Cellular immune mechanisms of psychiatric disorders and the stress response

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Date of submission:

January 2022

This thesis is submitted for the degree of Doctor of Philosophy

Preface

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text. I further state that no substantial part of my thesis has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. It does not exceed the prescribed word limit for the Degree Committee of the Faculty of Clinical Medicine.

For the flow cytometry dataset (**Chapter 2**), Dr Lori Turner designed the immunophenotyping panel and Dominika Wlazly, I-Shu (Dante) Huang, Anviti Vyas, Naghmeh Nikkheslat, Alison McColl and Alex Hatton performed blood sample processing and data collection at each study site. I performed the flow cytometry data gating and the data analysis. The BIODEP study team members at each clinical site administered the clinical assessments.

For the mouse model of chronic stress (**Chapter 3**), some of the data were generated by Alison McColl, Sam Listwak and Dr Stacey Kigar: S Kigar generated the meningeal single cell sequencing dataset, the meningeal microarray dataset and the $CD19^{-/-}$ meningeal RNA sequencing dataset. S Kigar and A McColl performed the IL10 stimulation assays and behavioural testing of $CD19^{-/-}$ animals. For the MAGPIX™ ELISA, I collected the samples, but the assay itself was run by S. Listwak. For all experiments, I performed the analysis.

Summary

Cellular immune mechanisms of psychiatric disorders and the stress response

Mary-Ellen Lynall

Multiple psychiatric disorders have been associated with abnormalities in the immune system. As I summarise in my opening chapter (**Chapter 1**), evidence from human and animal studies suggests that the immune system may be implicated in the pathogenesis of these disorders, at least in a subset of patients. However, the direct evidence for a causal role of immune mechanisms is limited. Moreover, there are currently no good biomarkers that allow us to identify which patients with psychiatric disorders have immune dysfunction, and thus might benefit from alternative treatment approaches. I outline the limits of what is known about the causality of immune dysfunction in psychiatric disorders from the existing literature, much of which focuses on soluble biomarkers in peripheral blood in observational case-control studies. This stimulates consideration of more mechanistically refined biomarkers, with a focus on which immune cell subsets, and what cellular mechanisms, might play a causal role in psychiatric symptoms. In this thesis, I use human genetic data, human immunophenotyping and animal models to investigate whether psychiatric disorders and stress are associated with dysfunction in particular immune cell subsets, and the evidence for a causal, pathogenic role of different immune cells.

In **Chapter 2** I describe an analysis of a flow cytometry study of peripheral immune cell subsets in people with depression and age- and sex-matched controls. I used univariate and multivariate analyses to investigate the immunophenotypes associated with depression and depression severity. I found that depressed cases, compared to controls, had significantly increased immune cell counts, especially neutrophils, CD4+ T cells and monocytes, and increased inflammatory proteins. Depressed cases were partitioned into two subgroups by forced binary clustering of cell counts: the inflamed depression subgroup had increased myeloid and lymphoid cell 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 were associated with increased inflammatory proteins and more severe depression, but differed in terms of myeloid

and lymphoid cell counts, raising the possibility that there may be more than one type of 'inflamed depression'.

Stress is one putative cause of immune dysfunction contributing to the pathogenesis of multiple psychiatric disorders, and recent work has highlighted the potential role of the meningeal compartment of the immune system in behaviour. As described in **Chapter 3**, I used an animal model to investigate the effects of stress on the peripheral and meningeal immune compartments (the latter being poorly accessible in humans). Using flow cytometry and transcriptomic (including single cell) analyses, I demonstrated dysregulation of both myeloid and lymphoid immune cells in the periphery and meninges, and showed that B cells may influence behaviour by regulating meningeal myeloid cell activation.

In **Chapter 4**, I investigated the implications of genome wide association studies (GWAS) of psychiatric disorders for cellular immunity. I tested for enrichment of GWAS variants associated with multiple psychiatric disorders (cross-disorder or trans-diagnostic risk), and 5 specific disorders (cis-diagnostic risk), in regulatory elements in immune cells. For this analysis, I used three independent epigenetic datasets representing multiple organ systems and immune cell subsets. Trans-diagnostic and cis-diagnostic risk variants (for schizophrenia and depression) were enriched at epigenetically active sites in brain tissues and in lymphoid cells (T, B and NK cells), especially stimulated $CD4^+$ T cells. There was no evidence for enrichment of either trans-risk or cis-risk variants for schizophrenia or depression in myeloid cells. This suggests a model where stimuli, e.g., stress or infection, activate T cells to unmask the effects of psychiatric risk variants, contributing to the pathogenesis of mental health disorders.

In summary, the results from the human studies highlight the involvement of both the innate and adaptive immune system in psychiatric disorders. They further suggest that there are likely both shared and distinct contributions of cellular immunity to the pathogenesis of different psychiatric disorders. The results from the mouse model support the role of psychological stress in contributing to immune abnormalities in psychiatric disorders and suggest that the effects of stress may in part be mediated by stress-induced alterations in the meningeal immune system. These results are summarised in a concluding chapter (**Chapter 5**) which highlights outstanding questions, and priorities for future research, in the current understanding of the role of the immune system in mental health disorders.

Acknowledgements

First and foremost, I would like to thank my supervisors Professor Ed Bullmore and Professor Menna Clatworthy for their invaluable support, training and advice. Conversations with Professor Bullmore thirteen years ago inspired me to pursue academic psychiatry. I am hugely grateful for his thoughtful and caring mentorship throughout my clinical and academic training. I have learned an enormous amount from working across the traditional academic and institutional boundaries between psychiatry and immunology: I am very grateful to Professor Clatworthy for welcoming an ectopic psychiatrist into her dynamic and stimulating immunology laboratory. I would also like to thank my advisor Professor Peter Jones for his mentorship during my PhD, and for many thought-provoking exchanges on psychiatry and neuroscience.

I am very grateful to have had supportive and brilliant colleagues. I particularly want to thank the following for their generous help, encouragement and friendship: Dr Alex Riding, Dr Kevin Loudon, Dr Lori Turner, Dr Stacey Kigar, Dr John Ferdinand, Ciara O'Donnell, Junaid Bhatti and Linda Pointon. I would also like to express my thanks to all the research participants who have contributed to this thesis, and to the Medical Research Council for funding this work.

Finally, a special thanks to my partner, Ruari Hamlin, for his unwavering support, encouragement and humour, and to my mother, Annette Lynall, for a lifetime of intellectual encouragement.

I look forward to further pursuing the work presented in this thesis and I hope that these findings will stimulate others to investigate cellular immunity in psychiatry. Ultimately, I hope that this work will contribute to the development of better peripheral blood biomarkers of inflammation, and thus improve diagnosis and treatment for patients with psychiatric symptoms.

Publications

Publications arising from work in this thesis

- The material in chapter 2 has been published as: **Lynall ME**, Turner L, Bhatti J, Cavanagh J, de Boer P, et al. Peripheral Blood Cell-Stratified Subgroups of Inflamed Depression. Biol Psychiatry. 2020;88(2):185-96. https://doi.org/10.1016/j.biopsych.2019.11.017
- The material in chapter 3 has been published as: **Lynall ME**, Kigar SL, Lehmann ML, DePuyt AE, Tuong ZK, Listwak SJ, et al. B-cells are abnormal in psychosocial stress and regulate meningeal myeloid cell activation. Brain Behav Immun. 2021 Oct;97:226-238 https://doi.org/10.1016/j.bbi.2021.08.002
- The material in chapter 4 has been published as: **Lynall ME**, Soskic B, Hayhurst J, Schwartzentruber J, Levey DF, Pathak GA, et al. Genetic variants associated with cross-disorder and disorder-specific risk for psychiatric disorders are enriched at epigenetically active sites in peripheral lymphoid cells. Nat Commun 13, 6102 (2022) https://doi.org/10.1038/s41467-022-33885-7

Publications by collaborators to which I have also contributed

- Barbu MC, Huider F, Campbell C, Amador C, Adams MJ, **Lynall ME**, et al., Methylome-wide association study of antidepressant use in Generation Scotland and the Netherlands Twin Register implicates the innate immune system. Mol Psychiatry 2021 https://doi.org/10.1038/s41380-021-01412-7
- Aruldass AR, Kitzbichler MG, Morgan SE, Lima S, **Lynall ME**, et al. Dysconnectivity of a brain functional network was associated with blood inflammatory markers in depression. Brain Behav Immun. 2021 Nov;98;299-309
- Dutcher EG, Pama EAC, **Lynall ME**, Khan S, Clatworthy MR, Robbins TW, et al. Early-life stress and inflammation: A systematic review of a key experimental approach in rodents. Brain Neurosci Adv. 2020;4:2398212820978049
- Osimo EF, Perry BI, Cardinal RN, **Lynall ME**, Lewis J, Kudchadkar A, et al. Inflammatory and cardiometabolic markers at presentation with first episode psychosis and long-term clinical outcomes: A longitudinal study using electronic health records. Brain Behav Immun. 2021;91:117-27
- Cole JJ, McColl A, Shaw R, **Lynall ME**, Cowen PJ, de Boer P, et al. No evidence for differential gene expression in major depressive disorder PBMCs, but robust evidence of elevated biological ageing. Transl Psychiatry. 2021;11(1):404

Code availability

Code used to perform the analyses and generate the figures presented in this thesis are available as follows:

- Chapter 2: https://github.com/maryellenlynall/2019_depression_flow_cytometry
- Chapter 3: https://github.com/maryellenlynall/2019_bcell_stress
- Chapter 4: https://github.com/maryellenlynall/psychimmgen2021

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Chapter 1: Introduction

1.1 Inflammation in depression

Depression affects millions of people worldwide, but the underlying pathogenesis is poorly understood and current therapeutic interventions have limited efficacy (Kessler et al 2005a). Depression is a relapsing-remitting disorder involving a constellation of psychological and somatic symptoms. Its core symptoms, which must be present for at least two weeks, are (1) low mood (2) decreased energy and (3) decreased interest or pleasure in activities (World Health Organization 2004). Diagnostically, depression can occur in the context of Major Depressive Disorder (MDD), as part of another psychiatric disorder, e.g., bipolar disorder, or "comorbid" with a medical disorder. Major depressive disorder (MDD) affects 10-15% of the population (Kessler et al 2005b), and at least one third of patients with depression do not respond to conventional antidepressants that target serotonin or other monoamines (Rush et al 2006). Treatment-resistant depression (TRD) is associated with increased hospitalisation and suicide attempts (Amital et al 2008) but the mechanisms of treatment-resistance are unclear and novel treatments are urgently needed.

Immuno-psychiatry - a combined approach drawing on immunology, neuroscience and psychiatry - has recently emerged as an inter-disciplinary framework for the mechanistic understanding of depressive disorders. Some patients with MDD have increased circulating levels of inflammatory cytokines, chemokines and C-reactive protein (CRP) (Kohler et al 2017a, Valkanova et al 2013). Moreover, 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 (Miller & Raison 2016), and from human studies demonstrating that treatment (for hepatitis) with a pro-inflammatory cytokine (interferon) is followed by increased incidence of MDD (Pinto & Andrade 2016). Convergently, meta-analytic reviews of clinical trial data have consistently demonstrated that anti-inflammatory drugs can significantly improve comorbid mood and fatigue symptoms, measured as secondary endpoints, in patients with medical inflammatory disorders (Kappelmann et al 2018, Kohler et al 2014, Wittenberg et al 2019). These and other findings support the hypothesis that depressive symptoms – whether part of MDD or associated with inflammatory or autoimmune conditions – could be caused or exacerbated by activation of the immune system.

In this introductory chapter, I will summarise our current understanding of the relationship between the immune system and the brain; summarise the immune abnormalities that have been associated with depression; examine the evidence that this immune dysregulation contributes causally to symptoms; outline the possible sources of inflammation in depression; consider the evidence for an inflamed subgroup of patients with depression; and examine how inflammation relates to (potentially transdiagnostic) symptoms or endophenotypes in psychiatry.

1.2 The immune system and its relationship with the brain

In order to further consider the role and sources of inflammation in depression, I will first recap the principal functions and features of the immune system, and our current understanding of the relationship between the immune system and the brain. The function of the immune system is to protect the body from pathogens and other forms of tissue damage. The immune system generates inflammatory responses to pathogens and tissue injury, but also plays a critical role in the regulation and resolution of inflammation (Mauri & Menon 2017, Sakaguchi et al 2020, Serhan & Levy 2018). Cells in the immune system can circulate in the blood or reside in tissues or lymphoid organs (including the thymus, spleen, bone marrow and lymph nodes) (Lewis et al 2019, Masopust & Soerens 2019, Randolph et al 2017).

The immune system is composed of an innate and an adaptive arm. The innate system provides the initial response to injury – this response is rapid, and is non-antigen specific, instead triggered by generic classes of molecules present on pathogens (PAMPs, pathogen-associated molecular patterns) or associated with tissue injury (DAMPs, damage-associated molecular patterns) (Seong & Matzinger 2004). The innate immune system includes phagocytic cells such as neutrophils and monocytes, and soluble factors such as the complement system, acute phase proteins and pro-inflammatory cytokines (e.g., interleukin-6, IL-6), although adaptive immune cells also secrete many of the same cytokines (Commins et al 2010). Monocytes develop in the bone marrow, circulate and mature in the blood, and can travel to tissues where they mature into macrophages (Guilliams et al 2018). Tissue macrophages can be derived from circulating monocytes or, alternatively, seeded into tissues prenatally. Neutrophils are short-lived cells (several days) and are normally found in the blood. They are some of the first responders to an insult: signals from a tissue indicating damage or the

presence of pathogens cause neutrophils to migrate to the site of injury (Liew & Kubes 2019). Acute phase proteins are markers of inflammation which are increased in blood during acute inflammation and include C-reactive protein (CRP). Acute phase proteins have many and varied functions, which include tagging microbes for destruction, altering blood clotting, and mediating negative feedback on the inflammatory response (Schrödl et al 2016).

The adaptive system, which includes T and B cells, provides a delayed but specific response to infection by recognising and targeting particular antigens (molecular shapes) on invading pathogens. The adaptive system generates highly specific memory of previous infections, based on gene rearrangements in B and T cells, so that subsequent encounters with the pathogen generate a stronger and more rapid response (the basis of vaccination) (Farber et al 2014, McHeyzer-Williams & McHeyzer-Williams 2005). The innate immune system also has memory, but this is based on epigenetic changes rather than on gene rearrangement, and is less specific than adaptive memory – an initial immune challenge can alter the innate response to subsequent, unrelated challenges (Dominguez-Andres & Netea 2019).

Traditionally, the immune system and the brain/mind were considered distinct systems, separated by the blood-brain barrier. There were known to be myeloid immune cells resident in the brain, but these microglia, the most numerous immune cells in the central nervous system, are derived from embryonic yolk sac primitive macrophages which invade the brain in early embryogenesis and proliferate in situ (Ginhoux et al 2010, Schulz et al 2012). In rodents, fate-mapping studies demonstrated that yolk-sac derived microglia persist into adulthood with no substantial contribution of peripheral blood monocytes to the microglial pool under homeostatic conditions (Ginhoux & Prinz 2015), although whether the same is true in humans is less clear. Moreover, until recently, the brain was also thought to lack a lymphatic system (the drainage system by which tissue fluid is monitored by lymph nodes, a key part of the immune system). All this seemed to reinforce the idea that the brain is segregated from the peripheral immune system. It makes sense that the brain is protected from inflammation to a degree – neurons are, for the most part, not regenerated: the collateral damage and cell death that accompany inflammation are thus particularly problematic in the brain. Moreover, the brain sits in an enclosed box (the skull), so the oedema that usually accompanies inflammation could cause dangerous compression of the brain. However, it is increasingly clear that the immune system and brain are deeply entwined: immune cells regulate the development and activity of the nervous system, and the nervous system regulates immune responses

throughout the body (Dantzer 2018). Moreover, it has recently been discovered that the brain has its own local immune system (in addition to microglia and perivascular macrophages) in the meninges, the lining of the brain (Rua & McGavern 2018), and that the brain does have its own lymphatic drainage system, externalised to the meninges (Aspelund et al 2015, Louveau et al 2015). The meningeal immune and lymphatic systems provide additional mechanisms by which the body's immune system can affect and be affected by the brain.

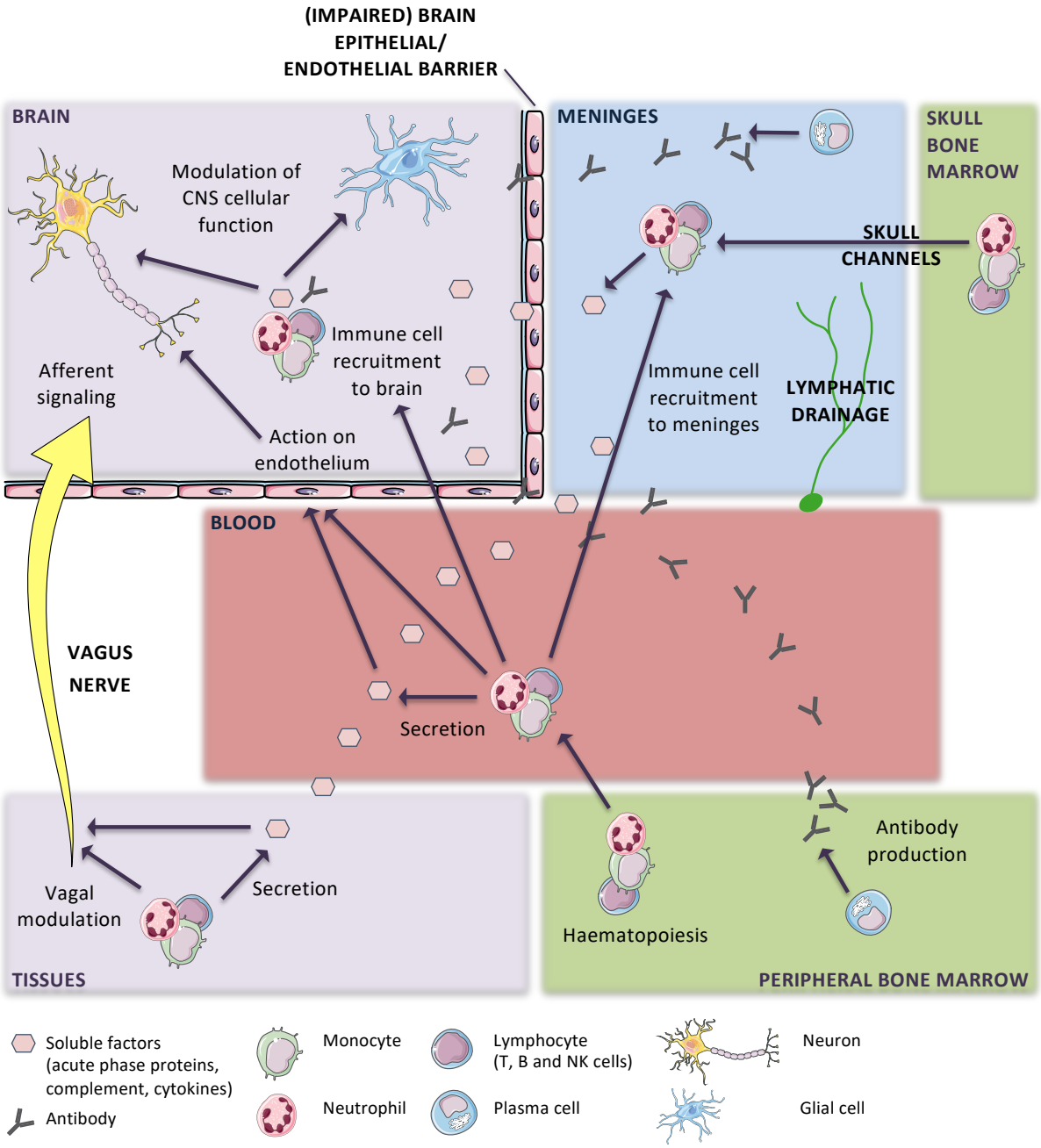


Figure 1-1 *Pathways by which the immune system may act on the brain to induce behavioural change. Effects of immune cells and inflammatory mediators on the brain and behaviour may be mediated by direct action of cells, antibodies and soluble components on central nervous system (CNS) cells, with infiltration of cells and cytokines across the blood-brain barrier. Alternatively, cells and soluble components may act indirectly on the brain via effects on brain epithelium/endothelium; via the meningeal immune system; by the action of immune cells or cytokines on afferent vagal nerve signalling; or via the effects (not shown) of inflammation on neurotransmitter metabolism (see text for more details). Immune cells can be recruited to the CNS from the peripheral bone marrow, passing via the peripheral circulation, or be recruited directly from the skull bone marrow to the meninges, via channels in the skull.*

Mechanistic studies, primarily in rodents, have revealed that there are multiple pathways by which the immune system can act on the brain to induce behavioural change (see **Figure 1-1**), which include:

1. Cytokines crossing the blood-brain-barrier to act directly in the brain (Menard et al 2017).
2. Cytokines acting on brain endothelium / epithelium (Blank et al 2016).
3. Afferent neural signalling by immune cells or cytokines modulating vagus nerve activity (Bluthe et al 1996).
4. Effects of inflammation on the metabolism of neurotransmitters or the generation of neurotoxic metabolites (Dantzer 2018, O'Connor et al 2009b).
5. Infiltration of peripheral immune cells into the brain (Wohleb et al 2013).
6. The effects of antibodies on the brain, as suggested by associations between autoantibodies (especially anti-NMDA receptor antibodies) and psychiatric symptoms (Kayser & Dalmau 2016) and the effects of passive transfer of patient anti-NMDAR antibodies on rodent brain function (Jezequel et al 2017, Planaguma et al 2015).
7. Effects mediated by the meningeal immune system (see **Section 1.2.1** below).

In this thesis, we often refer to 'inflammation' and its association with different exposures or disorders. Inflammation is a broad term, originally encompassing the four cardinal signs of infection – redness, swelling, heat and pain, as described by the Roman scholar Celsus in the first century AD – but with no agreed-upon modern definition. It has generally come to mean *'a protective response of the organism to stimulation by invading pathogens or endogenous* signals' (Netea et al 2017), but in clinical studies, molecular markers associated with this (e.g., increases in plasma cytokines) are often used as a proxy indicator of inflammation. Here, we use the term in this modern and broad sense. We allow 'inflammation' to include both markers of the acute phase of inflammation, as well as mediators important in the resolution of inflammation, given that, in many studies, it is not possible to determine whether a given marker or cell type has a pro- or anti-inflammatory role.

1.2.1 The meningeal immune system and its potential role in psychiatric symptoms

Until recently, the brain was viewed as an immune-privileged site, and with the exception of perivascular macrophages and microglia the healthy brain was thought to be essentially devoid of immune cells. However, recent data demonstrating immune cells within the meninges in the steady state challenged this dogma (Kipnis 2016, Rua & McGavern 2018). Healthy meninges

contain both innate and adaptive immune cells, including all major haematopoietic lineages (Ajami et al 2018, Korin et al 2017, Schafflick et al 2021). Most meningeal immune cells are sited behind the blood-brain-barrier (or blood-meningeal-barrier) as, like most cells in the central nervous system, they are not labelled by high-molecular weight markers injected into the vascular system (Korin et al 2017, Schafflick et al 2021). The extent to which there is recirculation between peripheral and meningeal compartments in health is unclear, although there is evidence from parabiosis experiments of long-term meningeal tissue-residency for at least some myeloid cells and B cells in the meninges (Cugurra et al 2021, Schafflick et al 2021), and some meningeal T cells express surface markers associated with long-term tissue-residency (Urban et al 2020). Moreover, meningeal immune cells need not be recruited via the blood – they can traffic directly from the skull bone marrow to the meninges via specialized channels crossing the inner skull bone (Brioschi et al 2021, Cugurra et al 2021, Herisson et al 2018). The meninges also contain a system of lymphatics which are physically related to the dural venous sinuses and drain to the deep cervical lymph nodes (Aspelund et al 2015, Louveau et al 2015). Immune cells around these sinuses monitor the cerebrospinal fluid (CSF) draining the brain for antigens and support local immune effector functions within the meninges (Rustenhoven et al 2021).

Experiments in rodents suggest that meningeal immune cells can have an important role in homeostatic behaviour even in the absence of infection or peripheral inflammation; such investigations have primarily focused on the role of meningeal T cells. Data suggest that meningeal $CD4^+$ T cells can impact cognition, social behaviour, fear memory, and responses to stress in rodents (Cohen et al 2006, Derecki et al 2010, Filiano et al 2016, Herz et al 2021). These effects can occur via immune cell cytokine signalling to neurons, including the action of T cell IFN-γ (Filiano et al 2016) or T cell IL-4 (Herz et al 2021) on neurons. In addition, meningeal γδ T cells have been shown to regulate homeostatic anxiety levels via IL-17 signalling to neurons (Alves de Lima et al 2020). Such studies have demonstrated relevant cytokine production by meningeal T cells during the studied behavioural tasks, and some have used cell specific genetic knock-outs or chimeric animals to demonstrate the importance of cytokine production by a specific cell type (i.e. T cells) or the importance of cytokine signalling to a specific cell type (e.g., neurons). However, without effective tools for spatially targeting or selectively depleting specific immune cells in the meninges, we cannot conclusively infer that it is meningeal (rather than peripheral) T cells which are crucial for the observed effects on behaviour in these studies.

1.3 Evidence of inflammation in depression

1.3.1 Peripheral soluble inflammatory markers in depression

Biomarker research in immuno-psychiatry to date has mainly focused on blood proteins such as cytokines and CRP. Increased blood levels of pro-inflammatory cytokines and acute phase reactants have been repeatedly reported in case-control studies of major depressive disorder (MDD) compared to non-depressed controls, with meta-analytic evidence for increased interleukin(IL)-6, tumour necrosis factor (TNF)-α, IL-10, soluble IL-2 receptor, IL-13, IL-18, IL-12, IL-1 receptor antagonist, soluble TNF receptor 2 and CRP, but decreased interferon(IFN)-γ, in MDD (Chamberlain et al 2018, Kohler et al 2017a, Syed et al 2018, Valkanova et al 2013). Depression (in people who are otherwise physically well) is also associated with altered peripheral blood chemokines, with increased CCL2, CCL3, CCL11, CXCL7 and CXCL8 and lower levels of CCL4 compared to controls (Leighton et al 2018). However, absolute levels of these proteins are low; the effect sizes are modest; and there are many sources of variation in blood cytokines and chemokines that can confound the association with depressive symptoms. Importantly, only some MDD patients show evidence of inflammation by these measures: for example, only ~30% of MDD cases have CRP greater that the upper limit of normal (3mg/L) (Chamberlain et al 2018).

1.3.2 Peripheral cellular immunophenotypes in depression

As described above, 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. 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, an increased neutrophil to lymphocyte ratio, and an increased ratio of $CD4^+$ to $CD8^+$ T cells (Maes et al 1992c, Mazza et al 2018, Zorrilla et al 2001). However, there are less consistent results concerning regulatory T cells, Th17 cells, natural killer (NK) cells, monocytes, and B cells (Grosse et al 2016a, Grosse et al 2016b, Hasselmann et al 2018, Maes et al 1992b, Patas et al 2018, Pavon et al 2006, Suzuki et al 2017). Notably, many 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.

1.3.3 Peripheral immune transcriptional phenotypes in depression

A number of studies have examined gene transcription in peripheral blood samples, primarily in whole blood or PBMCs, which can easily be separated from blood using density gradient centrifugation. Analysis of candidate genes in whole blood samples by qPCR has consistently demonstrated increased inflammatory gene expression (interleukin 1-beta, *IL1B*; interleukin 6, *IL6*; tumour necrosis factor-alpha, *TNF*; macrophage migration inhibitory factor, *MIF*) and decreased glucocorticoid receptor (*GR*) expression in MDD cases compared with healthy controls (Cattaneo et al 2013). In an independent sample, absolute expression of *IL1B* and *MIF* were increased in patients with TRD compared to treatment-responsive MDD (Cattaneo et al 2016). Other studies have used microarray or RNA sequencing (RNAseq) to analyse gene expression more broadly and have generally demonstrated increased expression of genes in innate immune pathways and decreased expression of genes in adaptive immune pathways in MDD compared to controls, although the specific genes over- or under-expressed in depression are highly variable across different studies (Wittenberg et al 2020). The results of case control studies of gene expression in mixed cellular samples are summarized in **Table 1-1**.

Findings of MDD-associated transcriptomic changes raise a key question: what is the cellular source of these gene expression changes? Are depression-related changes in *IL1B* and *GR* expression coupled within individual cells or within a specific subset of abnormal cells? Transcriptional analyses of mixed samples implicate especially cells of the myeloid lineage (monocytes, neutrophils and dendritic cells) (Leday et al 2018), but almost all genes are expressed in multiple cell subsets, so cannot easily be attributed to a particular cell subset. A second limitation of gene expression analyses of heterogeneous mixtures of cells is that between-group differences in gene expression may not reflect changes in cell-intrinsic gene expression, instead simply reflecting group differences in the proportion of component cellular subsets (Lyons et al 2010). To mitigate this problem, measured or bioinformatically estimated cell numbers can be used as covariates in models of the effects of depression on gene expression, but this cannot adequately adjust for differences in cell proportions across samples. Moreover, in heterogeneous samples, transcriptomic changes in one cell subset may be diluted or obscured by signals (which may be opposite in direction) from other cell subsets

(Lyons et al 2010). Thus, transcriptomic analysis of sorted cell subsets, or single cells, may be necessary to uncover disease-related pathways in MDD, as has been the case for other conditions (Lee et al 2011, Martin et al 2019, McKinney et al 2010). To date, there have been no genome-wide analyses of gene expression in sorted immune cell subsets in MDD, and no studies of single cell gene expression in immune cells in MDD. A small number of studies have examined expression of candidate transcripts in sorted monocytes and T cells. These are summarized in **Table 1-2**, and are suggestive of an increase in monocyte proinflammatory signalling, increased monocyte chemotaxis, and monocyte glucocorticoid resistance in MDD, as well as potential alterations in T cell receptor diversity in MDD.

Table 1-1 *Gene expression in peripheral blood samples (whole blood and PBMCs) in depression (MDD) vs. controls.*

Includes only studies with >20 samples per group. Excludes studies testing candidate genes by qPCR-only. PBMC, peripheral blood mononuclear cells. MA, microarray; RNAseq, RNA sequencing; WGCNA, weighted gene co-expression network analysis; rMDD, recurrent MDD, MDD-R, MDD treatment responders; MDD-NR, MDD non-treatment responders; BPD, bipolar disorder; DE, differentially expressed; IPA, Ingenuity Pathway Analysis; RBC, red blood cell; BMI, body mass index; FDR, false discovery rate.

Publication	Sample size	Assay	Comments on clinical sample	Covariate adjustment	Findings
Whole blood					
(Spijker et al 2010)	21 MDD 21 CTL Replication: 13 MDD 14 CTL	Collection in heparin-coated tube; transferred to PAXgene within 60 mins of venipuncture or simulation. Stimulation of whole blood with LPS (10 ng/mL), 5-6hrs, 37°C 44K Agilent whole genome array, RT-qPCR validation.	MDD = unmedicated, no comorbid physical diagnosis, currently depressed	Matched for age, sex, smoking, (for females) reproductive/menstrual state. No covariate adjustment.	Signature of top 12 DE probes/genes distinguished MDD from control in both basal and LPS-stimulated samples: *FLJ23556, ITGB3, LMNA, NBR1, AL833005, CAPRIN1, CLEC4A, KRT23, MLC1, PLSCR1, PROK2, ZBTB16* 12-gene signature replicated in second cohort for LPS-stimulated (but not basal) samples. qPCR validated findings (same direction of change) for the following 7 transcripts (of 8 transcripts tested): - Upregulated: *CAPRIN1, CLEC4A, KRT23, PLSCR1, PROK2* - Downregulated: *MLC1, ZBTB16*
(Mostafavi et al 2014)	463 MDD 459 CTL	Collection in PAXgene tubes; Library preparation with polyA selection (i.e. mRNA). Illumina HiSeq 2000; 50/51-bp single end reads, average depth 70M reads/sample. Filtered for protein-coding genes only and	MDD = medicated and unmedicated	Yes – 39 covariates included in model, including BMI, gender, smoking, age.	2 DE genes at FDR P<0.1 (Fig. S5): *COPG* upregulated and *MICOS10* (*MINOS*, mitochondrial inner membrane protein) downregulated in MDD. MDD associated with upregulation of Reactome IFN-α/β pathway. No genome-wide significant differential isoform ratios.

		minimum expression. 14k genes analysed.			
(Jansen et al 2016) and (de Kluiver et al 2019) NB. same sample	882 depressed MDD 635 remitted MDD 331 CTL Including: N=246 depressed MDD + increased appetite/weight, N=341 depressed MDD + decreased appetite/weight.	Heparin tube, then transfer to PAXgene within 60 minutes. Affymetrix U219 array 18k genes analysed.		Yes – models included sex, age, smoking, RBC count, ± BMI	Jansen 2016 analysis: DE changes in current MDD vs. CTL (129 genes at FDR P<0.1) >> DE in remitted MDD vs CTL (where no DE genes at FDR P<0.1) Following 18 genes DE at FDR<0.05 for current MDD vs. control: - Upregulated in MDD: *LRRC4*, *PVRL1*, *DYSF*, *TNFRSF10C,* SSH2 - Downregulated: *KLRD1*, *IL2RB*, *GZMB*, *CALM1*, *TGFBR3, SNRPD3, APOBEC3G, PTPN4, KLRK1, NCALD, ERG28 (C14orf1), COX18* Pathways: increase in IL-6 signalling; decrease in NK cytotoxicity in MDD. De Kluvier 2019 analysis: DE in MDD subgroups, stratified by changes in appetite/weight. MDD+increased appetite/weight subgroup vs. CTL showed more substantial DE than subgroup with decreased weight/appetite. Top pathways implicated in MDD+increased appetite/weight were NK mediated cytotoxicity, IL-3, IL-5 and GM-CSF signalling, caspase pathway, NFAT transcription factor pathway, GATA3 pathway. BMI adjustment attenuated signals.
(Guilloux et al 2015)	34 MDD 33 CTL Subanalyses: 18 MDD-R v.s 18 CTL 14 MDD-NR v.s. 14 CTL	PAXgene collection tubes Illumina HT12-v4.0 array. 12k probes analysed. qPCR validation	MDD = currently depressed, unmedicated, non-psychotic, with comorbid anxiety; subsequent treatment response assessed.	Samples age- and gender-matched..	MDD vs. CTL: 303 DE genes at P<0.01: MDD-NR v.s CTL – 842 DE genes at P<0.01, abs(FC)>20%, implicating inflammation and leucocyte recruitment pathways (IPA). MDD-R v.s. CTL – 89 DE genes qPCR MDD-NR vs. CTL: the following genes (total 21 tested) showed significant change (uncorrected P<0.05) in same direction in qPCR and microarray: - Downregulated: *ADSL, CD3D, GZMA, RPL4, RPL5, RPL17, TIMM23*

					- Upregulated: *ARHGEF11, IL17RA, OSCAR, TBXAS1*
(Yamagata et al 2017)	Cohort 1: 21 MDD v.s. 30 CTL Cohort 2: 18 MDD v.s. 19 CTL	Collection tube not specified; QIAamp RNA Blood Mini Kit	Cohort 1 age ≥50 Cohort 2 age ≥20	Groups unbalanced for age	Genes downregulated in current MDD compared to CTL in cohort 1: *SLC35A3*, *HIST1H2AL*, *YEATS4*, *ERLIN2*, and *PLPP5*. Decrease in *HIST1H2AL* in current MDD validated by qPCR in 2nd cohort.
(Ciobanu et al 2018)	83 MDD (includes 27 rMDD); 438 CTL	PAXgene collection tubes; Illumina HT-12 v4 Array; 9k genes analysed.	Relatively healthy older adults (70-90yo)	Yes – age, BMI, sex	WGCNA: no modules associated with lifetime or current MDD; 1 of 24 modules was associated with rMDD at FDR P<0.2. This module was not associated with covariates. Implicates downregulation of protein processing in ER and Coat Protein 2 mediated vesicle transport.
(Hori et al 2018)	Cohort 1: 47 currently depressed (47 MDD, 7 BPD) 14 remitted depression (12 MDD, 2 BPD) 54 CTL Cohort 2: 59 currently depressed MDD 60 CTL	PAXgene collection tubes; Agilent 4x44K array; 28k probes used for differential expression analysis. qPCR validation.	Majority of participants medicated.	Cohorts matched for age and sex.	Candidate approach: focus on 8 candidate ribosomal genes taken from study of stress vulnerability showed increase in *RPL17*, *RPL34*, *RPL36AL* in currently depressed patients in Cohort 1 (not corrected for multiple comparisons). Association of current depression with increase in *RPL17* and *RPL34* replicated by qPCR in an independent cohort (Cohort 2) and not driven by medication use.
(Leday et al 2018)	Cohort 1: 128 MDD 64 CTL Cohort 2: 94 MDD 100 CTL	Affymetrix U133 plus 2.0 array 19k probes analysed.	Subset of MDD had comorbid anxiety disorder.	Yes – age, sex, batch, anxiety ± BMI included in models.	165 genes DE in both studies with same direction of fold-change (P value thresholds chosen as largest threshold associated with twice as many DE genes as expected by chance). Pathways: increase in innate immune pathways; decrease in adaptive immune pathways. Differences attenuated by inclusion of BMI as covariate.

PBMCs					
(Glahn et al 2012)	Used pedigree information from total 1122 individuals, of whom 215 had rMDD	Isolated 'Lymphocytes' using Histopaque gradient i.e. sample is PBMCs. Illumina Sentrix WG-6 Series 1 BeadChips Analyzed 11k genes (those with heritability >0.2)	Blood sample collected 12-15 years prior to ascertainment of MDD status.	Yes – age and sex included in model.	13 transcript levels showed significant genetic correlation with rMDD at FDR P<0.1. Top transcripts: *RNF123* (E3 ubiquitin-protein ligase family), *PDXK, ZFP64, RWDD2A, B4GALT7, MARK2, GADD45A, PIGN, HTT, ABHD12*
(Savitz et al 2013)	29 depressed (21 MDD and 8 BPD) 24 CTL	PBMC isolation by Ficoll-Paque. Illumina HT-12 v4 Expression BeadChip RT-qPCR validation.		Groups balanced for age, sex, BMI.	26 DE genes at FDR P<0.05; fold-change>1.25. Upregulated in MDD: *ADM, APBB3, CITED2, HLA-H, Hs.572649, IER5, NFKBIZ, NR4A2, RNU1-3, RNU1-5, RNU1F1, RNU1G2, SERTAD1, SNHG1, SNORD12C, SNORD31, TNF* Downregulated in MDD: *BRI3P1*, *CD160*, *CFD*, *CTSZ*, *GAPDHL6*, *LOC100008589* (ribosomal RNA), *LOC100132394*, *LOC100134364*, *NUCKS1* RT-qPCR for the 12 known protein-coding genes confirmed direction of change for *ADM*, *APBB3*, *CD160*, *CITED2*, *IER5*, *NR4A2*, *NUCKS1*, *SERTAD1*, and *TNF* but not *CTSZ*, *CFD* and *NFKBIZ*. Ingenuity Pathway Analysis of DE genes implicated: 1) NFkβ / TFGβ / ERK network and 2) cell cycle/kinase network.
(Le et al 2018)	78 MDD 79 CTL	PBMC isolation using Cell Preparation Tubes. Library preparation for RNAseq: Illumina Truseq Stranded mRNA (i.e. coding and noncoding RNA)	Unmedicated (≥3 weeks), no major medical comorbidities, no psychosis.	Yes – model included age, sex, batch, BMI ± smoking.	WGCNA: 2 modules were associated with depressive symptoms across all participants (MDD and CTL pooled). Modules were enriched for apoptosis, BCR signalling and PI3K activity. *Note samples were mapped to antisense strand in error.

		Sequencing: Illumina HiSeq 3000 150bp paired-end. Average depth 30M reads/sample. Filtered to obtain autosomal genes only, and to exclude lowly expressed genes. 6k genes analysed.			
(Cole et al 2021)	187 MDD (94 treatment-resistant; 47 treatment-responsive; 46 untreated) 44 CTL	PBMC isolation by density centrifugation, Library preparation for RNAseq: Illumina TruSeq stranded mRNA Sequencing: Illumina Hiseq 4000 75bp paired-end. Average depth 54.5M reads/sample. Filtered to obtain coding genes only and exclude lowly expressed genes.	Mixture of medicated and unmedicated, no major medical comorbidities, no psychosis.	Yes – model included age, BMI, sex, batch ± other clinical factors	Only 1 DE gene in MDD vs CTL participants (decreased *HIST1H2AE* in MDD) No DE genes between CTL and MDD subgroups. Evidence of increased biological ageing in MDD vs HC (using transcriptional signature of 888 age-associated genes).

Table 1-2 *Gene expression in peripheral blood cell subsets in depression (MDD) vs. controls.*

11β-HSD-1, 11β-hydroxysteroid dehydrogenase type 1; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; GILZ, glucocorticoid-induced leucine-zipper; TCR, T cell receptor.

Publication	Sample size	Assay	Clinical sample comments	Covariate adjustment	Findings
Monocytes					
(Carvalho et al 2014)	47 MDD 47 CTL	CD14+ cells MACS-sorted from cryopreserved PBMCs. RT-qPCR of 47 genes.	Unmedicated (≥1 week) MDD	Yes – age and gender (+BMI for GR ratio) included in models	Decreased GRα/GRβ isoform ratio in MDD vs CTL. Genes upregulated in MDD at Simes-corrected P<0.05: *CCL20*, *IL1B*, *EREG*, *PDE4B*, *CXCL2*, *IL8*, *TNFAIP3*, *BTG3*, *PTX3*, *ADM*, *CDC42*, *ATF3*, *MAFF*, *BCL2A1*, *IL6*, *MAPK6*, *IL1A*, *MXD1*, *PTGS2*, *SERPINB2*, *CXCL3*, *IRAK2*, *EGR3*, *DUSP2*, *TNF*, *PU.1*, *RGC32*, *CCL2*, *IL1R1*, *TREM1*, *THBD*, *CCL7*, *STX1A*, *AREG*. No genes significantly downregulated in MDD The 47 genes tested fell into two co-expression clusters: a proinflammatory cluster and a chemotaxis/adhesion cluster.
(Grosse et al 2015)	56 MDD 31 MDD	CD14+ cells MACS-sorted from cryopreserved PBMCs. RT-qPCR	89% medicated MDD	Yes – age, BMI, gender and smoking included in model	Planned analysis: - No difference in composite gene expression score in MDD vs CTL – score composed of genes implicated in MDD in (Carvalho et al 2014). Score = count of genes outside of CTL mean ±1 s.d. value, summing +1 for upregulated and -1 for downregulated genes. Exploratory age-stratified analysis: - Increased gene expression score and decreased GRα/β ratio in MDD patients aged ≥28 years only. - Increased *IL1B* and *TNF* expression in MDD ≥28yo
(Hasselmann et al 2018)	35 MDD 35 CTL	CD14+ cells MACS-sorted from cryopreserved PBMCs. RT-qPCR	Unmedicated (≥2 weeks) MDD	Covariates not in model, but samples matched for age, sex, BMI, smoking, comorbidities.	Decreased *GR* mRNA and downstream target *GILZ* in MDD. No difference in expression of *MR* or *11β-HSD-1* (t-test; no multiple comparison correction).
(Hung et al 2019)	47 MDD 33 CTL	PBMC isolation by Ficoll-Paque then	Unmedicated (≥1 week) MDD	Yes – age, sex, smoking and BMI	6 microRNA regulators of TLF4-signaling tested. At uncorrected P<0.05, *miR-146a* and *miR-155* downregulated in pre-treatment MDD vs CTL

		fresh CD14+ magnetic selection. RT-qPCR		included in ANCOVA	
T cells					
(Hasselmann et al 2018)	35 MDD 35 CTL	CD3+ cells MACS-sorted from cryopreserved PBMCs. RT-qPCR	Unmedicated (≥2 weeks) MDD	Covariates not in model, but samples matched for age, sex, BMI, smoking, comorbidities.	No difference in T cell expression of *GR, GILZ, MR*, or *11*β-*HSD-1*.
(Patas et al 2018)	20 MDD 20 CTL	PBMC isolation by Ficoll-Paque CD4+ cells isolated from cryopreserved PBMCs by negative selection RT-qPCR and TCR sequencing	Unmedicated MDD.	Balanced for age, sex, BMI, smoking.	Expression of T cell subset transcription factors tested (*FOXP3*, *T-bet*, *GATA3*, *RORC*). Increased *FOXP3* in MDD vs. CTL. Flow cytometry demonstrated that this reflected increased Treg proportion of total CD4+ T cells in MDD. Sequencing of TCR beta chain CDR3 region in n=5 MDD, n=5 CTL (to confirm flow cytometry result) demonstrated increased Vβ 5.1 usage in MDD vs. CTL.
B cells, NK cell, dendritic cells, neutrophils					
No data available					

1.3.4 Central nervous system immunophenotypes in depression

As summarised in **Table 1-3**, multiple lines of evidence from both *in vivo* CSF and neuroimaging studies, as well as post-mortem studies, point to brain immune abnormalities in MDD. However, the relationship between peripheral blood and central (brain) immune abnormalities in MDD is unclear. In the existing data, there is little evidence of correlation between peripheral and central markers of inflammation in MDD and some evidence of lack of correlation between peripheral proinflammatory markers and microglial activation as indexed by translocator protein (TSPO) binding (Enache et al 2019, Schubert et al 2021). However, most studies of central inflammation have been small and most have not measured peripheral and central inflammation concurrently. More generally, animal work has shown that the fidelity of the transcriptional response in blood compared to tissues is dependent on both the pathway implicated and the nature of the immune insult (Singhania et al 2019). Nonetheless, it is clear that in humans, low-grade inflammation in the periphery can alter the CNS immune compartment. For example, intravenous administration of low-dose endotoxin (LPS), a pathogen-associated molecular pattern, induces increases in not only peripheral, but also central (CSF), IL-6 with greater increases in CSF IL-6 (but not peripheral IL-6) being associated with a greater LPS-induced deterioration in mood (Engler et al 2017).

Table 1-3 *Central nervous system immunophenotypes in depression.*

Assay type	Evidence
CSF cytokine measurements	Analyses of cytokine levels in cerebrospinal fluid (CSF) have been limited, with small sample numbers, but there is meta-analytic evidence for increased IL-6 (effect size, ES = 0.4) and IL-8 (ES = 0.57) in CSF in MDD (Wang & Miller 2018).
Positron emission tomography imaging of translocator protein (TSPO) binding	There is meta-analytic evidence for elevations in TSPO binding (ostensibly indicating microglial activation) in MDD in anterior cingulate cortex (ACC), temporal lobe, prefrontal cortex (PFC), insula and hippocampus – areas of the brain functionally implicated in emotion regulation and mood disorders – but not in occipital cortex, parietal cortex and thalamus (Enache et al 2019, Schubert et al 2021). In vivo TSPO ligand binding correlates with pro-inflammatory microglial and astrocyte polarization in rodents (Pannell et al 2020), although what TSPO binding reflects in humans remains controversial (Perry 2018).

Post-mortem brain transcriptomic analysis	A meta-analysis of the association between MDD and gene expression in post-mortem brain samples (anterior cingulate, dorsolateral prefrontal cortex and amygdala) demonstrated divergent MDD-associated signatures in males and females, with increases in microglia-related genes in males, but decreases in females (Seney et al 2018). Some of the pathways identified as altered might relate to changes described in studies of peripheral blood samples: both sexes showed an under-expression of inner mitochondrial membrane genes in MDD, and females (but not males) showed a decrease in antigen processing pathways. The brain samples were heterogeneous mixtures of cells and the cellular source of these alterations is unclear. Another study found a similar divergence of MDD-associated differential expression in males and females (Labonte et al 2017).
Post-mortem myeloid cell histology	In MDD associated with suicide, compared to controls, there is evidence of an increased proportion of activated/neurotoxic microglia as quantified by microglial morphology (Torres-Platas et al 2014) (in ACC), density of activated (HLA-DR+) microglia (Steiner et al 2008) (in ACC and dorsolateral PFC), and density of microglia positive for the inflammation-induced tryptophan metabolite quinolinic acid (Steiner et al 2011) (in ACC). One study also found an increased density of blood-vessel-associated Iba-1+ macrophages in ACC in MDD associated with suicide compared to controls (Torres-Platas et al 2014).

1.4 Evidence that inflammation contributes causally to psychiatric symptoms in depression

As described above, there is a large body of evidence showing that depression is associated with altered immune function. However, for immune dysfunction to be compelling for biomarker development or as a treatment target, we require evidence that inflammation – or some aspect of it – contributes causally to symptoms in depression. Sources of evidence which speak to causality include clinical observational studies; longitudinal cohort studies demonstrating that inflammation precedes depression; randomized controlled trial data demonstrating that treating inflammation can improve symptoms; genetic association data; animal models; and human experimental and mechanistic studies demonstrating the pathways by which inflammation can affect brain function and symptoms. We consider here each of these in turn.

1.4.1 Clinical observational studies

Clinical observations have suggested a potential role for inflammation in the development of mood symptoms. We know that medical treatments that activate the immune system (e.g., IFN-α and IL-2) substantially increase the risk of depressive symptoms (Capuron et al 2001, Pinto & Andrade 2016). This suggests a causal role for inflammation in mood, albeit in non-physiological circumstances. Inflammation – as measured by pro-inflammatory cytokine levels – has also been associated with reduced responsiveness to standard (especially serotoninergic) antidepressant treatment, both cross-sectionally and in some prospective studies (Arteaga-Henriquez et al 2019, Haroon et al 2018, Jha et al 2019, Jha et al 2017, Lanquillon et al 2000, Strawbridge et al 2015); and inflammation has also been prospectively associated with reduced responsiveness to psychological therapy (Strawbridge et al 2020). Inflammation in depression may thus indicate a pathological process which is not amenable to treatment with current therapies, or which counteracts the mechanism of action of current therapies (e.g., inflammation affecting metabolism of serotonin precursors (O'Connor et al 2009b), affecting the efficacy of SSRIs). However, it is also possible that inflammation is merely indexing another factor independently associated with treatment resistance, for example, childhood trauma or disease severity.

1.4.2 Longitudinal cohort studies in non-clinical populations

Longitudinal cohort studies in non-clinical populations have shown that immune dysregulation can be detected prior to onset of depression – a necessity, if inflammation is to be causal. There is meta-analytic evidence that both increased CRP and IL-6 are prospectively associated with subsequent depressive symptoms (Mac Giollabhui et al 2021). These data are supportive of immune causality in depression, since if inflammation precedes depression, the inflammation cannot simply be a consequence of the disorder or of behaviours caused by its symptoms. However, such results could reflect the effects of risk factors for depression which precede its onset - such as high body mass index (BMI), childhood trauma, or family environment – on both the immune system and the brain. For such risk factors, inflammation may or may not mediate their effects on depression risk. Observational cohort studies cannot entirely distinguish between these possibilities, although studies which have carefully controlled for many key risk factors still find evidence that inflammation precedes depression onset (Khandaker et al 2014).

1.4.3 Randomized controlled trials

In randomized controlled trials (RCTs) of patients given immunomodulatory treatments for conditions other than depression, anti-inflammatory treatments improve mood scores (Wittenberg et al 2019). This effect could not be entirely attributed to mood improvements related to improvements in patients' medical symptoms: mood scores improved following treatment with anti-IL6 or anti-TNF therapy even in those whose physical symptoms did not improve (Wittenberg et al 2019). Turning to randomised controlled trials (RCTs) where depression was the treatment target, adjunctive treatment with the non-steroidal anti-inflammatory drugs (NSAID) celecoxib has shown efficacy against depressive symptoms (Abbasi et al 2012, Akhondzadeh et al 2009, Majd et al 2015, Muller et al 2006), including at a meta-analytic level (Kohler et al 2014), supporting a causal contribution of inflammation to symptoms. It is likely that only a subset of patients (those with inflammation) will benefit from immunomodulation. There is substantial evidence from secondary analyses of trial data that patients with depression and evidence of inflammation are most likely respond to immunomodulatory treatments – often, such treatments do not show overall efficacy in unselected patients with depression. One randomized controlled trial used infliximab to inhibit TNF-alpha in patients with depression without medical comorbidities (Raison et al 2013). An exploratory analysis showed that those with high CRP improved following treatment, but those

with a low CRP had worse outcomes following TNF blockade, compared to placebo, with the estimated threshold point for response at a CRP of 5mg/L. Another trial of infliximab, but for depression in the context of bipolar disorder, showed a reduction in overall depressive symptoms only in those reporting childhood maltreatment (McIntyre et al 2019): a reanalysis of the data showed that infliximab was particularly effective against anhedonic symptoms, especially in participants with high baseline TNF-α (Lee et al 2020). Trials of adjunctive minocycline for depression (minocycline is an antibiotic known to decrease microglial activation, among other anti-inflammatory effects) have yielded mixed results (Dean et al 2017, Husain et al 2017, Nettis et al 2021, Savitz et al 2018), but, as with infliximab, there is evidence that patient stratification using markers of inflammation can help to select those patients most likely to respond. In an RCT of adjunctive minocycline for treatment-resistant depression an exploratory analysis showed evidence of response in participants with high CRP, with an estimated threshold point for response at a CRP of 2.8mg/L (Nettis et al 2021). Similarly, in an RCT of adjunctive minocycline (± low-dose aspirin) for bipolar depression, minocycline was particularly effective in participants with higher baseline IL-6 concentrations (Savitz et al 2018).

