows the full distribution of data.

**Figure 3: Cellular predictors of MDD status and symptoms severity**

**(A)** Partial least squares discriminant analysis (PLS-DA) for the predictors of case-control status. Response variable is major depressive disorder (MDD)/control status (purple point), predictor variables are the 14 absolute cell counts from **Figure 1A** (green points). Analysis includes all participants (n=283). A single component PLS model (Component 1) is significantly predictive of MDD status by permutation testing ($P$ = 0.002). Of the 14 cell types, only those with significant weights in the model are labelled: neutrophils, eosinophils and $CD4^+$ T cells (bootstrapped Z-score >3, see **Methods**). See also **Figure S4E**.

**(B)** Partial least squares regression (PLS-R) for the predictors of depressive symptom severity within the MDD group. Response variable is the matrix of symptoms scores (shown in purple), predictor variables are the 14 absolute cell counts (green points). A single component PLS model (Component 1) is significantly predictive of MDD severity by permutation testing ($P$ = 0.001). Of the 14 predictor cell types, only those with significant weights in the model are labelled: neutrophils, NKT cells and B cells (bootstrapped Z-score >3, see **Methods**). Analysis includes MDD cases only (n=199 with full clinical scores available). SHAPS = Snaith-Hamilton Pleasure Scale. HAM = Hamilton Depression Rating Scale.

**Figure 4: Theoretically driven immune cell stratification into inflamed and uninflamed MDD subgroups**

**(A)** Gaussian finite mixture modelling of the cellular phenotypes for MDD cases (n=206). Forced two-way mixture modelling identified two clustered immunophenotypes, uninflamed depression (UD, n=125 cases, grey) and inflamed depression (ID, n=81, red). Plot shows the PCA scores for each case on cellular PC1 and PC2 (**Figure S5A**), with cluster membership indicated by color.

**(B, C, D)** Comparisons between the two clusters. Boxplots show median and interquartile range for each cluster, with the outer violin shape showing the full distribution of data. Effects of cluster were tested by Mann-Whitney U or (for unemployment) $\chi^2$, **P*<0.05, ***P*<0.01, ****P*<0.01. **(B)** Absolute cell counts (inputs to clustering). **(C)** Peripheral blood markers: C-reactive protein (CRP), interleukin-6 (IL-6) and triglycerides (not used as inputs to clustering). **(D)** Clinical phenotype of participants in each cluster. Bar annotations indicate participant numbers. See **Table S4** and **Figure S5** for statistics, further clinical and demographic measures and item-level scores corresponding to MDD 'typicality'.

**(E)** Sensitivity analysis: principal components analysis (PCA) of the 14 residual cell counts after linear regression of body mass index (BMI), age, sex, current tobacco use and recent infection on each cell count (MDD cases only, N=206). Left-hand panel shows the eigenvector for the first principal component of the residual cell counts (PC1). Right hand panel shows the results of forced two-way clustering of the residual cell counts, which identified two immune cell-stratified subgroups of cases: uninflamed depression (N=104, grey) and inflamed depression (N=102, red), overlaid on a scatterplot of PCA scores.

**(F)** Depression rating scores for inflamed and uninflamed cases identified by binarization of residual immune cell counts. Inflamed vs uninflamed subgroup differences were tested by Mann-Whitney U, **P*<0.05, ***P*<0.01. HAM-D = Hamilton Depression Rating Scale (practitioner-administered), BDI = Beck Depression Inventory (self-report).

**Figure 5: Data-driven immune cell stratified MDD subgroups**

**(A)** Data-driven Gaussian finite mixture modelling of the cellular phenotypes for MDD cases (n=206) identified four discrete clusters (immunophenotypes). Plot shows the PCA scores for each participant on cellular PC1 and PC2, with cluster indicated by color. The arbitrary cluster numbers and colors are used consistently throughout this figure to designate each cluster (cluster 0, grey, n=58 cases; cluster 1, orange, n=10; cluster 2, blue, n=100; cluster 3, red, n=38).