1.4.4 Genetic data

Genetic data can also shed light on the potential causal contribution of the immune system to depression. The random segregation of alleles during reproduction and the stability of the genetic code over the lifespan means that genotype-phenotype associations can be used to identify genetic loci likely to causally contribute to disease pathogenesis. In psychiatry, the most common methodological approach to finding such loci is through the meta-analysis of large case-control genome-wide association studies (GWAS). GWAS studies in MDD have mainly implicated variants in non-exonic regions of the genome, with pathway analysis of genes near these variants primarily suggestive of effects on synaptic structure and neurotransmission, but also of effects on cytokine production (Howard et al 2019, Wray et al 2018). Notably, associations with classical human leukocyte antigen (HLA) alleles, seen in classical autoimmune diseases, have not been observed in depression (Glanville et al 2020). By seeking evidence of greater than chance overlap between GWAS risk variants and the genomic regions known to be epigenetically active in different cells type or tissues, we can infer which cell types/tissues are likely to be pathogenically important in a given condition. Such approaches have been extensively used to investigate the brain cell types and regions

implicated by depression risk variants (Finucane et al 2018, Li et al 2018a). No studies to date have demonstrated enrichment of depression risk variants in any specific immune cell type; one study showed lack of enrichment of MDD risk variants in immune cell subsets (Finucane et al 2018), although this study drew on GWAS results from 2016 which have now been superseded by much better powered studies of depression. It is not surprising that the key signals from GWAS studies to date have primarily implicated the brain. Given that depression is first and foremost a condition of the mind, we would expect MDD-related genetic signals in the immune system to be smaller, or to be relevant in only a subset of patients. The extent to which depression risk variants affect immune function is only beginning to be investigated.

As discussed above, depression has been associated with multiple alterations in the immune system in clinical cohort studies, but it is difficult to infer causality from such studies because of the risk of confounding. For example, observed inflammation in depression may be due to the consequences of a depression diagnosis, e.g., decreased ability to self-care or other pro-inflammatory lifestyle changes, which are a consequence, rather than a cause, of depression. One way to overcome these confounding effects is to use genetic risk scores as a proxy for susceptibility to disease (Lohoff 2010). The effects of a genetic risk score on an outcome of interest (e.g., pro-inflammatory cytokines) can be assessed independent of case-control status (e.g., by using cohorts of controls or by controlling for the effect of diagnosis), mitigating the potentially confounding effects of common disease comorbidities or the consequences of disease on the outcome measures. MDD is highly polygenic, with a heritability of ~40-50%: summary statistics from genome-wide association studies can thus be used to generate polygenic risk scores (PRS) for individuals, capturing their susceptibility to disease due to assayed common genetic variants. Studies have shown some modest effects of high depression PRS on the standard markers of peripheral inflammation typically assayed in research studies of inflammation in depression. A high depression PRS has been robustly associated with increased peripheral white cells counts (Sealock et al 2021, Sewell et al 2020), but not with pro-inflammatory cytokines (Palmos et al 2021), and with mixed results for CRP (Palmos et al 2021, Sewell et al 2020). Depression PRS scores are also (modestly) predictive of case status for several autoimmune diseases (psoriasis, inflammatory bowel disease and type 1 diabetes) (Glanville et al 2021). Genetic correlations between disorders can also be used to identify disorders with shared genetic pathophysiology – notably, there is a genetic correlation

between depression and inflammatory bowel disease and depression and psoriasis (Glanville et al 2021, Howard et al 2019).

Another genetic approach to evaluating potential causality is mendelian randomization (MR), which tests exposure-outcome associations by using genetic variants as instruments reliably associated with the exposure of interest which, because of random allele segregation, should not relate to confounding variables. For example, genetic risk variants for high white cell count (WCC) can be used to analyse the potential causal role of increased WCC in depression. MR studies of inflammation in depression have supported a causal role for increased WCC and increased IL-6 in risk for depression and depressive symptoms (Perry et al 2021, Sealock et al 2021, Ye et al 2021), but a (counterintuitively) protective effect of increased CRP against depressive symptoms (Ye et al 2021).

1.4.5 Animal models

Several animal models have been used to investigate the effects of inflammation on behavioural phenotypes relevant to depression, and the mechanisms uncovered support causal effects of the immune system on the brain and behaviour which may also be important in humans with depression. As these models rely on mimicking the risk factors for depressive symptoms (e.g., psychosocial stress, infections), they will be discussed in **Section 1.5** on the proposed sources of inflammation in depression, and **Section 1.6** on the effects of stress on cellular immunity.

1.4.6 Human experimental and mechanistic studies

We have discussed evidence that inflammation is associated with depressive disorder, with some evidence that the association is causal. But if such a link is to be believed, it is important that we have plausible mechanisms for how this could occur at an immunological and neurobiological level: *How* can peripheral inflammation lead to depressive symptoms? Crucially, how does inflammation affect brain regions and neural circuits known to be important for depressive symptoms?

Studies of human participants undergoing experimental or clinical induction or antagonism of inflammation have demonstrated important within-person effects of inflammation on mood-

relevant circuitry and mood-relevant neurotransmitter systems, as outlined in **Table 1-4**. Such studies have especially implicated the ventral striatum – important for reward processing and motivation – and the insula – important for interoception and punishment sensitivity – potentially linking inflammation to the maladaptive motivational changes observed in depression (Savitz & Harrison 2018). Importantly, looking across participants, the magnitude of the inflammation-related circuit changes detected has often been found to correlate with the changes in mood or anhedonic symptoms induced by these paradigms.

Table 1-4 *Human experimental studies involving induction or manipulation of inflammation: effects on mood-relevant brain circuitry.*

Experimental paradigm	Findings
Endotoxin administration	Low-dose endotoxin (LPS) administration acutely decreases ventral striatal activity to reward cues, and this ventral striatal activity correlates with the extent of endotoxin-induced depressed mood (Eisenberger et al 2010). Low-dose endotoxin also increases glucose metabolism in the insula, as detected by PET, and this correlates with the endotoxin-induced change in social interest (Hannestad et al 2012).
Vaccination	When inflammation is induced experimentally in human using typhoid vaccination, participants experience an acute deterioration in mood (at 3 hours) which correlates with increased activity in the anterior cingulate cortex (ACC) during an emotional face-processing task (Harrison et al 2009a), as well as an acute increase in fatigue and confusion which correlates with increased activity in interoception-related regions during a Stroop task (Harrison et al 2009b). Vaccination has also been shown to lead to acute microstructural alterations in the insula which correlate with fatigue (Harrison et al 2015); to lead to increased intra-insula functional connectivity which correlates with fatigue (Stefanov et al 2020); and to acutely enhance punishment (compared to reward) sensitivity, correlating with decreased coding of reward prediction error in the ventral striatum and increased coding of punishment prediction error in the insula (Harrison et al 2016).
Interferon administration	IFN-α administration acutely enhances amygdala reactivity to sad faces, and the extent of this change is predictive of the effect of chronic IFN-α administration on mood (Davies et al 2021). IFN-α has also been shown to decrease ventral striatal

	activity during a reward-based gambling task, and to decrease striatal update and turnover of dopamine precursors (consistent with decreased striatal dopamine release); these changes correlated with the chronic mood, anhedonic and fatigue-related effects of IFN-α (Capuron et al 2012). IFN-α induces acute changes in striatal microstructure which correlate with subsequent development of chronic IFN-α related fatigue (but not mood) symptoms (Dowell et al 2016). Chronic IFN-α administration has also been shown to lead to increases in glutamate concentrations in the ACC and basal ganglia (as measured by magnetic resonance spectroscopy), with changes in basal ganglia glutamate correlating with IFN-α-associated decreases in motivation scores (Haroon et al 2014). The effects of IFN-β administration (another type I interferon which induces sickness behaviours) have been much less investigated, but a study assessing changes in appetitive motivation and reactivity to negative emotional stimuli in healthy participants receiving 8 consecutive days of IFN-β found that IFN-β decreased appetitive motivation and decreased ventral striatal responses (consistent with altered reward processing), but did not increase negative affect, or alter amygdala reactivity to emotionally salient stimuli (Coch et al 2019).
Cytokine blockade	TNF-α antagonism acutely decreases amygdala reactivity to sad faces, and the extent of this change is predictive of the positive effect of chronic TNF-α antagonism on mood (Davies et al 2021). Circulating TNF-α levels also correlate with brainstem serotonin transporter (5-HTT) availability in the brain as measured by SPECT imaging, and inhibition of TNF-α with etanercept has been shown to decrease brainstem 5-HTT availability (Krishnadas et al 2016).

1.4.7 Causal role of peripheral (vs central) inflammation in depression without medical comorbidity

As a final perspective on our consideration of immune causality in MDD, it is useful to consider the evidence for a distinct causal contribution of the *peripheral* immune system to psychiatric symptoms (as distinct from the potential contribution of immune cells in the brain and meninges). If peripheral immune activity were to causally contribute to psychological symptoms, this would open the door to new ways of treating psychological disorders – we may be able to 'repurpose' existing (already approved) therapies which target peripheral immune cells or molecules to help some patients with psychological symptoms.

The idea that peripheral inflammation induced by inflammatory stimuli or inflammatory conditions can affect mood is not controversial. Along with the evidence discussed above, such causal effects are also supported by the efficacy of (peripherally restricted) anti-cytokine drugs for mood symptoms in chronic inflammatory diseases, independent of improvement in physical symptoms (Kappelmann et al 2018, Wittenberg et al 2019). Whether peripheral inflammation contributes causally to mood in MDD that is *not* comorbid with medical disorders is less clear. One hypothesis is that the peripheral inflammation observed in MDD simply reflects or is a consequence of changes (inflammatory or otherwise) in the brain. If this is the case, peripheral samples might provide useful biomarkers, but peripheral immune abnormalities would not constitute useful direct therapeutic targets. Evidence that TNF blockade (infliximab) improves symptoms in patients with MDD and raised CRP goes against this, as infliximab does not cross the blood-brain barrier, suggesting a causal role for peripheral TNF in mood symptoms not associated with medical disorders (Raison et al 2013). Animal studies also demonstrate that peripheral inflammation resulting from psychological (rather than immunological) stressors makes a causal contribution to psychological symptoms. For example, depletion of IL-6 from peripheral haematopoietic cells only (using $IL6^{-/-}$ bone marrow chimeras) confers resilience to stress, suggesting that stress-induced peripherally-derived IL-6 is important for behaviour (Hodes et al 2014). Moreover, transfer of peripheral (lymph node) leucocytes from chronically stressed mice to unstressed mice was shown to have an antidepressant effect in recipient mice, highlighting that the peripheral immune system may also contribute to behavioural resilience to stress (Brachman et al 2015).

1.5 Proposed sources of inflammation in depression

There are multiple proposed sources of the inflammation associated with psychiatric disorders including stress, infection, comorbid or subclinical inflammatory disorders, obesity, environmental insults, lifestyle factors, genetics, gut microbial dysbiosis, and altered gut barrier integrity. Several of these mechanisms may be relevant, or interact, in a given individual. The extent to which these sources of inflammation converge on a common pathology underlying inflammation in psychiatric disorders is unclear. In the table below (

Table 1-5), I consider the evidence for each of these potential contributing factors, and some insights into the role of these risk factors from animal models, with a more in-depth focus on the potential role of stress in the following section (**Section 1.6**).

Table 1-5 *Proposed sources of inflammation in depression.*

Source	Evidence / proposed mechanism
Infection	*Human data:* In epidemiological surveys, both severe and minor infections have been shown to increase the risk of depression and suicide, with risks highest in the two years following infection, but persistently raised above baseline even many years later (Benros et al 2013, Kohler et al 2017b, Lund-Sorensen et al 2016). Whether this reflects a failure to completely resolve inflammation (i.e., an ongoing proinflammatory state) or damage occurring at the time of infection in unclear. Human studies of the effects of pathogen associated molecular patterns (e.g., endotoxin) and infection-associated cytokines on brain and behaviour are described in **Section 1.4.6**. *Animal models*: In rodents, infection-induced depressive symptoms are frequently modelled by exposing animals acutely to pathogens; to pathogen-associated molecular patterns such as endotoxin/lipopolysaccharide (LPS) and the viral mimetic poly(I:C); or to cytokines known to be produced during inflammatory responses (e.g., interferons). Such investigations have revealed multiple mechanisms by which pathogens can elicit sickness- or depressive-like behaviours including via the action of interferon on brain endothelium/epithelium (Blank et al 2016); via the effects of LPS-induced IL-1 on brain endothelium (Liu et al 2019); and via LPS-induced or IFN-γ-induced upregulation of the tryptophan-degrading enzyme IDO (O'Connor et al 2009a, O'Connor et al 2009b). Inflammation-associated depressive symptoms are associated with microglial activation, and new chemogenetic tools to selectively modulate microglial activity show that striatal microglial activation is sufficient to cause depressive behaviours and necessary for LPS-induced aversive behaviour, with microglial/myeloid cell IL-6 and prostaglandin secretion being key to the effect (Klawonn et al 2021). Interestingly, stress can exacerbate the effects of peripheral inflammation on the immune system: for example, chronic stress potentiates LPS-induced prefrontal and hippocampal NF-κB activation in rats (Munhoz et al 2006). Non-human primates have also been used to investigate the effects of peripheral inflammation on depression-relevant mood circuitry: in vivo microdialysis and PET experiments have demonstrated that chronic IFN-α administration leads to anhedonia and decreased striatal dopamine release (Felger et al 2013).

Comorbid chronic inflammatory disease	*Human data:* Epidemiological studies demonstrate that chronic autoimmune disorders increase the risk of subsequent mood disorder (Benros et al 2013). There is evidence that in patients with inflammatory or autoimmune diseases, the associated inflammation makes a causal contribution to mood: meta-analytic analysis of the effects of anti-cytokine treatments on mood scores in patients with inflammatory disorders showed a positive effect on mood of IL-6 blockade, even when only considering those whose primary disease did not respond to treatment (Wittenberg et al 2019). Incipient, undiagnosed or subclinical inflammatory disease may also contribute to depression that is ostensibly not associated with medical co-morbidity, but the clinical importance of this effect is unclear. *Animal models:* Animal models of chronic inflammatory disorders recapitulate some of the neurobiological changes seen depression and suggest mechanisms underlying the effects seen in humans. For example, in a rat model of rheumatoid arthritis (RA) which is associated with anhedonia (adjuvant-induced arthritis), cortical and hippocampal BDNF were decreased (Pedard et al 2018). In another model of RA (human TNF-alpha transgenic mouse model), chronic RA-like pathology led to TNF-dependent myeloid cell activation in the cortex, changes also seen on post-mortem histology from humans with RA (Suss et al 2020).
Obesity	*Human data:* Obesity may causally contribute to mood symptoms via psychological mechanisms (e.g., negative self-appraisal). However, obesity may also contribute to depressive symptoms via its proinflammatory effects. Notably, obese adipose tissue releases pro-inflammatory cytokines including TNF-α and IL-6 (Ouchi et al 2011), as well as leptin, a hormone best known for signalling energy availability, but which also has pro-inflammatory effects, stimulating both innate and adaptive immune cells (Naylor & Petri 2016). In observational studies, BMI statistically explains some of the observed association between inflammation and depressive symptoms (Chamberlain et al 2018), but given the pro-inflammatory effects of adipose tissue, obesity cannot be considered a simple confounder of the relationship between inflammation and depression. Obesity-induced inflammation may also, in part, mediate the effects of stress on symptoms: human epidemiological studies and primate studies of the experimental effects of stress suggest that chronic stress increases upper body obesity (Jayo et al 1993). Moreover, there is evidence for an immunometabolic subtype of depression associated with increased appetite, altered energy homeostasis and obesity (Milaneschi et al 2020)(see **Section 1.7.1**).

	Animal models: Animal models using high fat diet (HFD) to mimic human diet-induced obesity show that HFD/obesity-induced inflammation is dependent on a vicious cycle of interactions dependent on CD8+ T cells and macrophages within adipose tissue (Nishimura et al 2009).
Pro-inflammatory environmental insults / lifestyle factors	There is evidence that smoking, alcohol, low cardiorespiratory fitness, sleep disruption and the effects of environmental pollution all contribute to peripheral inflammation (O'Connor et al 2009c, Tsai et al 2019). For example, air pollution exposure has been associated with higher rates of subsequent depression (Roberts et al 2019) and also with increased peripheral CRP and IL-6 (Li et al 2017). Like the effects of obesity, these cannot simply be considered confounders to be factored out, as the inflammation they cause may contribute to symptoms. Equally, however, symptoms of depression and known risk factors for MDD (e.g., low socio-economic status) make many of these exposures more likely. The causal pathways between mood symptoms, lifestyle-related behaviours and inflammation remain underexplored.
Gut microbiome and gut barrier integrity	*Human data:* Immune dysregulation in MDD may also reflect microbial dysbiosis or altered gut physiology. Depression has been associated with replicated increases and decreases in multiple taxa of bacteria, corresponding to a decrease in anti-inflammatory butyrate-producing bacteria (e.g., *Faecalibacterium* and *Coprococcus*) and an increase in pro-inflammatory butyrate-depleting bacteria (e.g., *Eggerthella*) (Nikolova et al 2021b). Depression and anxiety have also been associated with a gut microbiome over-representative of LPS biosynthetic genes, increased plasma levels of LPS and LPS-binding protein, and increased plasma markers of impaired gut epithelium tight junction barrier integrity (zonulin and FABP2) (Alvarez-Mon et al 2019, Stevens et al 2018), possibly reflecting increased bacterial translocation from the gut to the blood in MDD. *Animal models:* Germ-free mice, mice treated with antibiotics, and faecal microbiota transplants have been extensively used to investigate the effects of microbiota on behaviours relevant to depression, demonstrating that microbiota are essential for appropriate stress-responses and social behaviour, and can modulate anxiety/depressive behaviours (Nagpal & Cryan 2021). Such studies have demonstrated multiple immune and non-immune (neural, hormonal and metabolic) pathways by which gut microbiota can affect symptoms. Immune mechanisms of

	depressive symptoms include gut bacteria-induced accumulation of Th17 cells in the hippocampus (Medina-Rodriguez et al 2020) as well as microbiome-dependent increases in stress-induced peripheral IL-6 (Bailey et al 2011) and hippocampal IL-1β (Pearson-Leary et al 2020). Notably, in mice, chronic stress causes T cell- and IFN-γ-dependent increases in gut permeability, and an increase in bacterial translocation to the liver (Ferrier et al 2003), convergent with the findings of altered gut barrier integrity in human depression described above. Studies using faecal microbiota transplants from humans to rodents have shown that depressed compared to non-depressed microbiota can induce depression-related phenotypes in rodents, providing evidence for a causal contribution of microbial dysbiosis to symptoms (Chinna Meyyappan et al 2020).
Reduced exposure to non-pathogenic microbes	The absence in many modern human environments of 'old friends', i.e., microbes with whom we co-evolved, is hypothesized to impair immunoregulatory responses, with evidence that humans raised in urban environments without pets (vs. rural environments with animals) show delayed resolution of stress-induced increases in peripheral leucocytes and decreased IL-10 (an immunoregulatory cytokine) following psychosocial stress (Bobel et al 2018).
Genetic propensity	See discussion in **Section 1.4.4**
Psychosocial stress	See **Section 1.6** (next section) for a discussion of the effects of stress on cellular immunity, including acute stress, chronic adult stress and early life stress, all of which are hypothesized to contribute to immune abnormalities in depression.

1.6 The effects of stress on immunity

There is strong meta-analytic evidence that the risk of depression is increased both by stressful life events in adulthood (Kendler et al 1999) and by adverse childhood experiences (Teicher & Samson 2013). There also is evidence that stress is associated with similar immunological changes to those observed in some patients with depression, supporting the hypothesis that inflammation may be a link in the causal pathway from stress to depression. For example, there is meta-analytic evidence from observational studies that childhood adversity is associated with increases in adult CRP, IL-6 and TNF-α (Baumeister et al 2016) and some evidence from observational studies (variably reproduced) that various forms of chronic stress (e.g., caregiver stress) are associated with increases in CRP and IL-6 (Rohleder 2019). Acute/subacute stress has also been associated with changes in peripheral blood cell counts. For example, intensive care doctors on- compared to off-shift show increased blood white cell

counts (Heidt et al 2014). In terms of adaptive immunity, in observational studies, subacute naturalistic stressors (e.g., exams) tend to be associated with suppressed measures of cell-mediated immunity (e.g., T cell proliferative responses); preserved or increased immunoglobulin titres; and decreased antibody and cellular responses to vaccination (Glaser et al 1992, Madison et al 2021, Segerstrom & Miller 2004). Chronic stress (e.g., caregiver stress) tends to be associated with suppression of both T cell and B cell immunity, including decreased T cell proliferative responses, as well as decreased antibody and cellular responses to vaccination (Kiecolt-Glaser et al 1996, Madison et al 2021, Segerstrom & Miller 2004).

Experimental studies of induced stress allow a clearer interpretation of the causal relationship between stress and immune changes, with the limitation that – for ethical reasons – only acute (and not chronic) stress can be studied in this way in humans. Experimentally-induced acute stress has been shown to lead to immunological changes similar to those seen in depression, with meta-analytic support for acute stress-induced increases in IL-6, IL-1β, IL-10 and TNF-α (Marsland et al 2017). One method of inducing acute stress experimentally is the Trier Social Stress Test (TSST), where participants give a presentation and perform mental arithmetic in front of unsympathetic judges. A study in teachers showed that the TSST increased IL-6 production from peripheral blood cells (IL-6 was measured following stimulation of whole blood *in vitro* with LPS) (Bellingrath et al 2013), although which specific immune cells were responsible for this increased IL-6 production is unclear. Teachers with high levels of chronic stress (perceived effort to reward ratio) showed higher baseline levels of IL-6 prior to the TSST. Mirroring findings in observational studies of the relationship between subacute stress and peripheral cell counts, participants exposed experimentally to socially evaluated cold pressor stress (a mixed psychological and physiological stressor) showed increased relative counts of peripheral monocytes (as a percentage of total live cells) (van de Wouw et al 2021). Similar to findings in observational studies of participants with subacute/chronic stress, experimentally induced acute stress leads to decreased lymphocyte responses to *in vitro* stimulation (Segerstrom & Miller 2004). Which (if any) of these immune consequences of stress contribute to the behavioural effects of stress in humans remains unclear.

In order to address how stress-induced immune changes causally contribute to behavioural susceptibility to stress, the mechanisms of immune responses to stress, and the effects of stress on tissues that are poorly accessible in humans, animal models of stress have been

extensively investigated (Tsyglakova et al 2019). These include models of acute psychological stress, early life stress and chronic stress. Like in humans, both acute and chronic stress produce an increase in peripheral IL-6 in rodents. However, while in humans, acute stress has been shown to increase IL-6 production from immune cells (Bellingrath et al 2013), a study in rodents showed that, at least in mice, the key source of acute stress-induced IL-6 is brown adipocytes and not immune cells (Qing et al 2020). For chronic stress, however, experiments using $IL6^{-/-}$ bone marrow chimeric mice have shown that haematopoietic cell IL-6 is critical for the induction of stress-induced behavioural abnormalities (Hodes et al 2014).

Animal models of chronic stress – including repeated social defeat (RSD), chronic unpredictable stress (CUS) and chronic restraint stress models – have suggested that multiple immune mechanisms contribute to the negative behavioural effects of stress. These include: microglial activation (Kreisel et al 2014); microglial oxidative stress (Lehmann et al 2019); endothelial IL-1 receptor dependent priming of microglia (Wohleb et al 2014); inflammasome activation (Iwata et al 2016); type 1 IFN activity (Tripathi et al 2021); and increased blood-brain barrier permeability and consequent entry of peripheral IL-6 into the brain (Menard et al 2017). As summarised in **Figure 1-2A**, there is also a large body of animal work showing that psychosocial stresses cause release of inflammatory monocytes into the circulation from reserves in the bone marrow and spleen (Weber et al 2017), mirroring the findings in humans discussed above. The release of these $Ly6C^{hi}$ inflammatory monocytes is hypothalamic-pituitary-adrenal (HPA) axis-dependent (Niraula et al 2018) and the monocytes released are distinguished by expression of pro-inflammatory cytokine genes, glucocorticoid resistance, and surface expression of receptors for MCP-1 (CCL2) and other chemokines (Weber et al 2017). There is some evidence that IL-1-dependent trafficking of inflammatory monocytes to the brain, recruited by microglia, contributes to the detrimental effects of stress on neuronal function and behaviour (McKim et al 2018, Wohleb et al 2014, Wohleb et al 2013), although this remains controversial (Lehmann et al 2016).

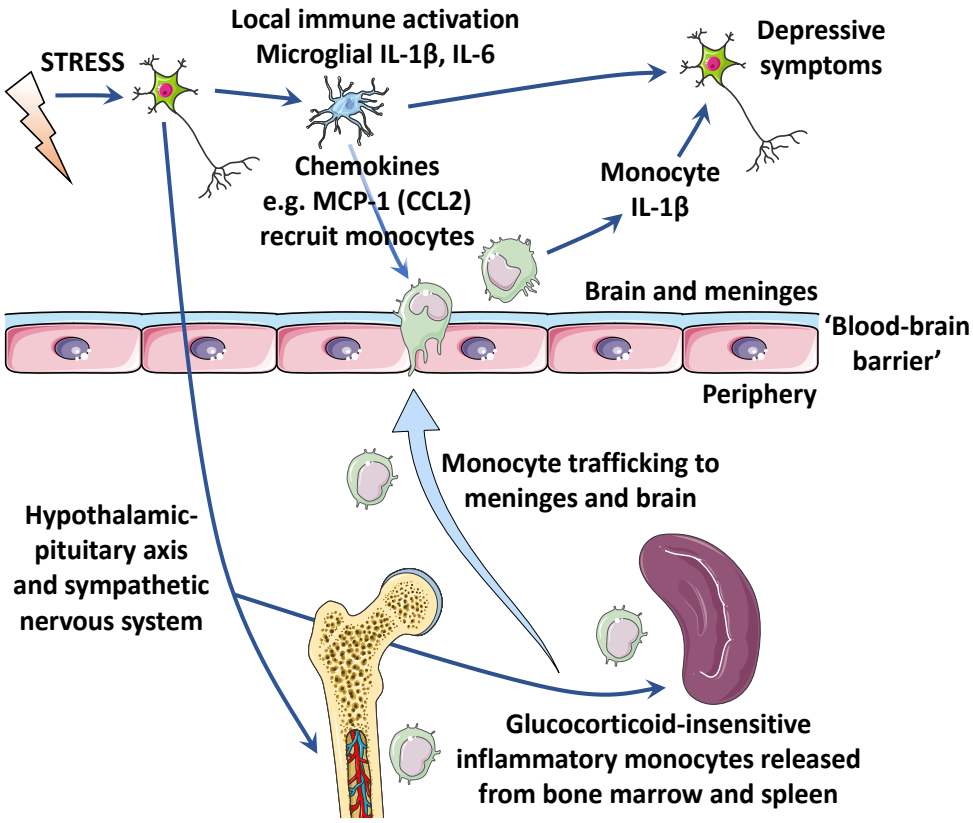


Figure 1-2 *Effects of stress on the immune system in the brain and periphery*

Recruitment of pro-inflammatory, glucocorticoid-insensitive monocytes to the central nervous system may mediate the link between stress and depression (figure created based on (Weber et al 2017)). Parts of the figure were drawn by using pictures from Servier Medical Art, which is licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).

The contribution of adaptive immunity to the behavioural response to stress has been less investigated, but mouse experiments modulating the adaptive immune system by genetic manipulations, antibody depletion of adaptive immune cells, or adoptive transfer of immune cells between animals have shown that brain-reactive T cells are necessary for behavioural resilience to stress (Cohen et al 2006); that microbiota-induced Th17 cells can promote susceptibility to learned helplessness in response to acute footshock stress (Beurel et al 2013, Medina-Rodriguez et al 2020); and that adoptive transfer of lymphocytes from chronically stressed mice can induce an anti-depressant phenotype in naïve or stressed recipient mice (Brachman et al 2015, Scheinert et al 2016). Acute stress has been shown to increase T cell infiltration to the brain and choroid plexus (Lewitus et al 2008), and high levels of glucocorticoid signalling at the choroid plexus block the stress-induced infiltration of Th2 and

Treg cells to the brain (Kertser et al 2019), although the contribution of these brain-infiltrating cells to the behavioural response to stress remains unclear.

Early life stress in rodents, although less investigated from an immunological perspective than chronic stress, has also been associated with immune abnormalities. Neonatal repeated maternal separation (RMS) in rodents, a model of parental neglect, leads to increases in tissue IL-6 and TNF-α and increased microglial activation in the days/weeks immediately following RMS (Dutcher et al 2020). These increases do not tend to persist into adulthood without rechallenge, but RMS rodents who are re-exposed to stress in adulthood show greater pro-inflammatory tissue cytokine responses (in both brain and non-brain tissues) and increased microglial activation compared to adult-stressed animals not exposed to RMS (Dutcher et al 2020).

In terms of the effects of stress on the meningeal immune compartment, one T cell-focused study showed that neither subacute nor chronic stress affected IL-17a expression in meningeal γδ or αβ T cells (Alves de Lima et al 2020), but there has otherwise been no investigation of the effects of acute stress, chronic stress or early life stress on meningeal immune cells in either humans or animal models.

1.7 Diagnostic considerations

1.7.1 Evidence for an immune subtype in MDD, and associations of inflammation with specific symptoms or endophenotypes

There are no universally accepted subtypes of MDD, but a distinction akin to the contrast between 'typical' and 'atypical' depression (from the Diagnostic and Statistical Manual of Mental Disorders) has recently gained traction as biologically meaningful (Lamers et al 2010). In the NESDA cohort study, patients with typical depression did not show immune activation, while atypical patients – characterized by increased appetite and weight gain – had increased levels of CRP and IL-6 (Lamers et al 2013, Milaneschi et al 2020). This effect could be seen even when groups were matched for BMI (Simmons et al 2018). Atypical (but not typical) depression also shows genetic correlation with obesity (Milaneschi et al 2017). These and other data support an 'immunometabolic'/atypical subtype of depression with greater prominence of symptoms related to altered energy expenditure (Milaneschi et al 2020). The previously

described RCT data demonstrating responses to adjuvant infliximab or minocycline only in MDD patients with evidence of inflammation also support the hypothesis of an immune subgroup in MDD (Nettis et al 2021, Raison et al 2013).

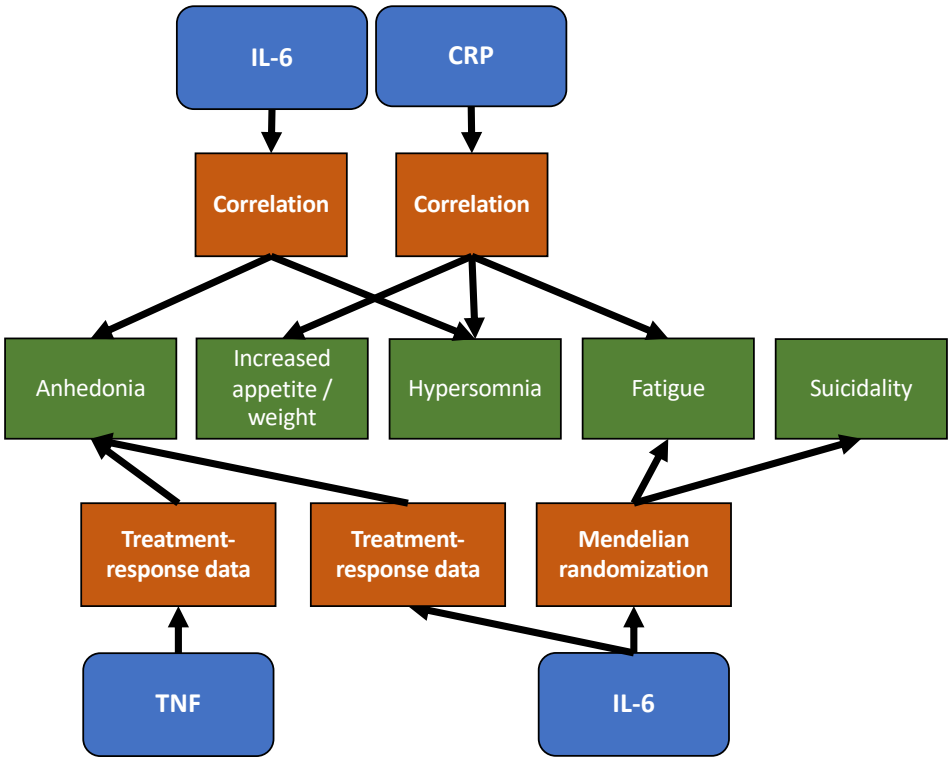


Figure 1-3 *Inflammatory markers with strong evidence for links to specific depressive symptoms. Acute phase reactants and pro-inflammatory cytokines (blue boxes) have been particularly linked to anhedonia and neurovegetative symptoms. Methods supporting these links (red boxes) include correlations between symptoms and inflammatory markers in observational studies; mendelian randomization studies; and evidence from randomized controlled trials that immunomodulatory treatment show particular efficacy against specific depressive symptoms. See text for further details. CRP, C-reactive protein; IL-6, interleukin-6; TNF, tumour necrosis factor.*

In a complementary approach, other studies have sought not to identify patient subgroups, but assessed whether inflammation relates to particular symptom domains or cognitive endophenotypes. Peripheral inflammation, as indexed by CRP and IL-6, is not uniformly associated with all depressive symptoms (see **Figure 1-3**). Consistent with the evidence for an immunometabolic/atypical subgroup in MDD, studies have tended to associate inflammation with atypical or 'neurovegetative' symptoms (changes in appetite or sleep, or psychomotor slowing) (Milaneschi et al 2020). CRP has been particularly associated with fatigue, increased

sleep, and increased appetite, while IL-6 has been associated with anhedonia, increased sleep and decreased appetite (Milaneschi et al 2021); inflammation was less associated with anxiety symptoms. Mendelian randomization (MR) analyses further support a causal role for increased IL-6 specifically in fatigue (Milaneschi et al 2021). Another MR analysis supported a causal role for IL-6 in suicidality (Kappelmann et al 2021). MR analyses have not supported a specific causal link between CRP or IL-6 and anhedonia (Kappelmann et al 2021). Randomized controlled trial data, however, do support a specific role for inflammation in anhedonia. In treatment-resistant depression, anti-TNF-α therapy (Raison et al 2013) and anti-IL-6 therapy (Salvadore et al 2018) were associated with specific improvements in anhedonia compared to placebo, even where (for anti-IL-6 therapy) there was no evidence of efficacy for depressive symptoms overall. Likewise, in an RCT of anti-TNF therapy for bipolar depression, anti-TNF-α was particularly effective against anhedonia, especially in participants with high baseline TNF-α (Lee et al 2020). This is in keeping with the experimental evidence in humans described in **Section 1.4.6**, which demonstrates a particularly strong link between modulation of inflammation and altered activity in reward circuitry – the circuitry most implicated in anhedonic symptoms (Husain & Roiser 2018).

Together, the above findings suggest that inflammation may contribute specifically to neurovegetative symptoms and anhedonia rather than to overall risk of depression diagnosis. However, these symptoms can be present in numerous neurological and psychiatric disorders. For example, while anhedonia is a cardinal symptoms for depressive disorders, it is also a symptom of schizophrenia, traumatic brain injury, post-traumatic stress disorder, substance use disorder, Parkinson's disease, and Alzheimer's disease (Husain & Roiser 2018). Such data challenge the simplistic view that inflammation contributes to depression as a discrete disorder, instead prompting consideration of the idea that inflammation has a transdiagnostic effect on cognition and symptoms, a hypothesis we now move on to consider.

1.7.2 Evidence for transdiagnostic immunopathology in psychiatric disorders

Clinical diagnosis for mental health disorders is comprised of multiple, categorically distinct clinical syndromes such as schizophrenia, MDD, and bipolar disorder. However, symptoms overlap between different psychiatric diagnoses, and comparative investigations of psychiatric disorders have revealed both shared and specific genetic (Cross-Disorder Group of the Psychiatric Genomics Consortium 2019, Lee et al 2021, Peyrot & Price 2021) and

environmental risk factors (Kessler et al 1997, Schmitt et al 2014), and brain transcriptomic profiles (Gandal et al 2018). Thus, some genetic and environmental risks operate trans-diagnostically across multiple psychiatric syndromes, rather than being cis-diagnostically aligned to a specific syndrome, as would be expected if each disorder was a biologically discrete disease entity. These and other data support a general predisposition to psychopathology or 'p factor' which captures an individual's likelihood of developing any psychiatric disorder (Caspi et al 2014).

Immune system abnormalities have been observed in case-control studies of many psychiatric disorders, including schizophrenia (Khandaker et al 2015, Miller & Goldsmith 2017), MDD (Miller & Raison 2016), bipolar disorder (Rosenblat & McIntyre 2017), autism spectrum disorder (ASD) (Meltzer & Van de Water 2017), and attention deficit hyperactivity disorder (ADHD) (Hoekstra 2019). Among the most consistently reported findings, across multiple disorders, are increased C-reactive protein (CRP) (Yuan et al 2019), increased pro-inflammatory cytokines (Goldsmith et al 2016, Yuan et al 2019), increased white blood cell counts in both myeloid and lymphoid lineages (Barbosa et al 2014, Breunis et al 2003, Fernandez-Egea et al 2016, Grosse et al 2016b, Jackson & Miller 2020, Miller et al 2013, Munkholm et al 2018), and inflammasome activation (Alcocer-Gomez et al 2014, Kim et al 2016, Saresella et al 2016). Polygenic risk scores for both depression and schizophrenia have been associated with increased peripheral white cell counts, although MDD PRS was more associated with increased neutrophil counts and schizophrenia PRS was more associated with increased lymphocyte counts (Sewell et al 2020). Mendelian randomization studies support a causal role for IL-6 in both depression and schizophrenia (Perry et al 2021) and multiple psychiatric disorders show genetic correlations with immune disorders (Tylee et al 2018). The gut dysbiosis associated with psychiatric disorders also shows a consistent transdiagnostic pattern across depression, bipolar disorder, schizophrenia and anxiety disorders (Nikolova et al 2021b). Moreover, environmental exposures that elicit an immune response are risk factors for multiple psychiatric disorders, including *in utero* or parental infections (Al-Haddad et al 2019, Lydholm et al 2019), childhood and adult infections (Benros et al 2011, Benros et al 2013, Breithaupt et al 2019, Kohler-Forsberg et al 2019), childhood adversity (Hostinar et al 2015), and acute or chronic stress (Rohleder 2019). Finally, there is some evidence from observational studies that anhedonia and psychomotor slowing correlate with peripherally measured inflammation in disorders beyond MDD. For example, in PTSD, peripheral inflammation, especially IL-6, has

been associated with anhedonia and altered reward circuitry connectivity (Mehta et al 2020); and in schizophrenia, peripheral inflammatory cytokines correlate with psychomotor slowing (Goldsmith et al 2020) and predict subsequent development of negative symptoms including anhedonia (Goldsmith et al 2019).

On the basis of this evidence, it is conceivable that the immune system could be implicated in the pathogenesis of multiple psychiatric disorders. However, the limited immunophenotyping that has been applied across multiple disorders (only plasma CRP/cytokines and coarse peripheral blood cell counts have good cross-disorder data available for comparison) as well as the lack of transdiagnostic immunophenotyping studies mean that the degree to which the immune contribution to different psychiatric disorders is shared or distinct remains an open question. Moreover, it is unclear whether immunopathology is simply an additive risk factor increasing susceptibility to multiple diagnoses; makes a specific trans-diagnostic contribution to symptoms such as anhedonia and fatigue; affects prognosis (e.g., by contributing to treatment resistance) across multiple disorders; or reflects a trans-diagnostic subgroup of patients with an immune pathogenesis.

1.8 Summary

I have outlined the large body evidence that depression is associated with altered protein, cellular and transcriptional immunophenotypes, and the growing body of evidence from multiple sources that inflammation makes a causal contribution to symptoms in depression. I have also explored the possible sources of inflammation in depression, focusing especially on the marked effects of stress on the immune system. Key gaps in our knowledge, however, remain. In particular, studies have tended to focus on genes or proteins in whole blood, or on coarse-grained cellular subsets. Deeper cellular immunophenotyping is required to determine the specific cell subsets and pathways implicated in depression and the stress response. The causal contribution of cellular immunophenotypes to symptoms remains unclear, as does the extent to which there is common or distinct cellular immunopathology contributing to different psychiatric disorders. Moreover, the role of the recently discovered meningeal immune system in stress and depression has hardly been explored. The analyses and experiments in the chapters to follow aim to address these outstanding questions.

Chapter 2: Peripheral blood flow cytometry immunophenotypes in health and depression

2.1 Introduction

As discussed in **Chapter 1**, inflammatory mechanisms could be plausible targets for repurposing or *de novo* development of immunomodulatory drugs for anti-depressant efficacy in cases of '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 immune-targeted anti-depressant drugs, which should be precisely focused on the cases most likely to have a favourable benefit:risk response to treatment. This motivates the search for peripherally accessible biomarkers of inflammation which could be used to guide stratified treatment for patients with depression. The identification of such biomarkers to guide treatment has long been a goal of psychiatry research, but at present, no biomarkers are used to guide treatment for any psychiatric condition.

Most work on peripheral biomarkers in depression (summarised above in **Section 1.3**) has focused on soluble components of the immune system such as cytokines and acute phase proteins. A major limitation of such measurements is that blood levels of proteins reflect the combined output of multiple different cell and tissue types, both rendering the measurements noisy as biomarkers, and making it difficult to attribute depression-associated changes in these markers to any particular immunopathological mechanism. For example, while peripheral blood IL-6 is often presumed to come from myeloid cells (e.g., (Bellingrath et al 2013)), it is also produced by adaptive immune cells (Barr et al 2010) as well as many 'non-immune' tissues and cells, e.g., adipocytes (Qing et al 2020). This limitation has prompted investigation of cellular phenotypes as biomarkers in depression, aiming to associate depression with abnormalities in particular immune cell subsets. The simplest cellular phenotypes are counts of different immune cell subsets in venous blood samples. In terms of peripheral blood cell counts,

depression has been reproducibly associated with leucocytosis, neutrophilia, an increased neutrophil to lymphocyte ratio, an increased ratio of $CD4^+$ to $CD8^+$ T cells (Maes et al 1992c, Mazza et al 2018, Zorrilla et al 2001), and increased numbers of intermediate monocytes (Alvarez-Mon et al 2019, Hasselmann et al 2018, Nowak et al 2019). Other findings have mainly been inconsistent or seen in only small studies. Depression has been associated with both decreased (Cai et al 2017, Zorrilla et al 2001) and unchanged (Demir et al 2015) lymphocyte counts and both increased or unchanged numbers of non-classical monocytes (Alvarez-Mon et al 2019, Hasselmann et al 2018, Nowak et al 2019). One study associated depression with increased memory CD4+ T cell counts (Maes et al 1992a) and some patients with depression also show increased CD8+ T cells and B cells ((Maes et al 1992a) and (Maes et al 1992b)). Depression has also been associated with reduced numbers of regulatory B cells in two small studies (Ahmetspahic et al 2018, Duggal et al 2016), but associations between depression and regulatory T cells and other T cell polarization states have been inconsistent (Alvarez-Mon et al 2019, Grosse et al 2016b, Hasselmann et al 2018, Jahangard & Behzad 2020, Patas et al 2018, Suzuki et al 2017).

One issue in interpreting cellular immunophenotyping data is that many prior studies have measured the relative frequency of each immune cell subset in proportion to the superset of peripheral blood mononuclear cells (PBMCs), or some other cellular superset (e.g. the percentage of classical monocytes of total monocytes) (e.g., (Grosse et al 2016b, Nowak et al 2019)). Such relative cell counts are difficult to interpret since a decrease in the relative proportion of any particular subset may reflect either an absolute decrease in their number or an absolute increase in the number of another subset. It is thus preferable to analyse absolute count data where possible. A further challenge is that with modern cytometry, it is now possible to analyse counts of a large number of immune cells subsets (10s-100s) from a single blood sample. If depression-associated changes are assessed independently for each immune cell subset, the number of univariate statistical tests creates a substantial multiple comparisons problem, necessitating increasing sample sizes, and motivating multivariate approaches to the data. The variable immunophenotyping findings from case-control studies may also in part stem from patient heterogeneity, reflecting the inclusion of depressed participants both with and without inflammation, and potentially with differing underlying immunopathologies.

2.2 Hypotheses

We hypothesize that in patients with major depressive disorder, analysis of cellular immunophenotypes in peripheral blood samples can identify one or more subgroups of patients with evidence of inflammation and generate hypotheses about the underlying pathophysiology of depression in these patients, as well as potential treatment targets.

2.3 Methods

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 (Lamers et al 2013, Raison et al 2013) 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.

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 Structured Clinical Interview for DSM-5 Disorders (SCID) screening questionnaire (First et al 2016), 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 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. All study assessments were completed at one of five UK centres as part of the Biomarkers in Depression (BIODEP) study (Chamberlain et al 2018), 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.

Medical comorbidity

One exclusion criterion for the BIODEP study was a lifetime history of any serious medical disorder likely to compromise the interpretation of immunological data. Of the 283 participants included in this cohort, 276 participants completed a self-report medical questionnaire and had a complete medications list ascertained. Participants with any condition requiring treatment with regular oral corticosteroids were excluded. Participants with mild inflammatory disorders were not excluded. One participant reported regular NSAID use. The self-report medical questionnaire, which asked about past or current conditions, indicated that 41 participants possibly had a current inflammatory disorder as follows: 28 reported asthma (mostly not requiring treatment: 7 requiring inhalers, none requiring oral medication), seven reported psoriasis (none requiring any topical or oral treatment), three reported type II diabetes mellitus (one diet-controlled only; none insulin-dependent), two reported coeliac disease and two reported arthritis (not requiring any treatment). Conservatively, these participants were deemed to have 'minor inflammatory disease' for the analysis presented here. Thyroid stimulating hormone was measured (normal range 0.4-4.5 mIU/L): based on this, 15 participants had evidence of biochemical hypothyroidism and 3 participants had evidence of biochemical hyperthyroidism. 6 participants were euthymic but treated with thyroxine or levothyroxine. TSH levels were not significantly different between controls and MDD participants (MWU $P = 0.8$).

Assessments

Participants completed the following clinical assessments and self-report questionnaires: Hamilton Depression Rating Scale (Hamilton 1960); Beck Depression Inventory v2.0 (Beck et al

1996); Chalder Fatigue Scale (Chalder et al 1993); Snaith-Hamilton Pleasure Scale (Snaith et al 1995); State-Trait Anxiety Inventory (Spielberger et al 1983); Childhood Trauma Questionnaire (Bernstein et al 1994); and Life Events Questionnaire (Brugha & Cragg 1990). 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 using a standard clinical haematology panel (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 at each study centre on fresh PBMCs using live-dead stain (BioLegend 423106) and the following antibodies: PE anti-CD3 (BD 555333), V500 anti-CD4 (BD 560768), PerCPCy5.5 anti-CD8 (BD 560662), APC anti-CD19 (BD 557744), AF488 anti-CD56 (BD 557699), PB anti-CD14 (BD 558121) and PECy7 anti-CD16 (BD 557744). We manually gated all data in FlowJo™, blind to case/control status of each participant, according to the gating strategy in **Figure 2-1**.

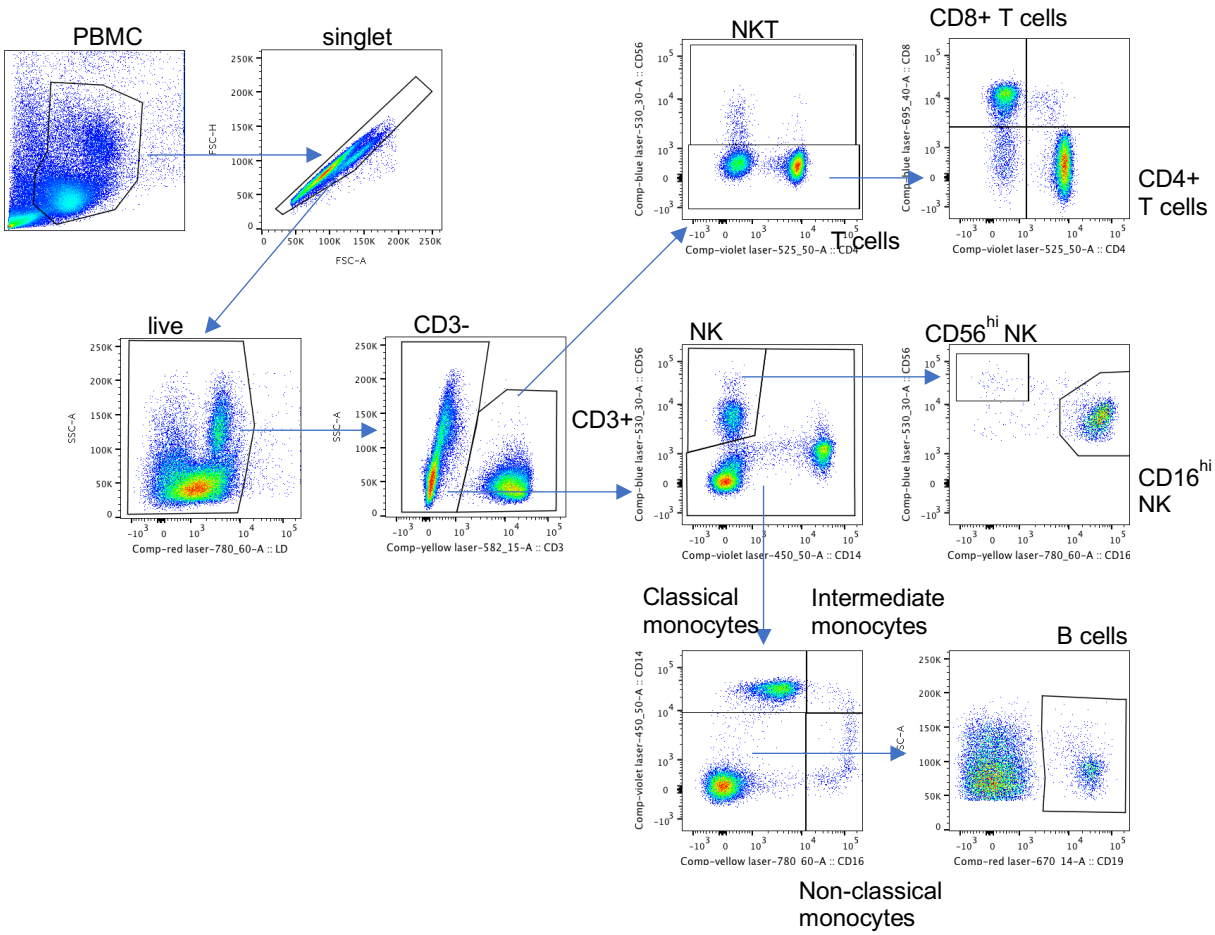


Figure 2-1 *Peripheral blood mononuclear cell flow cytometry gating strategy.*

Plots show recorded flow cytometry events, where each dot represents one recorded event (interpreted as one cell), and colour represents density of events. Arrows and black boxes show selection and sequential hierarchical gating (filtering) of events, using forward and side scatter (FSC and SSC) and the intensity of fluorophore-conjugated antibody staining (x and y axes as labelled) to select groups of cells corresponding to known immune cell subsets, e.g., CD4+ T cells are here defined as live-dead (LD) staining negative, $CD3^{+}CD56^{-}CD4^{+}CD8^{-}$.

Calculation of absolute cell counts

To derive absolute cell counts for the 14 cell types used in this analysis, flow cytometry counts (gated as percentages relative to larger cell subsets) were multiplied by the relevant absolute cell counts from the haematology counts (see **Figure 2-2** for a schematic of the processing pipeline). More specifically:

(1) Gated flow cytometry data were imported to [R] using [R] package flowWorkspace (Finak & Jiang 2018). The data were used to generate percentage features as outlined in the "multiplier" column of **Table 2-1**. These "multiplier" counts are proportions which are multiplied with the absolute cell counts from the hematology panel in order to generate absolute cell counts of different cell subtypes. For example, a participant's absolute CD4+ T cell count = proportion of lymphocytes which are CD4+ T cells (the multiplier, derived from flow cytometry) * the absolute lymphocyte count (from the hematology panel).

(2) Missing flow cytometry multiplier counts and hematology cell counts were imputed by multiple imputation under a multivariate normal model using [R] package mix (Ripley 2017) via the function ImputeData from [R] package mclust (Scrucca et al 2016). In total, only 0.33% of counts in this analysis required imputation.

(3) Percentage multipliers and absolute counts were tested for batch effects of clinical center (5 centers) using a Kruskal-Wallis (KW) test for each cell count. All the percentage multipliers showed marked batch effects related to clinical center, consistent with the fact that flow cytometry was performed locally at each center. For the hematology panel (local venepuncture but assayed centrally), only the absolute lymphocyte count ($P = 0.005$) and monocyte count ($P = 0.08$) showed evidence of a center-related batch effect.

(4) For those features showing significant center effects at $P < 0.1$ by KW test, the features were Box-Cox transformed to normalize the counts using the [R] caret package (Kuhn 2008) preProcess function; debatched for the effect of clinical center using [R] limma package (Ritchie et al 2015) removeBatchEffect function; then inverse Box-Cox transformed to return the data to their original distribution. To avoid the introduction of bias which may occur with unbalanced groups, no design matrix was included in the removeBatchEffect function.

(5) The debatched multipliers (from flow data) were multiplied by the debatched absolute cell counts (from the hematology panel) as per **Table 2-1** to generate the 14 absolute

cell counts used in this analysis: neutrophils, eosinophils, basophils, red blood cells, platelets, $CD4^+$ T cells, $CD8^+$ T cells, classical monocytes, intermediate monocytes, non-classical monocytes, $CD16^{hi}$ NK cells, $CD56^{hi}$ NK cells, NKT cells and B cells.

These 14 endpoints were selected as the 14 cell subsets which could reliably be discriminated based on the flow cytometry panel and haematology data acquired.

Table 2-1 *Derivation of fine-grained absolute cell counts from coarse absolute count data and flow cytometry data.*

Counts for each cell type were obtained by multiplying the value of the "multiplier" column with the "input count". Where the "multiplier" is non-unity, this is a percentage derived from flow data. The "input count" data are taken from the haematology panel.

Final cell count	Multiplier (Percentage feature from flow data)	Input count
Neutrophils	1	Absolute neutrophil count
Eosinophils	1	Absolute eosinophil count
Basophils	1	Absolute basophil count
Red blood cells	1	Absolute red blood cell count
Platelets	1	Absolute platelet count
CD4+ T cells	CD4+ T cells / (CD3+ cells + NK cells + B cells)	Absolute lymphocyte count
CD8+ T cells	CD8+ T cells / (CD3+ cells + NK cells + B cells)	Absolute lymphocyte count
Classical monocytes	classical monocytes / (classical monocytes + intermediate monocytes + non-classical monocytes)	Absolute monocyte count
Intermediate monocytes	intermediate monocytes / (classical monocytes + intermediate monocytes + non-classical monocytes)	Absolute monocyte count
Non-classical monocytes	non-classical monocytes / (classical monocytes + intermediate monocytes + non-classical monocytes)	Absolute monocyte count
CD16(hi) NK cells	CD16(hi) NK cells / (CD3+ cells + NK cells + B cells)	Absolute lymphocyte count
CD56(hi) NK cells	CD56(hi) NK cells / (CD3+ cells + NK cells + B cells)	Absolute lymphocyte count
NKT cells	NKT cells / (CD3+ cells + NK cells + B cells)	Absolute lymphocyte count
B cells	B cells / (CD3+ cells + NK cells + B cells)	Absolute lymphocyte count

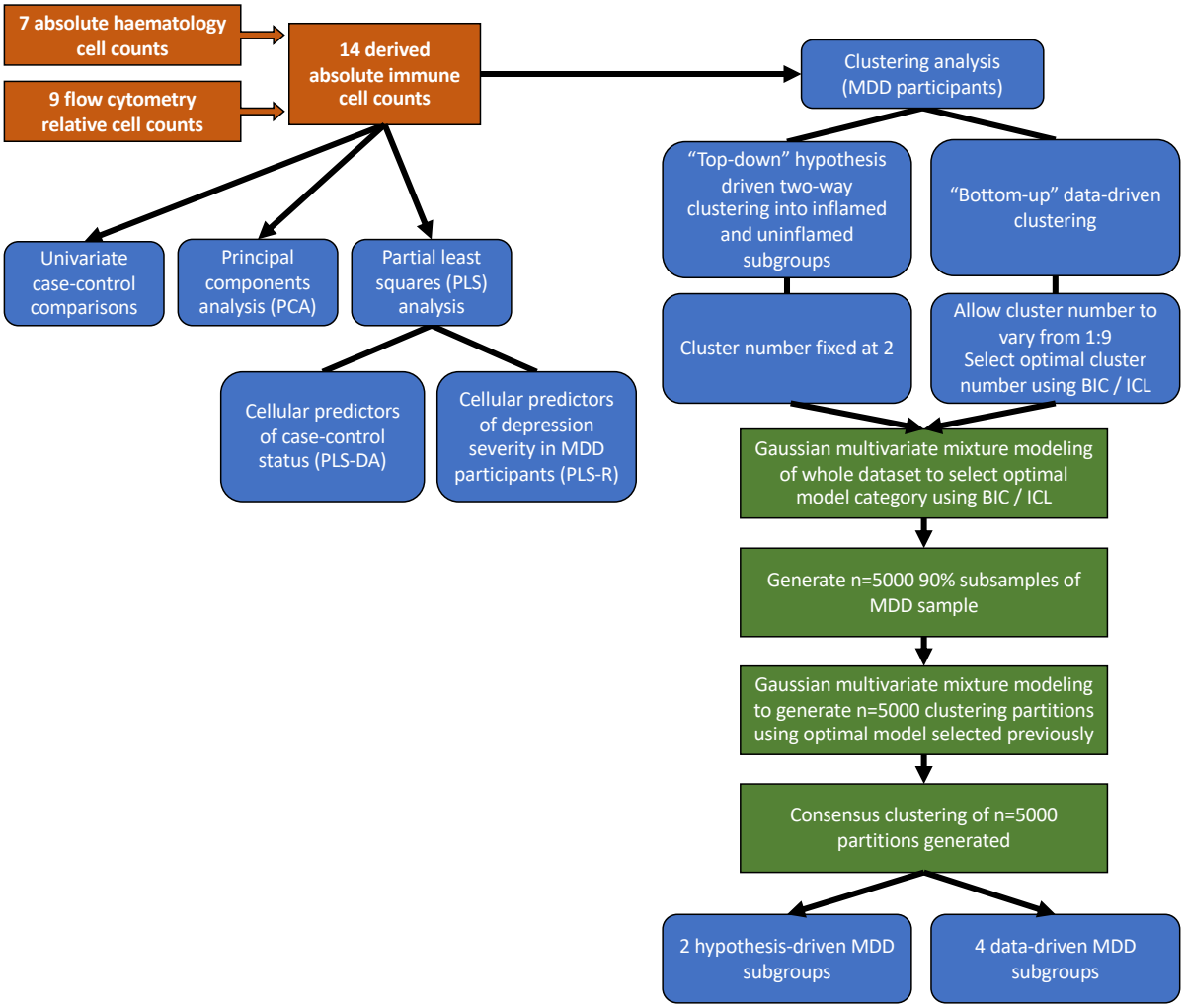


Figure 2-2 *Schematic of analysis pipeline for cell count data.*

Schematic shows the univariate and multivariate analysis techniques used to analyse the cell count data. See methods for more details. BIC, Bayesian Information Criterion; ICL, Integrated Complete Likelihood.

Statistical analysis

All analyses were performed in [R] version 3.5.1 (R Core Team, 2018). 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 χ^2 tests with FDR-corrected χ^2 tests for post-hoc pairwise comparisons.

Principal components and partial least squares analysis

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, uninformed by clinical phenotype. PCA was performed using [R] function prcomp with zero-centering and unit-variance scaling of data prior to singular value decomposition.

To identify the variability in the data which most relates to the clinical phenotype, we performed a partial least squares analysis. Partial least squares (PLS) is a statistical technique which finds the linear combinations of 'predictors' (here, the 14 cell counts) which most relate to variability in the 'response' variable (here, case vs. control status or a matrix of symptom scores). Where a component is found significantly to predict MDD status or its severity, the 'weights' in the PLS model indicate which of the different cell types most contribute to this ability of the model to predict the response, allowing interpretation of the model. 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. PLS was performed using the NIPALS algorithm via [R] package ropls (Thevenot et al 2015). For PLS-R, we used the default mean-centering and unit-scaling of predictor and response variables. For PLS-DA, we used weighted centering of predictors prior to PLS in order to avoid biases due to unequal group sizes (Brereton & Lloyd 2014). The number of significant PLS components was determined using the default ropls method. A component is only added to the PLS model if the P2Y metric (proportion of response explained) is > 1% and Q2Y > 0, i.e., if the predicted residual sum of squares of the model including the new component (estimated by 7-fold cross-validation) is less than the residual sum of squares of the model with the previous components only.

The cumulative Q2Y reflects the predictive ability of the PLS model. For both PLS-DA and PLS-R, the p-value for the predictive ability of the model (pQ2) was estimated by permuting group labels or clinical score sets (5000 permutations): pQ2 is the proportion of permuted cumulative Q2Y values above the original cumulative Q2Y for the model. We used the following method to test whether particular cell counts contributed significantly to the PLS model (i.e., testing the

significance of predictor weights): (1) use [R] package boot to resample the participant data and calculate the PLS model (and associated weights) using the resampled data (1000 resamples). (2) Generate a z-score for the cell count weight by dividing the cell count weight obtained in the original PLS model by the standard error of the bootstrapped PLS weights. (3) Significant weights are defined as those with an absolute z-score >3 as previously described (Fernandez-Egea et al 2016). PLS plots show only those cell count weights found to be significant by this metric. This method is more conservative than using VIP (Variable Importance in Projection) >1 to select significant weights. All cell counts designated significant in this analysis had VIP>1.

Clustering analysis

We used Gaussian finite multivariate mixture modelling, and consensus clustering, to identify subgroups of cases that shared an immune cell profile in common with each other and in contrast to the immune cells profile of cases in other subgroups (Hornik 2005, Scrucca et al 2016). See **Figure 2-2** for a schematic of the clustering analysis pipeline. We used Gaussian finite multivariate mixture modelling via [R] package mclust (Scrucca et al 2016) to detect clusters present in the data by using an initial hierarchical agglomeration clustering based on scaled, centred single value decomposition of the 14 cell counts, following by an expectation maximization algorithm to detect the components (clusters) present in the data. The optimal model type and the optimal number of clusters was determined using the Bayesian Information Criterion (BIC). For data-driven clustering, the number of components (clusters) was allowed to vary from 1 to 9 at every iteration of the mixture model and the best model was selected by BIC. To generate consensus partitions of participants into subgroups, we first performed Gaussian mixture modelling on the total dataset to select the category of models to be considered (e.g., VVI, EEI), then performed mixture modelling on 5000 randomly selected 90% subsets of the data (selection without replacement) using that model type to generate 5000 data partitions. To detect clusters (subgroups) which are more stable and not sensitive to particular data points, we performed consensus clustering of 5000 randomly selected 90% subsets of the data using [R] package clue (Hornik 2005). For each clustering partition generated, we used clue::cl_predict function to predict the cluster membership for the left-out 10% of the data. Finally, we used the clue::cl_ensemble function to find the consensus clustering which minimized the Euclidean dissimilarity of the 5000 clustering partitions generated. We also used an alternative goodness of fit metric - the Integrated Completed

Likelihood (ICL) - to test for the optimal model type and component number (Biernacki et al 2000). For our data, this selected the same model types and cluster numbers as the BIC metric.

Statistical comparison of subgroups

Following clustering, we then compared the clinical and demographic correlates of the four subgroups (immunophenotypes) detected. For continuous variables (e.g., cell counts, age, BMI, symptoms scores), we used a Kruskal-Wallis test for the overall effect of subgroup on the variable. For variables where the Kruskal-Wallis p-value was <0.05, we used [R] package PMCMRplus to perform post-hoc Conover non-parametric tests for significant differences between every pair of subgroups, with Benjamini-Hochberg FDR adjustment for multiple comparisons across all the subgroup pairs. The p-values shown on graphs comparing features subgroup-by-subgroup represent corrected p-values, and non-significant comparisons are not shown. For categorical variables (e.g., tobacco use, current antidepressant use), we tested for the overall effect of subgroup on the variable using a χ^2 test with simulated p-values via [R] function chisq.test. Where $P < 0.05$ for the overall χ^2 test, we performed post-hoc testing of which pairs of populations differ. A χ^2 test with simulated p-values was performed for each pair of subgroups, and the raw p-values were adjusted across all pairwise comparisons using Benjamini-Hochberg FDR correction. Only pairs with adjusted p-values <0.05 were deemed significant. Where there were only two subgroups, statistical tests performed to compare subgroups were simple χ^2 tests with simulated p-values (for categorical variables) or Mann Whitney U tests (for continuous variables).

Sensitivity analysis: generation of cell count residuals

To test whether there was evidence for depression-associated immune subgroups independent of some potentially confounding factors, we used linear regression to mitigate the effects of age, sex, BMI, recent infection history and tobacco use before analysis of residualised counts of the 14 immune cell subsets. In order to generate 'residual cell counts', absolute cell counts were Box-Cox transformed using [R] caret package preProcess as previously. The [R] MASS package function stepAIC was then used to perform model selection with default parameters. The initial model used for each cell count *c* was as follows, with recent infection and current tobacco use defined as binary yes-no variables:

$$c = \beta_0 + \beta_1 sex + \beta_2 BMI + \beta_3 age + \beta_4 recent\ infection + \beta_5 current\ tobacco\ use + \varepsilon \quad [Eq. 2.1]$$

For each of the 14 cell counts, the residuals of the chosen linear model were designated the ‘residual cell counts’ and used directly for further analyses (PCA, PLS and clustering). To allow stepwise model comparison and residual calculation, missing values for BMI (8 data points), tobacco use (4 data points) and the presence of recent infection (6 data points) were imputed at their median or modal value. These imputed values were only used to generate residual cell counts, and not used in statistical comparisons between cases/controls or between subgroups.

2.4 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 2-2**). 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.

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 2-3A**). 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.

Exclusion of the 41 participants with 'minor inflammatory disease' did not substantially alter the data structure: it remained true that the only cell type increased in MDD following FDR correction was the CD4+ T cell count (unadjusted $P = 0.002$), with trends towards increased neutrophils (unadjusted $P = 0.04$) and intermediate monocytes in MDD (unadjusted $P = 0.02$).

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 2-3B**). 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 (ρ = +0.25, FDR P = 0.0002).

Table 2-2 *Demographic and clinical characteristics of the study population.*

*P-values for comparison of control vs. MDD by Mann-Whitney (MWU, continuous variables) or χ^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).*

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**	χ^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

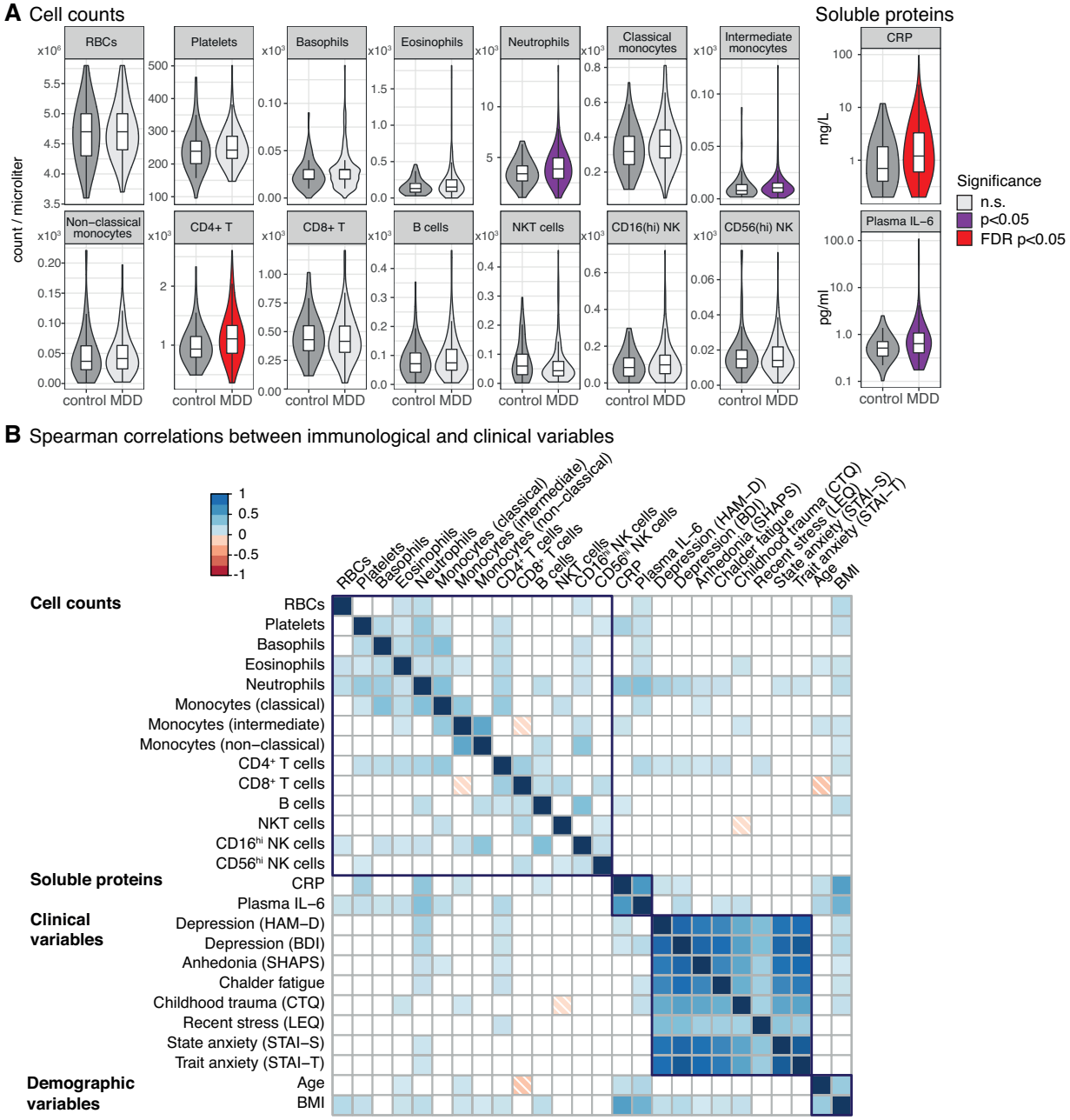


Figure 2-3 *Peripheral immunophenotypes in MDD and control participants.*

(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. Colour 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 2-3*** *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. Colour indicates the correlation coefficient (Spearman's ρ); dark blue outlines group together similar variables.*

Table 2-3 *Univariate comparison of immunological features in patients with MDD compared to controls. P-values for comparison of control (N=77) vs. MDD (N=206) by Mann-Whitney U (MWU) testing. * = FDR adjusted P<0.05 following correction for 16 multiple comparisons by Benjamini-Hochberg control of false discovery rate. MDD, Major Depressive Disorder; IQR, Interquartile Range; IL-6, interleukin-6. NB. Platelets are included as a 'cell count', although not strictly a cell type.*

Humoral factors	**Control (median, IQR)**	**MDD (median, IQR)**	**P value (MWU)**	**Effect size Z / √ n**
High sensitivity CRP (mg/L)	0.7 (0.4, 1.8)	1.2 (0.6, 3.3)	*0.003	0.18
Plasma IL-6 (pg/ml)	0.512 (0.346, 0.704)	0.635 (0.406, 1.071)	0.04	0.14
Absolute cell counts				
Red blood cells ($x10^6$ /µL)	4.7 (4.3, 5.0)	4.7 (4.4, 5.0)	0.7	0.03
Platelets (/µL)	239 (201, 270)	242 (217, 284)	0.7	0.08
Basophils ($x10^3$/µL)	0.020 (0.020, 0.030)	0.030 (0.020, 0.030)	0.2	0.08
Eosinophils ($x10^3$/µL)	0.130 (0.080, 0.200)	0.150 (0.090, 0.250)	0.09	0.10
Neutrophils ($x10^3$/µL)	3.400 (2.690, 4.180)	3.875 (2.935, 4.957)	0.01	0.15
Monocytes, classical ($x10^3$/µL)	0.318 (0.243, 0.405)	0.348 (0.281, 0.442)	0.1	0.10
Monocytes, intermediate ($x10^3$/µL)	0.008 (0.004, 0.014)	0.010 (0.006, 0.015)	0.02	0.14
Monocytes, non-classical ($x10^3$/µL)	0.037 (0.023, 0.063)	0.041 (0.024, 0.063)	0.6	0.03
CD4+ T cells ($x10^3$/µL)	0.934 (0.797, 1.146)	1.108 (0.862, 1.333)	*0.003	0.18
CD8+ T cells ($x10^3$/µL)	0.430 (0.334, 0.551)	0.415 (0.320, 0.548)	0.5	0.04
B cells ($x10^3$/µL)	0.071 (0.042, 0.109)	0.074 (0.049, 0.121)	0.3	0.07
NKT cells ($x10^3$/µL)	0.060 (0.030, 0.100)	0.043 (0.026, 0.075)	0.08	0.11
$CD16^{hi}$ NK cells ($x10^3$/µL)	0.084 (0.037, 0.136)	0.099 (0.051, 0.151)	0.2	0.08
$CD56^{hi}$ NK cells ($x10^3$/µL)	0.015 (0.010, 0.020)	0.014 (0.010, 0.022)	0.9	0.01

Principal component analysis was used 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 2-4A**). 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 2-5A**). Similar results were obtained when PCA was repeated with cases with minor inflammatory conditions excluded (**Figure 2-5B**), and for sex-specific subgroups of cases (**Figure 2-5C,D**).

PC1 scores were positively correlated with serum CRP (ρ = 0.26, FDR *P* = 0.00004), and IL-6 (ρ = 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 2-4B**) and PC1 scores were positively correlated with multiple measures of symptom severity including observer-rated depressive symptoms (HAM-D, ρ = 0.26, FDR *P* = 0.00004), self-reported depressive symptoms (BDI, ρ = 0.24, FDR *P* = 0.0002) and anhedonia (SHAPS, ρ = 0.23, FDR *P* = 0.0004), as well as BMI (ρ = 0.24, FDR *P* = 0.00004)(**Figure 2-4B**). A scatterplot of each participant's scores on both PCs (**Figure 2-4A**) 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. When participants with minor inflammatory conditions were excluded, PC1 remained associated with MDD diagnosis (*P* = 0.02) and HAM-D symptom severity (Spearman ρ = 0.23, FDR *P* = 0.002).

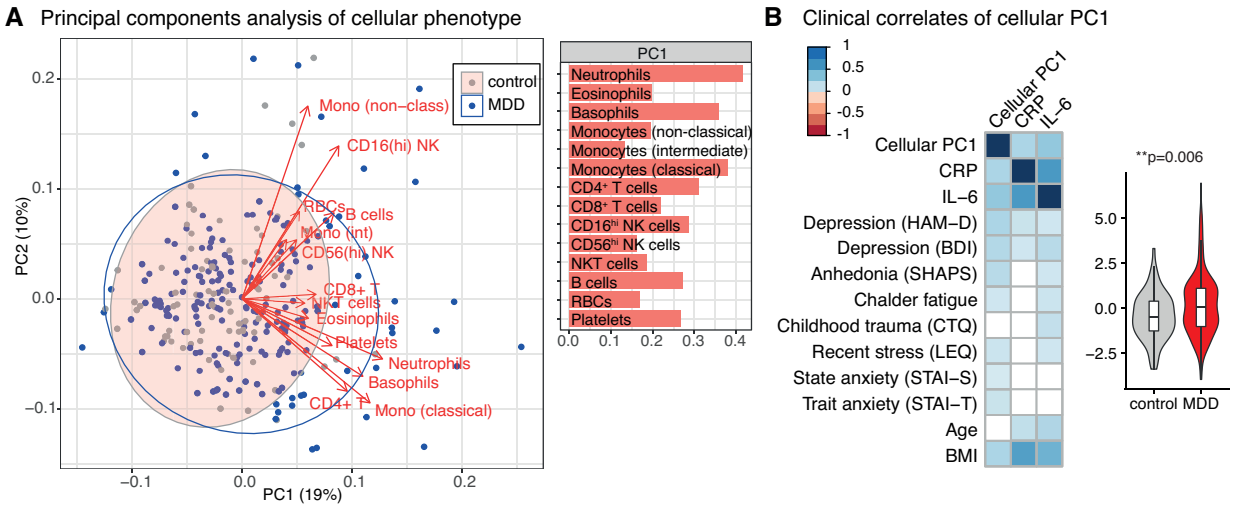


Figure 2-4 *Principal components analysis of cellular immunophenotypes.*

(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. (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. Colour indicates the correlation coefficient (Spearman's ρ). 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.

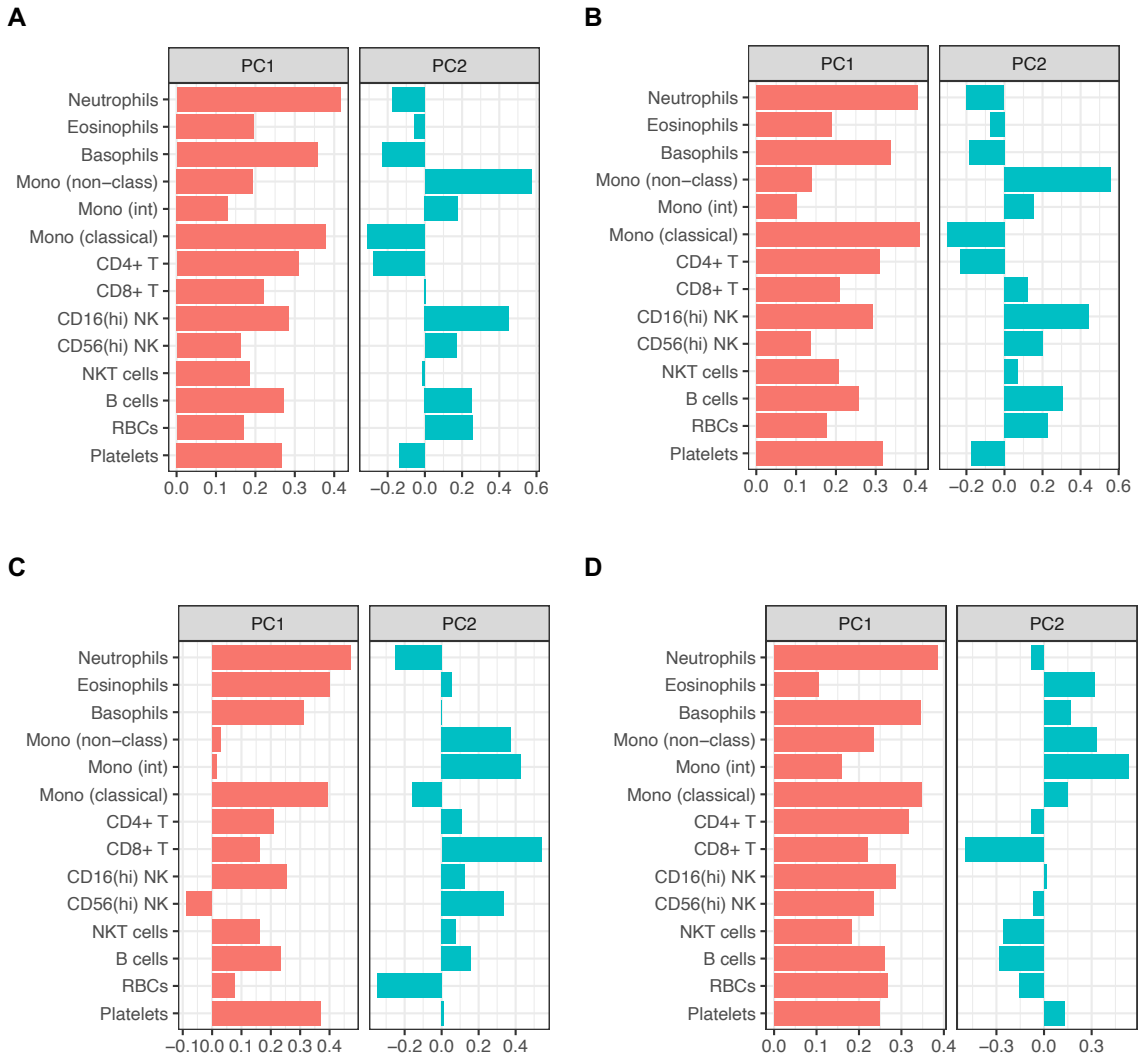


Figure 2-5 *Sensitivity of principal components analysis (PCA) to exclusion of minor inflammatory conditions and to sex.*

(A) Absolute counts: cellular PCA for all participants (N=283), corresponding to PCA shown in ***Figure 2-4A*** *(B) PCA where participants with minor inflammatory conditions have been excluded (N=242) (C) PCA for male participants only (N=88) (D) PCA for female participants only (N=195).*

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

We used partial least squares (PLS) discriminant analysis (**Figure 2-6A**) 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 next 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 2-6B**).

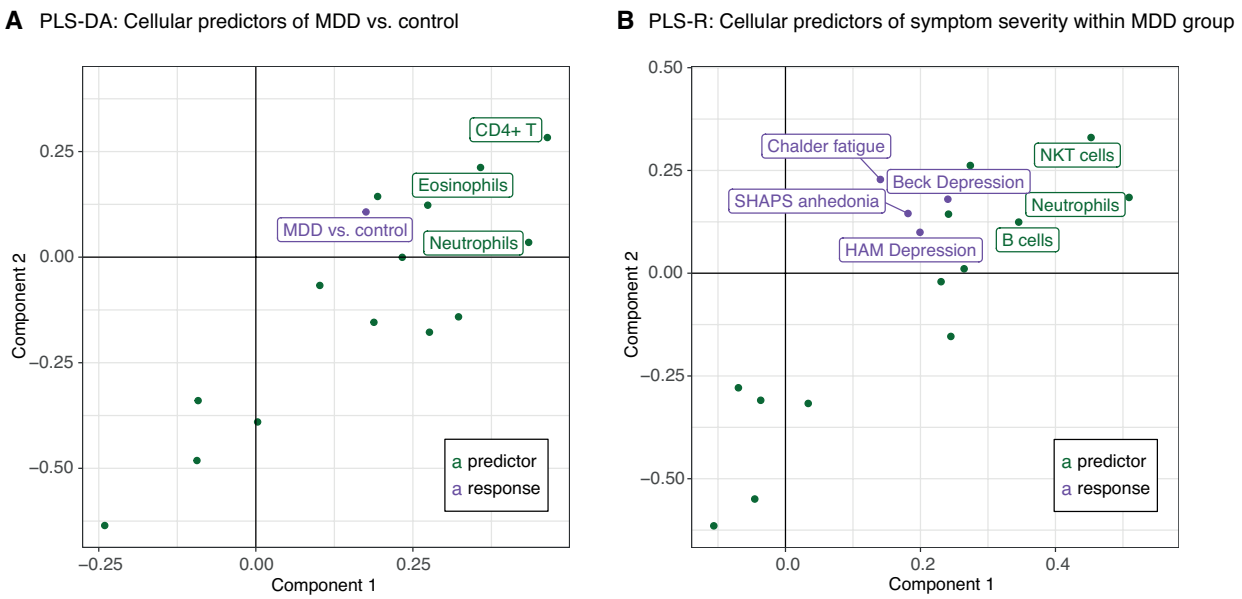


Figure 2-6 *Cellular predictors of MDD status and symptoms severity.*

(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 2-3A*** *(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****). (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.*

"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 modelling 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 2-7A,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 2-7C, Table 2-4**), 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 (**Figure 2-7D, Table 2-4**). 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 centre, current antidepressant use, alcohol or cannabis use, reported recent infection or minor inflammatory disease, or BMI (**Figure 2-8A, Table 2-4**).

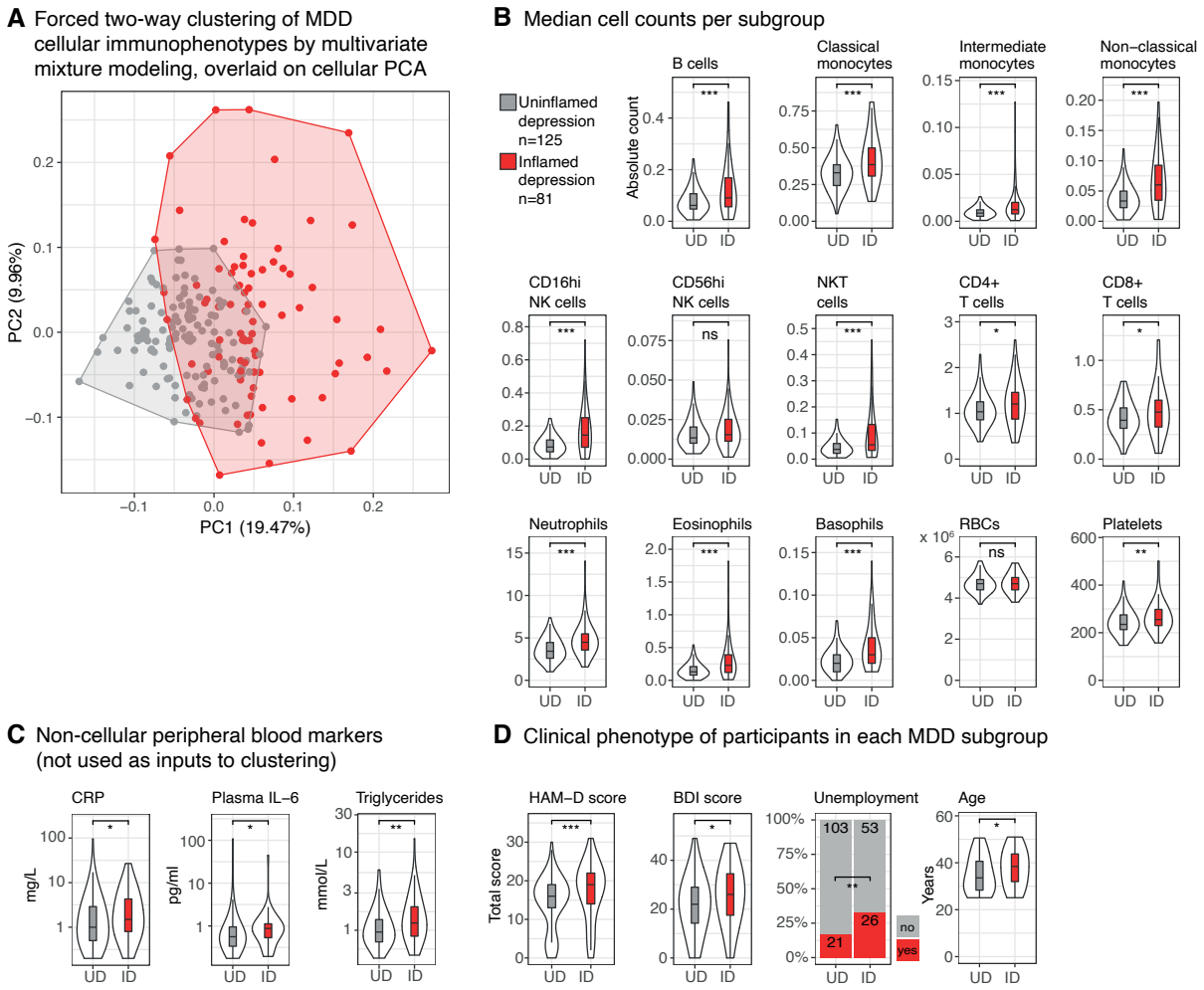


Figure 2-7 *Theoretically driven ("top-down") immune cell stratification into inflamed and uninflamed MDD subgroups.*

*(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 PC, with cluster membership indicated by colour. (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) χ^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.*

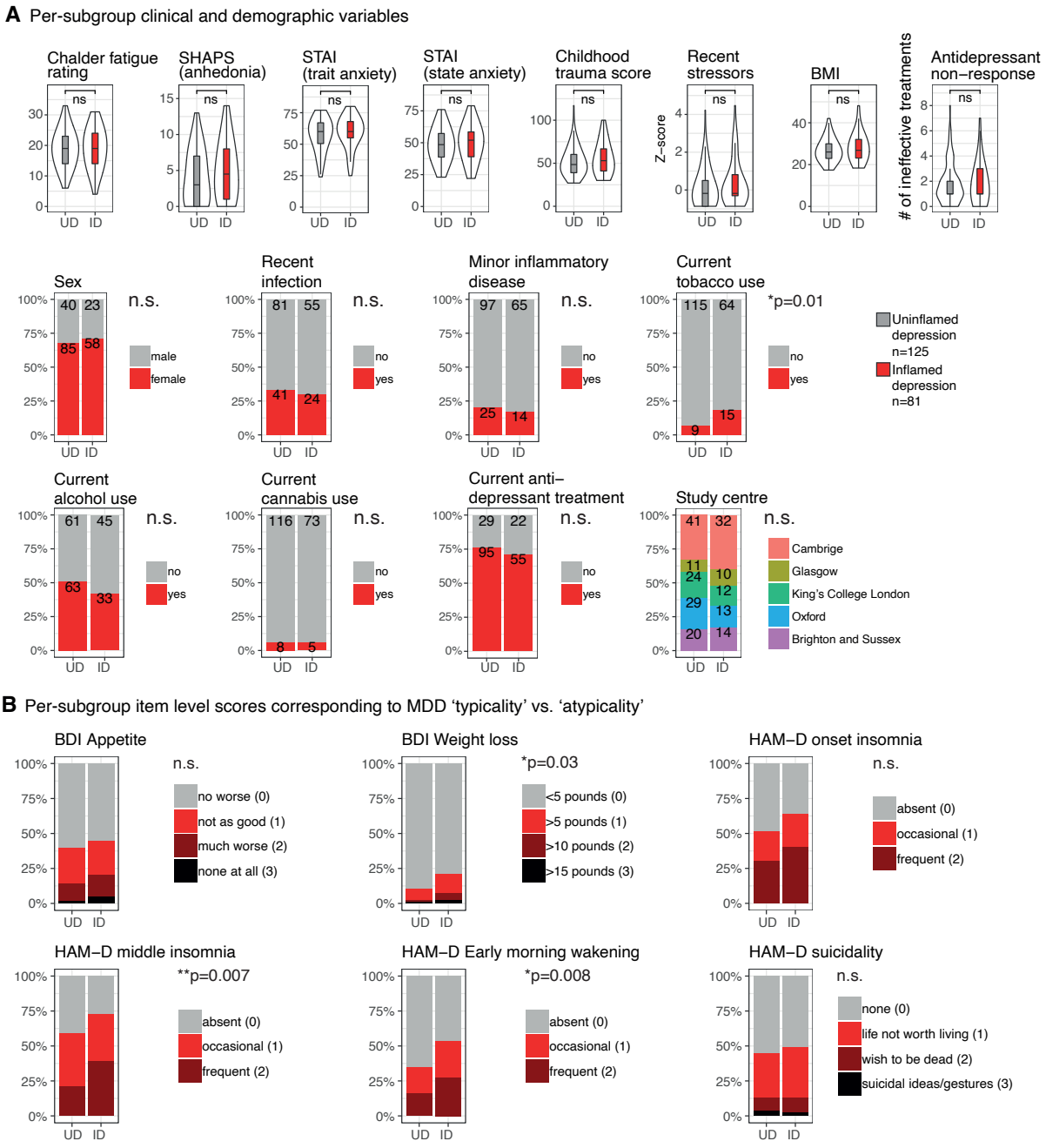


Figure 2-8 *Inflamed and uninflamed MDD subgroups: further clinical and demographic differences. (A) Results from forced two-way Gaussian finite mixture modelling of the cellular phenotypes for MDD participants as in* ***Figure 2-7****. Boxplots show median and interquartile range for each subgroup, with the outer violin shape showing the full distribution of data. Effects of subgroup were tested by Mann-Whitney U, *P<0.05, **P<0.01, ***P<0.01. Bar charts show demographic and clinical variables for each subgroup, indicating the percentage of participants in each subgroup; bar annotations indicate numbers of participants. The effects of subgroup were tested by* χ^2*. N.s. indicates that the effect of subgroup is not significant. SHAPS = Snaith-Hamilton Pleasure Scale, STAI = State-Trait Anxiety Inventory. (B) Participant item-level responses for questions corresponding to MDD 'typicality'. Bar charts indicate the percentage of participants in each subgroup giving each response. Effects of subgroup were tested by Mann-Whitney U, taking the question responses as an ordinal variable. N.s. indicates not significant.*

Table 2-4 *Clinical, demographic and serological features of the binary ("top-down") clustering of MDD immunophenotypes.*

*MDD, major depressive disorder; BMI, body mass index; IL-6, interleukin-6; BDI, Beck Depression Inventory; HAM-D, Hamilton Depression Scale; SHAPS, Snaith-Hamilton Pleasure Scale; STAI, State-Trait Anxiety Inventory. P-values shown are for Kruskal-Wallis (KW) or χ^2 tests of a significant effect of subgroup on the variable tested: *P<0.05, **P<0.01. The associated plots are shown in* ***Figure 2-7*** *and* ***Figure 2-8****. Data from N=206 participants.*

	Uninflamed depression (grey)	**Inflamed Depression (red)**	**P-value**
N per subgroup (all MDD)	125 (61%)	81 (39%)	
Cell counts	All cell counts low	Increases in all granulocytes, all monocyte subsets, T cells, B cells and $CD16^{hi}$ NK cells	See **Figure 2-7B**
Percentage			**P-value (χ^2)**
Female	68%	72%	0.7
Current antidepressant use	77%	71%	0.5
Current tobacco use	7%	19%	*0.01
Current alcohol use	51%	42%	0.3
Current cannabis use	6%	6%	1
Unemployed	17%	33%	**0.008
Recent infection	34%	30%	0.7
Minor inflammatory disease	20%	17%	0.7
Study center	-	-	0.6
Median			**P-value (KW)**
HAM-D	16	19	**0.0002
BDI	22	26	*0.01
Chalder fatigue	19	19	0.7
SHAPS (anhedonia)	3	4.5	0.1
STAI – state anxiety	48.5	52	0.4
STAI – trait anxiety	60	60	0.2
Childhood Trauma Questionnaire	48.5	53	0.2
Life Events Questionnaire z-score	-0.18	-0.18	0.1
Number of ineffective antidepressants	1	1	0.9
Age	33.6	38.4	*0.01
CRP	1	1.5	*0.03
Plasma IL-6	0.56	0.88	*0.02
BMI	26.0	26.8	0.2
Triglycerides	0.95	1.24	**0.002

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 as follows:

(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 (

(iii) **Figure 2-9**).

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 2-9A). 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 2-9B,D).

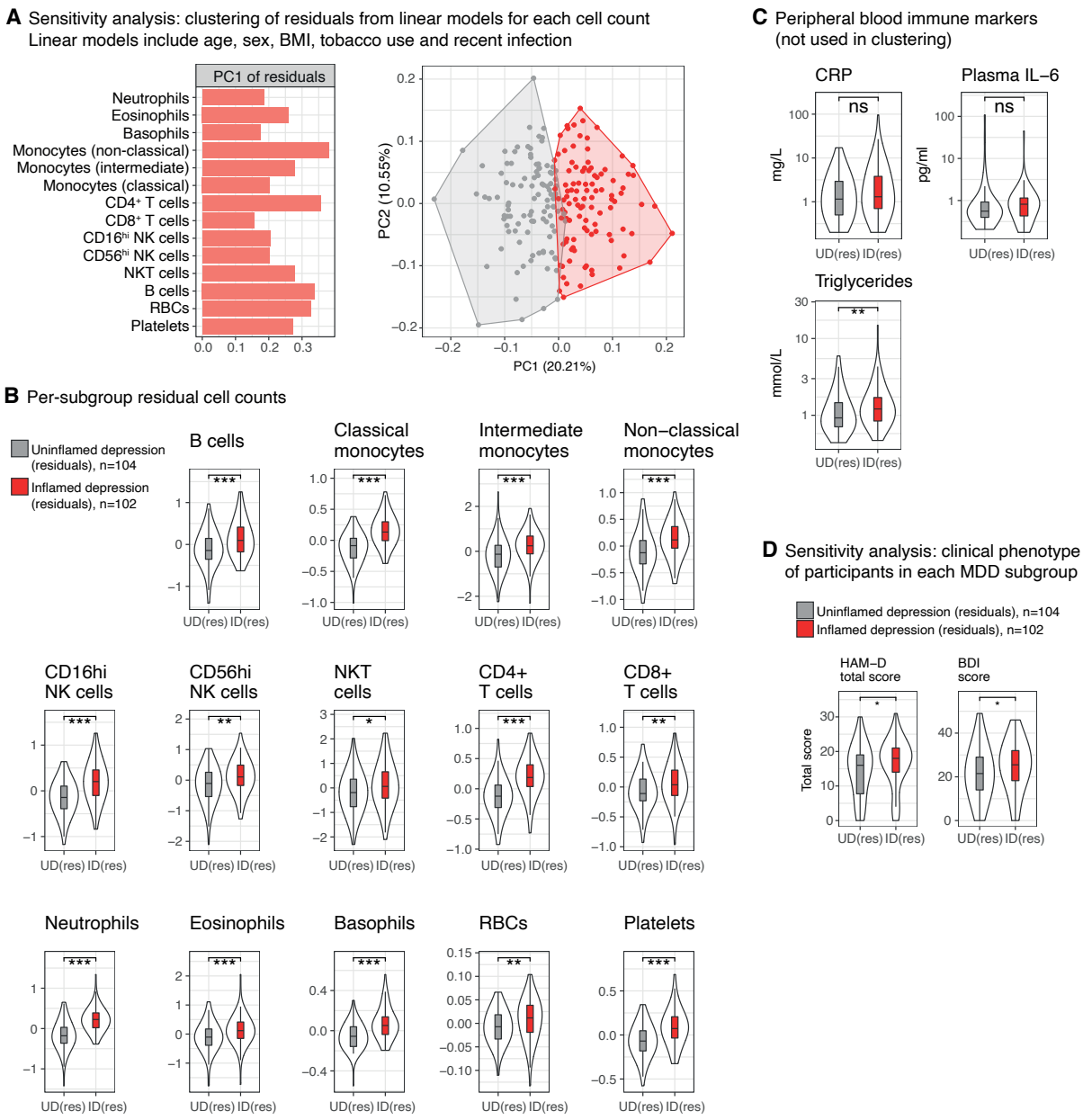


Figure 2-9 *Inflamed and uninflamed MDD subgroups: sensitivity analysis using adjusted ('residual') cell counts.*

(A) 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); see ***Equation 2.1****. 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. (B) Boxplots show the median and inter-quartile range of residual cell counts for participants in each subgroup, with the violin outline showing the full distribution of data. (C) Peripheral blood markers in each MDD subgroup generated from residual cell counts: C-reactive protein*

*(CRP), interleukin-6 (IL-6) and triglycerides. Boxplots show median and interquartile range, with the outer violin shape showing the full distribution of data. (D) Depression rating scores for inflamed and uninflamed cases identified by binarization of residual immune cell counts. HAM-D = Hamilton Depression Rating Scale (practitioner-administered), BDI = Beck Depression Inventory (self-report). For (B), (C) and (D), subgroups were compared by Mann-Whitney U testing: n.s. not significant, *P<0.05, **P<0.01, ***P<0.001. Data from N=206 participants.*

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

Next, we used Gaussian finite multivariate mixture modelling and consensus clustering, but without prior constraint on the number of distributions in the mixture. This identified 4 subgroups of cases (**Figure 2-10, Figure 2-11,**

Table 2-5), each characterized by a distinct profile of absolute immune cell counts (**Figure 2-10B, Figure 2-11A**). One subgroup comprised 58 cases (28%) with low counts for all cells and low CRP and IL-6 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 (**Figure 2-10C,D**), but they 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. 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.