**(B)** A radar plot shows the characteristic immune cell profile of each cluster of cases. Points represents the median value of the 14 absolute cell counts for each of the four clusters, rescaled onto a 0 to 1 range (with higher values on the outside of the plot) to highlight relative differences between clusters. Cluster differences are significant for all counts shown (Kruskal-Wallis $P<0.05$); red blood cell and $CD56^{hi}$ NK cell counts did not differ between the clusters and are not shown. See **Figure S7A** for underlying data and statistics.

**(C, D)** Inflammatory proteins, clinical and demographic data for each immune cell-stratified subgroup of cases. Cases in subgroup 3 (inflamed, myeloid-biased) had significantly increased observer-rated depressive symptoms (HAM-D, FDR $P = 0.004$), self-reported depressive symptoms (BDI, FDR $P = 0.006$), and anhedonia (SHAPS, FDR $P = 0.006$), compared to the uninflamed subgroup. Cases in subgroup 2 (inflamed, lymphoid-biased) likewise had significantly increased self-reported depressive symptoms (BDI; FDR $P = 0.003$), anhedonia (SHAPS; FDR $P = 0.004$), and fatigue ratings (CFS; FDR $P = 0.02$), compared to the uninflamed subgroup. Boxplots show the median and inter-quartile range of the relevant variable for each cluster, violin outline shows the full distribution of data. The effect of cluster on each continuous feature is

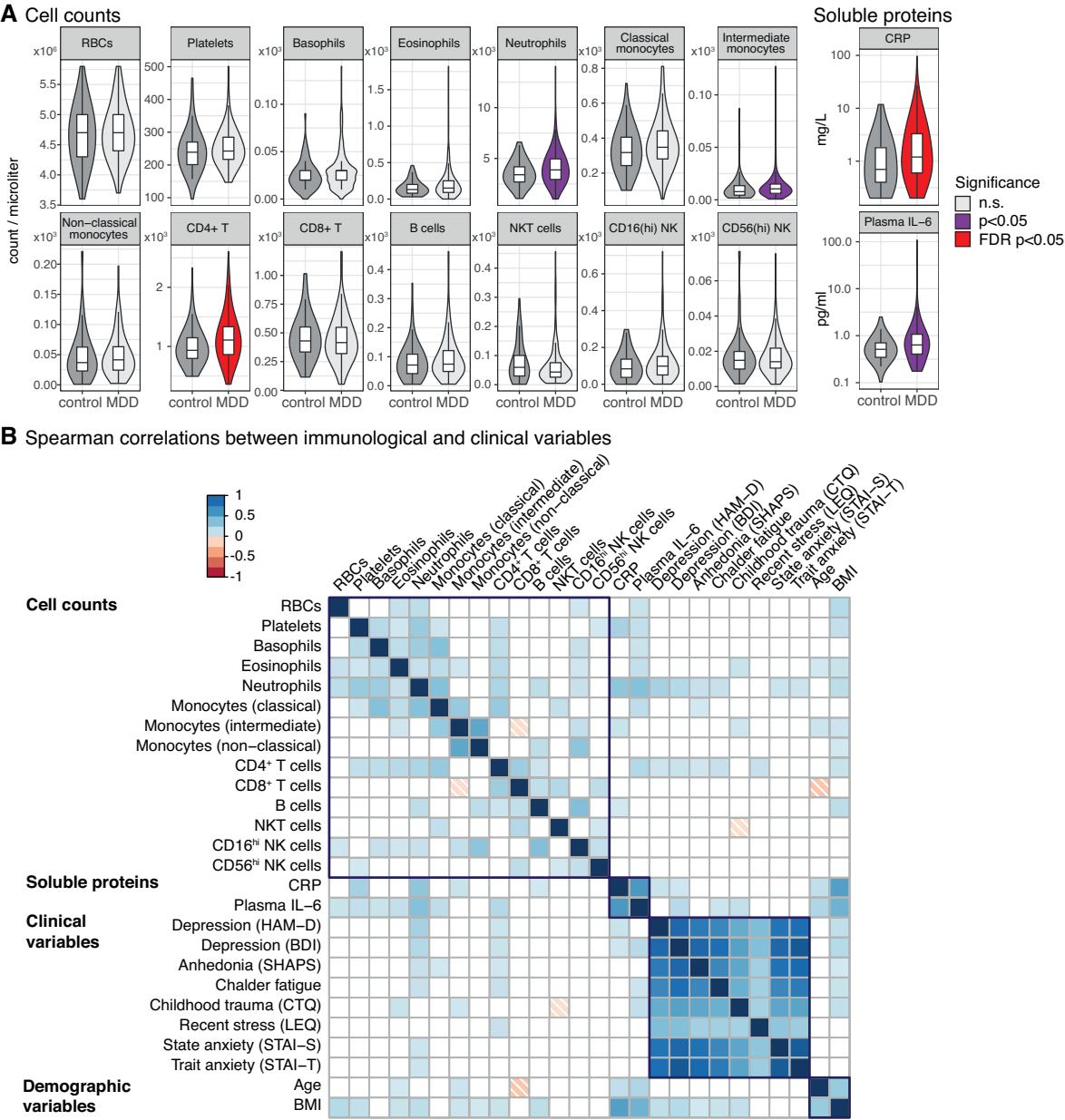
tested by Kruskal-Wallis testing. Where $P < 0.05$ for the overall Kruskal-Wallis test, we performed post-hoc Conover tests to identify which pairs of clusters differ for that feature – for these variables, each cluster was compared to every other cluster. For unemployment, the bar chart indicates the percentage of participants in each cluster and bar annotations indicate participant numbers. Clusters were compared by $\chi^2$ testing, with post-hoc $\chi^2$ tests to compare pairs of clusters. All p-values shown are corrected for the 6 pairwise cluster-cluster comparisons performed: FDR *P* *<0.05, **<0.01, and ***<0.001. Pairwise comparisons which were non-significant following FDR correction are not shown. For further statistics, demographics and item-level scores corresponding to MDD 'typicality' see **Figures S7, Table S5**. HAM-D = Hamilton Depression Rating Scale (practitioner-administered), BDI = Beck Depression Inventory (self-report), SHAPS = Snaith-Hamilton Pleasure Scale.