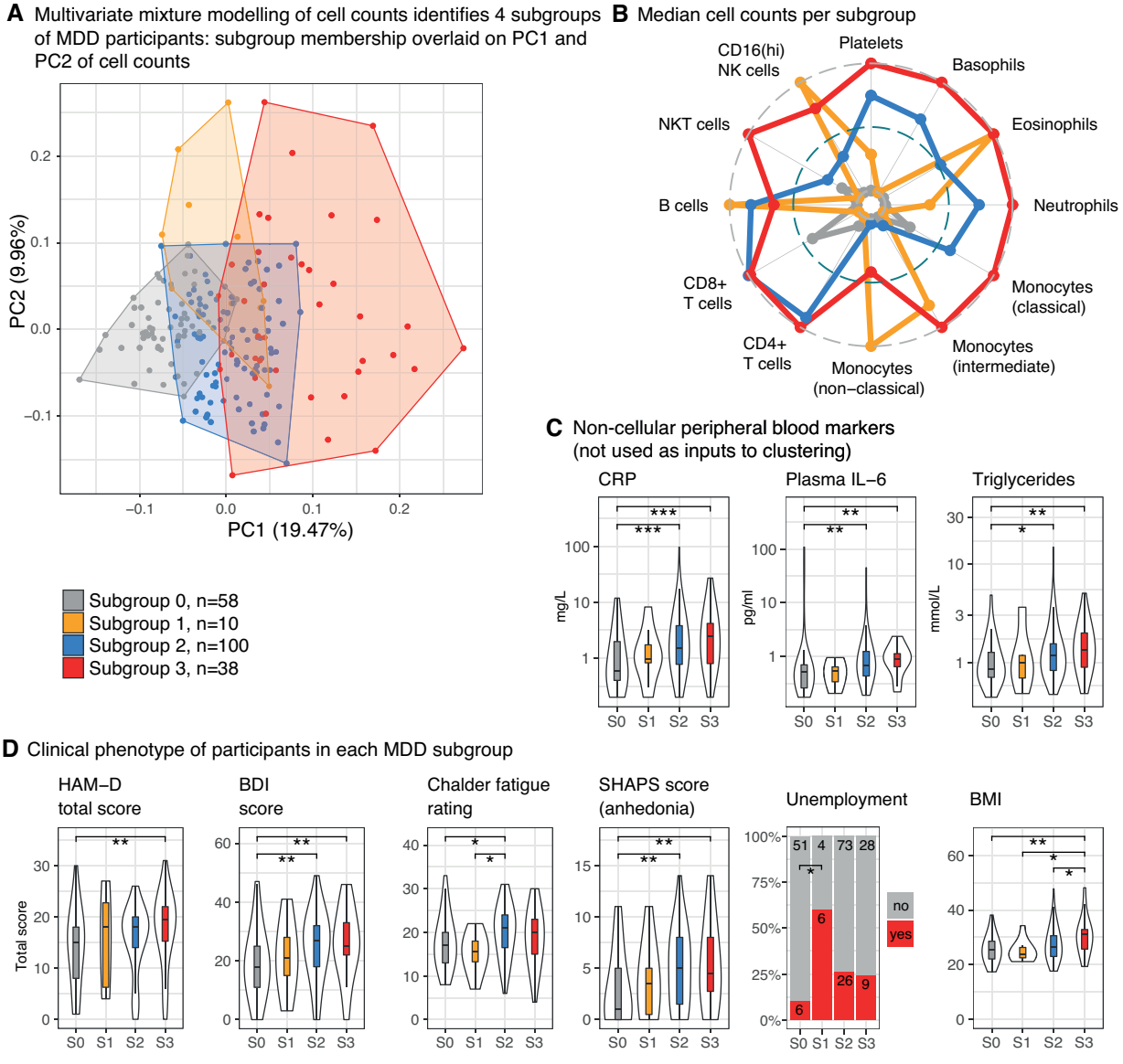


Figure 2-10 *Data-driven ("bottom-up") immune cell stratification of MDD patients identifies subgroups with differing symptom severity.*

(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 colour. The arbitrary cluster numbers and colours are used consistently throughout this figure to designate each cluster (subgroup 0, grey, n=58 cases; subgroup 1, orange, n=10; subgroup 2, blue, n=100; subgroup 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. (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 subgroup 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 subgroups differ for that feature – for these variables, each subgroup was compared to every other subgroup. For unemployment, the bar chart indicates the percentage of participants in each subgroup and bar annotations indicate participant numbers. Subgroups were compared by χ^2 testing, with post-hoc χ^2 tests to compare pairs of subgroups. All p-values shown are corrected for the 6 pairwise subgroup-subgroup comparisons performed: FDR P *<0.05, **<0.01, and ***<0.001. Pairwise comparisons which were non-significant following FDR correction are shown in* ***Figure 2-11****. HAM-D = Hamilton Depression Rating Scale (practitioner-administered), BDI = Beck Depression Inventory (self-report), SHAPS = Snaith-Hamilton Pleasure Scale.*

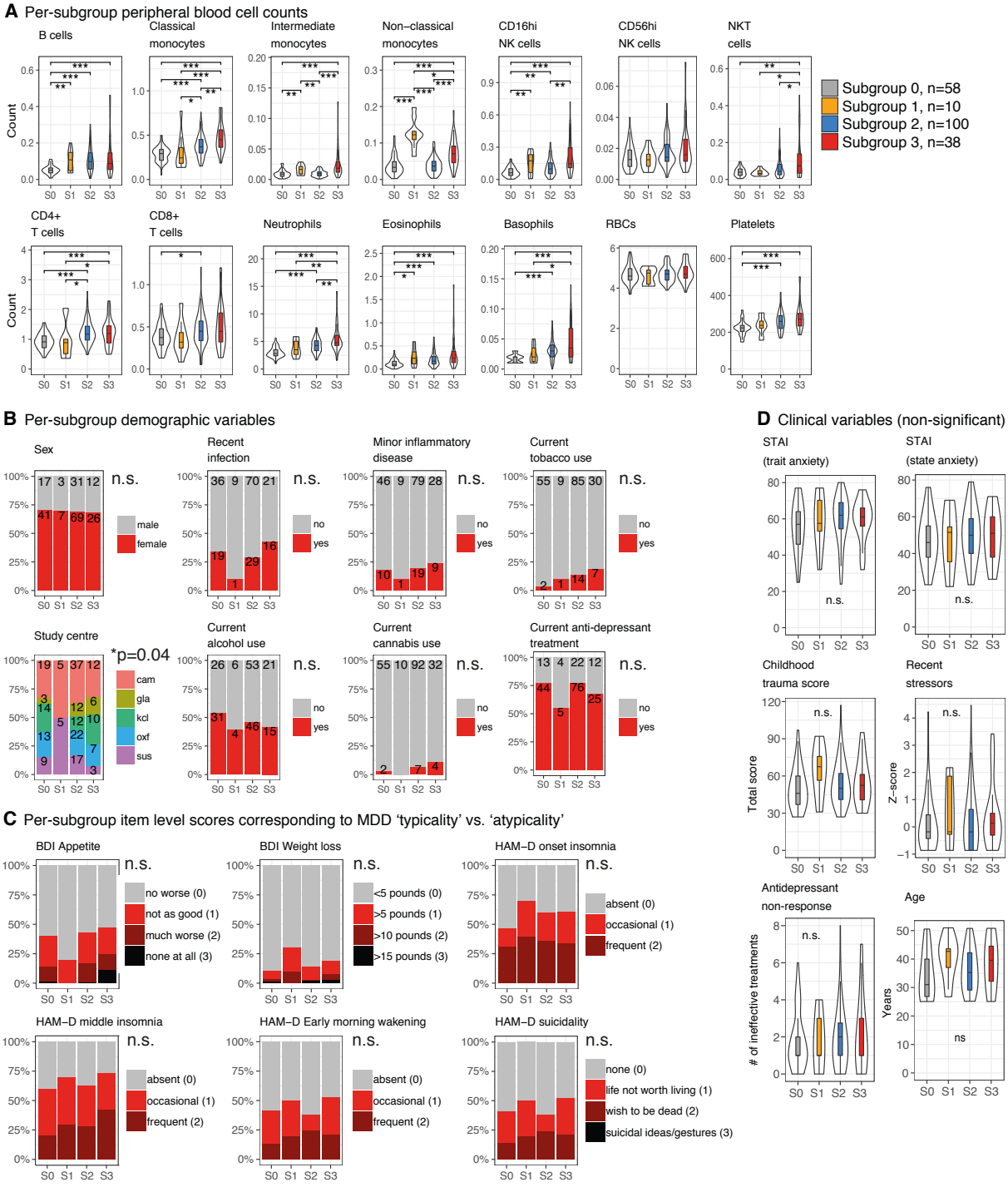


Figure 2-11 *Data-driven ("bottom-up") immune-cell stratified MDD subgroups: further comparison of immunological, clinical and demographic features.*

(A) Gaussian finite mixture modelling of the cellular phenotypes for MDD participants (N=206) identified four discrete subgroups (immunophenotypes) as shown in **Figure 2-10**. The arbitrary subgroup numbers and colours are used consistently throughout this figure and **Figure 2-10** to designate each subgroup (subgroup 0, grey, N=58 participants; subgroup 1, orange, N=10 participants; subgroup 2, blue, N=100 participants; subgroup 3, red, N=38 participants). Boxplots show the median and inter-quartile range of absolute cell counts in each subgroup, with the violin outline showing the full distribution of data. The effect of subgroup on each feature is tested by Kruskal-Wallis testing. n.s. indicates the overall effect of subgroup was not significant at P<0.05. Where P<0.05 for the overall Kruskal-Wallis test, we performed post-hoc Conover tests

for all possible subgroup pairs. P-values were corrected for the 6 pairwise subgroup comparisons performed (false discovery rate). Stars indicate adjusted p-values of *<0.05, **<0.01, and ***<0.001. Pairwise comparisons which were non-significant following correction are not shown. (B) Demographic features of each subgroup. Bar charts indicate the percentage of participants in each subgroup. Annotations on the bars indicate the number of participants in each subgroup. N.s indicates that the overall effect of subgroup on the feature is not significant by $\chi 2$ testing. * indicates P<0.05 by $\chi 2$ testing for the effect of subgroup. The results of post-hoc comparisons for each pair of subgroups are indicated in

***Table 2-5**. (C) Participant item-level responses for questions corresponding to MDD 'typicality'. Bar charts indicate the percentage of participants in each subgroup giving each response. Effects of subgroup were tested by Kruskal-Wallis, taking the response as an ordinal variable. As none of the effects of subgroup were significant (n.s.) post-hoc comparisons were not performed. (D) Clinical features not differing between subgroups (significantly different clinical scores are shown in **Figure 2-10**). Boxplots show the median and inter-quartile range of the relevant variable for each subgroup, violin outline shows the full distribution of data. The effect of subgroup on each continuous feature is tested by Kruskal-Wallis testing. STAI = State-Trait Anxiety Inventory.*

Notably, for the data-driven subgroups, there were no significant differences between the 4 immune cell-stratified subgroups in terms of multiple, potentially confounding demographic and clinical factors (**Figure 2-11B,D,**

Table 2-5). Moreover, this four-way, bottom-up stratification of cases was not simply nested within the top-down binarization (**Table 2-6**).

Table 2-5 *Data-driven ("bottom-up") immune-cell stratified MDD subgroups: clinical, demographic and serological features.*

This corresponds to the subgroups shown in ***Figure 2-10*** *and* ***Figure 2-11****. MDD, major depressive disorder; BMI, body mass index; IL-6, interleukin-6; BDI, Beck Depression Inventory; HAM-D, Hamilton Depression Scale; SHAPS, Snaith-Hamilton Pleasure Scale; STAI, State-Trait Anxiety Inventory. P-values shown are for Kruskal-Wallis (KW) or χ^2 tests of the effect of subgroup on the variable tested: *P<0.05, **P<0.01, and ***P<0.001. For continuous variables, the post-hoc comparisons are shown in* ***Figure 2-10C,D*** *and* ***Figure 2-11A,D****. For categorical variables, pairwise subgroup comparisons which are significant by post-hoc testing following correction for 6 multiple comparisons at P<0.05 are indicated in the table below as follows: (a) different from uninflamed depression subgroup, (b) different from subgroup 1, (c) different from subgroup 2, (d) different from subgroup 3. Data from N=206 participants.*

	Subgroup 0 (grey)	**Subgroup 1 (orange)**	**Subgroup 2 (blue)**	**Subgroup 3 (red)**	**P-value**
N per cluster (all MDD)	58 (28%)	10 (5%)	100 (49%)	38 (18%)	
Cell counts	All cell counts low	Increases in non-classical monocytes, $CD16^{hi}$ NK cells and B cells	Increases in adaptive immune cells (B cells and T cells), and some innate immune cells	Myeloid-dominant immune profile. Increase in innate and adaptive immune cells, with particularly marked increases in all monocyte subsets and in neutrophils	See **Figure 2-11A**
Percentage					**P-value (χ^2)**
Female	71%	70%	69%	68%	1
Current antidepressant use	77%	56%	78%	68%	0.3
Current tobacco use	4%	10%	14%	19%	0.1
Current alcohol use	54%	40%	46%	42%	0.6
Current cannabis use	4%	0%	7%	11%	0.4
Unemployed	11% (b)	60% (a)	26%	24%	**0.004
Recent infection	35%	10%	29%	43%	0.2
Minor inflammatory disease	18%	10%	19%	24%	0.7
Study centre	-	- (d)	-	- (b)	*0.04
Median					**P-value (KW)**
HAM-D	15	18	18	19.5	**0.009
BDI	18	21	27	25	**0.002

Chalder fatigue	17	15.5	21	20	**0.005
SHAPS (anhedonia)	1	3.5	5	4.5	**0.003
STAI – state anxiety	46	51.5	50	51	0.6
STAI – trait anxiety	57	57.5	62	61	0.2
Childhood Trauma Questionnaire	46	67.5	50	52.5	0.08
Life Events Questionnaire z-score	-0.18	-0.18	-0.18	0.13	0.4
Number of ineffective antidepressants	1	1	2	1	1
Age	31.0	42.7	35.3	39.6	*0.02
CRP	0.60	0.95	1.50	2.45	***0.0003
Plasma IL-6	0.53	0.54	0.69	0.89	**0.002
BMI	25.4	23.7	26.6	31.2	**0.002
Triglycerides	0.85	0.98	1.17	1.34	**0.006

Table 2-6 *Correspondence between forced two-way ("top-down") and data-driven ("bottom-up") MDD subgroups.*

		Forced two-way clustering	
		Uninflamed depression	**Inflamed depression**
Data-driven clustering	**Subgroup 0**	58	0
	Subgroup 1	0	10
	Subgroup 2	67	33
	Subgroup 3	0	38

2.5 Discussion

In this study, we confirmed previous reports of case-control mean differences in CRP and IL-6, 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 (Cattaneo et al 2013, Chamberlain et al 2018, Kohler et al 2017a, Zorrilla et al 2001). 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, and strongly weighted on the PLS functions that optimally discriminated cases from controls or predicted symptom severity. 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 (Maes et al 1992c, Mazza et al 2018, Surtees et al 2003, Zorrilla et al 2001). The hypercortisolemia observed in some depressed cases (Juruena et al 2018) may thus relate to the neutrophilia observed in these data and other studies (Jilma et al 1998, Manz & Boettcher 2014). Neutrophils can traffic to the brain and neutrophil depletion has been shown to mitigate the effects of inflammation on behaviour in animal models (Aguilar-Valles et al 2014).

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 (Cohen et al 2013); and myeloid antigen presenting cells are important for activating and polarizing $CD4^+$ T cells towards a terminally differentiated state (Steinman & Hemmi 2006). 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 (Leday et al 2018) that indicate coupled changes in peripheral whole blood expression of genes specialized for innate and adaptive immune functions.

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 modelling 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 (Chamberlain et al 2018).

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 IL-6.

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 relatively 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.

To conclude, we found that depression case status was associated with increased immune cell counts, especially neutrophils, $CD4^+$ T cells and monocytes, and that within the depressed group, increased neutrophil and B cell counts were associated with increased symptom severity. However, not all patients with MDD had evidence of inflammation, and a theoretically-driven ("top-down") binary clustering of MDD patients based on peripheral immune cell counts identified an inflamed subgroup of MDD associated with increased symptom severity. Our alternative data-driven ("bottom-up") clustering approach in fact identified four immune-cell stratified MDD subgroups, of which two were associated with increased cell counts, increased inflammatory proteins, and more severe depression. These two inflamed groups differed in their cellular immunophenotypes and lymphoid/myeloid bias, suggesting that there may be multiple mechanistically distinct subgroups of inflamed depression.

Chapter 3: The meningeal and peripheral response to psychological stress in mice

3.1 Introduction

In this chapter, I investigate the effects of stress on the peripheral and meningeal immune systems, using a mouse model of chronic stress (chronic social defeat, CSD). As outlined in **Chapter 1**, numerous cell types and pathways have been implicated in the response to chronic stress, but beyond one study on γδ T cells (Alves de Lima et al 2020), there has been no previous investigation of the effects of stress on the meningeal immune system. The work I present here particularly implicates peripheral and meningeal B cells, so I begin with a brief overview of B cells, and what is known about their contribution to mood and the stress response.

B cells are multifunctional and are best known as the precursors of antibody-producing plasma cells, but they also have antibody-independent functions such as antigen presentation to $CD4^+$ T cells (Crawford et al 2006); the production of pro-inflammatory cytokines, including IL-6 (Barr et al 2010, Menard et al 2007); and the production of monocyte and neutrophil recruiting chemokines (Rauch et al 2012, Zouggari et al 2013). In contrast to these pro-inflammatory functions, some B cell subsets have immunoregulatory properties, inhibiting pathogenic inflammation via IL-10 secretion (Rosser & Mauri 2015). Indeed, recent data show that regulatory B cells may control inflammation in autoimmunity and organ transplantation (Blair et al 2010, Clatworthy et al 2009). Regulatory B cells have been shown to control innate immune cell activation (Iwata et al 2011), recruitment to the brain in the context of stroke (Ren et al 2011), and viral encephalitis (Mutnal et al 2014). Their contribution to regulation of innate immunity in the meninges, however, is unknown.

B cell immunology and antibody responses have received very little attention in studies of depression or the stress response. Some peripheral blood phenotyping studies in human depression have shown higher numbers of circulating B cells (Maes et al 1992b) as well as reduced IL10-producing regulatory B cells in patients compared with non-depressed controls (Ahmetspahic et al 2018, Duggal et al 2016). These studies suggest that depression may be

associated with an expanded, dysregulated peripheral B cell compartment with an imbalance between immune activating versus regulatory functions. For the B cell response to stress, two longitudinal studies of students exposed to exam stress have shown stress to be associated with increased absolute or relative counts of peripheral B cells (Maes et al 1999, Turner et al 2020), although another longitudinal study found exam stress to be associated with decreased relative peripheral B cell counts (McGregor et al 2016). These apparent inconsistencies may in part reflect the difficulty interpreting relative B cell counts, which are affected not only by changes in the number of B cells, but also by changes in the numbers of other immune cells in the superset used to define the relative count: notably, the study showing increased relative B cell counts post-stress quantified B cells as percentage of total PBMCs (Turner et al 2020) while the study showing decreased relative B cells post-stress quantified B cells as percentage of lymphocytes (McGregor et al 2016). In terms of B cell function, there is a large body of literature showing that chronic stress in humans is associated with poorer antibody responses to vaccination (Madison et al 2021).

Observational studies of immune dysfunction in humans with stress and depression are useful, but limited in their capacity to deliver information on the immune system beyond the peripheral blood. Furthermore, they do not allow inference about the causality of the associations observed. To overcome this, we sought to investigate how immune cells, particularly B cells, changed in both the periphery and the meninges in the CSD mouse model of psychological stress, which produces anxiety and depressive-like behaviour in defeated animals (Brachman et al 2015).

3.2 Hypotheses

We hypothesize that chronic stress leads to changes in both the peripheral and meningeal immune cell compartments, including changes in B cell phenotypes and functions. We further hypothesize that this immune dysregulation is important for the pathogenesis of stress-related behaviours.

3.3 Methods

Experimental animals

C57BL/6J mice (Jackson Laboratories) or UBC-GFP mice (Jackson Laboratories, strain C57BL/6-Tg(UBC-GFP)30Scha/J) were bred in-house. CD-1 aggressor mice were obtained from Charles River Laboratories as retired breeders. $Cd19^{-/-}$ mice (C57BL/6 background) were bred from animals kindly donated by Thomas Tedder (Duke University, NC) (Sato et al 1996, Yoshizaki et al 2012). All $Cd19^{-/-}$, WT, and UBC-GFP animals were born, weaned and housed in the same room. Animals were housed in a reversed 12-h light/dark cycle (Lights OFF at 0900) and tested during the dark phase. All animals used were male, aged 7-28 weeks. All comparisons used groups of male animals matched for median age. Behavioural testing, chronic social defeat and tissue analysis for animals were performed using cohorts of animals balanced as far as possible for experimental conditions. Animal numbers and ages in each set of cohorts are shown in **Figure 3-1**.

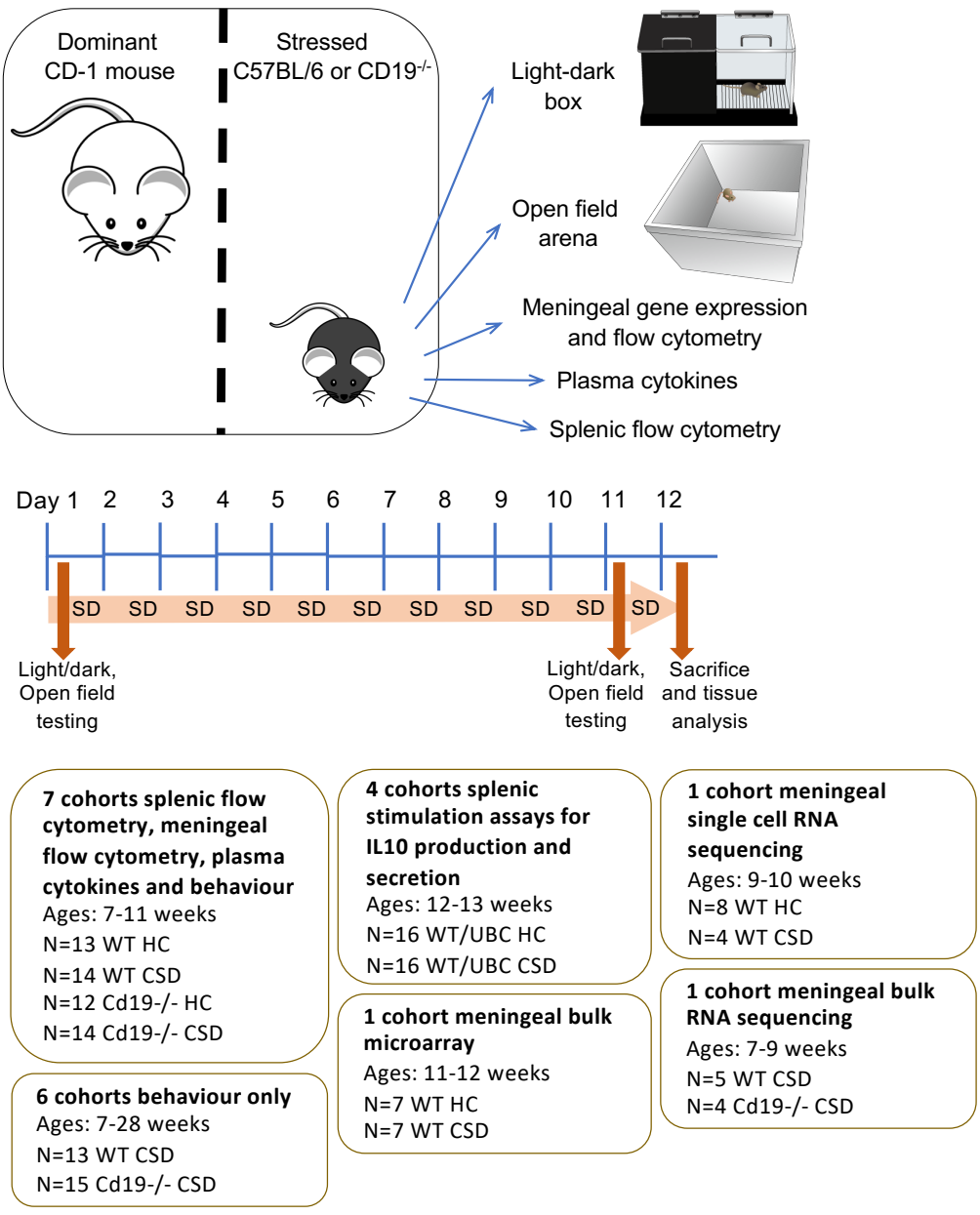


Figure 3-1 *Modelling stress using chronic social defeat.*

Male wild type (C57BL/6), UBC-GFP or Cd19$^{-/-}$ mice (C57BL/6 background) were co-housed with a large aggressive male CD-1 mouse for 11 days, separated by a transparent perforated partition, which was removed for 5 minutes each day (chronic social defeat condition, CSD). Control mice were housed with a conspecific, separated by a transparent perforated partition which remained in place throughout the experiment (homecage condition, HC). The lower panel shows the cohort of animals used for each assay, with cohorts balanced as far as possible for strain and condition. Ages indicate animal ages at baseline. The timeline shows the timing of social defeats (SD = 5 minutes social defeat), behavioural testing and sacrifice for tissue harvesting for each experimental cohort. All defeats and behavioural tests were performed in the dark phase.

Chronic social defeat (SD) paradigm

As previously described (Lehmann & Herkenham 2011), chronic social defeat was produced in an experimental intruder male mouse by co-housing with a dominant aggressor CD-1 male mouse. Animals were cohoused for 12 days. Aggressor CD-1 male mice were single-housed for >1 week prior to the experiment. In the SD condition, experimental mice were placed into the resident CD-1 mouse's home cage into which a perforated partition had been placed down the middle to separate the pair. The partition was removed for 5 min per day for 11 days allowing interaction between the pair. To prevent bite wounds, the lower incisors of CD-1 mice were trimmed weekly. In the control HC condition, male experimental animals (C57BL/6 or $Cd19^{-/-}$ mice) were housed with another male C57BL/6 or $Cd19^{-/-}$ mouse respectively, separated by a perforated partition, for the duration of the experiment.

Behavioural phenotyping

At baseline and on day 11 following SD or HC housing, mice underwent behavioural testing. On day 11, testing was performed approximately 18 h following the day-10 SD session. On testing days, mice were allowed 30 min to acclimatize to the testing room; tested in the light-dark box; allowed to recover for 30 min; then tested in the novel open field arena. Automated tracking of behaviour was recorded as previously described (TopScan; Cleversys (Lehmann et al 2017)). The experimenter left the room during testing periods. Light-dark box test was conducted in a 50 x 25 x 30 cm Plexiglas box divided into dark (one-third of total area) and light compartments with an open door. The number of transitions between compartments during 10 min was measured. Novel arena open-field testing was performed in a 50 x 50 x 50 cm open-field arena. The proportion of time spent in the central 50% of the arena during 15 min was measured.

Tissue processing for flow cytometry and cytokines

Mice in the SD group were euthanized the morning of day 12 at approximately 17 h following the 11^{th} defeat. Matched day-12 HC mice were euthanized simultaneously. Animals were weighed, then injected intravenously with 5 µL of anti-CD45-conjugated FITC in 200 µL sterile PBS to label circulating intravascular cells. Animals were anaesthetised 5 mins after CD45 injection using isofluorane, then euthanized by cardiac exsanguination, with blood samples collected in EDTA tubes. Animals were immediately perfused with cold PBS. Blood samples were centrifuged at >1600 g and the plasma supernatant frozen at -80°C for later cytokine analysis. Splenic dissection: the spleen was dissected, weighed, placed in ice-cold Roswell Park

Memorial Institute medium containing 10% foetal-calf serum (RPMI + 10% FCS), then passed through a 70 µm Nylon mesh (BD Biosciences) using a 1 mL syringe plunger. Erythrocytes were lysed in ammonium-chloride-potassium (ACK) buffer (Quality Biological 118-156-101). Meningeal dissection: following exsanguination and perfusion, the head was removed and placed in cold HBSS. Skin and muscle were cleaned from the skulls, which were kept cold in HBSS until meningeal dissections. The meninges (dura, arachnoid and pia) were dissected into ice-cold RPMI+10% FCS by removing the meninges from the skull cap, skull base, and brain surface with the aid of a dissecting microscope following a previously described protocol (Bowyer et al 2012). The meningeal tissue was passed through a 70 µm Nylon mesh (BD Biosciences 352350) using a 1 mL syringe plunger.

Meningeal and splenic samples were centrifuged at 350 *g*, resuspended in 300 µL staining volume, then stained with Fixable viability dye eFluor 780 (eBioscience 65-0865-14) for 10 min in the dark at room temperature (for live vs. dead cell gating). Samples were washed and the pellet resuspended in 20 µL Brilliant Violet stain buffer plus 0.5 µL mouse serum plus (for the lymphoid spleen panel only) 0.5 µL of purified rat anti-mouse CD16/32 antibody (BD 553141). Fc block was not used for the meningeal or myeloid panels because of the cross- blockade of CD64 by anti-CD16/32. Fluorophore-conjugated antibodies to surface antigens (**Table 3-1**) were added, and then PBS, to make a final staining volume of 50 µL. Samples were stained for 20 min on ice in the dark, washed once, then resuspended in fixative (1% formaldehyde, 0.02% sodium azide, and 2% glucose in PBS) prior to flow cytometry acquisition (see below).

Flow cytometry acquisition and manual gating

Flow cytometry data were collected on a BD Fortessa flow cytometer. For meningeal samples, the fixed cell suspension was diluted to > 0.7 mL and run in its entirety. For splenic samples, it was not necessary to collect the entire sample as percentage counts (of total live cells) were combined with splenic weight to estimate cell counts. Compensation was performed for each session using UltraComp eBeads (eBioscience 01-2222-42) conjugated to antibodies used in the sample panels except for Red-780/60, where a mixture of live and ethanol-killed splenocytes stained with Fixable Viability dye eFluor 780 (eBioscience 65-0865-14) was used as a compensation control. Cell subsets were defined by manual gating in FlowJo™ according to the gating strategies shown in **Figure 3-3** and **Figure 3-4**. The following meningeal immune populations were identified: B cells, $CD11b^{+}$ DCs, $CD11b^{-}$ DCs, pDCs, $Ly6C^{hi}$ monocytes,

$CD64^{+}MHCII^{-}$ macrophages, $CD64^{+}MHCII^{+}$ macrophages, meningeal microglia-like macrophages, neutrophils, NK cells, NKT cells, T cytotoxic and T helper cells. The following splenic cell populations were identified: Plasmablasts/plasma cells, IgD^{-} B cells, IgD^{+} B cells, $CD9^{+}$ B cells, cytotoxic T cells, helper T cells, NK cells, NKT cells, $CD11b^{+}$ cDCs, $CD11b^{-}$ cDCs, pDCs, neutrophils, $F4/80^{+}$ macrophages, $Ly6C^{hi}CD11b^{-}$ cells and $Ly6C^{hi}$ monoctyes. B220, a well-validated B cell marker, was used in flow cytometric studies (rather than CD19) so that B cells could be identified in both WT and $CD19^{-/-}$ mice, as in the latter, CD19 obviously cannot be used to define B cells.

Table 3-1 *Meningeal and splenic immunophenotyping panels.*
Three multi-parameter immunophenotyping panels were used for flow cytometric analysis of meningeal and splenic immune cells. The number of antigens targeted per panel is limited by the flow cytometer and its setup, so to allow analysis of a greater number of surface antigens, we optimized both a lymphoid-focused and a myeloid-focused splenic panel. Only a limited number of cells are aquired from the meninges, so only a single flow panel was used for meningeal samples. The fluorophore-conjugated antibodies to surface antigens used for each panel are indicated: the row entry indicates the surface antigen targeted for that panel, and the column heading indicates the fluorophore to which the antibody was conjugated, i.e., in the meningeal panel, a BUV395-conjugated anti-CD45 antibody was used to assay CD45 expression. LD = live-dead fixable viability stain. See ***Figure 3-3*** *and* ***Figure 3-4*** *for the combinations of surface antigens used to identify and quantify particular immune cell subsets using these panels.*

Panel	BUV395	BUV737	BV421	BV605	BV650	BV711	BV785	FITC	PerCP-Cy5.5	PE	PE-dazzle	PE-Cy7	APC	Alexa700	AP-eF780
Meninges	CD45	CD11b	NK1.1	CD3	CX3CR1	B220	CD69	$CD45_{venous}$	Ly6C	CD11c	CD4	Ly6G	CD64	MHCII	LD
$Spleen_{lymphoid}$	CD45	CD11b	NK1.1	CD3	CD138	B220	CD69	$CD45_{venous}$	CD25	IgD	CD4	CD62L	CD9	MHCII	LD
$Spleen_{myeloid}$	CD45	CD11b	CD68	F4/80	CX3CR1	B220	$CD3/CD19_{dump}$	$CD45_{venous}$	Ly6C	CD11c	CD103	Ly6G	CD86	MHCII	LD

Analysis of meningeal count data

Previous publications have assessed the effects of experimental manipulations by comparing cell counts between group as percentages of live CD45+ cells. This method is subject to bias because, for example, the manipulation may cause a decrease in one cell type that can falsely lead to an apparent increase in another cell type if only relative proportions are compared. However, it is difficult to compare absolute cell numbers in the meninges because the completeness of dissection will vary from animal to animal, introducing considerable noise. Increasing the number of animals in a single experiment to overcome this is stymied by the considerable time to dissect, dissociate and stain each meningeal sample. To overcome these problems, we performed meningeal dissections on 53 mice, performed in 7 cohorts, balanced

for both strain and condition (as far as possible), to avoid any systematic bias in dissection by mouse strain or experimental condition. I personally performed all dissections for which the data were pooled for flow cytometry analysis, avoiding any confound by experimenter. Data were combined across all cohorts by including cohort as a variable in the models used to compare cellular abundance and marker expression (**Equations 3.1, 3.3 and 3.4** below).

In multi-parameter flow cytometry, the analytical challenge of comparing multiple cell counts across groups is similar to the statistical challenge of analysing read counts in RNA sequencing datasets. We thus repurposed an [R] package for analysis of RNA read counts, DESeq2 (Love et al 2014) to compare group differences in cell counts using a negative binomial model. The problem of differing efficacies of meningeal dissection produces noise in cell count data analogous to the problem of different library sizes in RNA sequencing count data. In DEseq2, library size correction is used to normalize for differing sequencing depths across samples. The size factor used for normalization is calculated for each library as the median ratio of gene counts relative to the geometric mean per gene – this is preferable to using total summed counts as the library size factor, as summed counts are susceptible to bias from a few highly expressed and differentially expressed genes (Anders & Huber 2010). Here, we repurpose the DESeq2 median ratio library size correction to account for the effects of differing meningeal dissection efficacy while minimizing the influence of differentially abundant cell counts. We noted that results were not substantially different if library size correction was not used. The linear predictor for the negative binomial model for cell subset *i* with conditional mean μ_i was defined as:

$$\log(\mu_i) = \beta_0 + \beta_1 cohort + \beta_2 strain + \beta_3 condition + \beta_4 (strain \times condition) \qquad [Eq. 3.1]$$

Volcano plots show the effects of the relevant contrast (e.g., SD vs. HC or Cd19$^{-/-}$ vs. WT).

Analysis of splenic cell count data

Immune cell subset data is often analysed in terms of proportions of CD45+ cells. However, this approach is subject to significant bias - an apparent decrease in one cell subset may simply reflect an increase in another cell subset. It is thus preferable to analyse absolute counts. As it is not practical to immunophenotype an entire spleen for each animal, we combined proportional flow counts (gated as per **Figure 3-4**) with splenic mass to obtain estimated absolute cell counts as follows:

$$c_{ij} = 22.9 \times 10^5 \times m_i \times p_{ij} \qquad [Eq. 3.2]$$

where c = estimated absolute count, i = mouse identity, j = cell subtype, m = splenic mass (in milligrams) and p_{ij} = the proportion of live cells of cell subset j in mouse i's splenic cells, derived from flow cytometry data. The constant multiplier 22.9×10^5 was taken from a previous report of the number of cellular nuclei per milligram of mouse spleen (Mizen & Petermann 1952). Splenic cell counts for N=40 mice were then compared using package DEseq2 (Love et al 2014) as for meningeal cell counts (**Equation 3.1** above), but without the need for library size correction because splenic weight - unlike total meningeal weight - can be measured, so a correction for estimated completeness of dissection is not required. Automated clustering was not performed for splenic flow cytometry data as surface marker detection was split across two multicolour panels (lymphoid and myeloid panels), which precludes joint clustering analysis across all markers.

Automated clustering analysis of immunophenotyping data and comparison of per-cluster median fluorescence intensity

We confirmed our findings from manually gated meningeal flow cytometry data in a parallel, automated analysis of this data, modified from a workflow for analysis of mass cytometry data, CyTOF workflow (Nowicka et al 2017). In brief, we used [R] packages flowSOM (Van Gassen et al 2015) and ConsensusClusterPlus to perform clustering of meningeal extravascular immune cells (Wilkerson & Hayes 2010). See **Figure 3-2** and its legend for a schematic of the approach and further details of data analysis. Following clustering, cells were further downsampled to 1000 cells per sample to produce a combined-sample tSNE representation using [R] Rtnse (Krijthe 2015), onto which manual and automated clustering labels were overlaid for visualization.

Statistics: functional marker expression

Expression of functional markers was analysed from flow cytometry data in two ways. Firstly, for candidate functional markers of interest in a given cell subset (e.g., % $CD69^+$ of B cells), the percent of cells positive for a given marker were manually gated. Because of non-normality of the data, this was followed by using rank-based linear models (using [R] package Rfit (Kloke & McKean 2012)) to estimate the effect of animal cohort and condition or strain on these percentages. Secondly, the effects of strain or stress on biexponentially transformed median fluorescence intensity (MFI) of functional markers in each cell subset was tested by linear

modelling. For the effects of condition (tested in wild type animals only, SD vs. HC), the model used was:

$$MFI\ (or\ \%\ positivity) = \beta_0 + \beta_1 cohort + \beta_3 condition + \varepsilon \qquad [Eq. 3.3]$$

For the effects of strain (tested in homecage animals only, wildtype vs. *Cd19*$^{-/-}$), the model used was:

$$MFI\ (or\ \%\ positivity) = \beta_0 + \beta_1 cohort + \beta_2 strain + \varepsilon \qquad [Eq. 3.4]$$

See **Figure 3-2** for a schematic of the overall analytical approach. For both approaches, p-values for the effect of strain or condition were FDR-corrected across all markers tested.

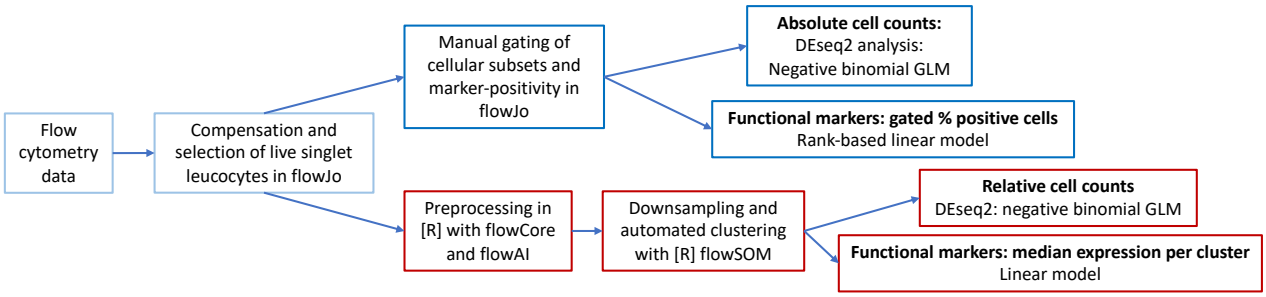


Figure 3-2 *Schematic summarizing manual and automated analysis of meningeal flow cytometry data. We used complementary manual (blue boxes) and automatic (red boxes) data analysis strategies. Manual gating was used to define absolute total meningeal counts, and to quantify the percentage of cells within a subset positive for functional markers, e.g., CD69. Automated clustering was used to detect clusters of cells, their relative counts, and median functional marker expression (MFI) in different experimental conditions. To perform automated gating, FlowJo workspaces, files, and manual gates (for comparison) were imported and manipulated using [R] flowWorkspace (Finak & Jiang 2018) and flowCore (Hahne et al 2009). Manually gated extravascular live singlet leucocytes (i.e., intravenous CD45-negative microglia-like macrophages and leucocytes) were selected for further automated processing (see gating strategy in* ***Figure 3-3****). [R] flowAI::flow_auto_qc was used to remove signal acquisition and dynamic range abnormalities to generate quality-controlled datasets (Monaco et al 2016). Prior to clustering, each of the 53 meningeal datasets were then downsampled to either the total number of extravascular immune cells or 20,000 extravascular immune cells, whichever was greater. Following downsampling, there was no overrepresentation of any animal group in the combined dataset of all cells (linear model was non-significant for the effect of strain and condition on the number of cells included). Downsampled concatenated cells from all 53 animals were automatically clustered into k = 1:20 clusters using [R] FlowSOM::BuildSOM (Van Gassen et al 2015) and [R] ConsensusClusterPlus with a Euclidean disease metric (Wilkerson & Hayes 2010) based on the following markers: Ly6G, CD11b, CX3CR1, Ly6C, MHCII, CD45, CD64, B220, CD11c, CD3, CD4, NK1.1. Clustering solutions were manually inspected and k = 18 was chosen for the best agreement with the granularity of manually gated cell subsets. The automated clusters generated were manually annotated by inspection of lineage marker expression in each cluster.*

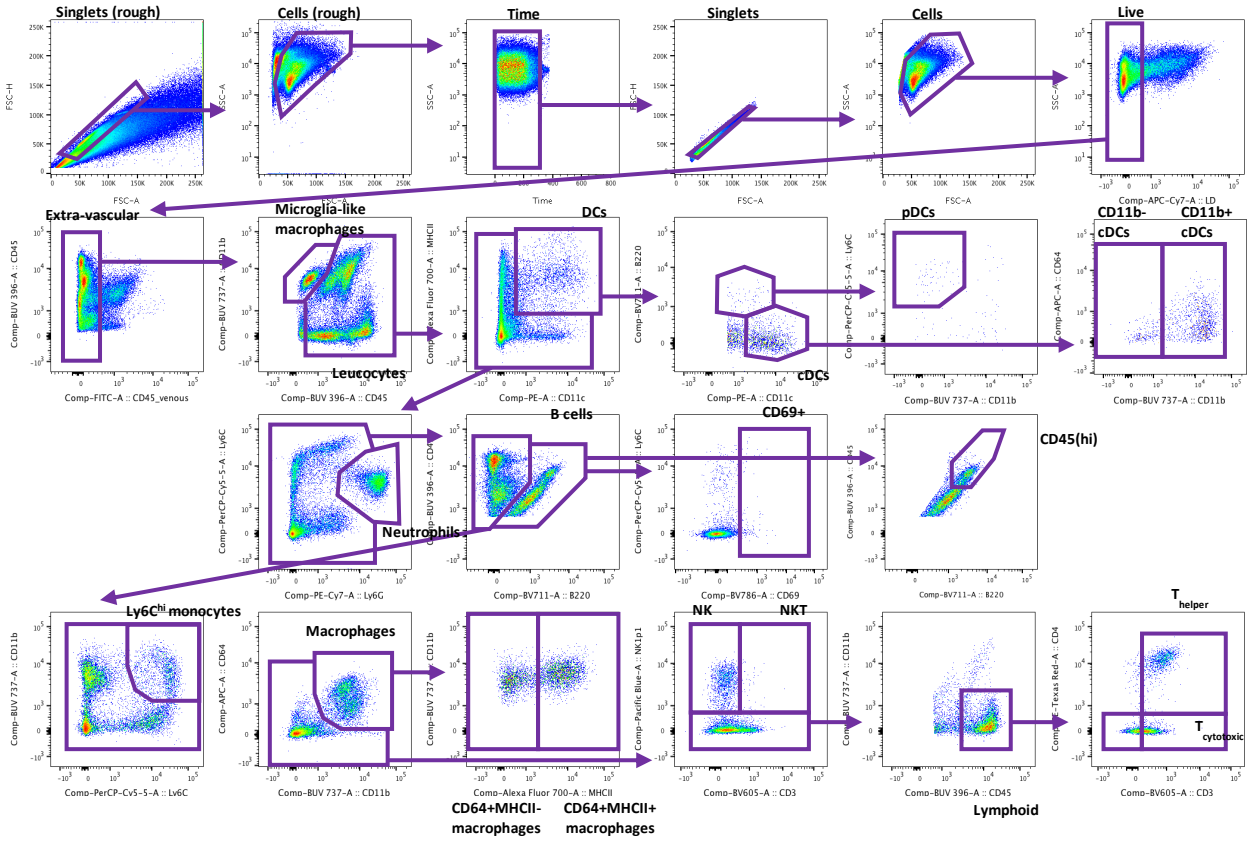


Figure 3-3 *Meningeal flow cytometry gating strategy.*

Gating strategy used to identify live, extravascular meningeal immune cells. Injection of intravenous labelled CD45-FITC was used to identify and exclude intravascular immune cells. The 'Time' gate was used to exclude low quality cells at the beginning and end of a flow cytometry run. Key cell types were identified as follows:

- Microglia-like macrophages: Live intravenous-CD45^{-} CD45^{lo}CD11b^{int}

- pDCs: Live intravenous-CD45^{-} CD45$^{int/hi}$CD11c^{hi}MHCII^{hi}B220$^{int/hi}$CD11b^{lo}Ly6C^{hi}

- cDCs: Live intravenous-CD45^{-} CD45$^{int/hi}$CD11c^{hi}B220^{lo}

- Neutrophils: Live intravenous-CD45^{-} CD45$^{int/hi}$Ly6G^{hi}Ly6C^{int}

- B cells: Live intravenous-CD45^{-} CD45$^{int/hi}$B220^{+}

- Macrophages: Live intravenous-CD45^{-} CD45$^{int/hi}$B220^{-}CD11b^{+}CD64$^{int/hi}$

- NK cells: Live intravenous-CD45^{-} CD45$^{int/hi}$B220^{-}CD3^{-}NK1p1^{+}

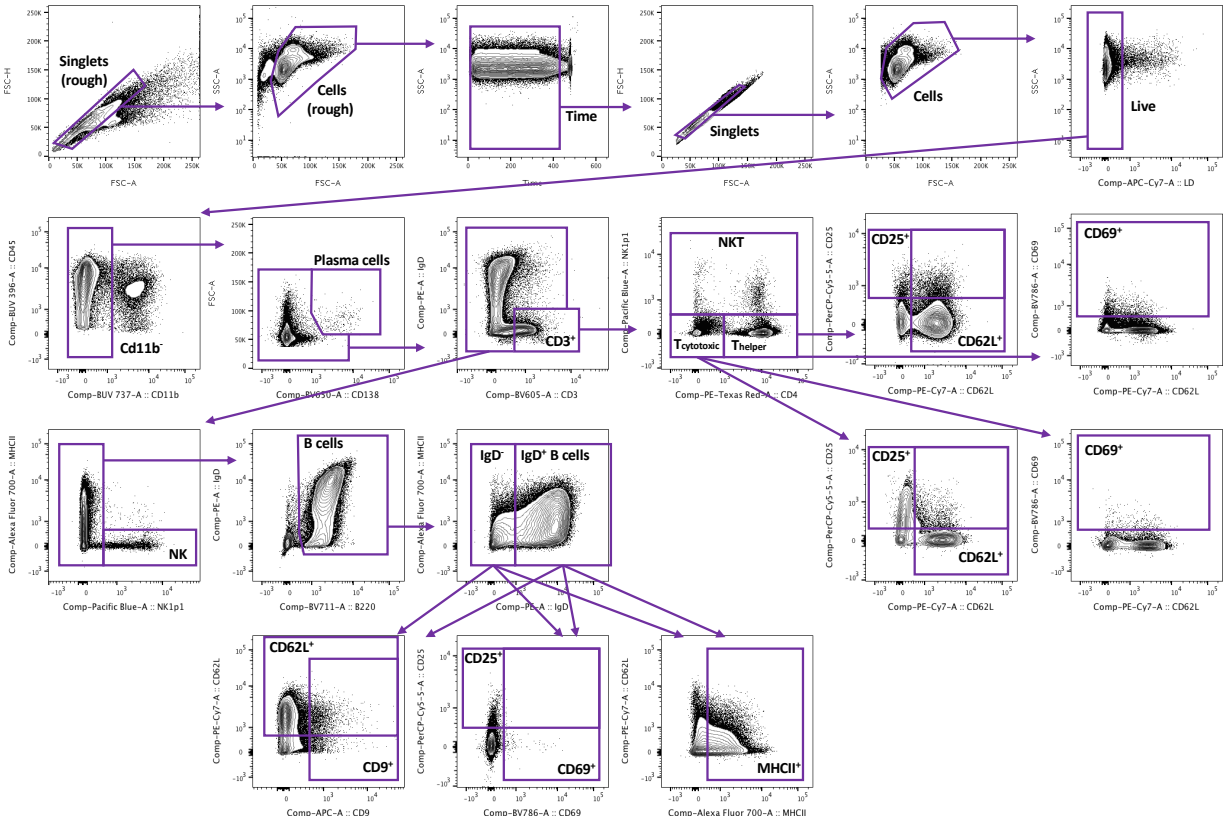
- NKT cells: Live intravenous-CD45^{-} CD45$^{int/hi}$B220^{-}CD3^{+}NK1p1^{+}

- Helper T: Live intravenous-CD45^{-} CD45^{hi}B220^{-} NK1p1^{-}CD3^{+}CD4^{+}

- Cytotoxic T: Live intravenous-CD45^{-} CD45^{hi}B220^{-} NK1p1^{-}CD3^{+}CD4^{-}

(see caption over)

A Splenic lymphoid panel gating strategy



B Splenic myeloid panel gating strategy

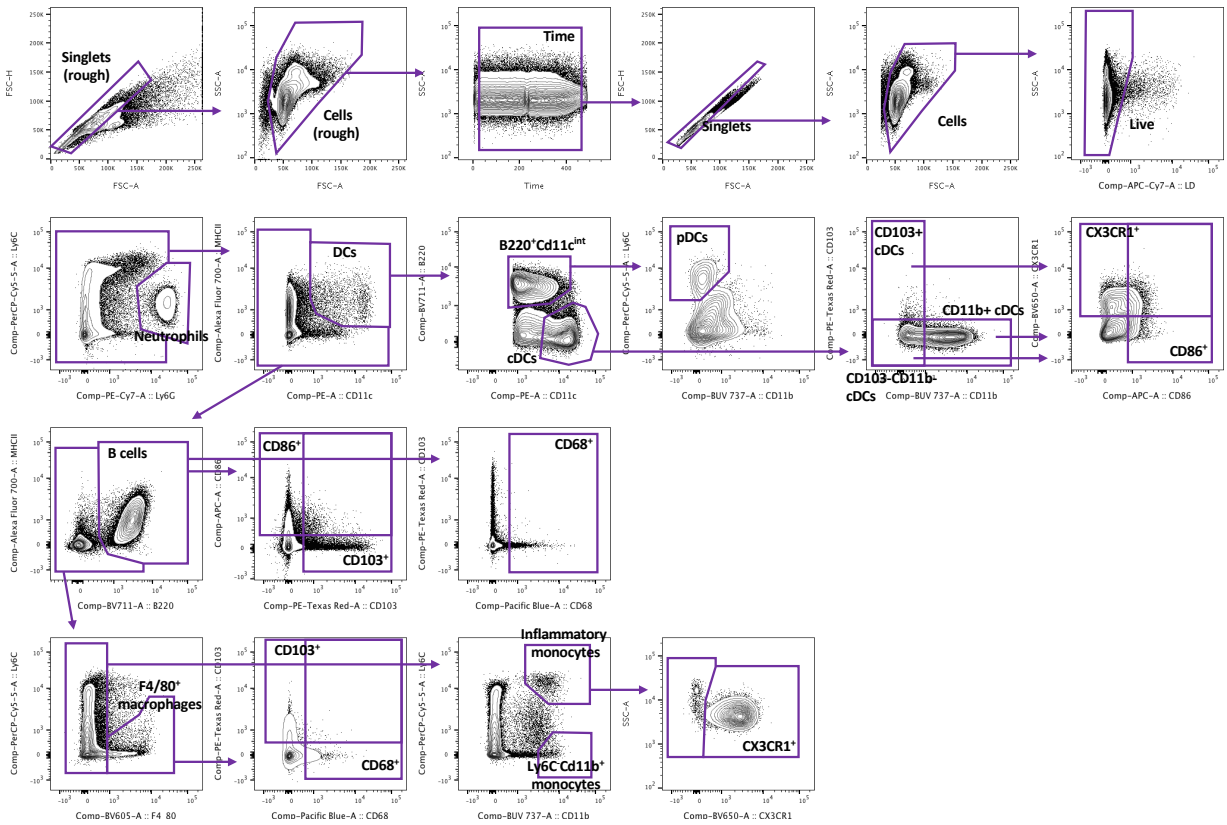


Figure 3-4 *Splenic flow cytometry gating strategies*

Gating strategies used to identify splenic immune cells using lymphoid-focused (A) and myeloid-focused (B) flow cytometry panels. The 'Time' gate was used to exclude low quality cells at the beginning and end of a flow cytometry run. Key cell types were identified as follows:

- Plasmablasts/plasma cells: Live $CD11b^{-}$ $CD138^{+}FSC^{int/hi}$

- B cells: Live $CD11b^{-}CD3^{-}MHCII^{-}NK1p1^{-}B220^{+}$

- NKT cells: Live $CD11b^{-}CD3^{+}IgD^{-}NK1p1^{+}$

- NK cells: Live $CD11b^{-}CD3^{-}MHCII^{-}NK1p1^{+}$

- Helper T cells: Live $CD11b^{-}CD3^{+}IgD^{-}NK1p1^{-}CD4^{+}$

- Cytotoxic T cells: Live $CD11b^{-}CD3^{+}IgD^{-}NK1p1^{-}CD4^{-}$

- Neutrophils: Live $Ly6C^{hi}Ly6C^{int}$

- Classical dendritic cells (cDCs): Live $CD11c^{hi}MHCII^{+}B220^{-}$

- Plasmacytoid dendritic cells (pDCs): Live $CD11c^{int}B220^{+}CD11b^{lo}Ly6C^{+}$

- $F4/80^{+}$ macrophages: Live $B220^{-}$ $Ly6G^{lo/int}Ly6C^{lo/int}F4/80^{+}$

- Inflammatory monocytes: Live $B220^{-}$ $Ly6G^{lo/int}Ly6C^{lo/int}F4/80^{-}Ly6C^{+}CD11b^{+}$

- $Ly6C^{-}CD11b^{+}$ monocytes: Live $B220^{-}$ $Ly6G^{lo/int}Ly6C^{lo/int}F4/80^{-}Ly6C^{-}CD11b^{+}$

Plasma cytokine data

Blood samples were acquired from mice by cardiac puncture at the time of sacrifice on day 12 of the chronic social defeat protocol. Samples were collected into EDTA tubes, centrifuged at >1600*g*, and stored at -80 degrees. At a later date, samples were thawed and the 53 samples (each in duplicate) were split across two plates, balanced for strain and condition. A logistic regression with the following link function did not show any significant relationship between plate, *y*, and any aspect of experimental design:

$$g(E(y)) = \beta_0 + \beta_1 cohort + \beta_2 strain + \beta_3 condition \qquad [Eq. 3.5]$$

Plasma cytokines were analyzed in duplicate according to manufacturer's instructions on a MAGPIX™ using Bio-Plex Pro™ Mouse Cytokine 23-plex Assay M60009RDPD, MAGPIX™ Calibration Kit MPX-CAL-K25 and Performance Validation Kit MPX-PVER-K25. Data were captured on the following cytokines: CCL11 (Eotaxin), G-CSF, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17A, CXCL1 (KC), CCL2 (MCP-1, MCAF), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES) and TNF-α. Values below the lower limit of detection were imputed at half the lower limit of detection, and cytokines for which >10% of values were below the lower limit of detection were removed from analysis. For cytokines included in analysis (CCL11, G-CSF, IL-1α, IL-6, IL-12 (p40), IL-17A, CXCL1 (KC), CCL3 (MIP-1α), CCL5 (RANTES) and TNF-α), group differences were compared by rank-based linear model using [R] package Rfit (Kloke & McKean 2012) to account for the non-parametric distribution of data and to minimise the bias from imputation of values below the lower limit of detection. P-values were adjusted for multiple comparisons (Benjamini-Hochberg method) across the 10 cytokines tested.

Intracellular IL-10

Spleens from UBC-GFP (cohort 1) or WT (cohorts 2-4) animals were first weighed, then mashed through a 70 µm cell strainer with the rubber end of a 3 mL syringe into a single cell suspension and pelleted. Red blood cell lysis with ACK buffer was performed for 5 min at room temperature, and the reaction stopped by diluting with HBSS + 0.1% BSA. Cells were pelleted, transferred through a 35 µm cell strainer, and live cells were counted on a hemocytometer via Trypan Blue exclusion. Stimulation for IL-10 production and intracellular IL-10 staining were performed similarly to as described elsewhere (Matsushita & Tedder 2011). Briefly, cells were diluted in RPMI-based medium, plated with equal density in triplicate (two stimulated wells and a negative control) before stimulation with LPS (10 mg/mL), PMA (10 ng/mL), ionomycin (1

µg/mL), and monensin (2 µM) for 5 h, then stained with antibodies to CD45, CD3, CD11b, CD19, CD9 and IL-10 prior to acquisition of flow cytometry data. B cells and B cell %IL-10 positivity were manually gated (B cells defined as live $CD45^{+}CD11b^{-}CD3^{-}CD19^{+}$ cells). The effect of stress on B cell IL-10 production was tested by linear model as follows:

$$IL10\ \%\ positive = \beta_0 + \beta_1 cohort + \beta_2 condition + \varepsilon \qquad [Eq. 3.6]$$

Supernatants from these experiments were tested in duplicate or triplicate for IL-10 protein levels by ELISA (mouse IL-10 DuoSet #DY417), performed as per manufacturer's instructions, with lower limit of detection 31.2 pg/ml.