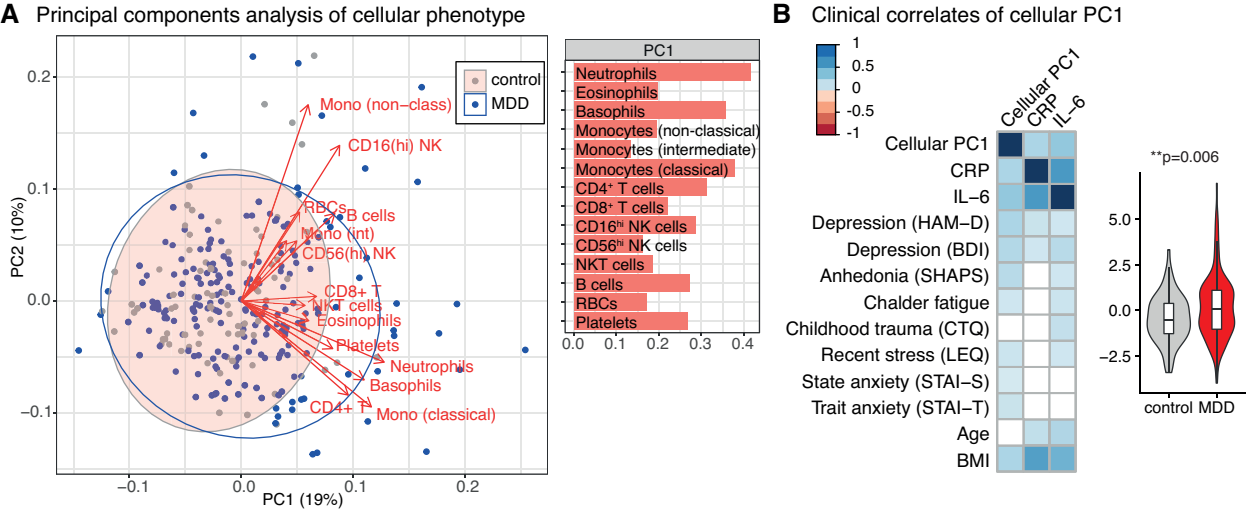
**Table 1: Demographic and clinical characteristics of the study population** (see caption over)