IL-10 stimulation conditions were as follows:

Stimulation medium composition (cells plated in triplicate):

Constituent	Product number	Final concentration
RPMI+ Glutamax	GIBCO # 61870-036	
Foetal Bovine Serum	Sigma # F4135	10%
Sodium Pyruvate	GIBCO # 11360	1mM
Hepes Buffer	Corning/Cellgro # 25-060-CI	10 mM
MEM Nonessential amino acids	Corning/Cellgro #25-025-CI	1x
Penicillin-Streptomycin	Gibco # 15140-148	Penicillin: 100U/ml, Streptomycin: 100 µg/ml
Beta-Mercaptoethanol	Sigma # M-7522	50 uM

Compounds used for stimulation:

Constituent	Product number	Final concentration
Phorbol-12-Myristate 13-Acetate (PMA)	Alexis # 445-004-M001	10 ng/ml
Ionomycin	Sigma # I0634	1 µg/ml
Monesin	Sigma # M5273	2 µM
LPS	Sigma # L2880	10 µg/ml
Brefeldin A	Sigma # B6542	10 µg/ml

Positive stimulation wells (2 per mouse) contained PMA + Ionomycin + LPS + Monesin + Brefeldin A. Unstimulated wells contained (1 per mouse) contained Monesin + Brefeldin A.

Meningeal microarray data

Meninges were dissected as described above for flow cytometry, then centrifuged and stored in Trizol. Samples were triturated using syringe needles, then total RNA was extracted using a Qiagen miRNeasy Mini kit (Cat:217004). Labelled probes were run on an Affymetrix GeneChip™

Mouse Gene 2.0 ST Array (Cat:902118) using protocols and processes recommended by the GeneChip manufacturer (Affymetrix, Inc). Data were RMA-normalized and limma (Ritchie et al 2015) was used to test for differential expression of genes in SD vs. HC conditions. Pre-ranked gene set enrichment analysis (ranking by t-statistic) using [R] clusterProfiler (Yu et al 2012) was performed to test for enrichment of Hallmark pathways (Liberzon et al 2015) in stress, with Benjamini-Hochberg FDR-correction of p-values across all tested pathways. xCell (Aran et al 2017) was used to estimate meningeal cell type composition in each sample, and estimated cell proportions in each condition were compared using Mann-Whitney U tests.

Debatching variables for the effect of cohort

As to be expected, there were substantial effects of mouse cohort on immunological and behavioural endpoints. To allow visualization of group differences, we generated debatched values for the following features: plasma cytokine levels; meningeal percentage positive and absolute counts; splenic percentage positive and absolute counts; and behavioural outcomes. Features were debatched for linear batch effects using limma's removeBatchEffect function (Ritchie et al 2015). As the original data were not normal, they were transformed prior to debatching to improve normality: count data were transformed using DESeq2::rlogTransformation; all other data were Box-Cox transformed using [R] package caret (Kuhn 2008). Following debatching, data were inverse transformed to return the data to their original scales. These debatched data were used for graphical representation and for correlating immunophenotypes with behaviour but were not used for statistical analysis of group differences (for group statistical analyses, cohort was simply included as a factor in the statistical models as described above).

Meningeal bulk RNA sequencing data

Meningeal samples were prepared as described above for flow cytometry. Care was taken to remove intact sheets of meningeal tissue, which were transferred to RNAlater and stored at -80°C. Samples were homogenized using a Precellys 24^{TM}. Total RNA was extracted using an RNeasy Plus Micro Kit (Qiagen, Cat:74023) and RNA quantity and integrity was assessed by Bioanalyzer (Agilent Inc). 500 ng of total RNA was used in conjunction with the TruSeq® Stranded Total RNA Library Prep kit (Illumina Inc, Cat:20020597). Library quality was checked by Bioanalyzer and quantitated by Qubit (ThermoFisher Scientific Inc). Equimolar quantities from each sample library were pooled and run on a Highoutput Next-Seq 550 kit. We used

Salmon, run in mapping-based mode (Patro et al 2017), to generate per-gene counts, initially aligning to the GENCODE transcriptome, taking advantage of transcript-resolution estimates to improve differential gene expression analysis (Soneson et al 2015). Preranked gene set enrichment analysis (ranking by DESeq2 Wald statistic) using [R] clusterProfiler v3.14.0 (Yu et al 2012) was performed to test for enrichment of Hallmark pathways (msigdbr v7.0.1) (Liberzon et al 2015) in stress, with FDR-correction of p-values across all tested pathways.

Meningeal single cell RNA sequencing

Droplet-based single cell RNA sequencing (10x Genomics' Chromium v2 platform) was performed on meningeal cells from 2 groups of HC mice and 1 group of SD mice, with each group consisting of cells pooled from 4 mice. Live, nucleated, singlet cells ($DAPI^-DRAQ5^+$) were sorted on a BD FACS Aria Fusion into HBSS + 10% FBS prior to droplet encapsulation. Transcript data were acquired with an Illumina NextSeq 550 sequencer and single cell feature counts were generated using the standard *Cellranger V2* pipeline. Single cell RNA sequencing data were then processed following the strategy shown in **Figure 3-5**. In brief, to obtain N = 6694 quality-controlled single cell transcriptomes, we performed the following steps. We defined cell-containing vs. empty droplets (cell calling) using DropletUtils::emptyDrops (Lun et al 2019). We then excluded cells that were outliers based on a high number of mitochondrial reads (>8.3% of total reads), which indicates likely damaged/dying cells, and excluded outlier cells with a very high or low number of detected genes per cell (range for included cells = 174 – 4548). We removed from analysis any genes which were not expressed with >1 count in >1 cell. Droplets likely containing more than one cell were identified and removed using the doublet detection method scrublet (Wolock et al 2019): doublet rates in the three samples were estimated as 6.9%, 5.1% and 3.4%. Gene expression libraries were normalised using deconvolution-based normalization via scran (Lun et al 2016); highly-variable genes (3599 genes) were selected by choosing those genes where biological variation across samples was > 0 (using scran::decomposeVar). Although all samples were run on the same 10X chip on the same day, there can be systematic differences between data from different 10X lanes. To combine data from the three lanes while correcting for any batch effects, we used mutual nearest neighbour (MNN) batch correction via batchelor::multiBatchNorm and fastMNN (Haghverdi et al 2018). To identify groups of similar cells, i.e., cell subsets, we performed clustering of the MNN-corrected gene expression PCA components across all single cells using the leidenalg clustering algorithm (Traag et al 2019). The cellular identity of each cluster was

manually annotated by comparing marker genes expressed in each cluster with those in existing single cell datasets. Two B cell clusters were identified. Differential gene expression between SD and HC cells within B cell clusters was performed as follows: read counts were renormalized within the cluster; genes differentially expressed between SD and HC in pseudobulk samples of empty droplets (i.e., likely representing ambient RNA) were removed as described elsewhere (Ernst et al 2019); genes expressed in ≤15% of cells in the cluster were removed; then gene expression in SD vs. HC cells was compared using a Mann-Whitney U test, with Benjamini-Hochberg FDR correction of p-values across all tested genes. To identify differentially up- and down-regulated pathways, pre-ranked gene set enrichment analysis was performed using clusterProfiler (Yu et al 2012) with genes ranked by -log10 (Mann-Whitney U test P-value) * sign(LFC). The cell cycle stage of each cell was estimated using scran::cyclone (Scialdone et al 2015).

Cellular stress-related genes

A list of cellular stress-related genes was generated by choosing the top 20 genes found to be upregulated in murine single cells following incubation at 37°C for 60 minutes (Adam et al 2017) which were also expressed in our dataset. The expression of these genes in each single cell was summarized using Seurat::AddModuleScore (Stuart et al 2019). Module scores were compared between HC and SD animals using a Mann Whitney U test.

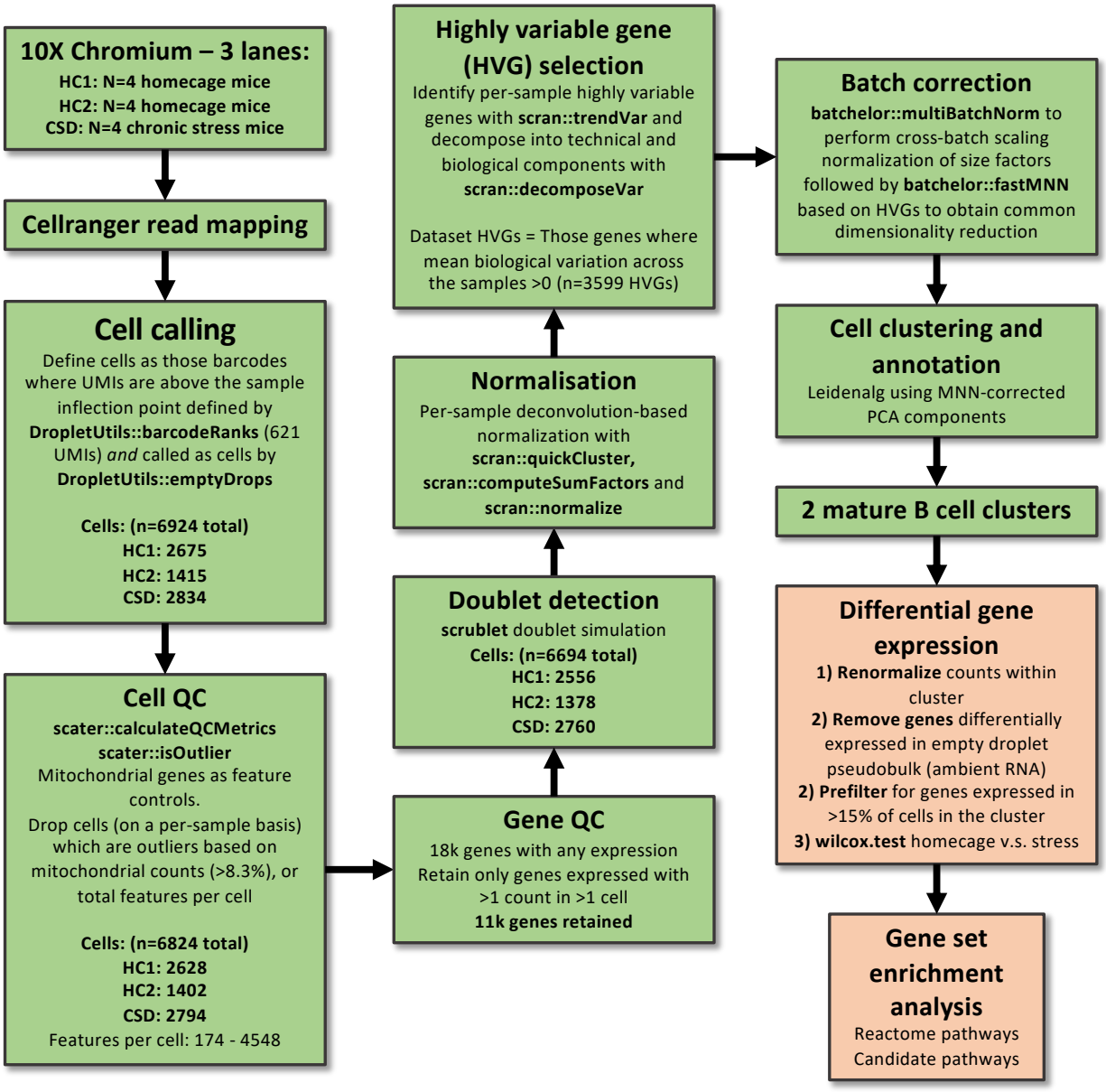


Figure 3-5 *Meningeal single cell RNA sequencing data processing pipeline.*

Three droplet-based single cell RNA sequencing libraries (10X Chromium), each including data from n=4 mice, were prepared and analysed as per the schematic. At each stage of processing, the numbers given for each library (HC1, HC2 and CSD) indicate the number of cellular transcriptomes passing the quality control (QC) filter indicated. See text for more details of analysis. UMI, unique molecular identifier (molecular barcode); MNN, mutual nearest neighbour.

3.4 Results

Social defeat (SD) stress leads to dysregulation of the peripheral B cell compartment occurs in mice

We performed chronic social defeat stress in wild type mice and collected tissue for flow cytometric analysis (**Figure 3-1**). As expected, on day 11 following the onset of the SD protocol, mice showed a behavioural phenotype consistent with anxiety and decreased motivation to investigate, with a reduction in the number of crosses to the light area in a light-dark box, and a reduction in the distance travelled in a novel open-field arena, in the defeated group compared to home cage controls (HC) (**Figure 3-6A**).

Flow cytometry showed that on day 12 following the onset of the SD protocol, there was a significant increase in splenic neutrophils, Ly6C^{hi} monocytes, and F4/80^{hi} macrophages compared to controls (**Figure 3-6B**), indicating innate immune system activation. Peripheral blood cytokines in SD animals were similar to those in controls, except for a significant increase in G-CSF, a neutrophil-mobilising cytokine (**Figure 3-6C**). Additionally, splenic plasmablasts/plasma cells (terminally differentiated B cells) were significantly increased in SD mice (**Figure 3-6B**), as were splenic CD9^{+} B cells (**Figure 3-6D**). CD9 has been described as a marker of IL-10-producing regulatory B cells (Sun et al 2015) and IL-10 secretion in B cells is enhanced by activating stimuli (Mauri et al 2003, Yoshizaki et al 2012). We therefore directly assessed B cell IL-10 production following stimulation ex vivo. We confirmed a significant increase in the percentage of IL-10^{+} regulatory B cells in the SD group, both at baseline and following ex vivo stimulation, and an associated increase in secreted IL-10 in the supernatants from these stimulations (**Figure 3-6E,F**). It was striking that the effect of stress on intracellular IL-10 was detectable without stimulation, suggesting that even without a classical immune stimulus, stress can alter B cell cytokine production. There was also evidence of B cell activation more broadly, with increased expression of CD25 on B cells in SD animals (**Figure 3-6G,H**). There were no differences in T cell number or activation in the SD group compared with controls (**Figure 3-6B,H**). Together, these data demonstrate that social stress in rodents is associated with abnormalities in the peripheral B cell compartment, with evidence of increased activation and an expansion of both IL-10-producing B cells and plasmablasts/plasma cells.

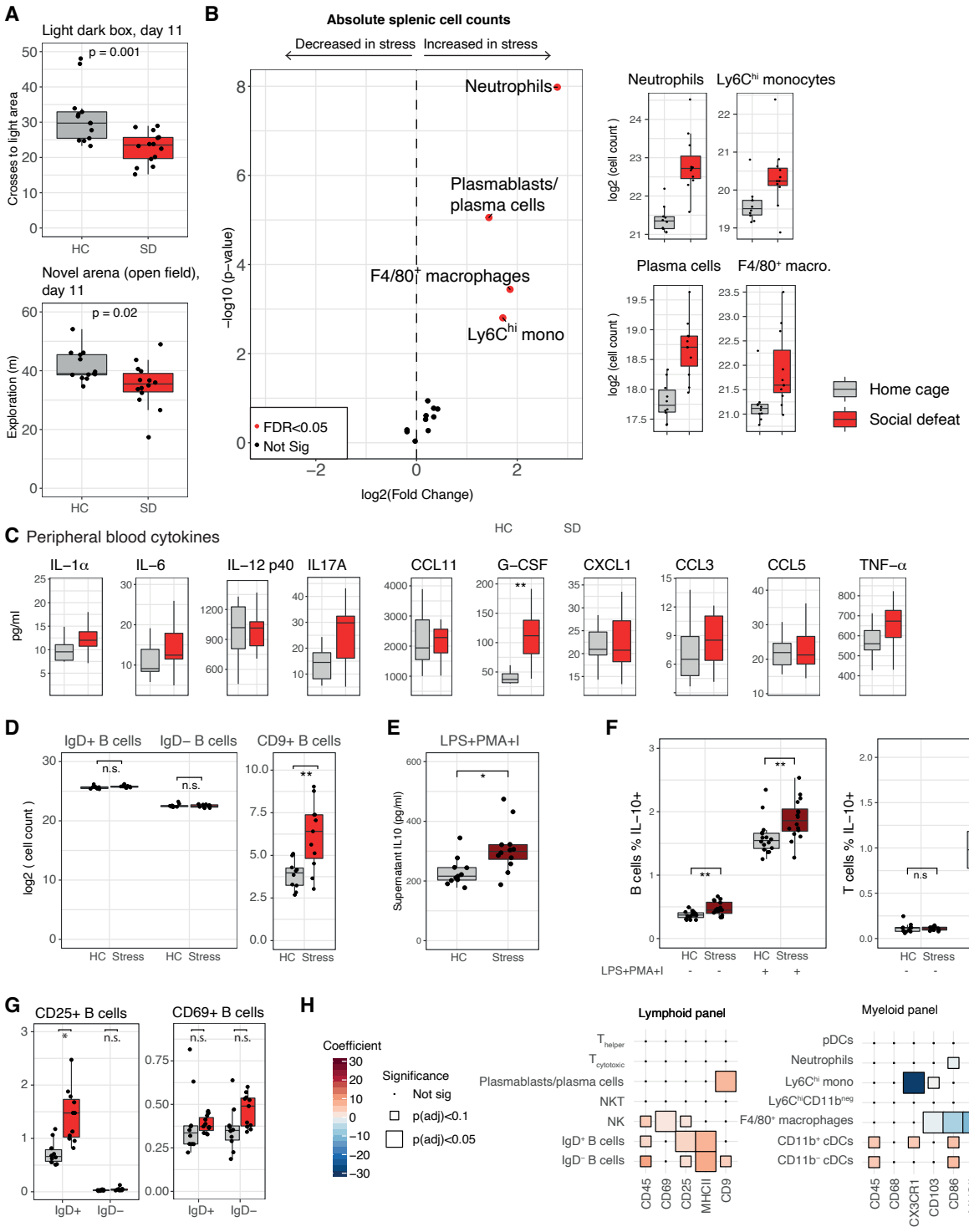


Figure 3-6 *Dysregulation of the peripheral B cell compartment in mice subjected to chronic stress. (A) Effects of chronic stress (11 days) on behaviour (median ±IQR). N = 13 control mice (HC = home cage), N = 14 social defeat (SD). (B) Volcano plot showing the effects of SD on splenic immune cell subsets. Results of a negative binomial model, FDR P < 0.05; each datapoint summarizes the effect of SD for that cell type across all mice, with the x-axis indicating the magnitude of change in absolute cell counts and the y-axis indicating the statistical significance of the change. The 14 subsets tested were IgD+ and IgD- B cells; NK cells; NKT cells; plasmablasts/plasma cells; helper and cytotoxic T cells;*

plasmacytoid dendritic cells (pDCs); CD11b^{+} and CD11b^{-} classical DCs (cDCs); neutrophils; Ly6C^{hi} monocytes, Ly6C^{hi}CD11b^{-} myeloid cells; and F4/80^{+} macrophages. N = 40 mice in overall model: count ~ animal cohort + strain + condition + strain:condition (see ***Methods****). Boxplots show absolute splenic cell counts corresponding to the data in the volcano plot. (C) Effects of stress on peripheral blood cytokines. Boxplots show the median and interquartile ranges for serum cytokines in each group (N = 13 homecage, N = 14 chronic stress). Groups were compared using rank-based linear models for the effects of experimental condition and mouse cohort with FDR-correction across the 10 tested cytokines. G-CSF levels were significantly increased (FDR P = 0.004) in stressed compared to homecage mice. There was a trend towards increased IL-6 (uncorrected P = 0.1) and IL-17A (uncorrected P = 0.1) in stress. (D) Effects of SD on splenic B cell subsets (median ± IQR; Mann-Whitney U test, **P < 0.01); N = 10 HC, N = 11 SD. (E) Stimulation of splenocytes from stressed and homecage animals by LPS, PMA and ionomycin: supernatant IL-10 measured by ELISA. IL-10 values were calculated based on a sigmoidal 4-parameter standard curve fit using Prism software. Linear models (log IL-10 ~ condition + batch) for stimulated samples showed an effect of stress on supernatant IL-10 (P=0.01). IL-10 was not detectable in the supernatant from unstimulated PBMCs. Each datapoint represents one animal, showing the average of 2-3 technical replicates. Data are debatched for the effect of cohort for visualization. Data from n=12 SD and n=11 HC animals assayed across 3 cohorts. (F) Effects of stress on regulatory B cells and T cells. Splenocytes were either stimulated with lipopolysaccharide (LPS), phorbol 12-myristate 13-acetate (PMA) and ionomycin (I) or unstimulated for 5 h. Linear models (IL-10 ~ condition + batch) for both stimulated and unstimulated samples showed an effect of stress on B cell intracellular IL-10 in both stimulated (P=0.004) and unstimulated (P=0.002) cells (n=16 CSD, n=16 HC animals) assayed across 4 cohorts. The effect of stress on intracellular T cell IL-10 was not significant in either stimulated (p=0.3) or unstimulated (p=0.96) cells (n=12 CSD, n=11 HC animals). Each datapoint corresponds to one animal and is the average of 2-3 technical replicates for that animal; **P<0.05. Datapoints are debatched for the effect of cohort for visualization, but statistics are performed on raw data. (G) Effects of SD on splenic cell subset functional marker expression, measured by percent positivity. Groups were compared using rank-based linear models for the effects of experimental condition and mouse cohort followed by FDR correction (for 27 marker percentages tested, only 3 comparisons shown here). (H) Effects of chronic stress on splenic cell subset functional marker expression, measured by median fluorescence intensity (MFI). Tile plots correspond to the two flow cytometry panels run for each mouse spleen. Tile colours indicate the linear model estimates (coefficients) for the effect of stress on MFI. Linear model includes condition (SD vs. HC) and animal cohort. Size of tile indicates significance following FDR adjustment for multiple comparisons in each panel (56 comparisons for myeloid panel, 35 comparisons for lymphoid panel). N = 10 HC, N = 11 SD. All replicates are biological.*

Psychosocial stress is associated with an increase in meningeal monocytes but a reduction in meningeal B lymphocytes

In addition to immune profiling in the spleen, we also investigated the effects of social stress on meningeal immune cells. Analysis of healthy mouse meninges by flow cytometry followed by either manual (**Figure 3-7A,B**) or automated gating (**Figure 3-7C,D**) of cell populations revealed heterogeneous immune cell populations, including innate and adaptive immune cells, as other investigators have described previously (Ajami et al 2018, Korin et al 2017). To ensure that intravascular immune cells were excluded, a CD45 antibody was administered immediately prior to tissue retrieval. B cells represented a major component of healthy mouse meninges, comprising ~25% of all immune cells (**Figure 3-7B**), consistent with recently published data (Brioschi et al 2021). Following SD, only two meningeal immune populations showed significant changes from their counts in homeostasis: B cells, which were significantly decreased, and Ly6C^{hi} monocytes, which were increased, with convergent results from the analysis of manually gated absolute counts (**Figure 3-7E**) and the analysis of automatically gated relative (proportional) cell counts (**Figure 3-7F**). There were trends towards increases in neutrophils and macrophages but no significant change in meningeal T cell counts (**Figure 3-7E,F**). In support of this finding, analysis of cell counts estimated by computational deconvolution of bulk meningeal transcriptomic data (microarray data) confirmed a reduction in naïve B cells and an increase in monocytes and neutrophils in meninges obtained from stressed mice (**Figure 3-8A**).

Of the residual meningeal B cells in SD mice, an increased proportion were activated in compared with meningeal B cells in control mice, as evidenced by expression of CD69 (**Figure 3-8B**), but there were insufficient cell numbers to perform ex vivo stimulation assays to measure IL-10 production. Meningeal Ly6C^{hi} monocytes, Ly6C^{int} monocytes, and macrophages also showed evidence of activation, with higher expression of CD11b (**Figure 3-8C**). Bulk transcriptomic analysis of stressed meninges confirmed that innate immune response pathway genes associated with myeloid cell activation, including 'TNF α signaling via NFkB' and 'IL6 Jak Stat3 signalling' were increased, as well as a number of other innate and adaptive immune pathways such as 'Complement, 'interferon γ response' and 'interferon α response' pathways (**Figure 3-8D**).

Within the stressed group, we observed a significant positive correlation between meningeal B cell numbers and the number of light-dark crosses (**Figure 3-8E**), showing that lower meningeal B cell numbers are associated with a more severe behavioural phenotype. Likewise, higher numbers of meningeal Ly6C^{hi} monocytes were associated with reduced distance travelled in a novel open-field arena (**Figure 3-8E**), indicative of reduced exploratory drive. Correlations between splenic and meningeal cell counts (assayed in the same animals) were minimal (**Figure 3-9**), arguing against the hypothesis that the meningeal cell counts simply reflect immune activity in the periphery. Together, these data demonstrate that social stress is associated with B cell activation in both the periphery and meninges, but that in contrast to the periphery, meningeal B cell numbers are markedly reduced in the context of stress.

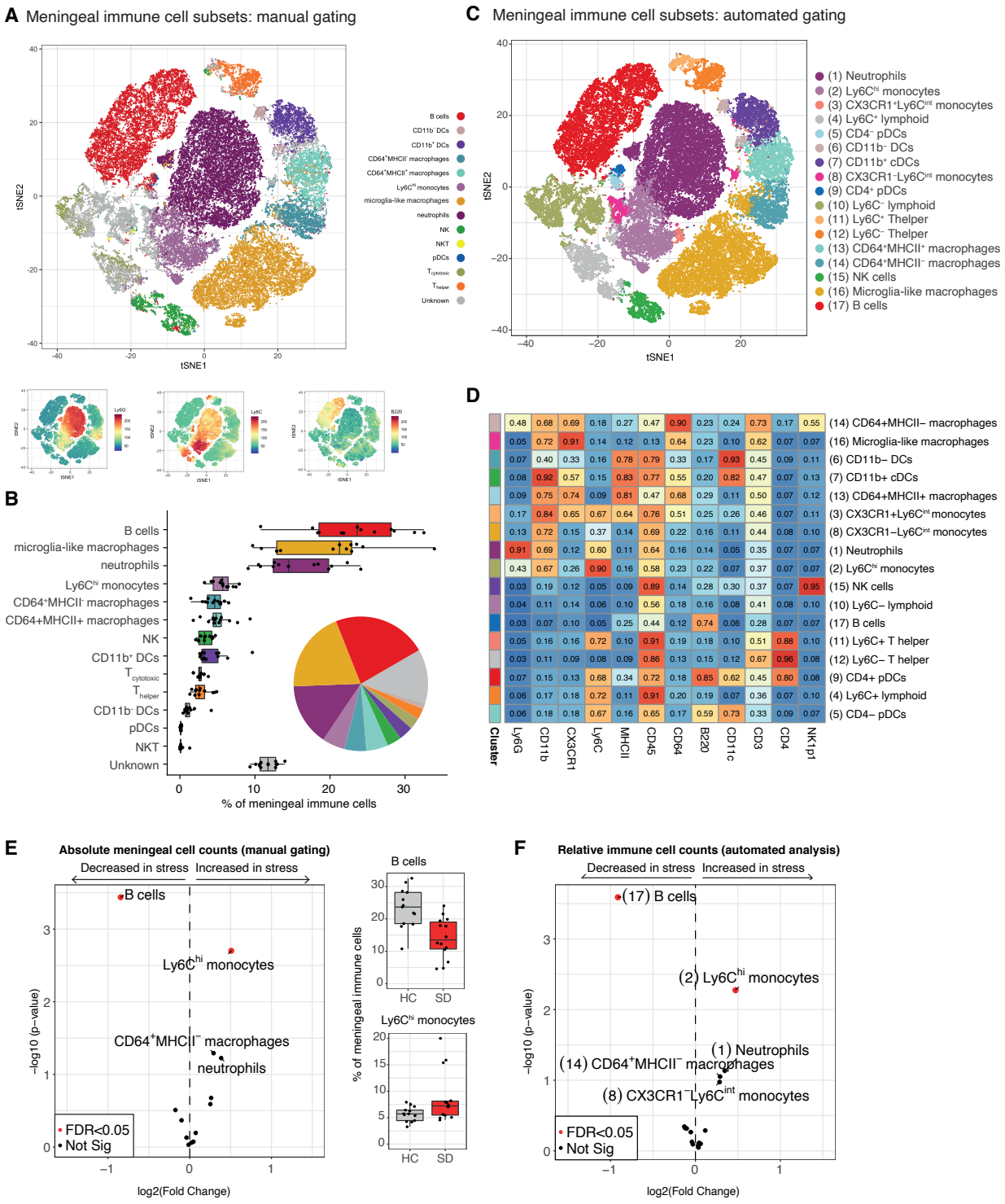


Figure 3-7 *Chronic stress is associated with an increase in meningeal myelomonocytic cells but a reduction in meningeal B lymphocytes.*

(A) Meningeal immune cell subsets (manually gated as per the gating strategy in ***Figure 3-3****) overlaid on a tSNE plot of downsampled cytometry data from the N = 53 mouse meninges used in the study. Insets show surface marker expression for B220, Ly6G and Ly6C overlaid on the tSNE plot. Intravascular immune cells were identified by intravenous CD45 labelling prior to harvest and excluded from analysis.*

(B) Relative proportions (median ± IQR) of extravascular immune cells identified in healthy meninges (manual gating). Pie chart indicates median proportion of each subset. N = 13 HC mice. (C) Automated clustering of meningeal flow cytometry data by flowSOM, visualized by tSNE; downsampled data from N = 53 mouse meninges, showing strong agreement with manual gating. (D) Annotation labels for automatically detected clusters were determined by surface marker expression profiles as shown. The colour in the heatmap represents the median of the 0-1 transformed marker expression calculated over cells from all the samples, varying from blue for lower expression to red for higher expression. The numbers indicate the actual expression values. (E) Volcano plot showing the effects of stress (negative binomial model) on absolute counts of meningeal extravascular immune cells (manual gating). N = 53 mice in overall model: count ~ animal cohort + strain + condition + strain:condition (see ***Methods****). The 13 cell subsets tested were Ly6C^{hi} monocytes; helper and cytotoxic T cells; CD11b^{+} and CD11b^{-} classical dendritic cells (cDCs); plasmacytoid DCs (pDCs); microglia-like macrophages; neutrophils; natural killer (NK) cells; NKT cells; CD64^{+}MHCII^{+} and CD64^{+}MHCII^{-} macrophages; and B cells. Each data point represents a cell subset, indicating the fold-change in that cell count in stress vs. homecage and the significance of that fold change. Cell subsets which are significantly different following correction for multiple comparisons are shown in red. Boxplots show percent of meningeal extravascular immune cells in wildtype home cage (N=13) and stressed (N=12) mice for the significant cell counts (B cells and inflammatory monocytes). (F) Volcano plot showing the effects of stress on relative counts of meningeal extravascular immune cell subsets (automated gating). Data show a significant increase in meningeal Ly6C^{hi} monocytes and a significant decrease in meningeal B cells in stress, equivalent to the results of the analysis using manually gated counts.*

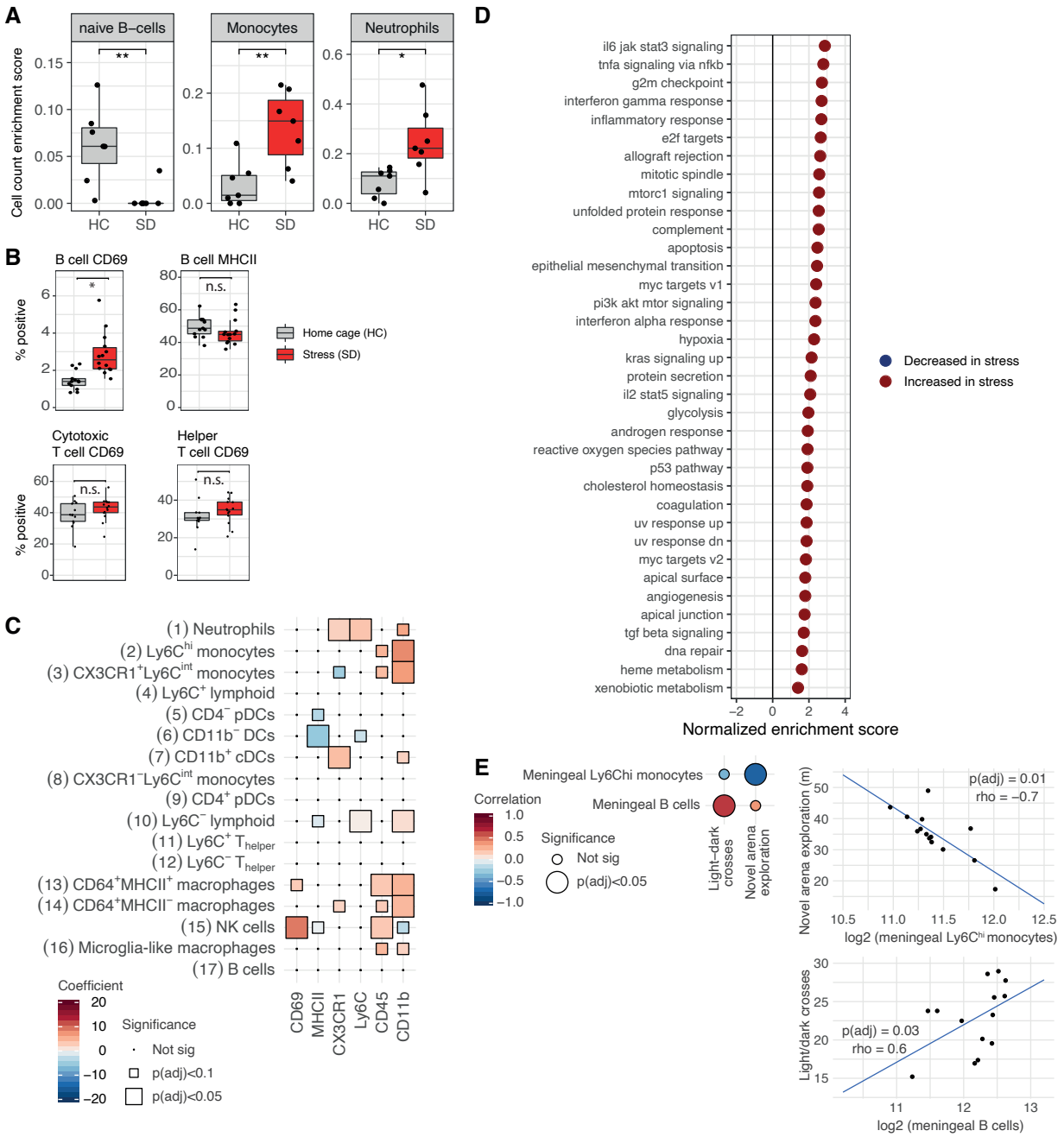


Figure 3-8 *Meningeal response to chronic stress.*

(A) Cell count enrichment scores (median ±IQR) from cellular deconvolution of meningeal microarray data support the flow cytometry results shown in ***Figure 3-7E,F.*** *Mann-Whitney U tests (unadjusted): *P < 0.05, **P < 0.01. N = 7 SD, N = 7 HC, biological replicates. (B) Effects of chronic stress on meningeal immune cell activation (manual gating for percent positivity). Rank-based linear models including condition and cohort. P-values corrected for 7 comparisons tested (4 shown), FDR P * < 0.05, ** < 0.01. (C) Effects of chronic stress on meningeal median functional marker expression (for* ***Figure 3-7C*** *clusters). Tile colours indicate linear model estimates (coefficients) for the effect of stress (model includes condition and cohort). Tile size indicates significance following FDR adjustment (102 comparisons). (D) Gene set enrichment analysis of bulk meningeal microarray data (same mice as* ***Figure 3-8A****): Hallmark gene sets enriched at FDR P < 0.05 in stressed (N=7) compared to control (N=7)*

meninges. (E) Spearman correlations between behavioural susceptibility to stress and immunophenotype in stressed wild-type animals. Correlations significant at FDR P < 0.05 are shown in inset. All replicates are biological.

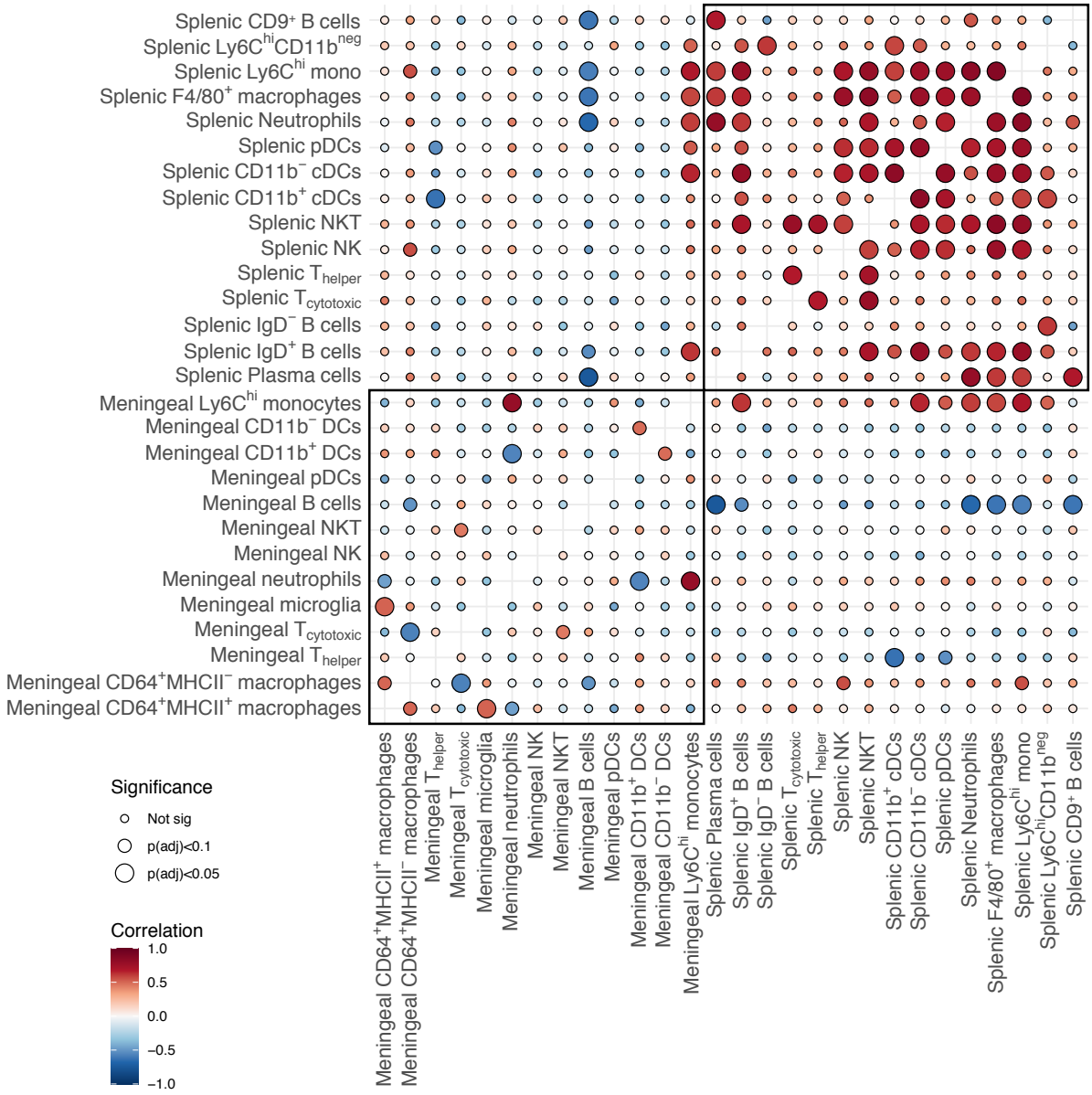
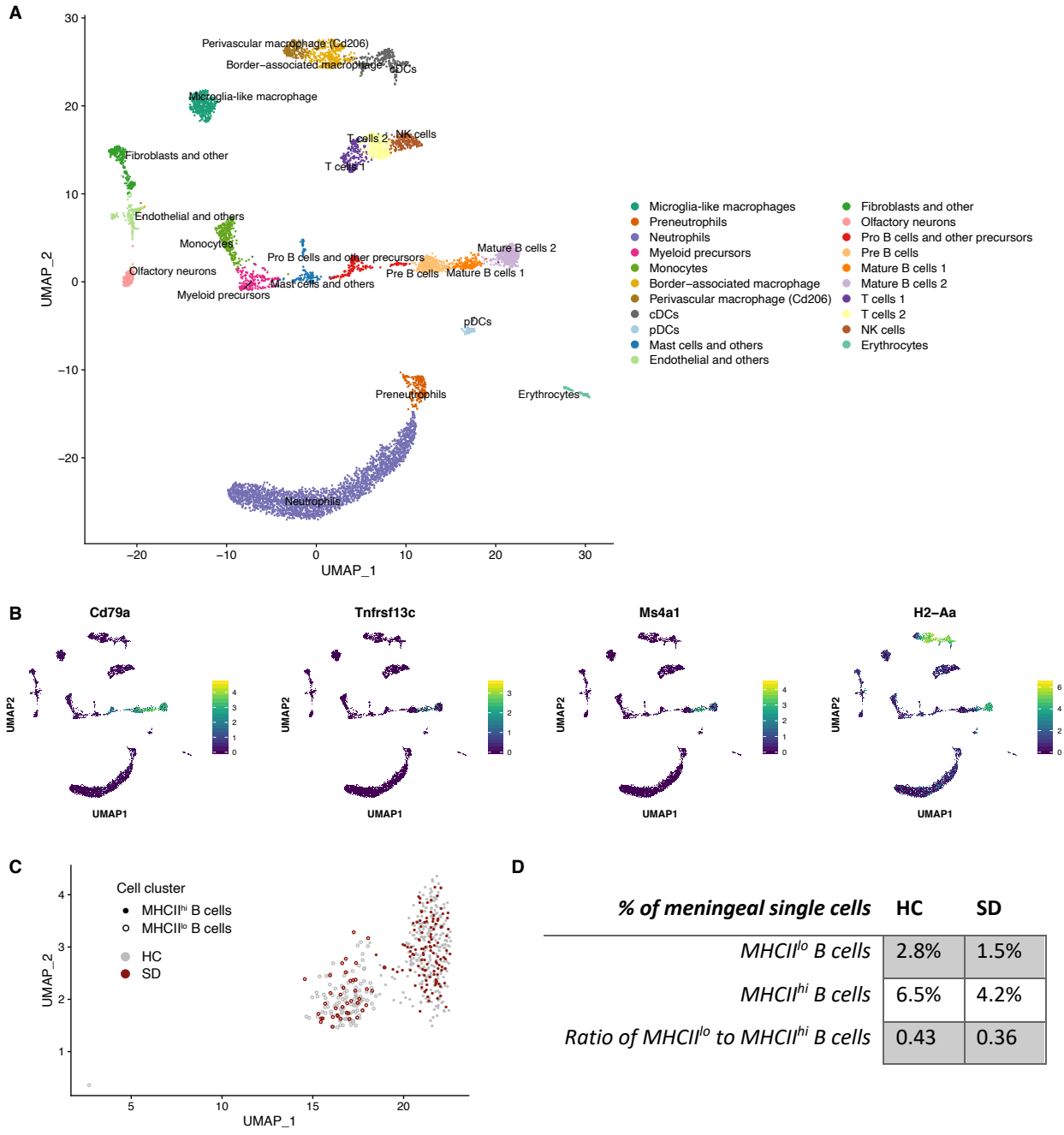


Figure 3-9 *Correlations between meningeal and splenic immune cell subsets.*
Spearman correlations between splenic and meningeal immune cell counts in wild-type animals. Blue indicates a positive correlation and red a negative correlation; size of circle indicates significance of the correlation. N = 27 for meningeal counts; N = 21 for splenic counts; includes both SD and HC animals.

Meningeal B cell response to stress

To better characterize B cell responses to stress, we used droplet encapsulation high throughput single-cell RNA sequencing (scRNAseq) (10x Genomics platform) to profile meningeal immune cells from non-stressed and stressed mice **Figure 3-10A**. Of the 21 clusters that were annotated based on their expression of canonical marker genes, we found two mature B cell clusters – an $MHCII^{lo}$ cluster (N = 150 cells) and an $MHCII^{hi}$ cluster (N = 369 cells) (**Figure 3-10B**), that were present in similar proportions in control and stressed meninges (**Figure 3-10C,D**).



% of meningeal single cells	HC	SD
MHCII^{lo} B cells	2.8%	1.5%
MHCII^{hi} B cells	6.5%	4.2%
Ratio of MHCII^{lo} to MHCII^{hi} B cells	0.43	0.36

Figure 3-10 *Single cell RNA sequencing of meningeal immune cells: clustering, identification of B cells and cluster abundances in stressed and unstressed animals.*

(A) Leiden clustering of quality controlled single cells (N = 6694) cells from N = 8 control mice and N = 4 chronically stressed mice overlaid on UMAP representation. (B) Expression of Cd79a (part of B cell receptor complex); Tnfrsf13c (BAFF receptor); Ms4a1 (CD20) and H2-Aa (MHCII gene) overlaid on the UMAP projection highlights the B cell clusters. The cluster "Mature B cells 1" (N = 150 cells) has low expression of MHCII genes (H2-Aa) and is referred to as the MHCII^{lo} B cell cluster while "Mature B cells 2" (N = 369 cells) is characterized by higher expression of MHCII genes and is referred to as the MHCII^{hi} B cell cluster. (C) UMAP projection of cells from the two mature B cell clusters (MHCII^{hi} B cells and MHCII^{lo} B cells). (D) Proportion of B cell subsets in SD (social defeat) vs. home cage (HC) mice. Both MHCII^{lo} and

MHCII^{hi} B cells are decreased in stressed (SD) vs. home cage (HC) mice as a percentage of total single cells, but the relative proportion of cells in each of the two B cells subsets is not altered in stressed vs. HC mice (Fisher's exact test; P = 0.3).

Differential gene expression analysis showed an increase in several innate immune response genes in the MHCII^{hi} B cell cluster in stressed meninges, including *Ngp*, *Lcn2*, *Camp*, and *S100a9* (**Figure 3-11A,B**). The proteins encoded by these genes have direct antimicrobial effects, but they can also regulate inflammation and myeloid cell activation, both positively and negatively. For example, *Ngp* encodes a cysteine protease that inhibits cathepsin (Boutte et al 2011), whilst Lipocalin 2, the protein encoded by *Lcn2*, promotes an anti-inflammatory M2 macrophage phenotype (Guo et al 2014) but can also promote M1 microglial activation (Jang et al 2013). *Camp* inhibits macrophage pro-inflammatory cytokine production (Torres-Juarez et al 2015) as well as IFN-γ-mediated activation of monocytes, macrophages, and DCs (Nijnik et al 2009). *S100a9* is an alarmin that can mediate anti-inflammatory innate immune cell re-programming (Ulas et al 2017) and promote the generation of myeloid suppressor cells (Dai et al 2017). The most downregulated gene in stressed B cells was *Cst3*, which encodes Cystatin-C, a cysteine protease inhibitor that can modulate neutrophil chemotaxis (Leung-Tack et al 1990). These data show that meningeal B cells undergo significant transcriptional changes during stress, with induction of innate immune transcriptional programs and the production of anti-microbial peptides that enable cross-talk with meningeal myeloid cells, with the potential to both activate and regulate these cells.

Gene set enrichment analysis (GSEA) of the meningeal scRNAseq B cell datasets showed an enrichment of Reactome pathways involved in mRNA quality control (particularly nonsense-mediated decay), ribosomal function and mRNA translation in stress (**Figure 3-11C**), suggesting that these meningeal B cells are geared towards increased protein production. There were no genes that were differentially expressed between SD and HC mice in the MHCII^{lo} B cell cluster in a significant manner, perhaps due to the lower number of cells in this cluster (and hence lower power to detect differential expression). B cell cytokine/chemokine and receptor genes (including *Il6*, *Tnf*, *Il10*, *Csf2*) were not generally sufficiently expressed for differential expression analysis (a common problem with the limited depth of sequencing generated by 10x Chromium methodology, see **Figure 3-11D**).

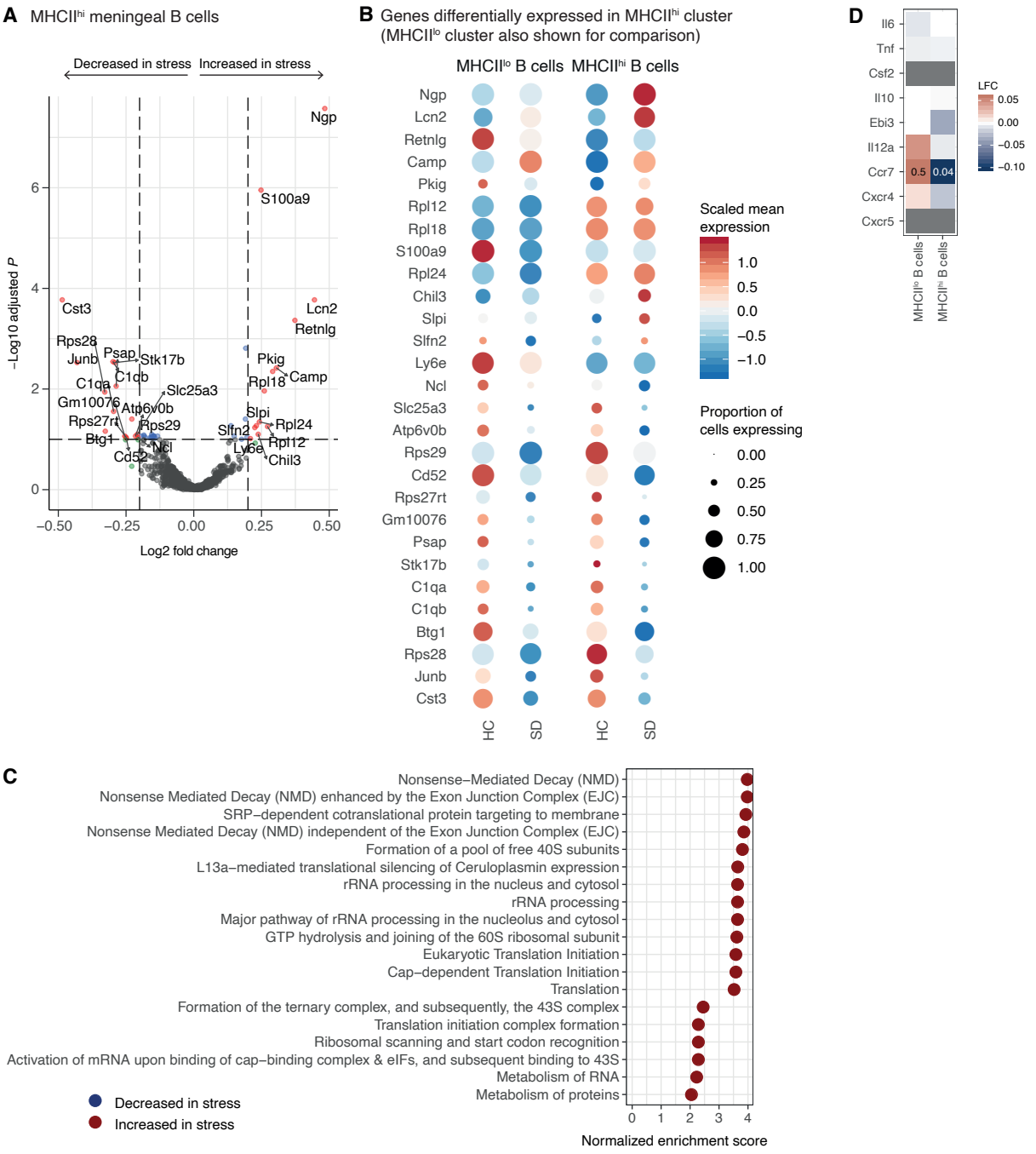


Figure 3-11 *Single cell RNA sequencing of meningeal immune cells: transcriptomic responses of meningeal B cells to stress.*

(A) Single cell differential expression analysis for cells in the MHCII^{hi} B cell cluster. N = 369 cells from N = 8 HC and N = 4 SD mice. Differential expression was tested by Mann-Whitney U with Benjamini-Hochberg correction for multiple comparisons. Dashed cut-off lines indicate FDR P < 0.1 and log fold change > 0.2. No genes were differentially expressed in the MHCII^{lo} B cell cluster. (B) Effect of stress on gene expression in B cells. Plot shows genes differentially expressed at FDR P < 0.1 and absolute log fold change (LFC) > 0.2 in MHCII^{hi} B cell cluster of single cells (as shown in volcano plot). Expression values are also shown for the MHCII^{lo} B cell cluster but no genes were significantly differentially expressed in

this cluster. Circle colour indicates row-scaled mean gene expression. Circle size indicates the proportion of single cells in the cluster expressing the gene. (C) Single cell analysis: Reactome pathways enriched in the MHCII^{hi} cluster of meningeal B cells in SD compared to HC mice at FDR P < 0.05. N = 369 cells. No pathways were enriched in the MHCII^{lo} B cell cluster. (D) Single cell analysis: plot shows differential expression of B cell cytokines and chemokines, and key B cell receptors, in meningeal B cells. Colour indicates log fold change of gene expression in SD vs HC mice in B cell clusters. Dark grey indicates no gene expression detected. Tile annotations indicate the FDR P-value for differential expression. Where no P-value is shown (i.e., all genes except Ccr7), gene expression was insufficient to test differential expression (criterion for testing: gene expressed in > 15% of cells in the cluster).