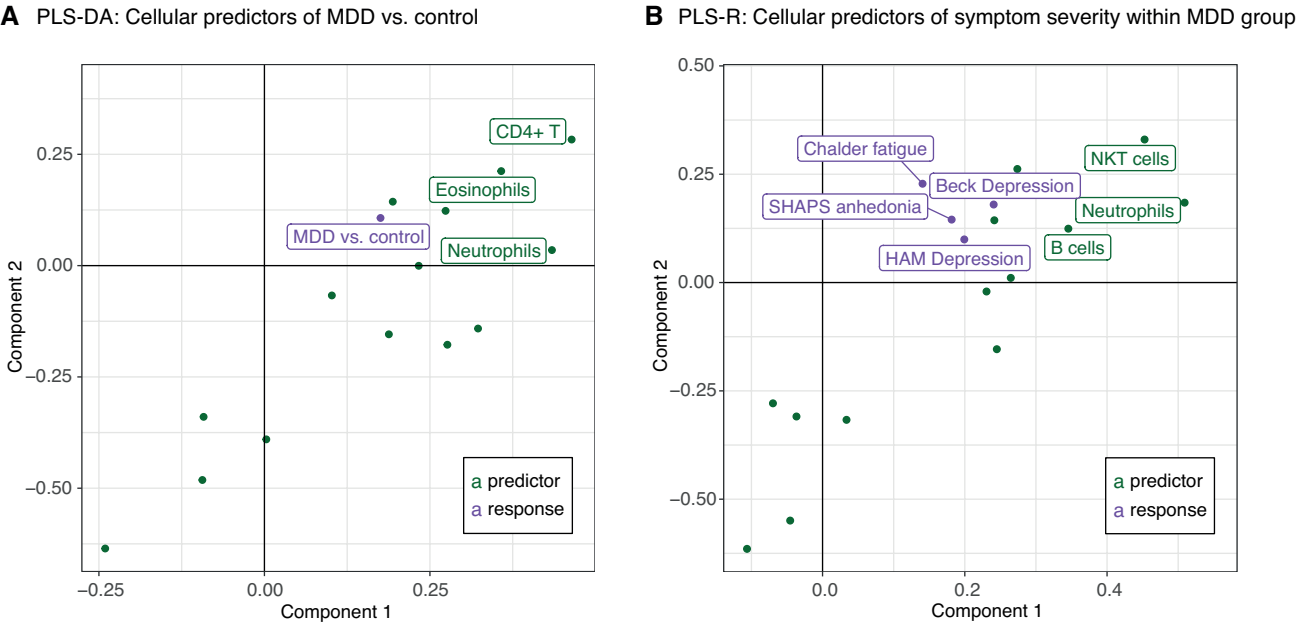
| Participant characteristics | Control (median, IQR) | MDD (median, IQR) | P-value (MWU) | Effect size $Z/\sqrt{(n)}$ | n (missing data) |
|---|---|---|---|---|---|
| **Number of participants** | N = 77 | N = 206 | - | | |
| **Age** | 32.5 (28.3, 39.1) | 35.3 (28.7, 42.9) | 0.09 | 0.10 | 0 |
| **BMI** | 23.5 (21.3, 27.6) | 26.6 (23.0, 31.2) | ***0.0008 | 0.20 | 8 |
| **Hamilton depression rating** | 0 (0.0, 1.0) | 17 (14.0, 20.0) | ***2E-53 | 0.74 | 0 |
| **Beck depression inventory** | 1 (0.0, 3.0) | 24 (15.0, 31.2) | ***8E-43 | 0.70 | 6 |
| **Chalder fatigue score** | 11 (8.0, 11.0) | 19 (14.0, 23.5) | ***4E-33 | 0.64 | 3 |
| **Snaith-Hamilton Pleasure Scale** | 0 (0, 0) | 4 (1, 7) | ***2E-24 | 0.58 | 4 |
| **STAI (state subscale)** | 25 (22.0, 29.0) | 50 (38.5, 57.5) | ***2E-39 | 0.68 | 3 |
| **STAI (trait subscale)** | 27 (24.0, 32.0) | 60 (52.0, 68.0) | ***1E-48 | 0.73 | 3 |
| **Childhood trauma score** | 35.0 (33.0, 38.5) | 49.5 (40.0, 62.0) | ***3E-19 | 0.51 | 6 |
| **Recent stressors (z-score)** | -0.9 (-0.9, -0.3) | -0.2 (-0.4, 0.5) | ***1E-10 | 0.37 | 4 |
| **Number of previous ineffective antidepressant treatments (<75% response)** | - | 1.0 (1.0, 3.0) | - | - | 7 |
| | **Control (percent)** | **MDD (percent)** | **P-value** | $\chi^2$ | **n (missing data)** |
| **Female sex** | 68% | 69% | 0.8 | 0.09 | 0 |
| **Unemployed (including for medical reasons)** | 0% | 23% | ***0.0005 | 21.4 | 3 |
| **Current tobacco use** | 11% | 12% | 0.8 | 0.09 | 4 |
| **Current alcohol use** | 33% | 48% | *0.03 | 4.81 | 5 |
| **Current cannabis use** | 3% | 6% | 0.3 | 1.57 | 5 |
| **Current antidepressant use** | - | 75% | - | | 5 |