Immune cells localise to tissue niches via chemokine-chemokine receptor interactions, for example the CXCL13-CXCR5 axis plays a central role in localizing peritoneal B1 cells, a predominantly tissue-resident B cell subset that is enriched for natural antibody production and regulatory B cells (Ansel et al 2002). In addition, cytokines such as B cell activating factor of the TNF receptor family (BAFF) are required for B cell survival in lymphoid organs and the peritoneal cavity (Mackay et al 2003). We therefore considered several explanations for the reduced number of B cells observed in the meninges of stressed mice; firstly, disruption of niche-localizing chemokine-chemokine receptor interactions leading to B cell migration out of the meninges, and secondly, reduced meningeal B cell survival due to decreased expression of survival factors within the niche or activation-induced cell death. To address the question of whether there was disruption of the chemokine or cytokine cues within the meninges, we assessed bulk microarray data obtained from meninges of SD or HC mice. Following SD, there was no significant reduction in *Cxcl13* or *Tnfsf13b* (the gene encoding BAFF); in fact the trend was towards an increased expression of these genes ($P < 0.05$ uncorrected for multiple comparisons, **Figure 3-12A**). These data suggest that the reduction in meningeal B cells observed in defeated mice was not due to a loss of known tissue 'niche' factors. There was a trend towards decreased *Ccr7* expression in the larger MHCII^{hi} B cell cluster in SD (Uncorrected $P = 0.04$, **Figure 3-11D**). Normally, B cell CCR7 promotes homing of B cells to lymph nodes, so this result goes against the hypothesis that the observed reduction in B cells was because of enhanced migration out of the meninges to draining lymph nodes.

Another possible explanation for reduced B cell numbers would be cell loss due to death, so we also examined the scRNAseq data for evidence that meningeal B cells may be undergoing

increased cell death during SD. We did not find any increase in cell stress-associated genes (**Figure 3-12B**), apoptosis or necrosis Reactome pathways in meningeal B cells in SD, nor was there an increased proportion of mitochondrial reads per cell, as would be expected in dying cells (**Figure 3-12C**). Additionally, stress was not associated with changes in the proportion of B cells estimated to be in each cell cycle phase (**Figure 3-12D**), suggesting the altered number of B cells is not due to suppression of local proliferation.

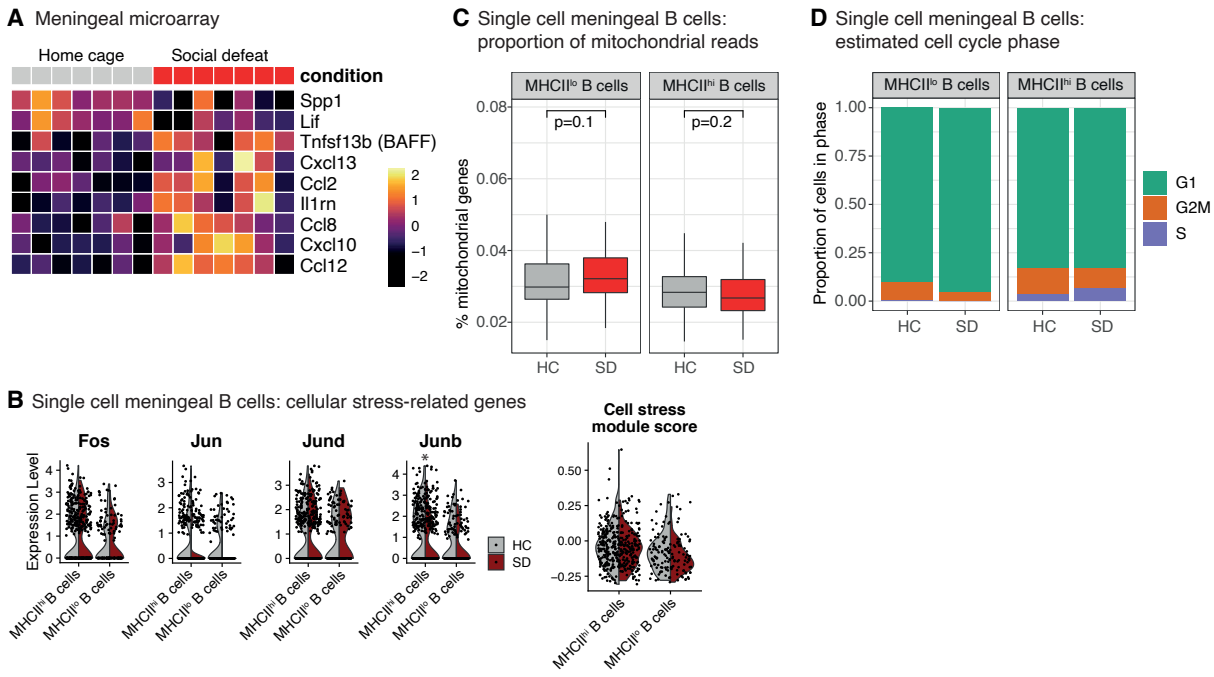


Figure 3-12 *Meningeal response to stress: B cell cytokines, cell cycle phases, and indicators of cellular stress in B cells.*

(A) B cell cytokines and chemokines differentially expressed in chronically stressed vs. control meninges (bulk microarray) at unadjusted P < 0.05. N = 7 HC, N = 7 SD; same mice as ***Figure 3-8A****. Genes for B cell cytokines BAFF (Tnfsf13b) and Cxcl13 were not reduced and showed a trend towards increased expression in stress. No cytokines or chemokines were differentially expressed following FDR adjustment. (B) Violin plots show the expression of selected genes related to cellular stress. Expression of Junb is significantly decreased in SD compared to HC (FDR P = 0.003). Right hand panel shows the effects of SD on the summary Seurat module score comprised of 20 cellular stress-related genes. There was no significant difference between HC and SD cells. (C) Boxplots show the percentage of mitochondrial reads per cell (median ± interquartile range) in MHCII^{lo} and MHCII^{hi} B cells. P-values show the results of a Mann Whitney U test for the effects of condition on the percentage of mitochondrial reads per cell. (D) Barplots show the fraction of cells in the MHCII^{lo} and MHCII^{hi} B cell clusters estimated to be in each cell cycle stage (using [R] package cyclone). There were no significant differences between conditions (HC = home cage; SD = social defeat) in the proportion of cells in each cell cycle stage (Fisher's exact test: P = 0.6 for MHCII^{lo} B cells, P = 0.3 for MHCII^{hi} B cells).*

Peripheral B cell deficiency results in an increase in baseline meningeal neutrophil number and in monocyte activation

To determine whether abnormalities in the B cell compartment might causatively affect meningeal myeloid cell activation and infiltration, we investigated *Cd19*$^{-/-}$ mice. *Cd19* encodes for a co-activating molecule expressed by B cells, and its deficiency results in mice with a reduced number of peripheral B cells. B1a cells, which include regulatory B cells, are particularly affected in this model (Engel et al 1995, Rickert et al 1995). *Cd19*$^{-/-}$ mice have been widely used to interrogate the potential immunoregulatory role of B cells (via secretion of IL-10), in contexts such as experimental autoimmune encephalitis and collagen-induced arthritis (Matsushita et al 2008, Yanaba et al 2008, Yoshizaki et al 2012). The *Cd19*$^{-/-}$ model of B cell deficiency lacks the more substantial effects on antibody secretion and other B cell effector functions that would be seen with antibody or genetic depletion of B cells. Analysis of the spleen in *Cd19*$^{-/-}$ mice confirmed a reduction in the total number of B cells (**Figure 3-13A**), including IgD^{+} (naïve) and IgD^{-} subsets, as well as a reduction in CD9^{+} B cells (a subset enriched for regulatory B cells) and plasma cells (**Figure 3-13A, B**). However, although there was a trend towards a reduction in meningeal B cells in *Cd19*$^{-/-}$ mice compared with their wildtype (WT) C57BL/6 counterparts, this did not reach statistical significance (**Figure 3-13A**). This model thus allowed us to assess the effects of a decrease in peripheral B cells, including regulatory B cells, on meningeal immunity in health and following social defeat. The lack of decrease in meningeal B cells also suggests that at least part of the meningeal B cell compartment is in disequilibrium with the peripheral B cell pool, a characteristic associated with a long-term tissue-resident cell phenotype (Masopust & Soerens 2019).

Unstressed *Cd19*$^{-/-}$ mice mirrored some, but not all, aspects of the peripheral immune activation we observed in stressed mice, with an increase in splenic neutrophils (**Figure 3-13B**), and in MHCII expression on splenic DCs (**Figure 3-13C**), as well as increased plasma G-CSF (**Figure 3-13D**). The residual splenic B cells in *Cd19*$^{-/-}$ mice also showed a more activated phenotype, with increased expression of CD25 and MHCII (**Figure 3-13C**). In the meninges, we observed an increase in neutrophils and cytotoxic T cells in *Cd19*$^{-/-}$ mice (**Figure 3-13E**) as well as higher expression of CD69 and CD11b on meningeal macrophages and monocytes, consistent with a more activated phenotype within the meningeal myeloid compartment (**Figure 3-13F**).

We next tested for behavioural differences between *Cd19*$^{-/-}$ and WT mice at baseline and following SD. At baseline, *Cd19*$^{-/-}$ mice showed reduced exploration of a novel environment compared with WT controls (**Figure 3-13G**). Following SD, there was no difference between *Cd19*$^{-/-}$ and WT animals on novel arena exploration or light dark testing (**Figure 3-13G**). Meningeal monocyte numbers significantly increased in WT animals following SD, but in *Cd19*$^{-/-}$ animals, basal monocyte numbers were already at an equivalent level to that observed in stressed WT animals and did not show a further significant increase (**Figure 3-13H**). Bulk RNA sequencing of meninges obtained from mice subjected to SD demonstrated an increase in 'interferon α response' and 'interferon γ response' pathways in *Cd19*$^{-/-}$ mice compared to WT counterparts (**Figure 3-13I**), pathways that we had also found to be increased by SD in the meninges in WT animals, suggesting convergent effects of psychological stress and CD19 deficiency on meningeal interferon signalling. We thus demonstrate that B cells play a role in negatively regulating meningeal myeloid cell activation in homeostasis and in the context of social defeat.

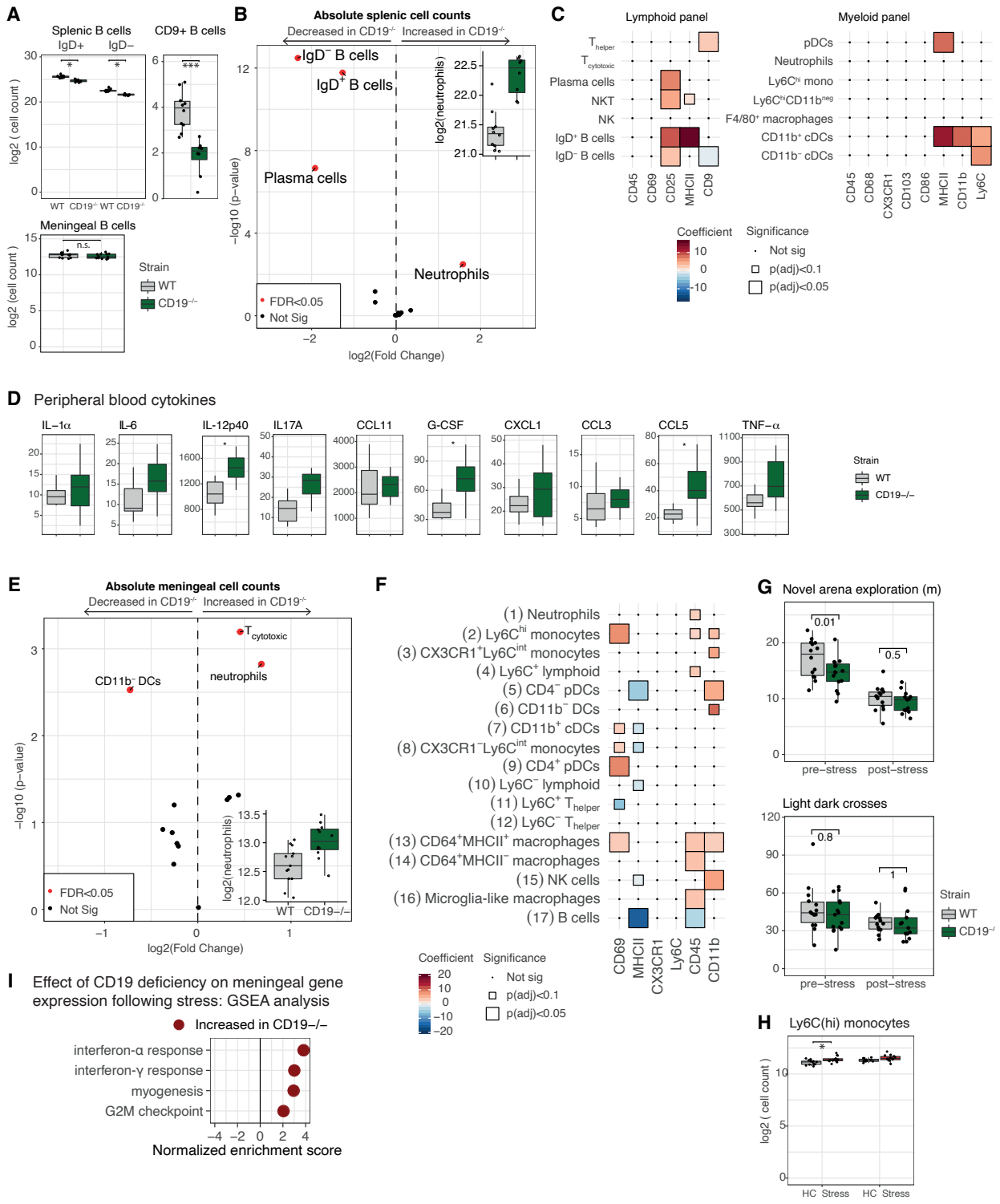


Figure 3-13 *Peripheral B cell deficiency results in an increase in baseline meningeal neutrophil number and in monocyte activation.*

(A) Effects of CD19 deficiency on B cell subsets (median ± IQR). Statistics for IgD+/- B cells are as per negative binomial model in ***Figure 3-13B****. CD9+ B cells: Mann-Whitney U test ***P < 0.001. Splenic data: N = 10 WT, N = 8 Cd19$^{-/-}$. Meningeal data: N = 13 WT, N = 12 Cd19$^{-/-}$. (B) Effects of CD19 deficiency (negative binomial model) on splenic immune cell counts. N = 40 mice in overall model (see Methods); 14 cell subsets tested: IgD+ and IgD- B cells; NK cells; NKT cells; plasmablasts/plasma cells; helper and*

*cytotoxic T cells; plasmacytoid dendritic cells (pDCs); CD11b+ and CD11b- classical DCs (cDCs); neutrophils; Ly6C^{hi} monocytes, Ly6C^{hi}CD11b^{neg} myeloid cells; and F4/80+ macrophages. (C) Effects of CD19 deficiency on splenic immune cell activation. Tile colours indicate linear model estimates (coefficients) for the effect of CD19 deficiency on MFI (model includes strain and cohort). Size of tile indicates significance following FDR adjustment for multiple comparisons in each flow panel (56 comparisons for myeloid panel, 35 comparisons for lymphoid panel). N = 10 WT, N = 8 Cd19$^{-/-}$ mice. (D) Effects of CD19 deficiency on peripheral blood cytokines. Boxplots show the median and interquartile ranges for serum cytokines in each group (N = 13 wildtype, N = 12 Cd19$^{-/-}$). Groups were compared using rank-based linear models for the effects of strain and mouse cohort followed by Benjamini-Hochberg correction for multiple comparisons across the 10 tested cytokines. *FDR P < 0.05. (E) Effects of CD19 deficiency (negative binomial model) on absolute counts of 13 meningeal extravascular immune cell subsets: Ly6C^{hi} monocytes; helper and cytotoxic T cells; CD11b^{+} and CD11b^{-} classical dendritic cells (cDCs); plasmacytoid DCs (pDCs); microglia-like macrophages; neutrophils; natural killer (NK) cells; NKT cells; CD64^{+}MHCII^{+} and CD64^{+}MHCII^{-} macrophages; and B cells. N = 53 mice in overall model. (F) Effects of CD19 deficiency on meningeal median functional marker expression (MFI). N = 13 WT, N = 12 Cd19$^{-/-}$. (G) Baseline and post-stress behaviour in WT (N = 14 pre- and post-stress) and Cd19$^{-/-}$ mice (N = 15 pre-stress; N=13 post-stress); littermate controls. Linear model including strain and cohort at each time point; brackets indicate significance of strain effect *P < 0.05. (H) SD-induced changes in meningeal monocytes in wild type and Cd19$^{-/-}$ mice, presented side-by-side for comparison. *FDR P < 0.05. (I) Positively enriched Hallmark gene sets in stressed Cd19$^{-/-}$ compared to stressed WT meninges (bulk RNAseq data) at FDR P < 0.05 (N = 5 WT; N = 4 Cd19$^{-/-}$).*

3.5 Discussion

Overall, the work described in this chapter shows that in the peripheral B cell compartment, social stress is associated with activation of B cells and an increase in (IL-10-producing) regulatory B cells and terminally differentiated B cells (plasmablasts/plasma cells) in the spleen. In the meninges, social stress was likewise associated with activation of local tissue B cells, and these meningeal B cells showed increased expression of genes encoding anti-microbial peptides with known immunomodulatory effects on myeloid cells.

The potential importance of the immunoregulatory effects of B cells on meningeal myeloid cells was demonstrated by the increase in meningeal neutrophils observed in unstressed *Cd19*$^{-/-}$ compared to unstressed WT mice, as well as the increased expression of activation markers such as CD69 and CD11b on meningeal monocytes and macrophages. Furthermore, compared with WT mice, CD19-deficient mice showed enrichment of IFN-γ response genes in the meninges following social stress, confirming the importance of B cells in regulating the effects of IFN-γ in the meninges. This could potentially occur via the increased B cell *Camp* expression demonstrated in the scRNAseq data following stress (Nijnik et al 2009). IFN-γ is a potent myeloid cell activator and has previously been implicated in the link between the immune system and behaviour, with evidence for both beneficial and detrimental effects. In animals, IFN-γ can activate the choroid plexus to promote CNS immune cell recruitment following injury (Kunis et al 2013). IFN-γ can also increase hippocampal neurogenesis, improve learning (Baron et al 2008), and modulate GABAergic neurotransmission to support social behaviour (Filiano et al 2016). Conversely, IFN-γ can mediate increased synaptic pruning in infection-associated inflammation (French et al 2019). Furthermore, in a murine chronic stress model, IFN-γ deficiency led to reduced corticosterone, cytokine, and behavioural responses to stress (Litteljohn et al 2010). In humans, increased plasma IFN-γ has been associated with stress-induced and generalized anxiety (Hou et al 2017, Maes et al 1998). Our data demonstrating the capacity of B cells to control IFN-γ signalling in the meninges thus suggest an important, clinically relevant facet of B cell function.

Other mechanisms by which B cells might modulate myeloid cells include the production of the regulatory cytokine IL-10. We observed an increase in splenic B cell IL10 production in stressed animals, however *Il10* expression in meningeal B cells was not sufficient to allow its detection using 10X Chromium scRNAseq (which provides relatively shallow sequencing depth). The

limited number of meningeal B cells present also meant that we were unable to assay the effects of stress on meningeal B cell IL-10 protein production. However, it may be that meningeal B cells also produce IL-10 as well as immunoregulatory anti-microbial peptides. The phenotype and contribution of crawling/intravascular meningeal B cells also remains an interesting question.

We have not identified the cause of the changes in B cells observed following stress, but there are several potential explanations for these findings. If there is continuous re-circulation between the meningeal and peripheral B cell compartments, it could be that stress increases B cell migration from the meninges to the circulation or deep cervical lymph nodes, without a corresponding increase in recruitment of B cells from the blood or skull bone marrow into the meninges. Stress has previously been shown to suppress recruitment of regulatory T cells to the brain via the choroid plexus gateway (Kertser et al 2019). Alternatively, it may be that changes in the peripheral and meningeal B cell compartment are not directly linked, and that meningeal B cells either die, or enter the brain in the context of stress, and that independent of this, peripheral B cells become activated, proliferate, and terminally differentiate. Indeed, while there were no transcriptional hallmarks of apoptosis present in the meningeal scRNAseq data set, the chronic nature of the stress means that meningeal B cell death could have occurred in the acute phase of the paradigm and be undetectable at the time point analysed. The baseline phenotype of the *Cd19*$^{-/-}$ mice sheds some light on this question; the fact that the marked reduction in peripheral B cell numbers observed in this strain is not mirrored by a similar reduction in meningeal B cells is instructive, suggesting that only a portion of the meningeal compartment is in continuity with the circulating B cell compartment. Therefore, the changes in meningeal B cells may represent a combination of these effects.

To conclude, we found, as summarized in **Figure 3-14**, that psychosocial stress was associated with changes in both the peripheral and meningeal B cell compartments, with activation and expansion of some peripheral B cell subsets and activation and contraction of meningeal B cells. While some of these changes may contribute to symptoms, other changes may be beneficial for tissue repair and restoration of the normal homeostatic state. Overall, these data suggest that B cells may affect behaviour by regulating meningeal myeloid cell activation and meningeal interferon responses, shedding light on the cellular networks at play in the immunological response to stress.

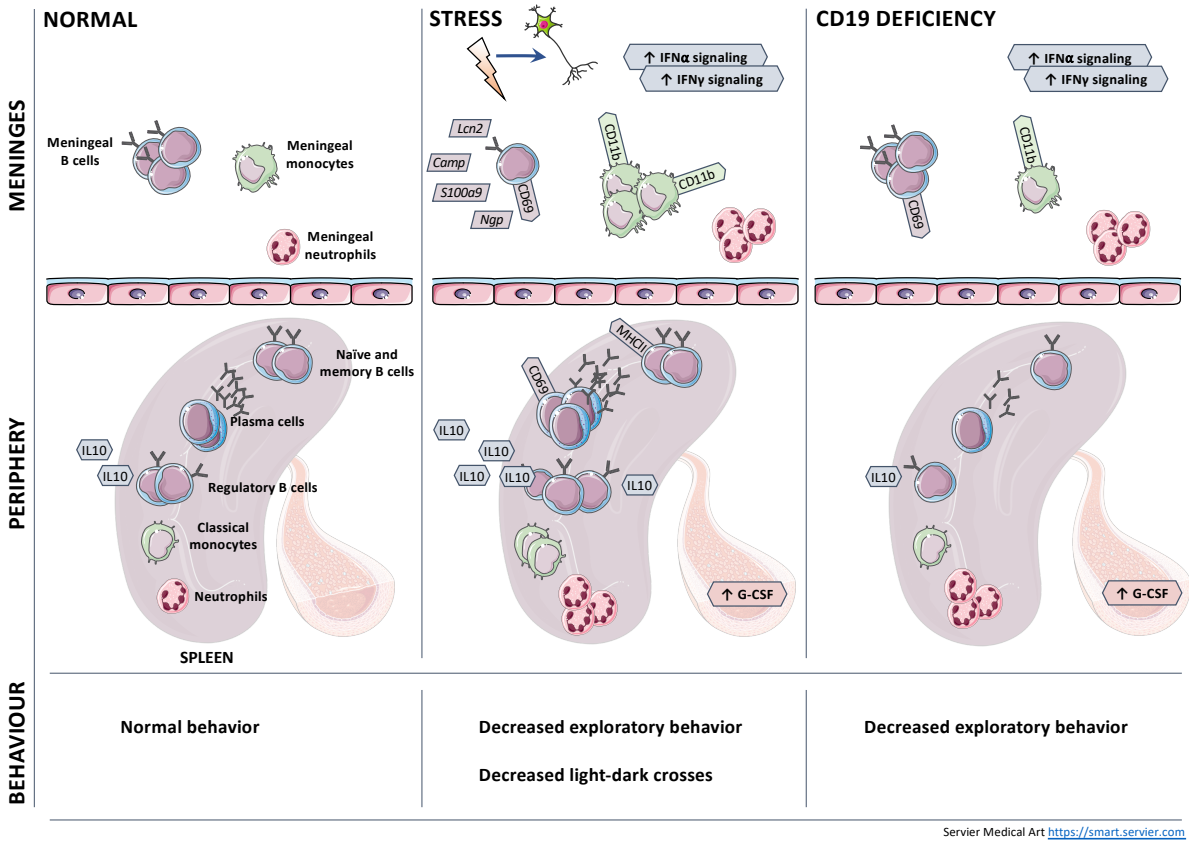


Figure 3-14 *B-cells are abnormal in psychosocial stress and regulate meningeal myeloid cell activation. In stressed mice (middle panel), in the periphery, we observed increased numbers of classical monocytes, neutrophils, plasma cells and regulatory B cells compared to unstressed mice (left hand panel). Stressed mice also showed increased plasma cell activation; naïve and memory B cell activation, and increased plasma G-CSF. In meninges from stressed mice, we observed increased numbers of monocytes and neutrophils; increased activation of meningeal myeloid cells; decreased numbers of B cells, which were activated and had increased expression of antimicrobial peptides; and increased interferon-α and interferon-γ signalling. These immunological changes were associated with decreased exploratory behaviour in a novel arena and anxious behaviour on light-dark box testing. In CD19 deficiency (right hand panel), in the periphery, we observed increased neutrophils; decreased naïve and memory B cells; decreased regulatory B cells; decreased plasma cells; and increased plasma G-CSF. In meninges from Cd19$^{-/-}$ mice, we observed increased meningeal B cell activation; increased meningeal myeloid cell activation; increased meningeal neutrophils; and increased interferon-α and interferon-γ signalling. These changes were associated with decreased exploratory behaviour. Parts of the figure were drawn by using pictures from Servier Medical Art, which is licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).*

Chapter 4: Genome-wide association studies of psychiatric disorders: implications for cellular immunity

4.1 Introduction

As discussed in **Chapter 1**, it is conceivable that the immune system could be implicated in the pathogenesis of multiple psychiatric disorders. Since germline genetic variants cannot be the consequence of disease, sequence variation associated with a disorder (or disorders) could shed light on the immune processes or cells likely to cause mental health symptoms. There is already some genetic evidence that a component of psychiatric risk is mediated by the immune system. Polygenic risk scores (PRS) for depression, bipolar disorder and schizophrenia are associated with increased lymphocyte counts (Sewell et al 2020); mendelian randomization studies support a causal role for IL-6 in both depression and schizophrenia (Perry et al 2021); and there are genetic correlations between immune disorders and multiple psychiatric disorders (Pouget et al 2019, Tylee et al 2018) (see **Section 1.4.4** for a fuller discussion of the genetic evidence for immune involvement in MDD). A pathway analysis of genes trans-diagnostically associated with schizophrenia, bipolar disorder and MDD has implicated neuronal, histone and immune pathways (Network & Pathway Analysis Subgroup of Psychiatric Genomics 2015); although a larger trans-diagnostic analysis did not implicate immune cells or pathways (Cross-Disorder Group of the Psychiatric Genomics Consortium 2019).

Most genetic variants associated with psychiatric risk are in non-coding regions of the genome, likely exerting their effects by altering the activity of regulatory elements (Gusev et al 2014) such as promoters or enhancers. Enhancers can be linearly distant (>10 kilobases) from the genes they regulate (Won et al 2016). Some regulatory elements control gene expression in multiple tissues, but others are specific to particular tissues, or particular cell states. For example, some enhancers are active in stimulated but not resting immune cells (Alasoo et al 2018, Chow et al 2014, Soskic et al 2019). The locations and activity status of putative enhancers and promoters in a given tissue can be identified through characteristic epigenetic modifications, such as histone modifications.

Epigenetic mechanisms have long been thought to be important in psychiatry, especially in mediating gene-environment interactions (Klengel & Binder 2015, O'Donnell & Meaney 2020). Epigenetic data from brain tissues have been extensively used to investigate the brain cell types and regions implicated by psychiatric risk variants (Girdhar et al 2018, Hauberg et al 2020, Li et al 2018a), by testing whether risk variants tend to be concentrated, or "enriched", in regions of the genome that are active in a given tissue. However, the enrichment of psychiatric risk variants in immune cell subsets has not been extensively explored. Studies to date have tended to use functional information from whole blood or immune organs, which obscures and dilutes possible effects in the myeloid and lymphoid immune cell subsets comprising these samples. There is some evidence of enrichment of risk variants for bipolar disorder in genes characteristic of neutrophils, T cells and haematopoietic stem cells; and for schizophrenia at genes in T cells and chromatin marks in T and B cells (Finucane et al 2018). To our knowledge, no studies have demonstrated enrichment of trans-diagnostic risk, or of cis-diagnostic risk for MDD or ASD, in any immune cell type (Alonso-Gonzalez et al 2019, Cross-Disorder Group of the Psychiatric Genomics Consortium 2019, Howard et al 2019, Stahl et al 2019), or tested whether immune cell enrichment is independent of brain tissue enrichment (rather than simply due to coincidental overlap of active genomic regions in brain and immune system cells).

4.2 Hypotheses

We hypothesize that some genetic risk variants for psychiatric disorders act via their effects on regulatory elements in specific immune cell subsets, thus potentially modulating the response of these cells to infections and other environmental stimuli. We further hypothesize that some of these immunogenetic mechanisms may represent a common pathogenic pathway to multiple psychiatric disorders.

To test these hypotheses, we integrated data on common genetic variants associated with trans- and cis-diagnostic risks for psychiatric disorder(s) with data on epigenetically active genomic regions in multiple human cell and tissue types. More formally, we tested the null hypothesis that a given set of risk variants was not co-located with tissue-specific marks of epigenetic activation more frequently than expected by chance in each of multiple tissues (Roadmap/ENCODE (Consortium 2012, Roadmap Epigenomics et al 2015)), in 19 sorted

immune cell subsets (BLUEPRINT (Chen et al 2016)), and in *ex vivo* stimulated naïve and memory $CD4^+$ T cells and macrophages (Soskic dataset (Soskic et al 2019)). To contextualise these results, we conducted parallel analyses of three "positive control" disorders: Alzheimer's disease, a brain disorder for which genetic risk has been associated with myeloid immune cells (Novikova et al 2021); rheumatoid arthritis, a canonical adaptive autoimmune disorder; and body mass index (BMI), a common comorbidity which may contribute to observed immune abnormalities in psychiatric disorders (McLaughlin et al 2021). To our knowledge, this is the first in-depth investigation of the immunological implications of GWAS variants conferring risk for psychiatric disorders.

4.3 Methods

Trans- and cis-diagnostic genetic risk variants for psychiatric disorders

The primary GWAS datasets used for the identification of trans- and cis-risk genes are listed in **Table 4-1**.

We used summary statistics from a meta-analysis of trans-diagnostic risk across 8 mental health or neurodevelopmental disorders (Cross-Disorder Group of the Psychiatric Genomics Consortium 2019): ASD, bipolar disorder, MDD, obsessive-compulsive disorder, schizophrenia, anorexia nervosa, ADHD, and Tourette syndrome. For analysis of cis-risk, i.e. cis-diagnostic risk of a specific psychiatric disorder, we separately tested 5 large primary genome-wide association studies (GWAS) of MDD (Levey et al 2021), bipolar disorder (Stahl et al 2019), schizophrenia (Schizophrenia Working Group of the Psychiatric Genomics 2014), autism (Grove et al 2019), and ADHD (Demontis et al 2019). For comparative purposes, we analysed GWAS results for BMI (Pulit et al 2019), Alzheimer's disease (Jansen et al 2019), and rheumatoid arthritis (Okada et al 2014). For all disorders except MDD, we selected the largest publicly available, predominantly-European GWAS dataset; for MDD, we used a larger recent European GWAS (Levey et al 2021).

Table 4-1 *Genome-wide association study details.*

Genetic variants associated trans-diagnostically with risk for 8 psychiatric disorders and cis-diagnostically with risks for each of 5 specific psychiatric / neurodevelopmental disorders, and 3 positive control disorders. Loci associated with risk were thresholded at $P < 5x10^{-8}$, then distance-based clumping was used to define independently significant loci.

Study	**Number cases**	**Number controls**	**Number of genome-wide independently significant loci**	**Download link**
Cross-disorder psychiatric risk (Cross-Disorder Group of the Psychiatric Genomics Consortium 2019)	162,151	276,846	115	https://pgcdata.med.unc.edu/cross_disorder/pgc_cdg2_meta_no23andMe_oct2019_v2.txt.daner.txt.gz
Depression (Levey et al 2021)	264,984	581,929	122	dbGaP Study Accession: phs001672.v6.p1
Schizophrenia (Schizophrenia Working Group of the Psychiatric Genomics 2014)	36,989	113,075	108	https://pgcdata.med.unc.edu/schizophrenia/ckqny.scz2snpres.gz
Bipolar disorder (Stahl et al 2019)	20,352	31,358	16	https://www.med.unc.edu/pgc/download-results/ File = daner_PGC_BIP32b_mds7a_0416a
Autism (Grove et al 2019)	18,382	27,969	2	https://pgcdata.med.unc.edu/autism_spectrum_disorders/iPSYCH-PGC_ASD_Nov2017.gz
ADHD (Demontis et al 2019)	19,099	34,194	10	https://pgcdata.med.unc.edu/adhd/adhd_eur_jun2017.gz
Body mass index (Pulit et al 2019)	806,834	NA	1023	https://zenodo.org/record/1251813#.X_iGVS-l1TZ File = bmi.giant-ukbb.meta-analysis.combined.23May2018.txt
Alzheimer's disease (Jansen et al 2019)	71,880	383,378	25	https://ctg.cncr.nl/documents/p1651/AD_sumstats_Jansenetal_2019sept.txt.gz
Rheumatoid arthritis (Okada et al 2014)	14,361	43,923	48	http://plaza.umin.ac.jp/~yokada/datasource/files/GWASMetaResults/RA_GWASmeta_European_v2.txt.gz

Testing for enrichment of genome-wide genetic risk at regulatory elements (ROADMAP data)

Stratified linkage disequilibrium score regression (s-LDSC) can be used to test whether genetic risk is concentrated or enriched in a genomic annotation, e.g., a set of active regulatory elements in a specific cell type (Finucane et al 2015). S-LDSC hinges on the fact that the disease association statistic for a given genetic variant depends on whether that variant is linked to the disease, but also whether variants in linkage disequilibrium (LD) with that variant are linked to the disease. By testing whether variants in LD with the annotation of interest tend to have higher association scores than variants elsewhere, we can calculate an enrichment score capturing the tendency of SNP-based heritability for that disease to be co-located with that annotation (Finucane et al 2015). We used this method to test for enrichment of psychiatric risk variants at active regulatory elements in 88 cell or tissue types (see **Figure 4-1** for analysis pipeline).

For a given tissue, CHiP-seq data assaying multiple histone marks can be integrated to segment the genome into annotations representing different functional epigenetic states, e.g., enhancers, promoters, repressed regions (Roadmap Epigenomics et al 2015). The IDEAS algorithm (Zhang & Hardison 2017) leverages shared features across cell types to improve this segmentation. Lacking a strong prior hypothesis about which particular regulatory elements in immune cells would be implicated by psychiatric risk, we generated a simple functional annotation of active states for each tissue in the Roadmap Epigenomics Dataset, which includes samples from all major organ systems including brain, heart, muscle, gut, adipose, skin, reproductive and immune tissues (Roadmap Epigenomics et al 2015). Data for a given tissue or cell type sometimes come from multiple donors – as is the case for most of the brain and immune samples – and sometimes from single donors (see https://egg2.wustl.edu/roadmap/web_portal/meta.html for metadata). Immune cell subsets were magnetically sorted from live donor blood samples; brain tissues were homogenized post-mortem samples. For each Roadmap tissue/cell type, we generated a whole genome binary annotation of active regulatory elements from IDEAS annotations based on 5 epigenetic histone marks (H3K4me3, H3K4me1, H3K36me3, H3K27me3 and H3K9me3). We combined the 6 IDEAS annotations representing active promoters and enhancers to generate a single binary annotation of active regulatory elements for each tissue. More exactly, we merged the IDEAS annotations for active transcription start sites (10_TssA); regions flanking active TSS (8_TssAFlnk); weak TSS (14_TssWk); enhancers (4_Enh); genic enhancers (6_EnhG); and genic

enhancers associated with transcription (17_EnhGA), following a previous definition of active states (Backenroth et al 2018). We generated partitioned linkage disequilibrium (LD) scores for each tissue as recommended, using HapMap3 SNPs (Finucane et al 2015).

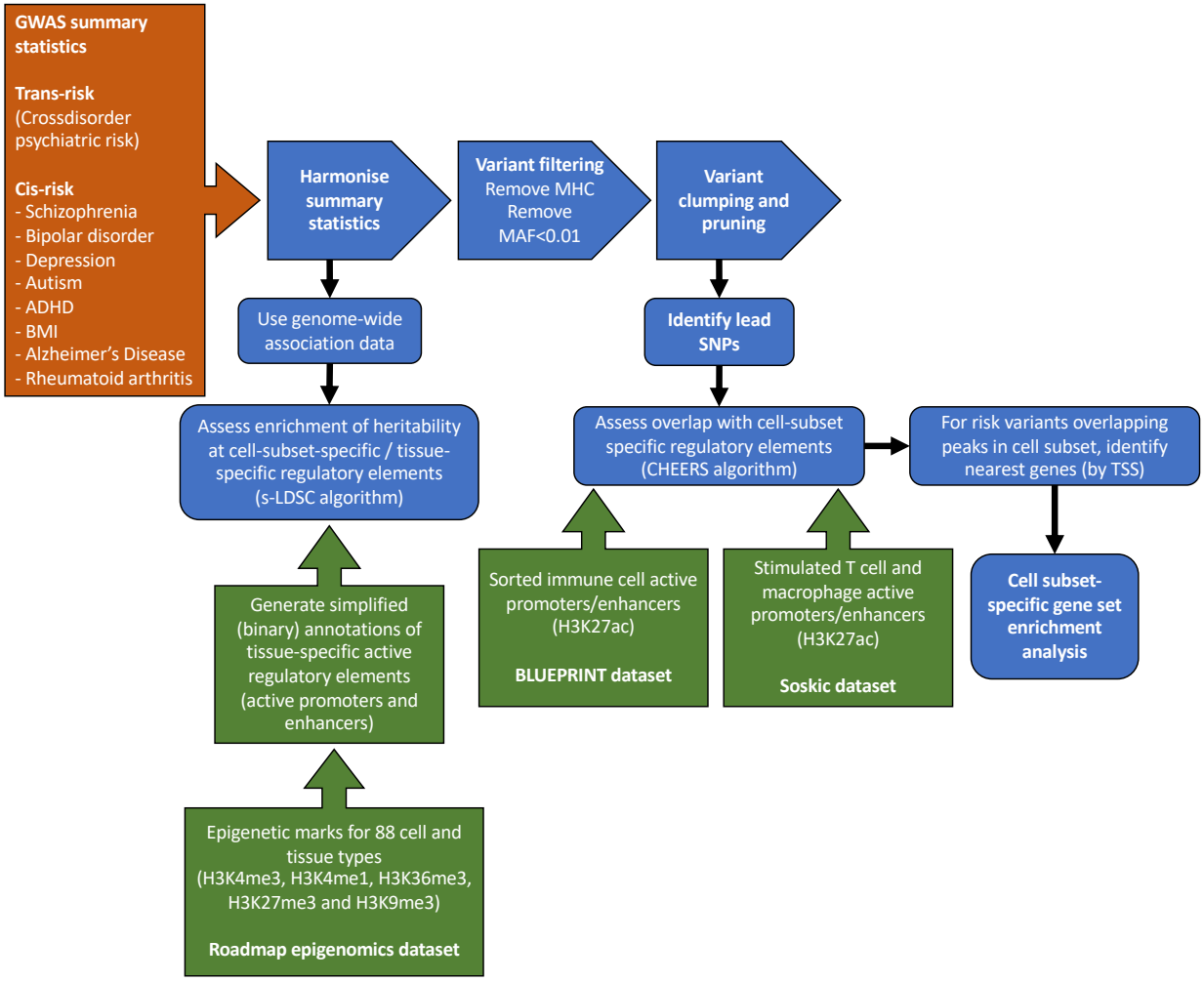


Figure 4-1 *Schematic of analysis pipeline used to assess enrichment of genetic risk variants at regulatory elements in different tissues and cell subsets.*
Genome-wide association study (GWAS) summary statistics (red box) were integrated with information on cell-subset-specific and tissue-specific active promoters and enhancers from three epigenetic datasets (green boxes). See methods for details. MAF, minor allele frequency; MHC, major histocompatibility complex; s-LDSC, stratified linkage disequilibrium score regression; SNP, single nucleotide polymorphism; BMI, body mass index; TSS, transcription start site.

We then used s-LDSC to test the enrichment of psychiatric risk variants in each cell type, using a separate model for each cell type, as is standard. Summary statistics were preprocessed using the LDSC recommended script munge_sumstats.py and we performed s-LDSC for each tissue in the Roadmap dataset, using recommended settings, excluding the MHC regions.

To further dissect the s-LDSC results for the active annotations, we also performed s-LDSC for the 3 types of genomic element comprising the active annotation: promoters, enhancers, and genic enhancers. We generated partitioned LD scores for the promoters (10_TssA, 8_TssAFlnk and 14_TssWk), enhancers (4_Enh) and genic enhancers (6_EnhG and 17_EnhGA) then performed s-LDSC using default settings for each of these annotations in the Roadmap immune tissues. The p-values from s-LDSC indicate the significance of the coefficient for the cell type specific annotations. P-values were corrected for multiple comparisons across tissues using Benjamini-Hochberg false discovery rate. Heatmaps are coloured by p-value rank to aid comparison across disorders or across annotations which are differently powered.

To account for the possible confounding effect of shared regulatory elements between brain and immune tissues, we also performed brain-conditioned enrichment analyses: for each tissue's s-LDSC model, we added terms for the active regulatory annotations for possibly confounding brain regions. In the LDSC model, τ (SNP-heritability) for a given genomic category/annotation (C) is estimated by regressing χ^2 (the SNP association statistics) against $\ell(j, C)$ (the linkage disquilibrium score for SNP j with respect to category/annotation C):

$$E[\chi^2] \sim \sum_{C=1}^{n} \tau_C \, \ell(j, C) \qquad [Eq.\ 4.1]$$

For the original s-LDSC models, the annotations (C) included in each multiple regression were the cell specific annotation of interest plus the standard non-cell type specific annotations (baseline v1.2, see https://storage.googleapis.com/broad-alkesgroup-public/LDSCORE/readme_baseline_versions). For the brain-conditioned models, the categories in each regression additionally included the annotations for the potentially-confounding brain regions.

SNP heritability *Z*-scores (heritability / standard error) and s-LDSC *Z*-scores (enrichment coefficient / standard error) were estimated using LDSC. To compare the results of the original and brain-conditional analyses, I used a one-sided two-sample *Z*-test as follows, where β_1 is the coefficient for the annotation in the original analysis and β_2 is the coefficient in the brain-conditional analysis. SE is the standard error of the coefficient for the original (SE_1) or conditional (SE_2) analysis. *Z*-scores were converted to p-values.

$$Z = \frac{\beta_1 - \beta_2}{\sqrt{(SE_1)^2 + (SE_2)^2}} \qquad [Eq.\ 4.2]$$

Testing for enrichment of genetic risk variants in cell-type specific active promoters/enhancers

To compare enrichment of genetic risk at regulatory marks in different immune cell subsets, and immune cells stimulated under different conditions, we used the CHEERS algorithm (Soskic et al 2019), see **Figure 4-1** for analysis pipeline. CHEERS quantifies the overlap of lead (independently significant) genetic risk variants with cell-specific epigenetic peaks. Crucially, CHEERS facilitates the comparison of similar cell types or conditions, which tend to have similar epigenetic profiles, by calculating peak specificity scores, indicating how specific a peak is to that cell type relative to other cell types, then quantifying cell-type enrichment as the specificity-weighted sum of overlaps of disease risk variants with these peaks. While s-LDSC leverages genome wide-information, CHEERS focuses on risk loci which meet genome-wide significance. In brief, CHEERS identifies peaks which overlap lead variants or variants in strong LD ($r^2 > 0.8$) with lead variants, then calculates the mean cell type specificity score (in that cell type) of those peaks, which captures the degree of enrichment of that cell type for a given disorder. One-sided p-values were reported from a discrete uniform distribution (reflecting the ranking of specificity scores within each cell type) and corrected for multiple comparisons across tissues using a Bonferroni correction. To identify lead disease risk loci, all summary statistics were processed consistently: liftover to hg38, harmonization, removal of MHC region, and distance-based clumping (see below for more detail). We applied CHEERS using two human H3K27ac ChIP-seq datasets: (i) BLUEPRINT consortium data from 19 sorted unstimulated immune cells subsets (Chen et al 2016) and (ii) the Soskic immune stimulation data from sorted and *ex vivo* stimulated immune cells (Soskic et al 2019). H3K27ac marks active (rather than inactive or poised) enhancer and promoter regions (Consortium 2012, Creyghton et al 2010). In the Soskic immune stimulation experiment, macrophages, naïve $CD4^+$ T cells and memory $CD4^+$ T cells were stimulated using different cytokine cocktails associated with autoimmunity or known to promote different cell fates. In addition, generic T cell receptor and CD28 co-stimulation signals were provided in all stimulated T cell conditions using beads coated with anti-CD3 and anti-CD28 antibodies. H3K27ac data were processed as described previously (Soskic et al 2019) to obtain cell-type specificity scores for H3K27ac peaks in each cell type or state. Here, we ran CHEERS using r^2 linkage disequilibrium values taken from unrelated European individuals from the 1000 genomes dataset (Lowy-Gallego et al 2019), calculated using PLINK (Purcell et al 2007).

To compare the enrichment between stimulated and unstimulated cell subsets, we used a one-sided, two-sample Z-test as follows, where x_1 is the mean specificity rank for the stimulated cell subset and x_2 is the mean specificity rank for the corresponding unstimulated cell subset. SE is the standard error of the mean and depends on the number of SNPs overlapping peaks. For a given disorder, SE is the same across different annotations, as peaks are called across the dataset as a whole. *Z*-scores were converted to p-values.

$$Z = \frac{x_1 - x_2}{\sqrt{2(SE)^2}} \qquad [Eq.\ 4.3]$$

Identification of independent risk loci

To identify independently significant loci for each disorder, we reprocessed all summary statistics consistently. Given the lack of well-matched linkage disequilibrium data for the populations underlying these studies, we aimed to conservatively identify independent lead variants without using LD information or conditional analysis within loci. We first lifted over the summary statistics (autosomal chromosomes only) and harmonized variants to the reference strand using the EBI summary statistics snakemake pipeline (https://github.com/EBISPOT/gwas-sumstats-harmoniser). Alleles with a minor allele count <10 were filtered out; where minor allele counts were not available, these were imputed from GnomAD v2.1.1 (Karczewski et al 2020) European frequencies lifted over to GRCh38. We then filtered all summary statistics to those variants also present at minor allele frequency > 0.01 in 1000 genomes phase 3 (unrelated European participants) called against GRCh38 (Lowy-Gallego et al 2019). To find independently significant lead loci, we used the Open Targets genetics finemapping pipeline (https://github.com/opentargets/genetics-finemapping) to filter summary statistics to variants with $P < 5 \times 10^{-8}$ (excluding MHC region chr6:28510120-33480577) and performed distance-based clumping of significant variants with a clumping distance of ±500kb. The number of lead SNPs identified for each disorder is shown in **Table 4-1**. ASD was excluded from downstream CHEERS analysis as only two significant loci were detected.

Over-representation analysis

Following CHEERS analysis, to test which biological pathways were implicated in T cells, we identified those T cell-specific peaks overlapped by disease risk variants, selected the genes overlapping those peaks or with transcription start sites nearest to those peaks, then

performed pathway analysis on those genes. More specifically, we selected (for a given disorder) the union of peaks highly specific (CHEERS specificity rank >0.9) to any T cell subset in the Soskic immune stimulation dataset which were also overlapped by risk variants for that disorder. For each peak, we used the ChIPseeker seq2gene function (Yu et al 2015) to identify the union of those genes overlapping the peak and those genes with a promoter region overlapping the peak, or (if no promoter overlapped the peak) the gene with the nearest transcription start site (up to a maximum of 10 kilobases away). The selected genes were tested for enrichment of GO biological processes and Reactome pathways using a hypergeometric test via the clusterProfiler enricher function, with Benjamini-Hochberg correction for multiple testing (Yu et al 2012).

4.4 Results

Trans-diagnostic psychiatric risk is enriched at active chromatin states in T cells

For trans-diagnostic risk of having any one of 8 major psychiatric disorders, we tested for enrichment of genetic risk at active regulatory elements in 88 cells or tissues from the Roadmap consortium, using stratified linkage disequilibrium score regression (s-LDSC). We found that three main tissue classes were significantly enriched for trans-diagnostic risk variants at regulatory elements, following correction for multiple comparisons: multiple adult and foetal brain regions; T cells; and pancreatic islets (**Figure 4-2**).

In the central nervous system (CNS), trans-risk variants were most strongly enriched at regulatory elements in foetal brain tissue samples. There was also significant enrichment ($P_{FDR} < 0.05$) at active regulatory elements in brain structures previously reported as abnormal in neuroimaging studies of psychiatric disorders: dorsolateral prefrontal cortex, angular gyrus, inferior temporal lobe, anterior caudate, cingulate gyrus, hippocampus and substantia nigra.

In the immune system, trans-risk variants were significantly enriched ($P_{FDR} < 0.05$) at epigenetically active genomic sites in multiple T cell subsets, including cytotoxic, helper and regulatory T cells in adult blood and T cells in cord blood. Conversely, there was no enrichment ($P > 0.05$) of trans-risk in myeloid cells (monocytes, neutrophils). We here use Benjamini-Hochberg correction for multiple testing, as the epigenomic profiles of different cell types are correlated rather than independent.