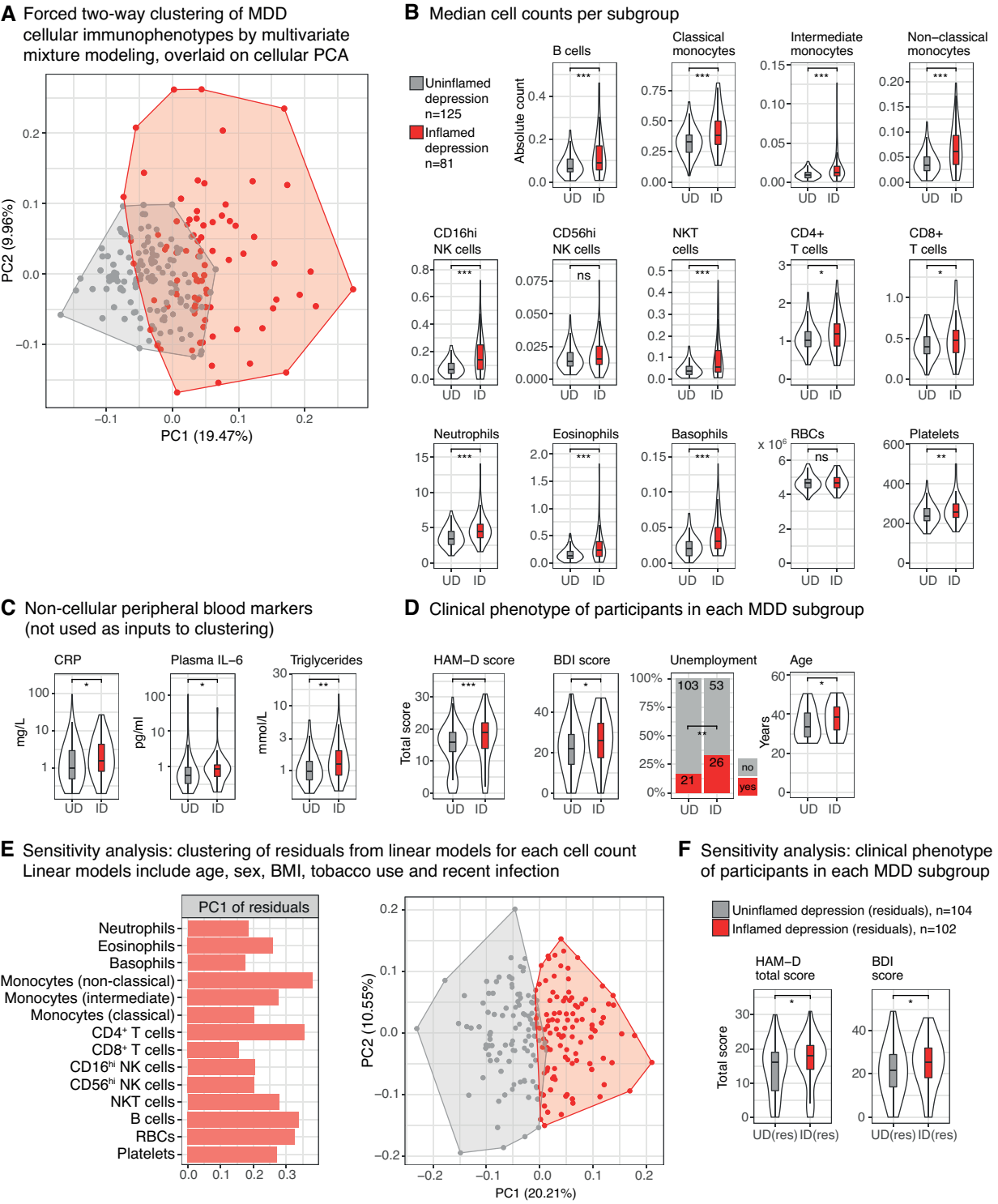
**Table 1: Demographic and clinical characteristics of the study population**