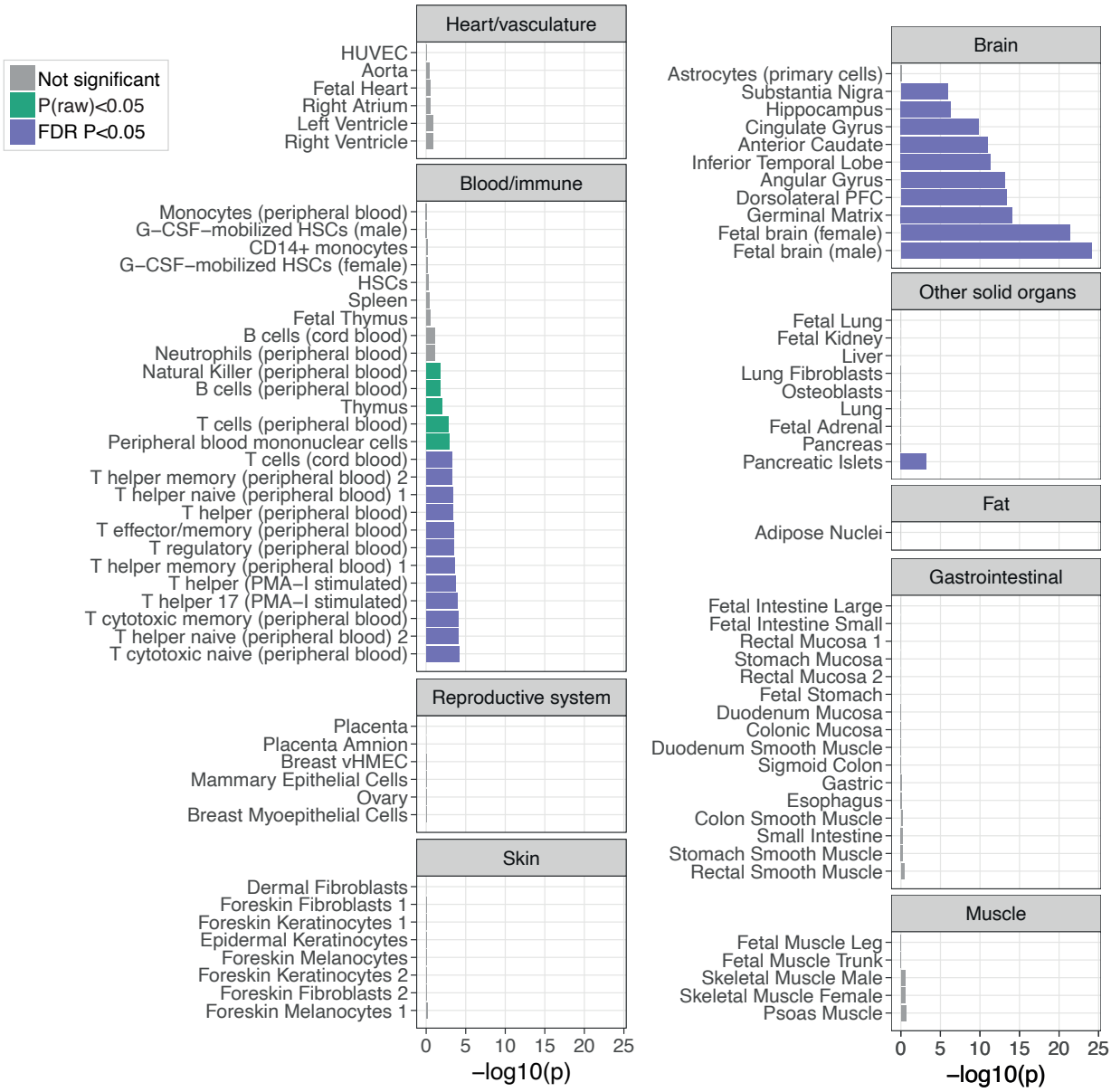


Figure 4-2 *Enrichment of trans-diagnostic risk at active regulatory elements (active promoters and enhancers) in 88 tissues from the Roadmap epigenomics consortium.*

P-values are shown for the results of stratified linkage disequilibrium score regression (s-LDSC) analysis, taking the union of active elements in a given cell type as the annotation of interest (see ***Methods****). The p-values from s-LDSC were used to test the null hypotheses that risk variants were not co-located with epigenetically activated sites more frequently than expected by chance, using the Benjamini-Hochberg false discovery rate to correct for multiple tests across N=88 tissues,* $P_{FDR} < 0.05$ *(purple). Tissues with nominally significant enrichment (P < 0.05, green) are also shown for context. HUVEC, human umbilical vein endothelial cells; vHMEC, variant human mammary epithelial cells; PFC, prefrontal cortex; HSC, hematopoietic stem cell; PMA-I, phorbol-myristate-acetate and ionomycin.*

Many regulatory elements are common to multiple tissues, so we reasoned that this pattern of CNS and immune system enrichment for trans-risk variants could be driven by coincidental overlap of brain and T cell active elements. In this case, the genetic risk would be theoretically expected to have pathogenic effects primarily by its modulation of epigenetically active sites in the brain, with no clearly independent pathogenic role mediated by T cells. We therefore repeated the s-LDSC analysis but included the active annotations for all 10 significantly enriched brain regions as extra terms in the s-LDSC models for every other cell type. In this brain-conditioned analysis, both helper and cytotoxic T cells remained strongly enriched for trans-diagnostic genetic risk (**Figure 4-3B**), while pancreatic islets did not. For enriched immune tissues in the original analysis (at $P_{FDR} < 0.05$), none showed significantly decreased enrichment following brain-conditioned analysis (two-sample *Z*-test, $P > 0.05$, **Table 4-2**). Conversely, including the annotation for male foetal brain (the brain tissue showing strongest enrichment) as an extra term in s-LDSC models significantly reduced trans-risk enrichment in all other brain regions (*Z*-test $P < 0.05$) except the substantia nigra ($P = 0.09$) and hippocampus ($P = 0.07$), reflecting some overlap of active elements between different brain regions at different developmental phases, and validating our statistical approach (**Figure 4-3A**). We showed the same effect for female foetal brain, the second most strongly enriched brain tissue (**Figure 4-3A**), excluding a potential effect of sex differences in brain development.

The global active annotation used as a binary marker of epigenetic activation combines trans-risk enrichment at three different classes of regulatory elements: active promoters, genic enhancers (enhancers found in gene bodies), and non-genic enhancers. To identify which classes were most enriched for trans-risk, we tested each class separately and found that the enrichment of trans-risk observed in terms of the global active annotation in T cells was not driven by a single class of regulatory element: there was enrichment of trans-risk at both active promoters ($P_{FDR} < 0.05$) and enhancers ($P_{FDR} < 0.05$ for genic enhancers) (**Figure 4-3C**).

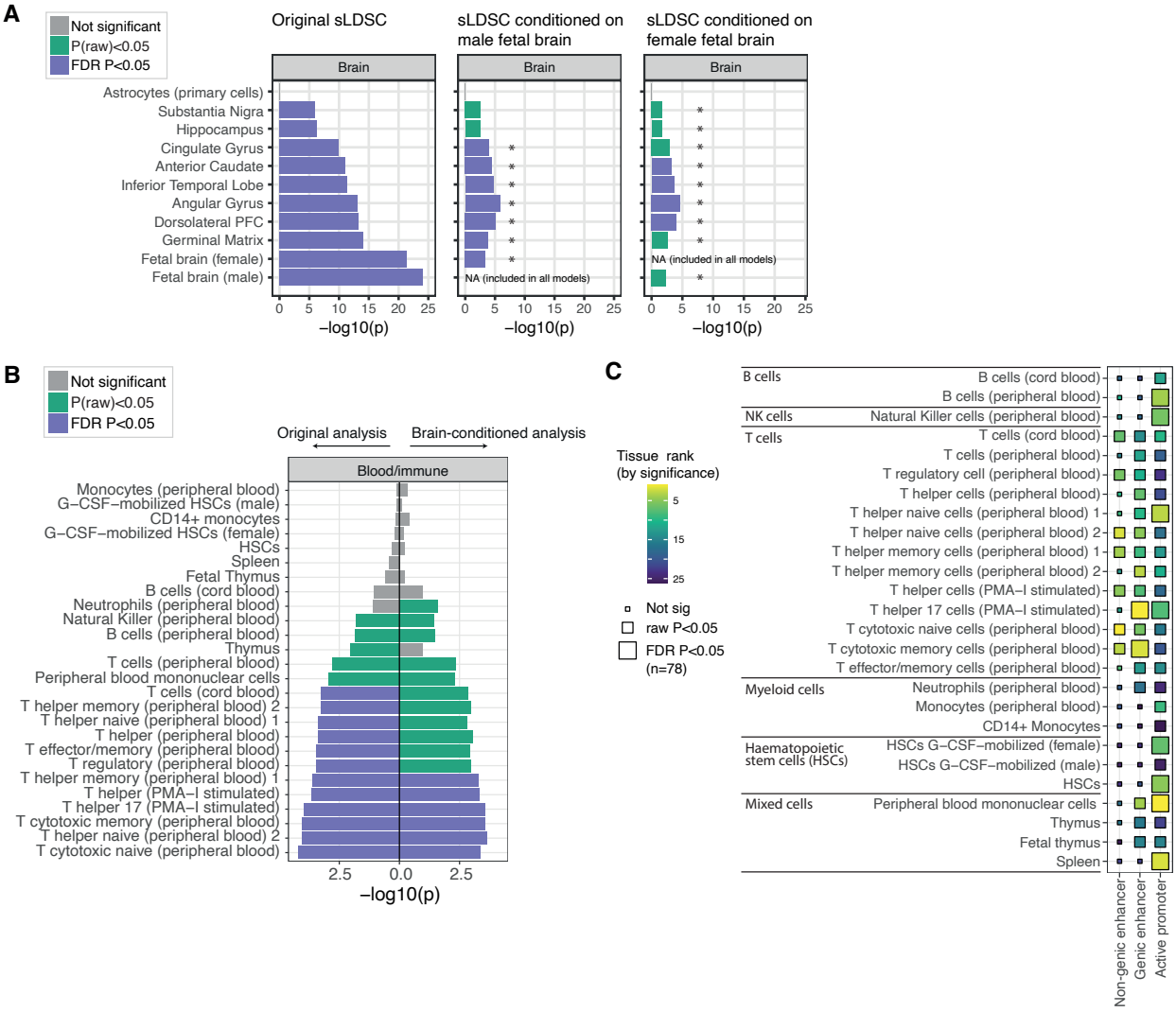


Figure 4-3 *Trans-diagnostic risk enrichment at epigenetically active sites in brain tissue and, independently, in T cells.*

(A) Validation of brain-conditioned analysis method: repeat of the analysis in ***Figure 4-2****, including the active regulatory annotation for foetal male brain as an additional term in the s-LDSC models for other cell types. LHS shows original s-LDSC for brain regions; RHS shows s-LDSC analysis with the addition of foetal male brain annotation to all models. Asterisks indicate those annotations showing significantly decreased enrichment in the conditional compared to the original analysis (two-sample Z-test P < 0.05). (B) Brain-conditioned analysis: right-pointing bars show repeat of the analysis in* ***Figure 4-2****, including the active regulatory annotations for all 10 significantly enriched brain regions as additional terms in each of the s-LDSC models for all other cell types. Left-pointing bars show the original results, as in* ***Figure 4-2****. Following conditional analysis, there was no significant decrease in enrichment significance for any immune tissues (P > 0.05). (C) Enrichment of trans-diagnostic risk in immune cell enhancers, genic enhancers and active promoters in Roadmap immune tissues. Tile size indicates s-LDSC significance: large tiles show results significant at* $P_{FDR} < 0.05$*, using the Benjamini-Hochberg procedure*

to correct for the 78 annotations tested; mid-sized tiles show nominally significant results (P < 0.05) for context. Tile fill indicates the P-value rank within each annotation across cell types.

Table 4-2 *Trans-diagnostic risk enrichment at epigenetically active sites in brain and T cells: statistical comparison of original vs. brain-conditioned stratified linkage disequilibrium score regression (s-LDSC) models by Z-test.*

Upper table: Comparison of original s-LDSC models and brain-conditioned s-LDSC models, which include the active annotations for all 10 significantly enriched brain regions as extra terms in the model for every other cell type. For immune tissues enriched for trans-diagnostic risk variants in the original analysis, none showed significantly decreased enrichment following brain-conditioned analysis. Lower table: Comparison of original s-LDSC models and conditional s-LDSC models including the annotation for male foetal brain (the brain tissue showing strongest enrichment for trans-diagnostic risk variants) as an extra term. Inclusion of male foetal brain reduced trans-risk enrichment in all other brain regions (except the substantia nigra and hippocampus), reflecting some overlap of active elements between different brain regions at different developmental phases and validating our statistical approach. Same effect shown for a conditional s-LDSC analysis including the annotation for female foetal brain, the second most strongly enriched brain tissue, as an extra term.

Immune cell subsets for which trans-risk showed significant enrichment (q < 0.05) in original s-LDSC model	Original model vs. conditional model including all 10 significantly enriched brain regions
T cytotoxic naive cells (peripheral blood)	z=0.34; p=0.37
T helper naive cells (peripheral blood) 2	z=0.20; p=0.42
T cytotoxic memory cells (peripheral blood)	z=0.21; p=0.42
T helper 17 cells (PMA-I stimulated)	z=0.19; p=0.42
T helper cells (PMA-I stimulated)	z=0.19; p=0.42
T helper memory cells (peripheral blood) 1	z=0.17; p=0.43
T regulatory cells (peripheral blood)	z=0.20; p=0.42
T effector/memory (peripheral blood)	z=0.26; p=0.40
T helper cells (peripheral blood)	z=0.20; p=0.42
T helper naive cells (peripheral blood) 1	z=0.28; p=0.39
T helper memory cells (peripheral blood) 2	z=0.18; p=0.43
T cells (cord blood)	z=0.23; p=0.41

	Original model vs. conditional model including foetal male brain	Original model vs. conditional model including foetal female brain
Brain Angular Gyrus	z=1.80; p=0.04	z=2.19; p=0.01
Brain Anterior Caudate	z=1.74; p=0.04	z=2.20; p=0.01
Brain Cingulate Gyrus	z=1.75; p=0.04	z=2.19; p=0.01
Brain Germinal Matrix	z=2.61; p=0.005	z=2.92; p=0.002
Brain Hippocampus Middle	z=1.50; p=0.07	z=1.99; p=0.02
Brain Inferior Temporal Lobe	z=1.80; p=0.04	z=2.19; p=0.01
Brain Dorsolateral Prefrontal Cortex	z=1.94; p=0.03	z=2.31; p=0.01
Brain Substantia Nigra	z=1.34; p=0.09	z=1.77; p=0.04
Foetal Brain (female)	z=1.73; p=0.04	NA
Foetal Brain (male)	NA	z=2.77; p=0.003

Cis-diagnostic risk is enriched at active chromatin states in T cells

Using data from the Roadmap Epigenomics Consortium, we next investigated the enrichment of cis-diagnostic risk variants at epigenetically active sites in brain tissues and immune cells for each of 5 mental health or neurodevelopmental disorders (schizophrenia, bipolar disorder, MDD, autism and ADHD) and each of 3 positive control disorders (Alzheimer's disease, obesity [BMI], and rheumatoid arthritis).

In the CNS, cis-risks for adult-onset mental health disorders (schizophrenia, bipolar disorder, MDD) were enriched in multiple foetal and adult brain tissues, and cis-risks for child mental health or neurodevelopmental disorders (autism, ADHD) were enriched more selectively in foetal brain tissue. Cis-risk for obesity (BMI) was also enriched for active sites across multiple foetal and adult brain tissues; but cis-risk for Alzheimer's disease was only (nominally) significantly enriched in hippocampus; and cis-risk for rheumatoid arthritis was not enriched in any brain tissue (**Figure 4-4A**).

In the immune system, similarly to trans-risk, cis-risks for schizophrenia, bipolar disorder, MDD and autism were enriched at globally activated sites in one or more T cell subsets (but with mainly nominal significance $P < 0.05$; **Figure 4-4B**). Cis-risk for rheumatoid arthritis was strongly enriched at globally active sites in multiple immune cell subsets; cis-risk for Alzheimer's disease was significantly enriched in myeloid cells and B cells, consistent with previous work (Kim et al 2021, Novikova et al 2021); and cis-risk for BMI was only enriched in one T cell class at $P < 0.05$ (**Figure 4-4B**).

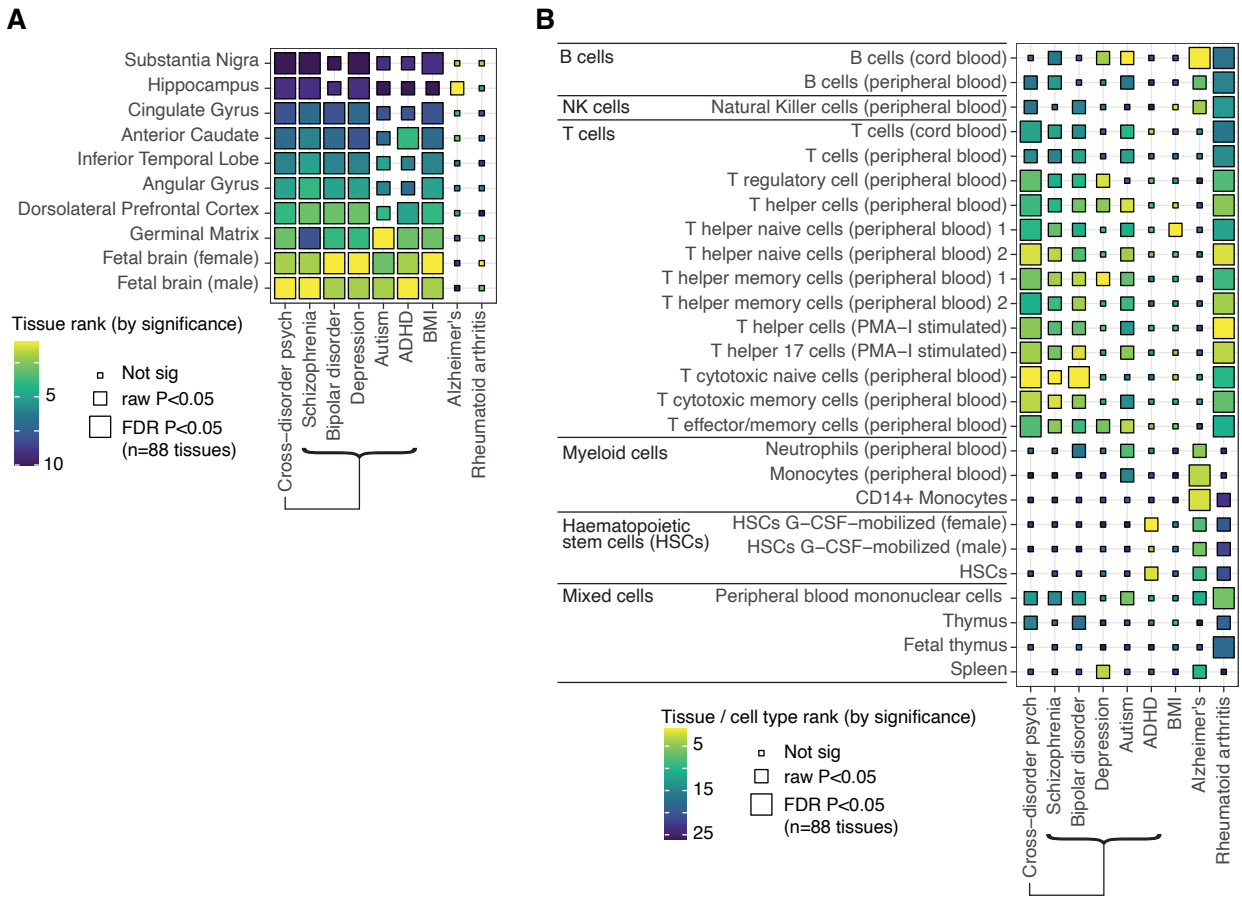


Figure 4-4 *Cis-diagnostic risk enrichment at epigenetically activated sites in adult and foetal brain tissue and immune cells for 8 specific disorders.*

For each of 5 mental health disorders (schizophrenia, bipolar disorder, major depressive disorder [MDD], autism, and attention deficit-hyperactivity disorder [ADHD]), and for each of 3 positive control disorders (obesity, Alzheimer's disease and rheumatoid arthritis), enrichment of cis-risk variants at active regulatory elements (active promoters and enhancers) was tested in (A) 10 brain tissue samples (3 foetal) and (B) 26 immune cell classes (3 foetal) (Roadmap Epigenomics et al 2015). P-values are shown for the results of stratified linkage disequilibrium score regression (s-LDSC) analysis, taking the union of active elements in a given cell type as the annotation of interest. Tile size, from large to small, indicates P-value thresholds from $P_{FDR} < 0.05$ (significant after Benjamini-Hochberg correction for all 88 tissues tested, including those not shown here), through $P < 0.05$ (nominally significant), to $P \geq 0.05$ (not significant). Tile fill indicates the P-value rank within each disorder across all cells/tissues to facilitate comparisons across results from differently-powered genetic association studies.

The statistical significance of enrichment results depends partly on the sample size of the underlying GWAS and the heritability and polygenicity of the disorder (factors influencing power, and captured by the SNP-based heritability *Z*-score) (Finucane et al 2015); but also on the strength of functional enrichment of the phenotype in that annotation. We hypothesized that, for immune enrichment in psychiatric disorders, the relationship between GWAS power and enrichment might not hold because (a) psychiatric disorders could differ in the degree to which genetic immune factors contribute and (b) immune-relevant genetic risk factors might only be important in a subgroup of patients, and the proportion of the subgroup of total cases would thus affect the immune enrichment detected. Therefore, for the two most enriched immune and brain annotations (naïve cytotoxic and helper T cells; foetal male and female brain), we tested the correlation between heritability *Z*-score and functional enrichment *Z*-score across the 9 disorders included in this study. Strikingly, we found a strong relationship between disorder heritability *Z*-score and detected brain enrichment (foetal male brain: Spearman's $\rho = 0.87$, $P = 0.005$; foetal female brain: $\rho = 0.87$, $P = 0.005$), but no correlation between heritability *Z*-score and immune enrichment (cytotoxic T cells: Spearman's $\rho = 0$, $P = 1$, helper T cells: $\rho = 0.03$, $P = 0.9$) (see **Figure 4-5**). This suggests that differences in GWAS power are not the primary driver of the different strengths of immune enrichment we observed for different disorders. Differences between disorders in the extent to which immunopathology contributes to symptoms, or the size of the patient subgroup with an immune pathogenesis, may be more important.

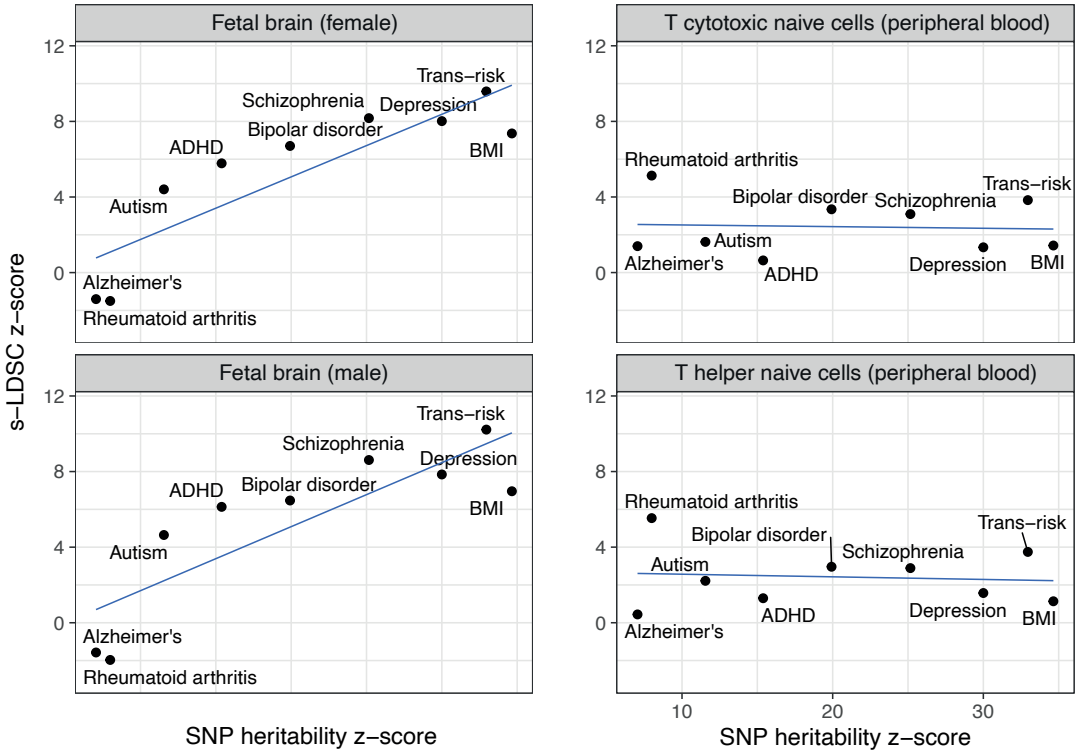


Figure 4-5 *Correlations between GWAS SNP heritability Z-scores and s-LDSC Z-scores across disorders. Correlations are shown for the two brain and two immune annotations most significantly enriched for trans-risk. Spearman's correlations with heritability z-score are as follows: foetal male brain $\rho = 0.87$, P = 0.005; foetal female brain $\rho = 0.87$ P = 0.005; cytotoxic T cells $\rho = 0$, P = 1; helper T cells: $\rho = 0.03$, P = 0.9.*

Trans- and cis-risk variants are enriched at active enhancers/promoters in lymphoid cells: BLUEPRINT data

To assess the generalizability of these results in an independent dataset, we tested for enrichment of trans- and cis-risk variants at active enhancer/promoter marks (H3K27ac) in sorted immune cell subsets from the BLUEPRINT consortium (Chen et al 2016), using the CHEERS algorithm (Soskic et al 2019). The CHEERS algorithm assesses enrichment of genetic risk variants at cell subset-specific epigenetic marks by calculating peak specificity scores, which indicate how specific an epigenetic peak is to that cell type relative to other cell types (see **Methods**). Cell-type enrichment is calculated as the specificity-weighted sum of overlaps of disease risk variants with these peaks, allowing effects in epigenetically similar cell types to be distinguished. These peak specificity scores are necessarily less correlated across cell subsets than the underlying epigenetic marks, so we here use Bonferroni correction to correct for multiple comparisons, as previously (Soskic et al 2019). We replicated our prior key finding from the Roadmap data, i.e., trans-risk was significantly enriched at epigenetically active sites in lymphoid cells; but not myeloid cells (**Figure 4-6A**). We also showed that cis-risk for schizophrenia and depression was significantly enriched after controlling for multiple comparisons ($P_{Bonf} < 0.05$) in lymphoid but not myeloid cells (confirming in this dataset the convergent, nominally significant results for these disorders in the Roadmap dataset). The lack of myeloid enrichment was not due to problems with the myeloid data, as we detected the expected enrichment of Alzheimer's Disease risk variants in macrophages (**Figure 4-6A**). As well as T cell enrichment, we also find enrichment of trans-risk and cis-risk for schizophrenia and (especially) depression in B cells, as well as enrichment of trans-risk and cis-risk for schizophrenia in NK cells. For ADHD and bipolar disorder (less well-powered GWAS studies with fewer independent significant loci available for analysis, see **Table 4-1**), no cell types were enriched at $P_{Bonf} < 0.05$ (**Figure 4-6A**). Despite both schizophrenia and depression showing strong lymphoid enrichment, the specific histone peaks overlapped by risk variants for these disorders were not generally shared between them (**Figure 4-6B,C**). This indicates that cis-risks for these two disorders were convergently enriched at a cellular level but distinct at the level of specific regulatory elements. It was also notable that cis-risk variants for obesity overlapped with a set of H3K27ac sites that was largely disjoint with the sets of regulatory elements overlapping with cis-risk variants for psychiatric disorders (**Figure 4-6B,C**).

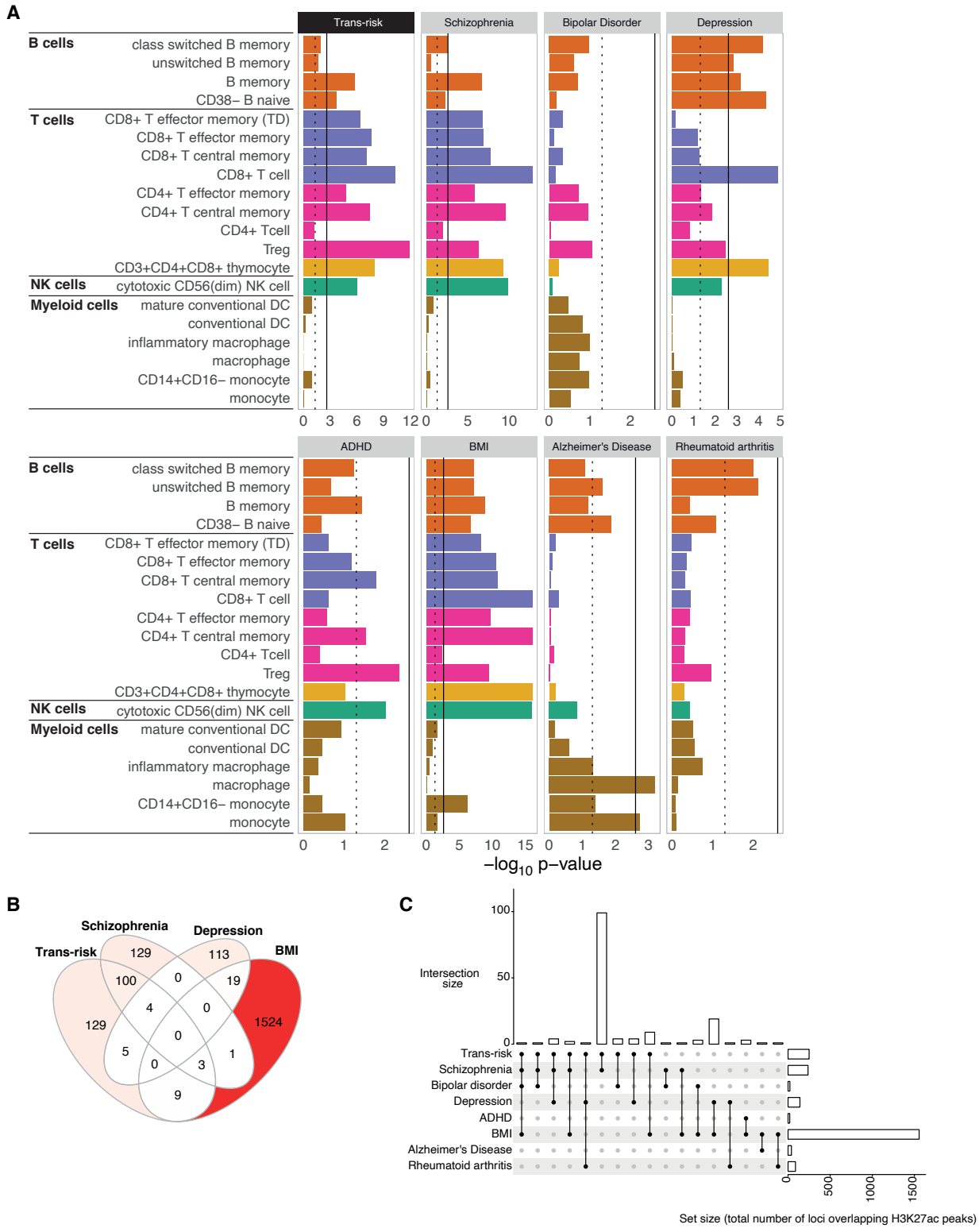


Figure 4-6 *Trans- and cis-diagnostic risk variant enrichment at histone-acetylated marks on adult immune cells in the BLUEPRINT dataset.*

(A) Bar plots show enrichment of genetic risk for each disorder at active promoters/enhancers (H3K27ac marks) in unstimulated, sorted immune cells. CHEERS was used to detect enrichment of risk loci at cell-type specific H3K27ac peaks (see ***Methods****). The dotted black line marks the nominal significance threshold, P < 0.05; the solid black line marks the Bonferroni-corrected significance threshold,* P_{Bonf} *<*

0.05. Note differing x-axis scales. (B) Venn diagram shows counts of variant-peak overlaps shared between disorders and unique to each disorder (each peak is only counted once even if overlapping multiple variants). (C) Upset plot for all BLUEPRINT immune stimulation H3K27ac immune peaks overlapped by risk variants for each disorder, showing counts (vertical bars) of shared peaks, compared to total peak number implicated by each disorder (horizontal bars).

Trans- and cis-diagnostic risk variants are enriched at histone-acetylated sites in stimulated T cells: Soskic immune stimulation dataset

Given that risk of mental health disorders is affected by both genetic variation and environmental factors, we reasoned that trans- and cis-risk variants could be most significantly enriched at sites that were epigenetically activated in immune cells stimulated by cytokines (mimicking environmental insults) towards different activated cell fates. To investigate this hypothesis, and to assess the robustness of our principal findings in a third independent dataset, we used CHEERS to test whether trans- and cis-risks were enriched at cell subset-specific regulatory elements (H3K27ac marks) active during immune cell activation, using a dataset of human naïve and memory $CD4^+$ T (helper) cells and macrophages stimulated *ex vivo* in the presence of 13 cytokine combinations. Chromatin activity was assessed at early and late timepoints after exposure to cytokine stimulations (16h and 5 days for T cells and 6h and 24h for macrophages), as well as in unstimulated cells (Soskic et al 2019). Both trans-diagnostic risk variants, and cis-risk variants for MDD, were most significantly enriched in memory T helper cells at day 5 following T cell stimulation with anti-CD3/anti-CD28 beads that mimic activation occurring with T cell receptor-crosslinking; trans-risk variants and cis-risk variants for schizophrenia were also significantly enriched in memory T helper cells at 16 h and in naïve T helper cells at day 5 only (**Figure 4-7**). The histone acetylation peaks that overlapped with cis-risk variants for MDD in late-activated memory T cells were almost completely disjoint with the peaks that overlapped with cis-risk variants for schizophrenia in late-activated memory T cells (**Figure 4-8A**), again demonstrating convergence of enrichment at the immune cell subset level, but divergence at the molecular level of specific regulatory elements. Similarly, although trans-risk and cis-risk for schizophrenia showed the most similar pattern of immune cell enrichment, most of the SNP-peak overlaps driving these results were not shared (**Figure 4-9A**), implying that trans-risk immune enrichment is not purely being driven by schizophrenia cis-risk variants. Trans-risk enrichment was generally greater for stimulated than unstimulated T cells, with smaller differences in the magnitude of enrichment between different cytokine stimulation

conditions (**Figure 4-7**). For 9 of the 10 cytokine conditions (all except Th17-cytokine polarizing condition), trans-risk enrichment was significantly greater (*Z*-test, $P < 0.05$) in stimulated compared to unstimulated late-activated memory T cells.

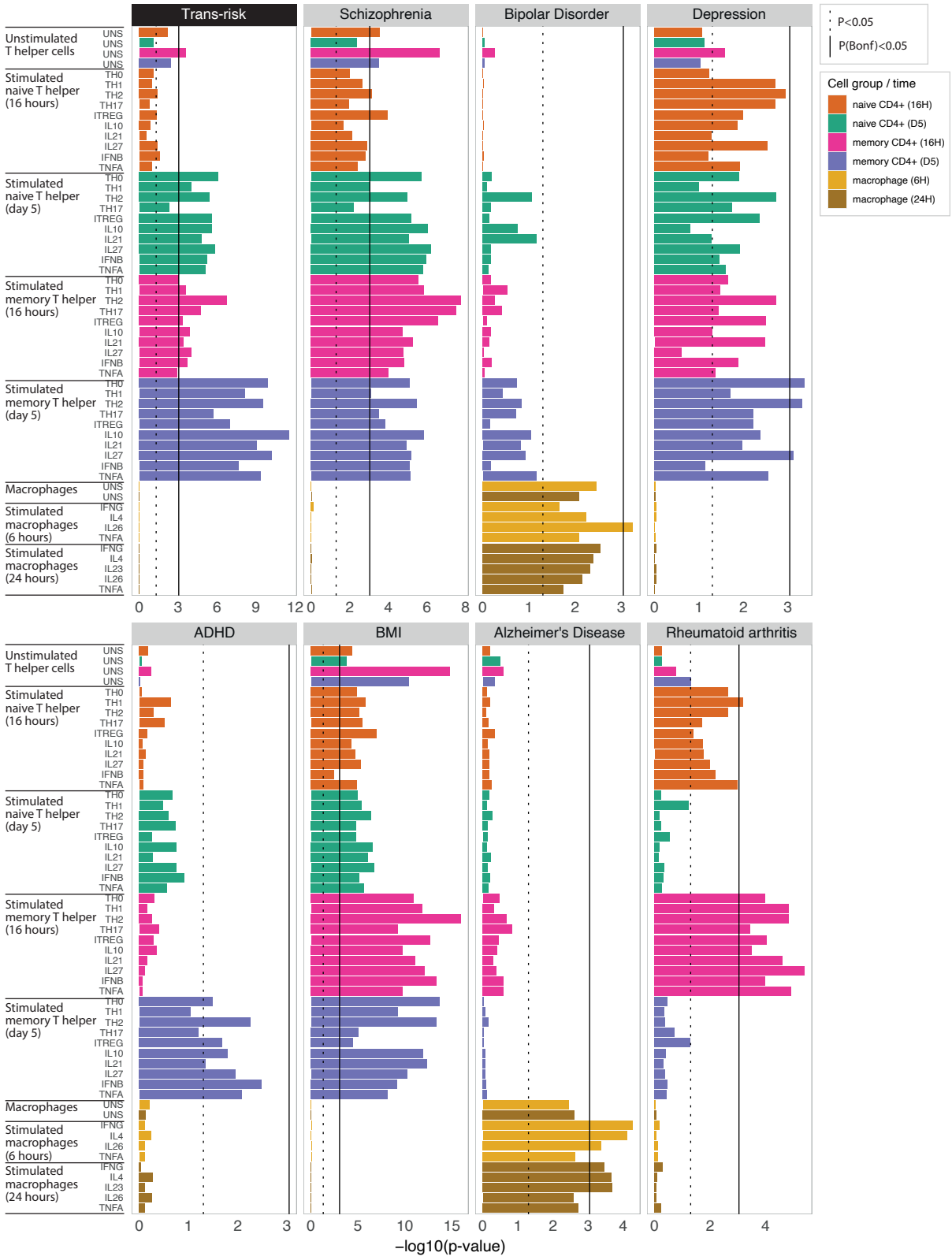


Figure 4-7 *Trans- and cis-risk variant enrichment at histone-acetylated marks on experimentally stimulated immune cells in the Soskic immune stimulation dataset.*

Bar plots show enrichment of genetic risk for each condition at active promoters/enhancers (H3K27ac marks) in sorted and unstimulated or ex vivo stimulated immune cell classes: macrophages, naïve CD4⁺ (helper) T cells and memory CD4⁺ T cells, assayed at both early and late timepoints after stimulation

with one of several different cytokine cocktails promoting differentiation to different T cell states (as shown in row labels). CHEERS was used to detect enrichment of risk loci at cell-type specific H3K27ac peaks (see Methods). The dotted black line marks the nominal significance threshold, P < 0.05; the solid black line marks the Bonferroni-corrected significance threshold, $P_{Bonferroni} < 0.05$. Note differing x-axis scales.

As in the two prior independent datasets, there was no enrichment for trans- or cis-risk of psychiatric disorders at epigenetically activated sites in myeloid cells, either stimulated or unstimulated, with the exception of enrichment of bipolar disorder risk in IL-26-stimulated macrophages (**Figure 4-7**). Cis-risk variants for obesity were enriched in unstimulated and stimulated T cell states (**Figure 4-7**), but only 9 of the 108 depression-associated H3K27ac peaks also overlapped with BMI risk variants (**Figure 4-9A,B**), indicating that cis-risks for obesity and depression were enriched at distinct regulatory elements in the same cell subsets.

For disorders showing enrichment in T cells, we performed pathway analysis (overrepresentation analysis) for those genes overlapping or with transcription start sites nearest to the T-cell specific histone acetylation peaks overlapped by risk variants (although we note that distance-based measures are limited in their ability to link epigenetic peaks with the genes to which they are functionally linked). Trans-risk and cis-risk for schizophrenia showed enrichment of pathways including epigenetic regulation, pre-notch processing, and oestrogen-dependent gene expression in T cells. In contrast, rheumatoid arthritis showed enrichment of lymphoid cell differentiation, activation, and response to antigenic stimulus (**Figure 4-10**).

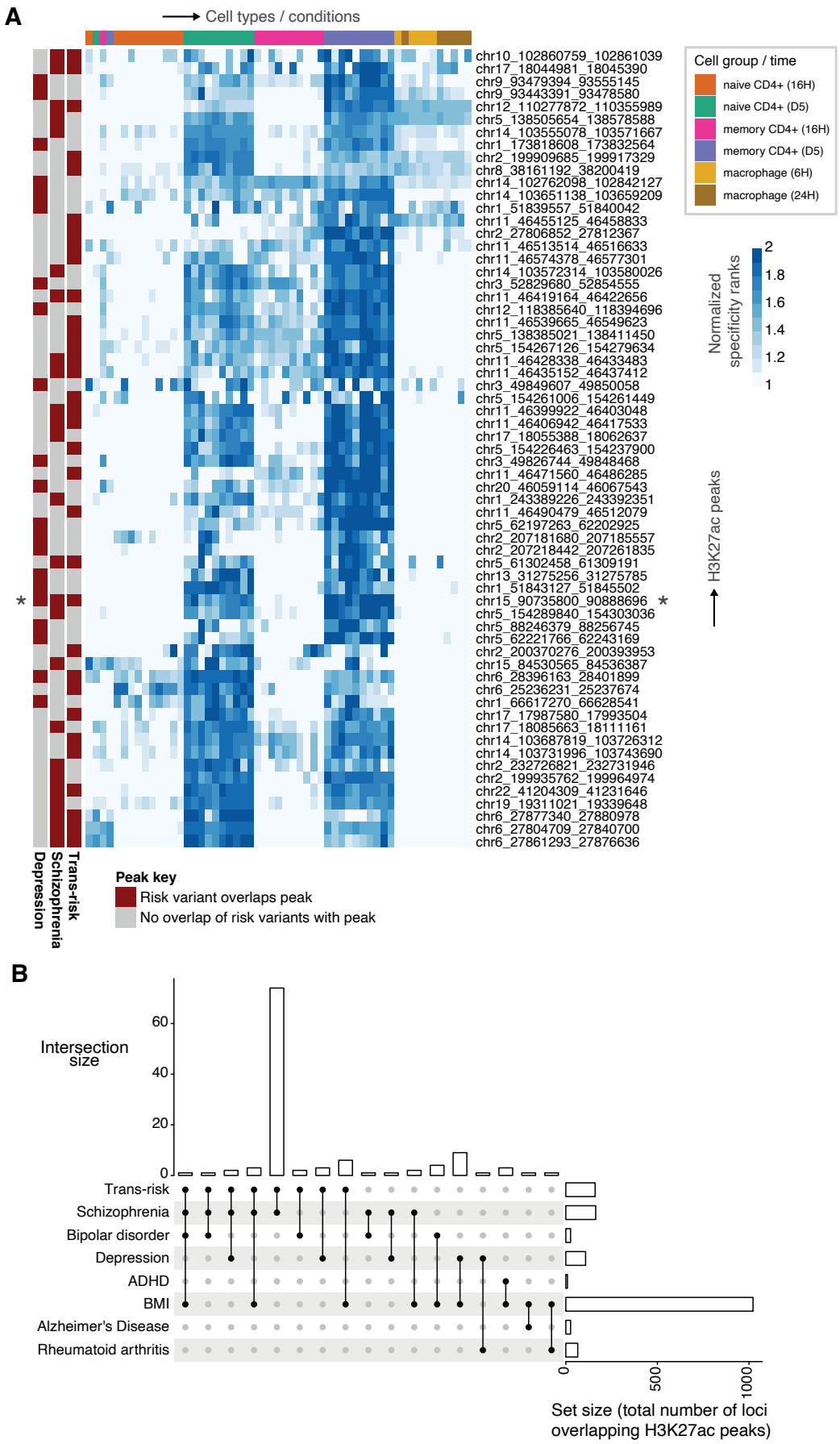


Figure 4-8 *Soskic stimulated immune cell dataset: overlap of H3K27ac peaks implicated by different disorders.*

(A) Heatmap shows the subset of peaks with specificity for late-activated naïve and/or memory CD4$^+$ T cells which are also overlapped by risk variants for either trans-risk, schizophrenia, or major depressive disorder. Each row corresponds to a H3K27ac peak overlapping a risk variant; each column corresponds to a different cytokine-induced cell state (see legend), ordered as in ***Figure 4-7****. Blue fill shade represents how specific each peak is to each cell state (specificity rank of the peak normalized to the mean specificity rank of all peaks). Row annotations indicate peaks which overlap (dark red) or do not overlap (grey) risk variants for the disorder indicated. Of the late-activation T cell specific peaks, only 1 (starred *) is overlapped by both schizophrenia and depression risk variants. (B) Upset plot for all Soskic dataset H3K27ac immune peaks overlapped by risk variants for each disorder, showing counts (vertical bars) of shared peak overlaps, compared to total number of peaks implicated by each disorder (horizontal bars).*

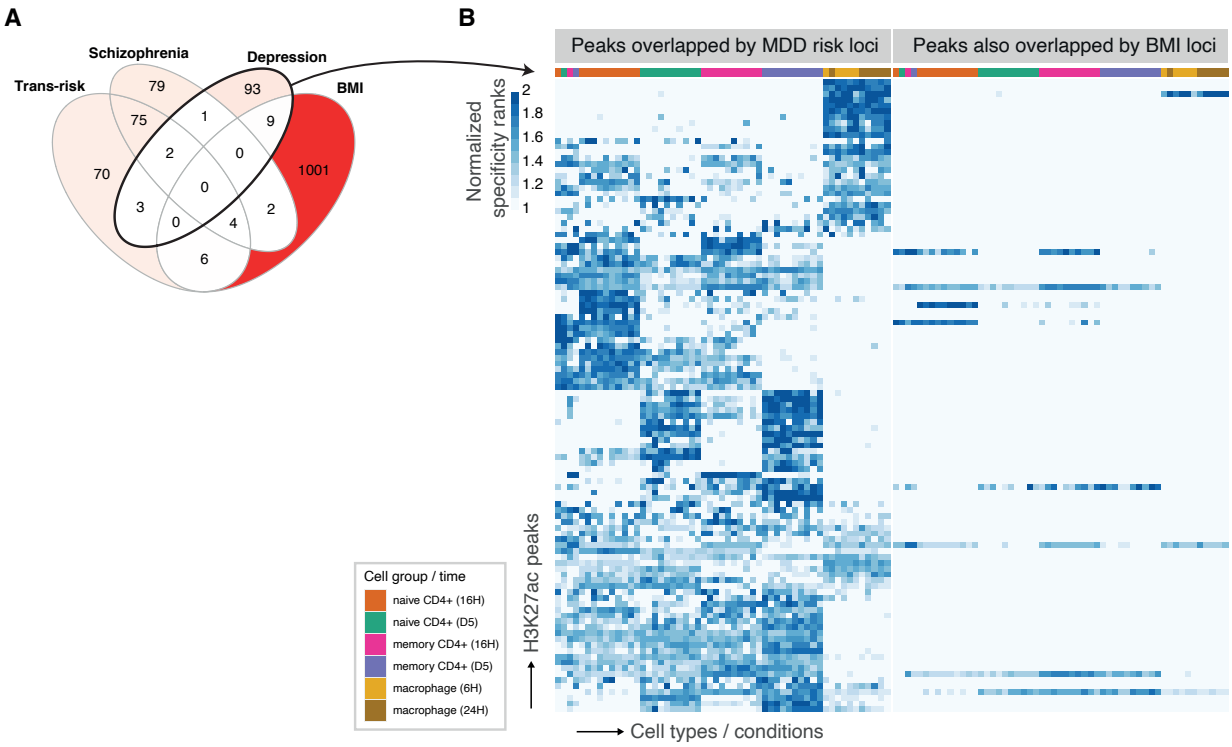


Figure 4-9 *Variant-peak overlaps shared between disorders and unique to each disorder (Soskic immune stimulation dataset).*

(A) Venn diagrams show counts of variant-peak overlaps shared between disorders and unique to each disorder. For an upset plot of peak overlaps across all disorders, see ***Figure 4-8B****. (B) All Soskic immune stimulation dataset peaks overlapped by risk variants for major depressive disorder. Each row corresponds to an H3K27ac peak overlapping a risk variant for MDD; each column corresponds to a different cytokine-induced cell state, ordered and coloured as in* ***Figure 4-7****. The blue fill shade represents how specific each peak is to each cell state (specificity rank of each peak normalized to the mean specificity rank of all peaks). Only 9 of the 108 MDD-associated H3K27ac immune peaks also overlap BMI risk variants.*

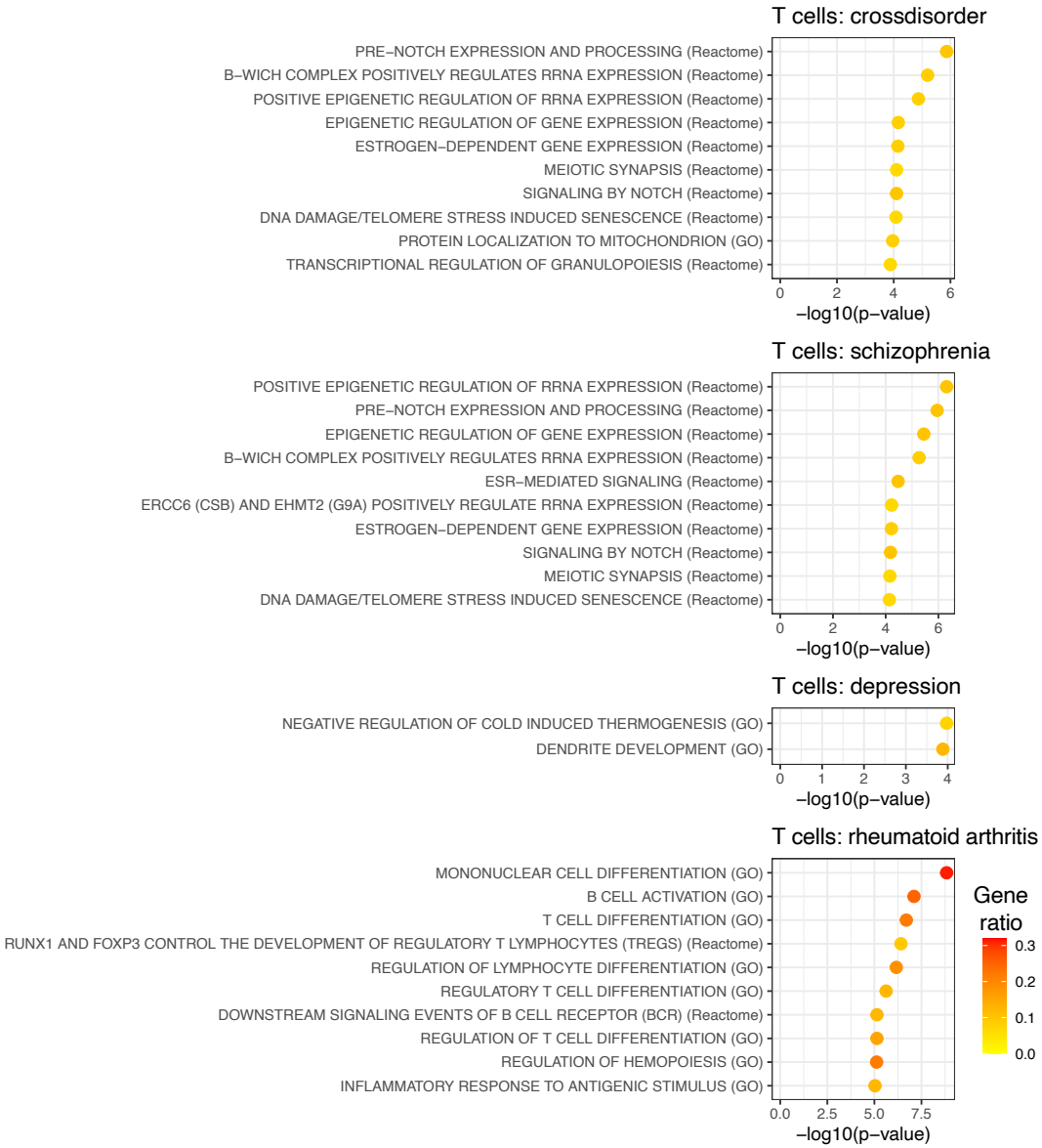


Figure 4-10 *Soskic stimulated immune cell dataset: pathway enrichment for genes nearest to peaks both specific to T cells and overlapped by risk variants.*

For peaks which were both highly specific to T cells (including both unstimulated and stimulated cells) and overlapped by trans-risk variants, nearest genes were identified and tested for enrichment for curated biological pathways (GO and Reactome) using a hypergeometric test. Results are shown only for those disorders which showed enrichment in T cell subsets. Only pathways with $P_{FDR} < 0.05$ are shown, with a maximum of 10 pathways shown per condition. Fill colour indicates gene ratio (number of test genes in the pathway / total number of test genes).

4.5 Discussion

We examined the enrichment of genetic risk variants for psychiatric disorders at epigenetically activated regulatory sites across multiple tissues. As expected, trans-diagnostic risk variants, commonly associated with multiple mental health and neurodevelopmental disorders, were significantly enriched at active regulatory sites in several adult and foetal brain tissue samples. Strikingly, we also found that trans-diagnostic risk variants were significantly enriched at an independent set of regulatory elements in peripheral blood lymphoid cells (but were not enriched in myeloid cells). Our key novel results – enrichment of trans-risk in T cells and lack of enrichment in myeloid cells – were statistically robust to multiple comparisons and replicated in three independent datasets, suggesting a previously unknown effect of trans-diagnostic genetic risk on T cells. Other lymphoid cells (for which fewer datasets were available) are likely also implicated in pathogenesis, as we also found enrichment of trans-risk in B cells and NK cells.

Further investigation of cis-diagnostic risk variants, specifically associated with one of 5 mental health or neurodevelopmental disorders, confirmed significant enrichment of genetic risks for schizophrenia and major depressive disorder at active promoters and enhancers in peripheral lymphoid cells (but not myeloid cells). Epigenetically activated sites in T cells, especially cytokine-stimulated $CD4^+$ T cells, were most consistently and significantly enriched for sequence variants associated with schizophrenia or MDD; however, the active regulatory elements overlapped by these cis-diagnostic variants were specific to each disorder. This suggests convergence of risk for schizophrenia and depression at a cellular level in the immune system, i.e. activated T cells, and raises questions about how the involvement of different specific regulatory elements in these two disorders might relate to the different phenotypic presentations of schizophrenia and depression. We also found strong enrichment of risk for depression in both naïve and memory B cells. To our knowledge, this is the first demonstration of enrichment of genetic risk for MDD at epigenetically active sites in lymphoid cells (or indeed any immune cell type). Notably, in all three datasets, immune enrichment of schizophrenia risk variants was much greater than for depression risk variants, despite the larger size of the depression GWAS dataset.