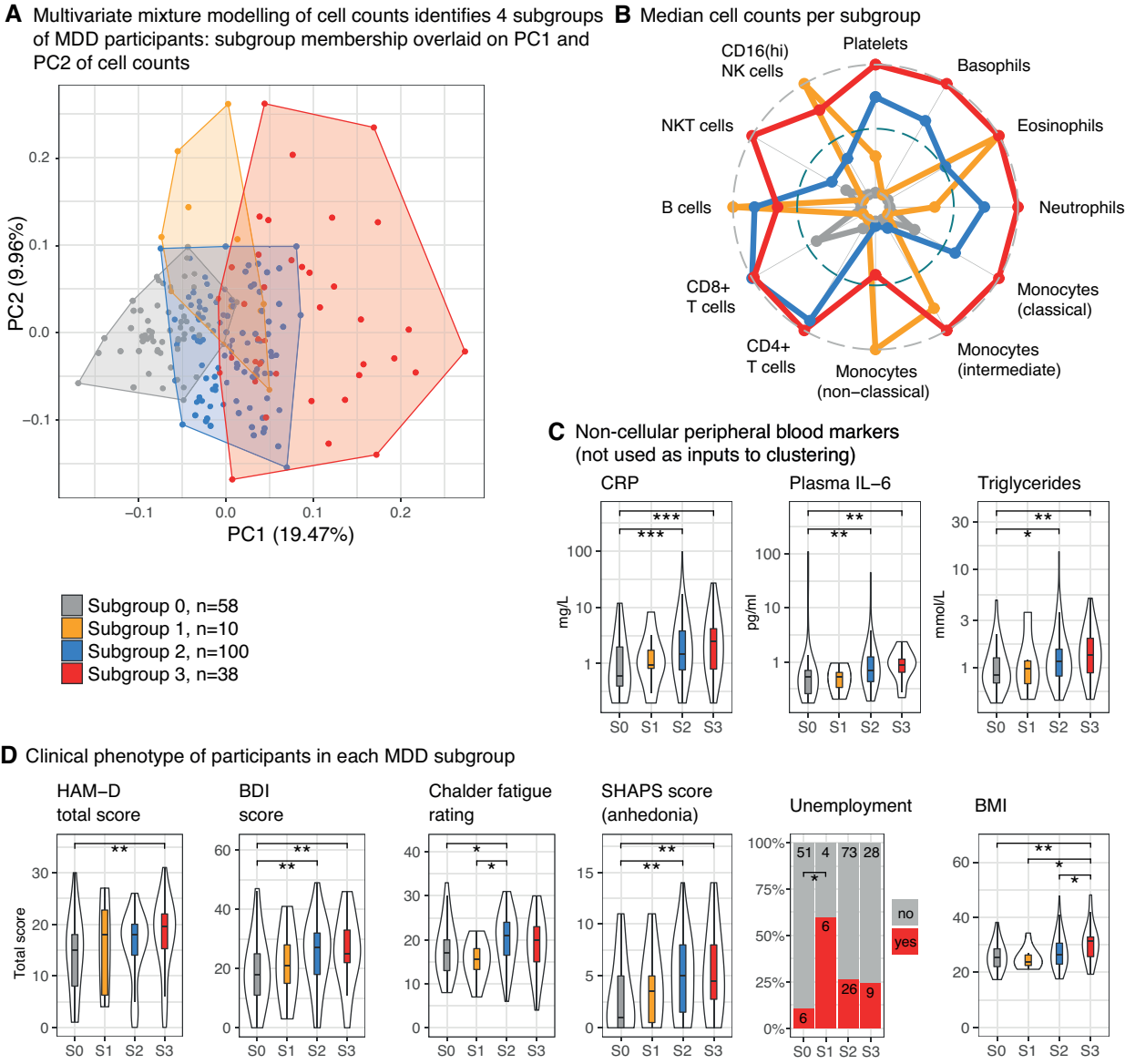
P-values for comparison of control vs. MDD by Mann-Whitney (MWU, continuous variables) or $\chi^2$ testing (categorical variables): *p<0.05, **p<0.01, and ***p<0.001. MDD, major depressive disorder; IQR, interquartile range; BMI, body mass index; STAI, Stait-Trait Anxiety Inventory. Number of missing data values for each variable are also shown (total n=283 participants).