The cis-diagnostic enrichment results for schizophrenia and MDD were statistically robust to multiple comparisons and in clear contrast to the comparable results for 3 positive control disorders. Cis-risks for Alzheimer's disease were significantly enriched at epigenetically activated sites in myeloid cells (but not lymphoid cells); cis-risks for rheumatoid arthritis were enriched at active sites in myeloid and lymphoid cells (but not brain tissue); and cis-risks for obesity (BMI) were enriched at active sites in brain tissue and (in some analyses) in immune cells, but with effects on regulatory elements distinct from those implicated by psychiatric disorders.

On this basis, we propose that genetic variants associated with increased risk for psychiatric disorders are likely to interact with epigenetic activation of specific and distinct regulatory elements in both the central nervous system and the adaptive immune system. This hypothesis-generating work immediately raises three key questions. What environmental exposures cause epigenetic modification at risk-enriched sites in T cells? How could atypical T cell phenotypes cause changes in the CNS that are ultimately manifest as mental health or neurodevelopmental disorders? What are the antigen presenting cells (our data suggest they may be B cells) which activate atypical $CD4^+$ T cells?

Infection is the most likely environmental stimulus to induce epigenetic activation in the immune system. There is also increasing evidence that psychosocial stress, especially in early life, can cause epigenetic modification of glucocorticoid receptor-related genes in animal models; and early life adversity has been associated with long-term changes in blood immune biomarkers in human longitudinal studies (Klengel & Binder 2015). However, here we focus on the abundant epidemiological evidence that foetal and post-natal infections increase the risk for multiple psychiatric disorders (Al-Haddad et al 2019, Breithaupt et al 2019, Kohler-Forsberg et al 2019, Lydholm et al 2019). The immune mechanisms by which early-life infection predisposes to later psychiatric symptoms are not known. But we do know that foetal or childhood infections can cause long-term changes in adaptive immune cell phenotypes, including T cell memory of antigens and B cell production of antibodies, that are crucial to development of adult immunity (Simon et al 2015). Thus, it is conceivable that the epigenetically activated sites enriched for trans- and cis-risks in T cells and memory B cells in these data were "marked" by exposure to infection or inflammation; and that genetic risk variants modulate the infection-induced activation of regulatory elements, leading to atypical T

or B cell phenotypes following infection in people at genetic risk of psychiatric disorder. There is already some epidemiological evidence for gene-by-environment interactions between infection and risk variants for schizophrenia (Borglum et al 2014, Clarke et al 2009, Demontis et al 2011) and MDD (Ye et al 2020). Many aspects of our data are compatible with this concept. For example, our finding that trans- and cis-diagnostic risk variants were enriched at sites epigenetically activated by delayed T cell responses to a wide range of pro-inflammatory cytokine stimuli seems consistent with the epidemiological finding that increased risk of multiple psychiatric disorders is found following a wide range of different infections (Benros et al 2011, Benros et al 2013, Brown & Meyer 2018, Meltzer & Van de Water 2017, Tioleco et al 2021) .

Atypical T cell phenotypes could conceivably have effects on the brain by at least two broad routes: via stimulus-driven T cell activation and via developmental pathways (**Figure 4-11**). Atypical T cells may impact on neuronal function via soluble inflammatory mediators (Alves de Lima et al 2020, Choi et al 2016); via contact-dependent mechanisms (Evans et al 2019); via depletion of metabolic precursors of monoamine neurotransmitters (Miyajima et al 2017); or via their effects on other immune or non-immune cells which in turn affect neurons (Evans et al 2019). Developmentally, T cells have an important physiological role in controlling microglial phagocytosis of synaptic terminals and neurites as part of normal childhood and adolescent neurodevelopmental programs of synaptic pruning (Pasciuto et al 2020). Thus atypical T cells in the meninges or brain could lead, via atypical synaptic pruning (Pasciuto et al 2020, Sekar et al 2016), to the disrupted brain connectivity seen in schizophrenia and other psychiatric disorders (Morgan et al 2019).

In contrast with autoimmune diseases, which tend to show greatest enrichment in early T cell activation states (Soskic et al 2019), the strongest enrichment for psychiatric risk variants was in T cells, especially late-activated memory $CD4^+$ T cells, and memory B cells. This may reflect abnormalities in the resolution (rather than onset) of immune responses to infection or social stress, potentially leading to chronic, low-grade peripheral inflammation seen in many psychiatric disorders (Goldsmith et al 2016, Yuan et al 2019).

The statistical significance of results for risk variant enrichment at epigenetically active regions reflects in part the sample size of the GWAS datasets used and the heritability and polygenicity

of the disorders. However, in contrast to our results for brain enrichment, we did not find any correlation between GWAS statistical power and immune enrichment. This suggests that while GWAS power can explain some differences between disorders in the observed significance of functional enrichment (as in the brain), differences in immune cell enrichment may in part reflect how frequently immune mechanisms are implicated in individual patients clinically diagnosed with a specific disorder. Thus, differences between disorders in the strength of immune enrichment seen here may be more indicative of between-disorder differences in how strongly immune mechanisms contribute to pathogenesis in general, or what proportion of cases have an immune pathogenesis. The immune enrichments we detected were significantly weaker than enrichments in brain tissues – this may reflect a weaker pathogenic contribution of epigenetically activated risk variants in the immune system (compared to the brain); or it may be that genetic immune mechanisms can have a larger effect but only in a subgroup of patients.

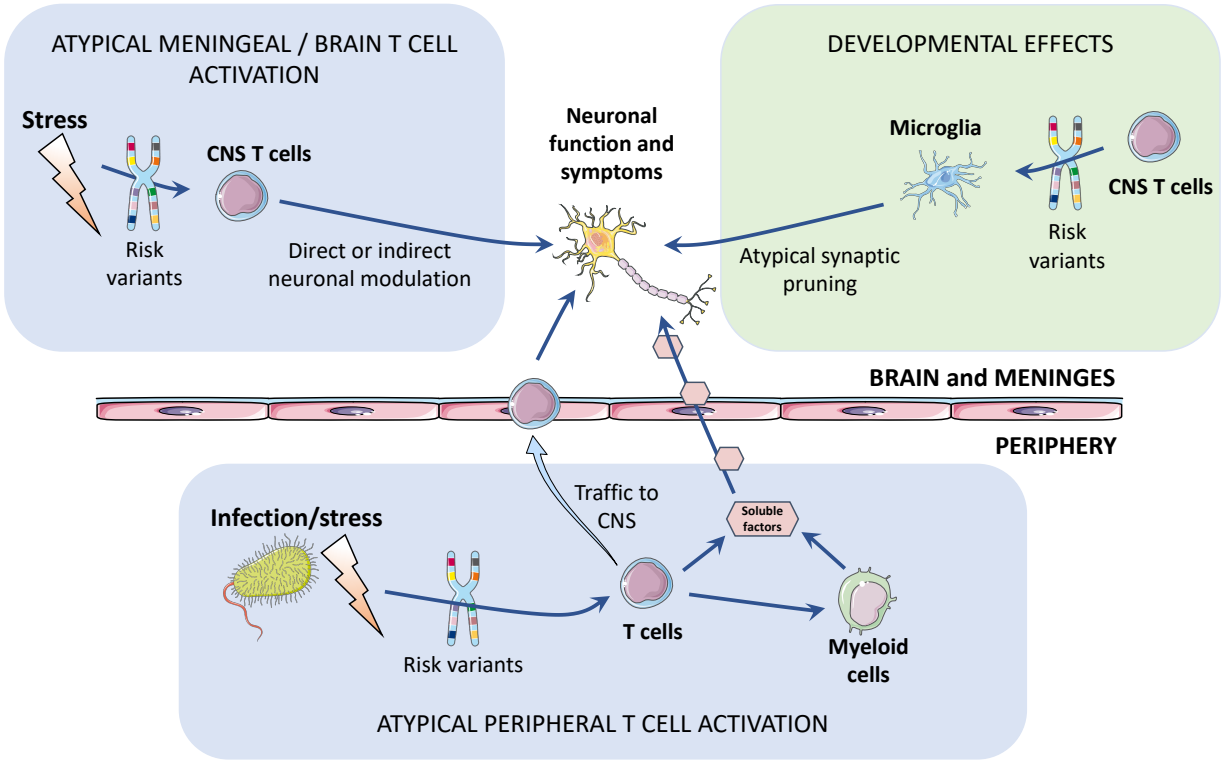


Figure 4-11 *Schematic of potential pathogenic pathways by which genetic risk variants enriched at epigenetically active sites in T cells could lead to neuronal changes and ultimately psychiatric disorders. Atypical stimulus-driven activation pathway (light blue boxes): Infection or other stressors can induce epigenetic activation of regulatory elements in T cells that are enriched for trans- or cis-diagnostic risk variants, proximally causing atypical T cell phenotypes, and distally causing increased inflammatory activation of innate immune (myeloid) cells in the periphery and CNS. Atypical activation of T cells resident in the meninges or brain, or trafficking into the meninges and brain from the periphery, could have direct adverse effects on neuronal function. Developmental pathway (light green box): T cells typically control microglial pruning of neuronal synapses as part of normative brain developmental programs in childhood and adolescence. Atypical T cells, potentially induced by infection or stress in genetically-at-risk individuals, could promote atypical microglial pruning of synapses, contributing to the formation of disconnected networks or circuits in the adult brain.*

We focused here on European ancestry genetic results, as the currently available datasets are from European participants, but the immunogenetics of psychiatric risk should be examined in other ancestries. In addition, the epigenetic datasets used here are predominantly adult: given the role of developmental insults in psychiatric risk, it will be important to investigate genetic enrichment in immune cells sampled at different developmental stages, including adolescence. Likewise, the immune cell states considered in this analysis are the canonical states associated

with infection and autoimmunity. It will also be important to explore whether genetic risk variants modulate immune cell phenotypes induced by exposure to non-infectious environmental stimuli, e.g., stress, especially given that childhood adversity and other social stressors are known to profoundly increase risk for multiple psychiatric disorders (Herzog & Schmahl 2018).

To conclude, we showed that genetic risk variants associated with multiple psychiatric disorders were significantly enriched at epigenetically active enhancers/promoters in adaptive immune cells, especially stimulated T cells. This enrichment at regulatory elements in adaptive immune cells was reproduced across multiple datasets; contrasted with a lack of enrichment at regulatory elements in myeloid cells; and our findings were not driven by genetic variants predisposing to high BMI. Overall, these data suggest a mechanistic role for adaptive immune cells in the pathogenesis of multiple psychiatric disorders, hypothetically by mediating the interaction between environmental exposures to biological or social threats and genetic risk variants.

Chapter 5: Discussion

5.1 Summary of findings

I here summarize our main findings, before moving on to discussing the broader implications of these results. In this thesis, we investigated the cellular immunophenotypes associated with stress, depression, and other psychiatric disorders in humans, and used animal models and genetic data to identify phenotypes and cellular subsets likely to causally contribute to psychiatric symptoms. Starting with an analysis of peripheral immunophenotypes in patients with depression, in **Chapter 2**, we used multi-parametric flow cytometry data to quantify 14 subsets of peripheral blood cells in 206 people with depression and 77 age- and sex-matched controls. We used univariate and multivariate analyses to investigate the immunophenotypes associated with depression and depression severity. Depressed cases, compared to controls, had significantly increased immune cell counts, especially neutrophils, $CD4^+$ T cells and monocytes, and increased inflammatory proteins (CRP and IL-6). Within-group analysis of cases demonstrated significant associations between the severity of depressive symptoms and increased neutrophil and B cell counts. Using forced binary clustering of cell counts, people with depression could be partitioned into two subgroups: the inflamed depression subgroup (N=81 out of 206; 39%) had increased myeloid and lymphoid cell 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. These groups differed in terms of their myeloid and lymphoid cell counts: while one subgroup of inflamed depression showed increases in all cell counts measured, the second subgroup showed marked increases in adaptive immune cells, but less pronounced increases in myeloid cells. Results were robust to potentially confounding effects of age, sex, body mass index, recent infection, and tobacco use. Peripheral immune cell counts could thus be used to distinguish inflamed and uninflamed subgroups of depression, and these results indicate that there may be multiple mechanistically distinct subgroups of inflamed depression.

In **Chapter 3**, in order to address the cellular mechanisms by which stress contributes to inflammation and behaviour, and in particular to assess the response of the meningeal

immune system to stress, we turned to a mouse model. We showed that in mice exposed to chronic psychosocial stress, there is widespread dysregulation of both the peripheral and meningeal immune compartments. Stressed mice showed increased splenic B cell activation and increased B cell secretion of the immunoregulatory cytokine IL-10. In the meninges, B cells were prevalent in homeostasis but substantially decreased following stress, whereas inflammatory ($Ly6C^{hi}$) monocytes increased following stress, and meningeal myeloid cells showed increased activation. Single-cell RNA sequencing of meningeal B cells demonstrated the induction of innate immune transcriptional programmes following stress, including genes encoding antimicrobial peptides that are known to alter myeloid cell activation. *Cd19*$^{-/-}$ mice, that have reduced B cells, showed baseline meningeal myeloid cell activation and decreased exploratory behaviour. Together, these data suggest that B cells may influence behaviour by regulating meningeal myeloid cell activation.

In humans, it is more difficult to address the causal contribution of different immune cell subsets to symptoms, but genetic association data from case-control studies of psychiatric disorders can shed some light on this question. Multiple psychiatric disorders have been associated with abnormalities in both innate and adaptive immune cells, but the role of these abnormalities in pathogenesis, and whether they are driven by psychiatric risk variants, remains unclear. In **Chapter 4**, we integrated genetic association data with tissue-specific epigenetic data to determine which immune cell subsets are likely to contribute to pathogenesis in psychiatric disorders. We tested for enrichment of GWAS variants associated with multiple psychiatric disorders (cross-disorder or trans-diagnostic risk), or 5 specific disorders (cis-diagnostic risk), in regulatory elements in immune cells. To test for enrichment, we drew on three independent epigenetic datasets representing multiple organ systems and immune cell subsets. Trans-diagnostic risk variants and cis-diagnostic risk variants (for schizophrenia and depression) were enriched at epigenetically active sites in brain tissues and in lymphoid cells (T, B and NK cells), especially stimulated CD4+ T cells. Strikingly, the finding that psychiatric risk variants were particularly enriched in adaptive immune cells was conserved across all three epigenetic datasets tested. There was no evidence for enrichment of trans-risk variants, cis-risk variants for schizophrenia, or cis-risk variants for depression in myeloid cells. This suggests a possible model where environmental exposures (e.g., infection or stress) activate T cells to unmask the effects of psychiatric risk variants, contributing to the pathogenesis of mental health disorders.

5.2 Implications for the role of innate and adaptive immunity in psychiatric disorders

Surprisingly, given the prior focus of the literature on innate immune abnormalities in psychiatric disorders, all three lines of work in this thesis implicated adaptive immunity. In the depression immunophenotyping study, both of the inflamed subgroups of patients associated with increased depression severity showed abnormalities in adaptive immune cells; in the mouse model of stress, stress led to dysregulation of both peripheral and meningeal B cells, with evidence that B cells contribute causally to behavioural phenotypes; and in the genetic analysis, the results implicated adaptive immune cells – both T and B cells – in the pathogenesis of multiple psychiatric disorders.

While the immunophenotyping study and mouse model of stress implicated myeloid cells as well as lymphoid cells, in the genetic analysis, epigenetically active sites in myeloid cells were not significantly enriched for trans-risk variants or for cis-risk variants for schizophrenia or MDD, with this finding reproduced across all three epigenetic datasets. What does this mean for the pathogenic role of myeloid cells in these disorders? It may be that genetic risk variants are indeed enriched at epigenetically active sites in myeloid cells, but only in cell states or under stimulation conditions not represented in the three datasets we analysed. Alternatively, it could be that myeloid abnormalities seen in psychiatric disorders are causally downstream of (i.e., secondary to) the effects of epigenetically activated risk variants in lymphoid cells. This would be consistent with the well-known role of T cells in coordinating and modulating innate immune function (Cohen et al 2013, Guarda et al 2009, Kim et al 2007, Rauch et al 2012), including via inflammasome inhibition (Guarda et al 2009). Thus atypical T cell phenotypes could promote or sustain low-grade inflammatory states of the innate immune system in the periphery, the meninges, and the brain, that have been robustly associated with MDD, schizophrenia and other psychiatric disorders (Alves de Lima et al 2020, Filiano et al 2016, Ziv et al 2006). A third possibility is that inflammatory responses of myeloid cells do have a primary causal role in the pathogenesis of psychiatric disorders but that these are driven by entirely environmental factors, e.g., early life adversity, rather than by genetic factors or gene-by-environment interactions.

It is also notable, and could seem somewhat contradictory, that while the animal stress model particularly implicated B cells, our genetic analysis demonstrated the most reproducible enrichment of psychiatric risk variants in T cells. However, this apparent contradiction may reflect the differences in scope of immunophenotyping between the animal and human datasets used for this analysis. On the one hand, the animal experiments were focused *a priori* on B cell phenotypes (although other animal studies have shown a T cell contribution to behavioural susceptibility to stress (Cohen et al 2006, Fan et al 2019)); and, on the other hand, the human data were focused on T cell subsets, with a relative paucity of B cell subsets available for our epigenetic analysis. The ROADMAP epigenetic dataset contained only two B cell datasets (compared to twelve T cell datasets) and there were no B cell data in the stimulated immune cell dataset. In the BLUEPRINT dataset where there was more even coverage of different immune cell subsets, trans-risk, cis-risk for schizophrenia and cis-risk for depression all showed enrichment in both T cells and B cells. Further work will thus be required to investigate the relative contribution of different adaptive immune cells to psychiatric disorders, including a fuller investigation of responses to stress in the meninges across the spectrum of adaptive immune cells, as well as analysis of the enrichment of psychiatric risk variants in a range of different stimulated adaptive immune cells.

In summary, the work presented in this thesis suggests that multiple psychiatric disorders are likely to be associated with altered adaptive immune cell phenotypes, and our genetic work particularly highlighted the potential transdiagnostic importance of T cell responses to stimulation. It is thus useful to consider what is already known about adaptive immune cell phenotypes from patient studies, especially responses to stimulation.

As summarised in **Table 5-1**, numerous psychiatric disorders have been associated with alterations in adaptive immune cells counts, cellular polarization, activation, signalling, and responses to stimulation. However, beyond investigations of simple immune cell counts (e.g., CD4+ T cell counts), such studies have mainly used small samples, are often confounded by medication use, and there is considerable variation in the experimental paradigms and stimulation conditions used. In addition, these studies have been primarily T cell focused, with little investigation of B cells (see **Table 5-1** for details and references). This makes it difficult to integrate data across primary studies, but as seen in **Table 5-1**, the most consistent findings across different psychiatric disorders, compared to controls, are of increased T helper 17

(Th17) cells and abnormal T cell responses to stimulation, both in terms of cellular proliferation and cytokine production. Th17 cells are a subtype of T helper cells that produce the canonical cytokines IL-17, IL-22 and GM-CSF. They are peripherally enriched at mucosal surfaces in homeostasis, and mediate immune responses to extracellular bacteria, but are also known for their ability to initiate and propagate brain inflammation and neurovascular dysfunction in the context of brain autoimmunity (Balasa et al 2020, Platt et al 2020). IL-17 has both homeostatic and pathogenic roles in the CNS: IL-17 has been shown to promote CA1 hippocampal synaptic plasticity, glial BDNF production and short-term memory in mice (Ribeiro et al 2019). But IL-17 has also been shown to inhibit synaptic plasticity and neurogenesis in the dentate gyrus of the hippocampus (Liu et al 2014) and to contribute to anxiety-like behaviour via its action on neurons (Alves de Lima et al 2020), and excess CNS IL-17 causes synaptic dysfunction and short term-memory deficits in a mouse Alzheimer's Disease model (Brigas et al 2021). There may thus be a bell-shaped relationship between brain IL-17 signalling and synaptic signalling, with this delicate balance potentially disrupted by the excess IL-17 signalling associated with psychiatric disorders. How the diverse findings on abnormalities in T cell activation across different disorders might relate to brain function and symptoms is less clear.

Table 5-1 *Adaptive immune cell phenotypes in different psychiatric disorders: cellular subsets, polarization, activation, responses to stimulation and other functional immune phenotypes.*

Disorder	Findings
Schizophrenia	**Cell subsets:** First-episode psychosis has been associated with increased total lymphocyte counts and increased CD4+ T cell counts at a meta-analytic level (Miller et al 2013). Schizophrenia has also been associated with increased proportions of CXCR5+ memory CD4+ T cells, but decreased proportions of central memory CD4+ T cells (Fernandez-Egea et al 2016). B cells have been much less investigated (van Mierlo et al 2019). To date, only one study has examined B cell subsets in schizophrenia, finding a decrease in naïve B cells in chronic schizophrenia compared to controls (Fernandez-Egea et al 2016). Polygenic risk score (PRS) for schizophrenia has been associated with an increased total lymphocyte count (Sewell et al 2020). **Polarization:** Schizophrenia has also been associated with altered immune cell polarization states, however, many findings are yet to be reproduced, or are inconsistent – for example, schizophrenia has been associated with increases, no changes, and decreases in Tregs across multiple studies (Corsi-Zuelli et al 2021). Slightly more consistently, schizophrenia has been associated with increases in the proportion of IL-17-producing CD4+ cells (Th17) cells, including in never-medicated patients with first episode psychosis, with the increase in Th17 cells corrected by antipsychotic treatment (Ding et al 2014, Drexhage et al 2011a). In another study, however, only patients with recent-onset psychosis/ultra-high risk for psychosis who also had a childhood trauma history showed increases in Th17 cells compared to controls (Counotte et al 2018). **Stimulation:** From experiments involving *ex vivo* stimulation, there is evidence of decreased T cell proliferative responses to stimulation, including in unmedicated patients (Craddock et al 2007, Matloubi et al 2007). There is also evidence of increased IFN-γ production from PBMCs stimulated either by the T cell mitogen PHA (Kozlowska et al 2019) or by anti-CD3/CD28 (mimicking TCR engagement by antigen presenting cells) (Sahbaz et al 2020). Another study stimulating PBMCs found an increase in PHA-stimulated IL-2, but not PHA-stimulated IFN-γ, in schizophrenia (Rapaport & Bresee 2010). **Activation:** There is also evidence that even unstimulated T cells are more activated in schizophrenia (as measured by CD25+ expression) (Sahbaz et al 2020), as well as histological evidence (based on cellular morphology) of increased activation of CSF lymphocytes in schizophrenia (Nikkila et al 2001).

Major depressive disorder	**Cell subsets:** Depression has been associated with both decreased (Cai et al 2017, Zorrilla et al 2001) and unchanged (Demir et al 2015) lymphocyte counts, but PRS for depression has been associated with increased total lymphocyte counts (Sewell et al 2020). Depression has also been associated with increased CD4+ T cell counts (**Chapter 2**, this thesis) and increased CD4/CD8 ratio (Zorrilla et al 2001) as well as increased memory CD4+ T cell counts (Maes et al 1992a). Some patients with depression also show increased counts of CD8+ T cells and B cells (**Chapter 2**, this thesis; (Maes et al 1992a) and (Maes et al 1992b)). In terms of B cells, depression has been associated with reduced numbers of peripheral IL10-producing regulatory B cells in two small studies (Ahmetspahic et al 2018, Duggal et al 2016). **Polarization:** Associations between depression and T cell polarization states, e.g., Tregs and Th17 cells, have been inconsistent (Alvarez-Mon et al 2019, Grosse et al 2016b, Hasselmann et al 2018, Jahangard & Behzad 2020, Patas et al 2018, Suzuki et al 2017). **Stimulation:** Following *ex vivo* stimulation of immune cells, there is meta-analytic evidence of decreased proliferative responses to the mitogens PHA, conA and PWM (Zorrilla et al 2001). There is also some evidence of decreased T cell IL-2 in MDD, with decreased production of IL-2 from PBMCs stimulated by the T cell mitogen PHA in MDD compared to control participants, including in unmedicated MDD patients (Lin et al 2018, Weizman et al 1994). Another study found that unmedicated MDD patients, compared to controls, showed increased proliferation of CD4+ T cells and decreased CD4+ T cell TGF-β production in response to stimulation with anti-CD3/CD28; these findings were not observed in medicated patients (Jahangard & Behzad 2020). **Mitochondrial dysfunction:** Another strand of work on adaptive immunity in psychiatry has highlighted the potential role of T cell mitochondrial dysfunction in psychiatric symptoms. In a mouse model, chronic stress caused T cell-specific dysregulation of mitochondrial fusion and decreased T cell mitochondrial respiration and glycolysis; transfer of CD4+ T cells from stressed to non-stressed mice was sufficient to cause anxiety-like behaviour (Fan et al 2019). Mirroring this, a recent human study found that patients with depression also show reduced T cell mitochondrial respiration and glycolysis (Gamradt et al 2021).

Bipolar disorder	**Cell subsets:** Bipolar disorder has been associated with increased lymphocyte counts (Munkholm et al 2018) and PRS for bipolar disorder has also been associated with an increased total lymphocyte count (Sewell et al 2020). Few studies have examined T or B cell subsets, but there is some evidence for increased CD4+ T cell proportions and decreased CD8+ T cell proportions in bipolar disorder (Barbosa et al 2014, Magioncalda et al 2018). **Polarization:** Similarly to MDD and schizophrenia, there are mixed results on frequencies of Tregs in bipolar disorder (Barbosa et al 2014, do Prado et al 2013, Drexhage et al 2011b). Bipolar disorder has been associated with either increased Th17 cells or no change in Th17 cells (Becking et al 2018, Drexhage et al 2011b, Magioncalda et al 2018, Poletti et al 2017). **Proliferation:** Bipolar disorder has also been associated (albeit in medicated patients) with decreased CD4+ T cell proliferation following stimulation of PBMCs with the T cell mitogen concanavalin A (Pietruczuk et al 2018). **Activation:** Bipolar disorder has been associated with increased frequencies of activated T cells as measured by CD25+ expression (Barbosa et al 2014, Breunis et al 2003), but no change or decrease in T cell activation as measured by the early activation marker CD69 (Breunis et al 2003, Maes et al 2021).
Autism	**Cell subsets**: In contrast to schizophrenia, depression and bipolar disorder, autism has been reproducibly associated with a *decrease* in CD4+ T cell counts (Ellul et al 2021), but no change in CD8+ T cell or B cell counts. **Polarization**: There is meta-analytic evidence that autism is associated with increased Th17 frequencies and decreased Treg frequencies (Ellul et al 2021). **Stimulation:** Following stimulation with the T cell mitogen PHA, children with autism compared to typically developing controls showed decreased expression of the activation markers CD134 and CD25 on T cells; decreased IL-12p40 production; and increased GM-CSF, TNFα, and IL-13 production (Ashwood et al 2011). Another study which activated T cells using a combination of staphylococcal enterotoxin B and anti-CD28 found an increase in IL-6-producing and IL-10-producing CD4+ and CD8+ T cells in children with autism (and their non-affected siblings) compared to controls, as well as an increase in stimulated CD4+ T cell IFN-γ production in children with autism compared to controls (Saresella et al 2009). Results on lymphocyte proliferative responses to stimulation have not been consistent: autism has been associated with both reduced (Stubbs & Crawford 1977,

	Warren et al 1986) and increased (Ashwood et al 2011) proliferative responses to stimulation with the T cell mitogen PHA.
Obsessive-compulsive disorder	There has been limited investigation of adaptive immune cell phenotypes in OCD-spectrum disorders, but one study found, similar to autism, increased Th17 and decreased Treg cells in children and adolescents with OCD compared to controls (Rodriguez et al 2019).

In addition to the investigations of adaptive immunity in specific disorders described in **Table 5-1**, there have also been some cross-disorder studies of adaptive immune cell function. One such study focused on lymphocyte signalling responses to stimuli across patients with ASD, bipolar disorder, MDD and schizophrenia, as well as control participants (Lago et al 2020). The authors used high content screening to measure the responses of different lymphocyte subsets (CD4+ T cells, CD4- T cells and CD3- lymphocytes i.e., predominantly B and NK cells) to stimulation by 14 different antigens, using phospho-specific flow cytometry to measure the activation of 42 intracellular signalling epitopes in response to these antigens. Across the disorders, 25 lymphocyte signalling nodes (cell subtype–epitope–ligand combinations) were significantly associated with different disorders, with abnormalities in both T cell and B cell signalling. Interestingly, there was substantial overlap between findings across the different disorders, with a spectral distribution of abnormalities on a continuum from MDD to bipolar disorder to schizophrenia to ASD, supporting the idea of transdiagnostic adaptive immune abnormalities in psychiatry. There has also been a cross-disorder investigation of metabolic markers on adaptive and myeloid immune cells in which the authors used flow cytometry to examine the expression of metabolic surface proteins (glucose receptor 1, insulin receptor and fatty acid translocase) on different PBMC subsets across ASD, bipolar disorder, MDD and schizophrenia (Lago et al 2021). The authors demonstrated alterations in these proteins on T cells and monocytes in schizophrenia relative to controls, but such differences were not seen in other psychiatric disorders, or on B cells. Finally, in one histological study of T cells and B cells in post-mortem brains in (pooled) patients with schizophrenia, unipolar and bipolar depression, increased B cells and T cells were seen in the hippocampal/parahippocampal region in patients compared to controls, with the B cell increase more prevalent in patients with mood disorders than in schizophrenia (Bogerts et al 2017).

The observational studies summarized above show widespread evidence of abnormal adaptive immunophenotypes in psychiatry, but cannot address the question of whether any of these changes contribute to pathogenesis or symptoms. Considering these data in the context of our new results suggests that some of these findings may be pathogenically important and prompts a renewed focus on this area. For example, it may be that the abnormal Th17 and T cell activation phenotypes observed in psychiatric disorders are pathogenic and have a genetic contribution from common variants that increase the risk of psychiatric disorders. The numerous T cell stimulation/activation abnormalities summarised in **Table 5-1** are especially tantalizing, given that our genetic analysis implicated stimulated T cells in the pathogenesis of multiple psychiatric disorders. Abnormal T cell responses to activation are thus strong candidates for being transdiagnostically pathogenic. How psychiatric risk variants mechanistically link to these observed T cell phenotypes will require further investigation, focusing on T cell activation phenotypes in participants with or without candidate risk variants (i.e., those psychiatric risk variants overlapping epigenetically active sites in T cells and/or close to genes involves in these processes). Our work also implicated other adaptive immune subsets, especially B cells, but the way forward here is less obvious, as there has been much less investigation of B cell phenotypes in psychiatric disorders (see again **Table 5-1**). However, given our findings of the effects of stress on regulatory B cells in the mouse model; the behavioural phenotype of mice with reduced regulatory B cells; and the prior literature showing reduced regulatory B cells in depression, dysfunction of regulatory B cells, with potential down-stream effects on both myeloid and T cell activation, may make a pathogenic contribution to psychiatric disorders. It would be interesting to investigate whether psychiatric risk variants are associated with altered regulatory B cell numbers or function in genetically stratified human studies.

5.3 Outstanding questions

The work in this thesis raises numerous further questions. Some key areas for future enquiry are as follows:

Can we identify and characterise pathogenic adaptive immune cell subsets and responses to antigen in psychiatric disorders?

Our findings particularly motivate further investigation of T cell and B cell phenotypes across multiple psychiatric conditions. Functional genomic analysis of adaptive immune cell subsets from patient cohorts will be particularly important to directly test for disorder- or symptom-associated alterations in DNA accessibility, histone modifications, enhancer-promoter interactions, gene expression, antigen receptor repertoire, and metabolic function. In terms of epigenetic profiles, we hypothesize that alterations will be found at those adaptive immune cell promoter/enhancer peaks identified in our analysis as overlapping psychiatric risk SNPs. However, there may be broader epigenetic consequences of risk variants, especially given that our pathway analysis of the genes near immune SNP-peak overlaps implicated epigenetic regulation processes; this epigenetic regulation may occur at genomic sites distant from the risk variants.

A key aspect of adaptive immune function not investigated in this thesis is antigenic specificity and antigenic receptor repertoires. Both B cell and T cell activation depend on the antigenic specificity of the B cell receptor (BCR) and T cell receptor (TCR) respectively, with intra-individual diversity in receptors generated by genetic rearrangement of receptor gene segments and (for BCRs) by somatic hypermutation, as well as by the nature of the antigen encountered, and the immunological context. Each T or B cell expresses a single receptor, and the range of receptors expressed across all immune cells in an individual is referred to as the repertoire. Regarding B cell receptors and immunoglobulin repertoires in psychiatry, investigations have focused on the presence of serum antibodies against specific antigens – there has been no broader molecular characterisation of B cell repertoires by BCR sequencing. Depression has been associated with increases in IgM antibodies against neoantigens generated by oxidative and nitrosative stress (Maes et al 2011) and an increased prevalence of anti-serotonin IgG/IgM antibodies (Maes et al 2012). In psychosis, a small proportion of patients have anti-NMDAR antibodies, with some evidence for pathogenicity of these (Jezequel et al 2017, Planaguma et al 2015), and some children with post-streptococcal neuropsychiatric

syndromes have antibodies against striatal antigens, again with some evidence for pathogenicity of these (Hyman 2021). In terms of TCR repertoires in psychiatry, there has been only limited analysis of these in some in small studies. Depression has been associated with a trend towards reduced CD4+ T cell receptor diversity (i.e. increased clonality) (Patas et al 2018), and in schizophrenia, there is some evidence for altered TCR V gene usage (Li et al 2018b) and for increased TCR repertoire diversity in a subgroup of patients (Luo et al 2021). The relationship between psychiatric phenotypes and antigen-specific responses is thus only beginning to be investigated.

Identifying pathogenic immunophenotypes in psychiatry is interesting not only from a mechanistic perspective, but also with a view to clinical biomarker development. To date, a few immune biomarkers have been used for the majority of psychiatric studies. In particular, high sensitivity CRP assays are widely used but also an unsatisfactory choice given the non-specificity of CRP as a marker of immunopathology, its confounding by many environmental and biological factors unrelated to psychiatry, and its temporal variability. Pathogenically-relevant biomarkers of immune dysfunction could be used in clinical populations to guide treatment pathways, potentially predicting responses to both current and novel pharmacological therapies, and to non-pharmacological therapies. For example, patients with psychiatric symptoms associated with immunopathology may respond better or worse to certain psychological therapies (Strawbridge et al 2020), or to alternative therapies such as exercise (Paolucci et al 2018, Rethorst et al 2015), vagal nerve stimulation (Bremner et al 2020), or treatment with probiotics (Nikolova et al 2021a, Park et al 2018) compared to patients without immunopathology. Immune biomarkers could also identify individuals at high risk of developing psychiatric symptoms. For example, biomarkers could be used to identify those exposed to severe stress or infection who are likely to develop psychiatric symptoms as a consequence, allowing preventative or early intervention. Our work suggests that adaptive cellular phenotypes may prove promising as biomarkers, and may be relevant trans-diagnostically and I outline our suggestions for future studies aimed at pinning down immunopathological changes in adaptive immune cells in **Section 5.4** below. However, even if a pathogenic immune cell subset or signalling pathway is identified, there will be considerable challenges in using this understanding to generate a practical and clinically useful biomarker. A cell subset specific marker requiring single cell technologies or cellular sorting to be detected would likely be prohibitively expensive, precluding its general use in psychiatry. Moreover,

many intracellular molecules (e.g., RNA and metabolites) begin to degrade within minutes/hours of blood sampling. The psychiatric hospitals and outpatient settings where psychiatric patients are usually assessed do not generally have the lab processing facilities necessary to rapidly process time-sensitive samples. Moving from a lab-based marker of immunopathology to a useable and cost-effective clinical marker which can serve as its proxy will thus require considerable further investigation and investment.

What is the role of meningeal and brain adaptive immune cells in psychiatric disorders?

The work presented in this thesis using a mouse model of stress, along with work from the Kipnis group on the contribution of meningeal T cells to behaviour, summarized in **Section 1.2.1**, suggests an important role for meningeal immune cells in normal behaviour and the response to stress in rodents. Moreover, rodent models have also demonstrated that peripherally restricted infection or immunization can lead to the accumulation of long-term resident T cells in the brain (Urban et al 2020), although the contribution of these cells to sickness behaviour is unclear. Along with our genetic findings implicating adaptive immune cells, these data suggest that adaptive immune cells located in the brain and meninges may be important in the immunopathogenesis of psychiatric symptoms. However, the contribution of brain and meningeal immune cells to behaviour in humans remains unclear. Two post-mortem histology studies have shown increases in T cells and B cells in the hippocampus in schizophrenia and mood disorders compared to controls (Bogerts et al 2017, Busse et al 2012) and studies of immune cell counts in CSF have shown a shift of CSF cells from lymphocytes to monocytes in psychosis/schizophrenia (Nikkila et al 1999, Rauber et al 2021). There has otherwise been little investigation of adaptive immune cells in the meninges or brain in the context of behaviour or psychiatric symptoms in humans. While it is practically very difficult to access the meningeal immune system and brain in humans with psychiatric disorders, it will be crucial to investigate evidence for a contribution of adaptive meningeal/brain immunity to behaviour and to psychiatric illness in humans.

Ultimately, it is unlikely that clinically used biomarkers will be based on meningeal, brain or CSF sampling for the majority of patients; in most countries, CSF sampling will not happen on a major scale in psychiatric services. Nonetheless, investigation of meningeal/brain immunophenotypes may highlight pathogenic cellular subsets which might then be detectable in the periphery with more focused immunophenotyping. Pathogenic cells trafficking to the

CNS via the blood will form only a small proportion of peripheral immune cells, but there is proof-of-concept evidence that such cells can be detected in the periphery: for example, in multiple sclerosis, a transcriptomic signature of a disease-associated CNS-homing CD4+ T cell subset which was identifiable in post-mortem brain tissue samples in multiple sclerosis could also be detected in immune cells from peripheral blood samples taken from living patients (Kaufmann et al 2021). Moreover, while neuroimaging of inflammation has to date focused on TSPO ligands (for which binding relates to microglial activation), there are multiple other PET ligands in development which will allow neuroimaging of other central immune targets (Meyer et al 2020), and methods for imaging meningeal inflammation continue to develop (Bhargava et al 2021). Magnetic resonance imaging (MRI) correlates of inflammation in psychiatric disorders – including functional MRI, magnetic resonance spectroscopy (a technique to detect tissue metabolites), and quantitative magnetization transfer (a microstructural marker of tissue composition) – are also activate areas of research and could potentially serve as proxy markers of CNS cellular immunopathology (Drevets et al 2022). These areas of ongoing innovation raise the prospect that neuroimaging biomarkers could be used to detect immunopathology initially observed in patient CNS tissue samples or predicted by translational models, but using more clinically accessible technology.

How does immunopathology evolve during development and over the course of psychiatric illness?

Practical considerations mean that both the human and animal work presented in this thesis focused on a single time point. The human flow cytometry study was an observational cross-sectional study, and data from the mouse stress model focused on a timepoint 11-12 days after the onset of chronic stress. In the future, it will be important to investigate the *dynamics* of the immune response to stress, and how immunopathology evolves over the course of psychiatric disorders. For example, in the human flow cytometry study, we identified several subgroups of inflamed depression. It is unclear whether these represent pathologically distinct disease processes, or whether these represent different phases of the same pathological process, and we are simply sampling the process at different points in its evolution. Longitudinal data will be needed to resolve this question in future.

Most psychiatric disorders have their onset in childhood, adolescence or early adulthood (Solmi et al 2021). However, there has been little investigation into immune-brain interactions

over the course of development. The investigations presented in this thesis likewise focused on mature humans and mice, and drew on epigenetic datasets primarily generated from adult participants, as there were no suitable developmental immune epigenetic datasets to interrogate. If risk factors for psychiatric conditions such as in utero infections, childhood infections, maternal stress and early life stress are to contribute to immunopathogenesis in psychiatric disorders, it is the responses of immune cells at the developmental stage of the insult which will likely be most relevant to pathogenesis. Thus, an understanding of immunopathogenesis in psychiatry will require a deeper characterisation of the normal development of the immune system and its interactions with the brain during gestation, childhood and adolescence, as well as investigations into how these interactions are disrupted following developmental insults and during the emergence of psychiatric symptoms. There is evidence from animal studies that both B cells and T cells are crucial for normal brain development, with important effects on oligodendrocyte and microglial development, and on synaptic pruning (Pasciuto et al 2020, Tanabe & Yamashita 2018). Thus atypical T or B cell activity during development could lead to the disrupted brain connectivity and white matter alterations seen in schizophrenia and other psychiatric disorders (Koshiyama et al 2020, Morgan et al 2019), motivating further investigation of the developmental role of the adaptive immune system in the emergence of psychiatric symptoms. As above, understanding how psychiatric immunopathology develops will require longitudinal data.

An understanding of how immunopathology emerges during development and in adulthood will necessarily include analyses of how risk factors for psychiatric disorders affect the immune system and its interactions with the brain. Multiple risk factors and environmental insults (as outlined in

Table 1-5) are associated with the inflammatory changes characteristic of psychiatric disorders, as most simply conceived, i.e., increases in pro-inflammatory cytokines, acute phase reactants and increased peripheral immune cell counts. However, such immune markers are highly non-specific and cannot be attributed to dysfunction of any particular immune cell subset without further investigation. It will be particularly interesting to investigate whether there is any convergence of the effects of these risk factors onto a shared cellular immunopathology upstream of (and preceding) psychiatric symptoms. It is also unclear why some people, but not others, exposed to a particular insult (e.g., a stressor or an infection) show prolonged inflammatory changes and behavioural susceptibility to its effects. One explanation for this is genetic susceptibility, i.e., gene-environment interactions. In the future, it will be crucial to understand what contribution genetics makes to our immunological and behavioural susceptibility to such insults.

Does immunopathology disrupt conventional psychiatric nosology?

Our current diagnostic symptoms draw a line between idiopathic psychiatric disorders and psychiatric disorders in the context of, or secondary to, medical disorders. For example, according to both the *Diagnostic and Statistical Manual of Mental Disorders*, fifth edition (DSM-V) and the *International Classification of Diseases*, tenth edition (ICD-10), the diagnosis of MDD (or for ICD-10, a depressive episode) is only to be applied to patients in whom there is no causative organic pathology. ICD-10 suggests a separate diagnosis of 'organic depressive disorder' if the symptoms are thought to be a consequence of *'cerebral disease, damage or dysfunction, or of systemic physical disorder known to cause cerebral dysfunction, including hormonal disturbances'* and DSM-V excludes a diagnosis of MDD if there is *'evidence from the history, physical examination, or laboratory findings that the disturbance is the direct pathophysiological consequence of another medical condition'*. Evidence of immunopathology in patients ostensibly with idiopathic psychiatric disease, as we describe in **Chapter 2**, is particularly disruptive to this traditional, but arbitrary, division into 'organic' and 'non-organic' psychiatric disorders. The evidence now suggests that immunopathology may contribute to symptoms both in the context of medical comorbidity and in patients without comorbidity.

At the cellular level, it remains unclear to what extent the immunopathology of psychological symptoms without medical comorbidity overlaps with immunopathology driving such symptoms in the context of medical comorbidity, e.g., rheumatoid arthritis or multiple

sclerosis. This lack of clarity is in part because studies tend to either exclude participants with medical comorbidity (necessarily so if a study focuses on the diagnostic category of MDD), or do not ascertain detail on medical comorbidities, or are not adequately powered to investigate patient subgroups with or without different comorbidites. Immunophenotypes can be compared across different studies, but the variation in assays, clinical phenotyping instruments and experimental paradigms used make this difficult. Nonetheless, there is some evidence of both shared and distinct cellular immunopathology between depression associated and not associated with medical comorbidity. For example, depression in the context of multiple sclerosis has been replicably associated with decreased $CD4^{+}CCR7^{low}$ T central memory cells and – unlike our findings in **Chapter 2** – not associated with higher lymphocyte counts, but – in keeping with our findings in idiopathic depression – associated with increased classical monocytes (Brasanac et al 2021).

A second way in which our findings disrupt conventional psychiatric nosology is in the finding that adaptive immune cells are implicated in the pathogenesis of multiple psychiatric disorders. If a given pathological process contributes to multiple disorders, patients with markedly different phenotypic presentations may benefit from a similar immune-targeting treatment approach, and this would open the door to immunologically-defined diagnoses in psychiatry, in stark contrast to our current symptom- and behaviour-based categories. Alternatively, these adaptive immune responses may differ across disorders – e.g., they may be antigen specific, with differing antigens triggering them in different disorders – and thus require different immunomodulatory treatment strategies.

Mechanistically, there are several (non-mutually exclusive) ways that a given immunopathology might contribute to multiple psychiatric disorders. Firstly, it may be that a given immunopathology is frequently shared across disorders, but that the symptoms presented depend on the individual's brain circuitry, with immunopathology lowering the threshold for whatever symptoms an individual is predisposed to express. Under this hypothesis, immune dysfunction could additively raise the risk of multiple psychiatric disorders, leading to clinical presentation of whichever psychiatric condition the immune-perturbed individual has the greatest baseline risk for (due to other genetic or environmental factors). Secondly, a given immunopathology could drive specific symptoms – for example, fatigue, anhedonia or altered sleep – and thus contribute to that symptom in a range of

different clinical presentations, ranging from depression to schizophrenia to psychological symptoms in the context of multiple sclerosis or neurodegenerative conditions. Thirdly, it may be that there is substantial heterogeneity in the immunopathology driving even one symptom or disorder, and that there are multiple distinct immunopathologies to be discovered. For example, low mood could be driven by either stress-induced meningeal monocytic infiltration, or by aberrant T cell activation and signalling to the vagus nerve, and we would need to perform laboratory investigations in each individual patient to determine which immunopathology was contributing to symptoms in that individual. Under this third model, an understanding of immunopathology in psychiatry becomes substantially more challenging, as studies of very large numbers of patients will be needed to tease apart the immune heterogeneity of psychiatric symptoms. The work presented in **Chapter 2** on the immunopathology of depression, which demonstrated multiple clusters of patients with inflamed depression, suggests that there may indeed be substantial heterogeneity of immunopathology in psychiatry.

In summary, our work, and immunopsychiatry more broadly, challenge both the organic / non-organic divisions used in our diagnostic criteria, and the individual disorder categories themselves. If immunopsychiatry is to lead to new diagnostic tools and treatment approaches, these will need to be developed and offered in a way that goes against the grain of current clinical approaches and cuts across often-siloed mental and physical healthcare settings. Moreover, such approaches will not intersect easily with current DSM/ICD diagnosis-based approval processes for novel treatments. Ensuring that patients benefit from our scientific findings will thus require considerable innovation both at the scientific level, and on a political and organizational level.

5.4 Key future approaches

Multiple approaches will be needed to address the challenges above, including innovations in both study design and in the immune endpoints measured.

5.4.1 Study design

Addressing the questions above will require longitudinal cohort studies which collect information on genetics, immunophenotypes, psychiatric symptoms and the exposome, with

the exposome broadly conceived to include pre- and peri-natal insults, developmental insults, and adult exposures, encompassing information about psychological, immunological and environmental exposures. Findings from existing cohort studies and animal models have emphasized the importance of collecting information on even very early (including prenatal) exposures (e.g., parental smoking) that are potentially correlated with stress or immune exposures: such exposures can have substantial and long-lasting effects on outcomes such as immune cell phenotypes and epigenetic profiles that will need to be disentangled from the effects of more recent exposures (Ladd-Acosta & Fallin 2019, Noor & Milligan 2018, Richmond et al 2018). It will be particularly important that cohort studies collect information on how the immune system and brain develop in tandem during foetal development, childhood and adolescence.

If there is indeed transdiagnostic immunopathology in psychiatry, as our work suggests, this further emphasizes the importance of clinical studies which recruit not on the basis of diagnoses, but by either population sampling; by cross-disorder sampling; or by sampling based on exposures (e.g., to infection or stress). In terms of genetic research, there is a strong body of transdiagnostic research in psychiatric genetics (Cross-Disorder Group of the Psychiatric Genomics Consortium 2019, Lee et al 2021). However, there are currently no inflammation-stratified genome-wide association studies in psychiatry, meaning that in current GWAS results, any immunogenetic signals will be diluted by signals from patients for whom the immune system does not contribute genetic risk. In future work, it will be important to investigate the genetic variants specifically associated with inflammation in psychiatric disorders.

5.4.2 Immunophenotyping

As discussed above, our mouse and human work motivates the further investigation of cellular (especially adaptive) immunophenotypes in patient groups. Deeper immunophenotyping could either serve as novel endpoints in traditional DSM case-control designs, or form part of the longitudinal cohort or transdiagnostic study designs described above. A major difficulty will be in deciding which cellular phenotypes or ‘omic’ technologies to prioritise in future immunophenotyping studies. Our genetic work highlighted the potential importance of histone marks of enhancer activity as a starting point for investigation in clinical cohorts, but this is partly a function of the datasets that were available for analysis. It is unclear whether cellular

proteomics, transcriptomics, methylation, chromatin accessibility, histone marks, metabolomics or some combination of these will be most informative. Another consideration for future clinical studies is the use of single cell technologies. As highlighted by the single cell analysis of mouse meninges presented in this thesis, single cell RNA sequencing has considerable advantages over bulk RNAseq in allowing detection of cell-subset specific changes in gene expression. More generally, single cell approaches have the advantages that they allow the detection of novel pathology-associated cell subsets; omic data in multiple cell subsets can be assayed simultaneously; the heterogeneity of omic data across different cells can be analysed; patterns of covariation in omic data can be explored at the single cell level; and results are not confounded by differences in the cellular composition of samples between patients and controls. Single cell technologies also have significant limitations: lowly-expressed genes (including many cytokines) are not easily detected; the technology is expensive (necessarily reducing sample size); and current methods used for library preparation generate batch effects which can be difficult to correct for. Because of the relative strengths and limitations of bulk and single cell analyses, future approaches to immunophenotyping in psychiatry will likely be most successful if they take complementary approaches using both single cell technologies as well as bulk analyses of separated immune cell subsets.

A key feature of adaptive immunity that we did not investigate is how stress and psychiatric phenotypes relate to antigenic receptor repertoires. TCR and BCR sequencing and analysis will allow in-depth investigation of this important aspect of adaptive immune function, and potentially prediction of the epitopes (i.e., the fragments of exogenous or endogenous antigens which bind to a TCR or BCR to generate a T or B cell response) which drive abnormal adaptive immune phenotypes in psychiatry, if such epitopes exist (Joglekar & Li 2021, Teraguchi et al 2020).

Our genetic results suggest that some of the immunophenotypes linked to genetic risk for psychiatric disorders may only be revealed under stimulation conditions. It will thus be important to assay stimulated immunophenotypes in patients with psychiatric disorders, with stimuli ranging from *in vivo* paradigms such as stress or vaccination to *in vitro* paradigms such as exposure of immune cells to stress hormones, pathogen-associated molecular patterns, or cytokines, using paradigms similar to those described in **Table 5-1**, but in larger cohort studies, and with a trans-diagnostic focus. Linked to this, by highlighting 'poised' or primed genetic

elements in unstimulated cells which can predict subsequent transcriptomic responses to stimulation (e.g., (Provencal et al 2020)), epigenetic analysis of immune cells may offer a proxy method to detect stimulation-related phenotypes in patient populations while avoiding the practical difficulties associated with implementing stimulation assays at scale or as part of clinical care.

As discussed, there has been very limited investigation of the CNS immune compartment in humans with psychiatric disorders. While practically difficult, it will be crucial to investigate the function and phenotypes of immune cells in the meninges and brain in order to better understand the role of the immune system in psychiatric symptoms. Such studies could include both post-mortem investigations of meningeal and brain immune cells, and studies of CSF immune cell phenotypes in live patients. These investigations should ideally be designed to allow paired analyses of immune cells in the CSF and periphery, so that the (more clinically useful) peripheral immune correlates of any central immune dysfunction can be identified, if possible.

5.5 Conclusions

I used human and animal models to uncover novel cellular immunophenotypes associated with depression and stress in the periphery and meninges. This work, along with the transdiagnostic genetic analyses presented, generated clear hypotheses about the immune cells likely to causally contribute to psychiatry symptoms, highlighting especially the importance of adaptive immune cells.

The new results presented in this thesis support the existence of trans-diagnostic adaptive cellular immunopathology, and, importantly, further suggest that this cellular immunopathology is likely to causally contribute to symptoms. These findings motivate a precision medicine approach to diagnosis and treatment, but one based not on the categorical diagnoses traditionally associated with psychiatry, but on trans-diagnostic immune biomarkers. Taken in concert with a growing understanding of trans-diagnostic effects in psychiatry beyond the immune system, this work poses a challenge to traditional approaches to psychiatric assessment, nosology and treatment. Incorporating our emerging neuroscientific and

immunopsychiatric understanding of disease pathogenesis into clinical practice will require a wholescale revision of our approach to patient investigation and care.

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