A Cell counts
RBCs
Platelets
Basophils
Eosinophils
Neutrophils
Classical monocytes
Intermediate monocytes
Non−classical monocytes
CD4+ T
CD8+ T
B cells
NKT cells
CD16(hi) NK
CD56(hi) NK
count / microliter
control MDD
Soluble proteins
CRP
mg/L
Plasma IL−6
pg/ml
Significance
n.s.
p<0.05
FDR p<0.05
B Spearman correlations between immunological and clinical variables
Cell counts
Soluble proteins
Clinical variables
Demographic variables
RBCs
Platelets
Basophils
Eosinophils
Neutrophils
Monocytes (classical)
Monocytes (intermediate)
Monocytes (non−classical)
CD4+ T cells
CD8+ T cells
B cells
NKT cells
CD16hi NK cells
CD56hi NK cells
CRP
Plasma IL−6
Depression (HAM−D)
Depression (BDI)
Anhedonia (SHAPS)
Chalder fatigue
Childhood trauma (CTQ)
Recent stress (LEQ)
State anxiety (STAI−S)
Trait anxiety (STAI−T)
Age
BMI


A
Principal components analysis of cellular phenotype
control
MDD
Mono (non−class)
CD16(hi) NK
RBCs
B cells
Mono (int)
CD56(hi) NK
CD8+ T
NK cells
Eosinophils
Platelets
Neutrophils
Basophils
CD4+ T
Mono (classical)
PC2 (10%)
PC1 (19%)
PC1
Neutrophils
Eosinophils
Basophils
Monocytes (non-classical)
Monocytes (intermediate)
Monocytes (classical)
CD4+ T cells
CD8+ T cells
CD16hi NK cells
CD56hi NK cells
NKT cells
B cells
RBCs
Platelets
B
Clinical correlates of cellular PC1
Cellular PC1
CRP
IL−6
Depression (HAM−D)
Depression (BDI)
Anhedonia (SHAPS)
Chalder fatigue
Childhood trauma (CTQ)
Recent stress (LEQ)
State anxiety (STAI−S)
Trait anxiety (STAI−T)
Age
BMI
**p=0.006
control
MDD


A
PLS-DA: Cellular predictors of MDD vs. control
CD4+ T
Eosinophils
MDD vs. control
Neutrophils
a predictor
a response
Component 2
Component 1
0.25
0.00
−0.25
−0.50
−0.25
0.00
0.25
B
PLS-R: Cellular predictors of symptom severity within MDD group
Chalder fatigue
Beck Depression
SHAPS anhedonia
HAM Depression
NKT cells
Neutrophils
B cells
a predictor
a response
Component 2
Component 1
0.50
0.25
0.00
−0.25
−0.50
0.0
0.2
0.4


A Forced two-way clustering of MDD cellular immunophenotypes by multivariate mixture modeling, overlaid on cellular PCA
PC1 (19.47%)
PC2 (9.96%)
B Median cell counts per subgroup
Uninflamed depression n=125
Inflamed depression n=81
Absolute count
B cells
Classical monocytes
Intermediate monocytes
Non−classical monocytes
CD16hi NK cells
CD56hi NK cells
NKT cells
CD4+ T cells
CD8+ T cells
Neutrophils
Eosinophils
Basophils
RBCs
Platelets
UD
ID
C Non-cellular peripheral blood markers (not used as inputs to clustering)
CRP
mg/L
Plasma IL−6
pg/ml
Triglycerides
mmol/L
D Clinical phenotype of participants in each MDD subgroup
HAM−D score
Total score
BDI score
Unemployment
103
53
21
26
no
yes
Age
Years
E Sensitivity analysis: clustering of residuals from linear models for each cell count
Linear models include age, sex, BMI, tobacco use and recent infection
PC1 of residuals
Neutrophils
Eosinophils
Basophils
Monocytes (non-classical)
Monocytes (intermediate)
Monocytes (classical)
CD4+ T cells
CD8+ T cells
CD16hi NK cells
CD56hi NK cells
NKT cells
B cells
RBCs
Platelets
PC1 (20.21%)
PC2 (10.55%)
F Sensitivity analysis: clinical phenotype of participants in each MDD subgroup
Uninflamed depression (residuals), n=104
Inflamed depression (residuals), n=102
HAM−D total score
BDI score
Total score
UD(res)
ID(res)


A Multivariate mixture modelling of cell counts identifies 4 subgroups of MDD participants: subgroup membership overlaid on PC1 and PC2 of cell counts
PC2 (9.96%)
PC1 (19.47%)
Subgroup 0, n=58
Subgroup 1, n=10
Subgroup 2, n=100
Subgroup 3, n=38
B Median cell counts per subgroup
Platelets
Basophils
Eosinophils
Neutrophils
Monocytes (classical)
Monocytes (intermediate)
Monocytes (non−classical)
CD4+ T cells
CD8+ T cells
B cells
NKT cells
CD16(hi) NK cells
C Non-cellular peripheral blood markers (not used as inputs to clustering)
CRP
Plasma IL−6
Triglycerides
mg/L
pg/ml
mmol/L
S0 S1 S2 S3
D Clinical phenotype of participants in each MDD subgroup
HAM−D total score
BDI score
Chalder fatigue rating
SHAPS score (anhedonia)
Unemployment
BMI
Total score
no
yes