B-cells are abnormal in psychosocial stress and regulate meningeal myeloid cell activation

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ABSTRACT

There is increasing interest in how immune cells, including those within the meninges at the blood–brain interface, influence brain function and mood disorders, but little data on humoral immunity in this context. Here, we show that in mice exposed to psychosocial stress, there is increased splenic B cell activation and secretion of the immunoregulatory cytokine interleukin (IL)-10. Meningeal B cells were prevalent in homeostasis but substantially decreased following stress, whereas Ly6C hi monocytes increased, and meningeal myeloid cells showed augmented expression of activation markers. 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.

1. Introduction

Depression affects millions of people worldwide, but the underlying pathogenesis is poorly understood, and current therapeutic interventions have limited efficacy (Kessler et al., 2005). There is increasing interest in the interplay between immune system activity and mood disorders; some patients with depression have features of immune dysregulation, with elevated circulating C-reactive protein and pro-inflammatory cytokines, such as interleukin (IL)-6 and TNFα, with both peripheral immune activation and the development of depression (Duggal et al., 2016; Maes et al., 1992; Voorhees et al., 2013). Furthermore, psychological stress is associated with both peripheral immune activation and the development of depression (Duggal et al., 2016; Maes et al., 1992; Voorhees et al., 2013). There is some evidence that inflammatory cytokines may directly cause behavioral abnormalities; for example, the administration of interferon (IFN) α, a type I interferon, induces depression in some humans and depression-like behavior in rodents via endothelial and microglial activation (Blank et al., 2016; Pinto and Andrade, 2016; Wachholz et al., 2016).

Until recently, the functional link between peripheral immunity and the central nervous system (CNS) has been obscure, with the brain historically viewed as an immune-privileged site and, with the exception of microglia, devoid of immune cells. However, recent data demonstrating resident immune cells within the meninges in the steady state challenge this dogma. Healthy meninges contain both innate and adaptive immune cells, including monocytes, T cells, and B cells (Ajami et al., 2018; Korin et al., 2017) but whether there is recirculation between peripheral and meningeal compartments in health is unclear. Data indicate that meningeal CD4+ T cells can impact cognition, social behavior, and responses to stress in rodents, potentially via cytokine production, including the canonical type II IFN, IFNγ (Cohen et al., 2006; Derecki et al., 2010; Filiano et al., 2016). IFNγ is a potent activator of myeloid...
Abnormalities in myeloid cell activation have been linked to the observed increase in peripheral inflammatory markers observed in stress. For example, chronic social defeat stress in rodents increased levels of circulating inflammatory cytokines and mobilized peripheral monocytes (Hodes et al., 2015). Changes in CD4+ and CD8+ T cells also contribute to altered affective behavior following chronic stress in animals and are associated with depression in humans (Herkenham and Kigar, 2017). In contrast, humoral immunity has received little attention in mood disorders, but causative associations between autoantibodies and psychosis (Dalmau, 2016) suggest a role for B cells. 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 IL6 (Barr et al., 2010; Menard et al., 2007); and 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 IL10 secretion (Rosser and 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

Fig. 1. Dysregulation of the peripheral B cell compartment in mice subjected to chronic stress. (A) Effects of chronic stress (11 days) on behavior (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+ classical DCs (cDCs); neutrophils; Ly6Chi monocytes, Ly6C+CD11b+ mesenchymal myeloid cells; and F4/80+ macrophages. N = 40 mice in overall model: control + animal cohort + strain + condition + strain × condition (see Methods). (C) Effects of SD on splenic B cell subsets (median ± IQR; Mann-Whitney U test, **P < 0.01); N = 10 HC, N = 11 SD (wild-type mice same as Fig. 1B); see Fig S1 for underlying count data for other cell subsets. (D) Stimulation of splenocytes from stressed and homecage animals by LPS, PMA and ionomycin: supernatant IL10 measured by ELISA. IL10 values were calculated based on a Sigmoidal 4-parameter standard curve fit using Prism software. Linear models (log IL10 ~ condition) were performed on raw data. For raw data for each cohort separately, see Fig S4. (E) Effects of SD on splenic cell subset functional marker expression, measured by percent positivity. Datapoints are debatched for the effect of cohort for visualization. Data from 10 HC, N = 11 SD (wild-type mice same as Fig. 1B); see Fig 1C for more detailed analysis. (F) Effects of SD 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 colors 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 correction for multiple comparisons in each panel (56 comparisons for myeloid panel, 35 comparisons for lymphoid panel); N = 10 HC, N = 11 SD, same mice as Fig. 1C. All replicates are biological.
cells have been shown to control innate immune cell activation (Iwata et al., 2011) and recruitment to the brain in the context of stroke (Ren et al., 2011) and viral encephalitis (Mutnal et al., 2014), but their contribution to regulation of innate immunity in the meninges, and their role in the stress response, is unknown.

Some peripheral blood phenotyping studies in human depression have shown higher numbers of circulating B cells (Maes et al., 1992) as well as reduced IL10-producing regulatory B cells in patients compared with non-depressed controls (Ahmetspahi 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. 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 the distinction of causative versus associative correlations.

To overcome this, we sought to investigate how immune cells, particularly B cells, changed in both the periphery and the meninges in the SD mouse model of psychological stress, which produces anxiety and depressive-like behavior in defeated animals (Brachman et al., 2015).

2. Results

2.1. Dysregulation of the peripheral B cell compartment occurs in mice subjected to social defeat (SD) stress

We performed chronic social defeat stress in behaviorally phenotyped wild type mice and collected tissue for flow cytometric analysis (Fig. S1A). As expected, on day 11 following the onset of the SD protocol, behavioral parameters (the number of light–dark crosses and distance travelled in a novel open-field arena) were significantly reduced in the defeated group compared to home cage controls (HC) (Fig. 1A), suggesting increased anxiety levels and decreased motivation to investigate.

On day 12 following the onset of the SD protocol, there was a significant increase in splenic neutrophils, Ly6Chi monocytes, and F4/80hi macrophages compared to controls (Fig. 1B), indicative of innate immune activation. Peripheral blood cytokines in SD animals were similar to controls, except for a significant increase in G-CSF, a neutrophil-mobilising cytokine (Fig. S1B). In addition, splenic plasmablasts/plasma cells (terminally differentiated B cells) were significantly increased in SD mice (Fig. 1B, S1C), as were splenic CD9+ B cells (Fig. 1C). CD9 has been described as a marker of IL10-producing regulatory B cells (Sun et al., 2015) and IL10 secretion in B cells is enhanced by activating stimuli (Mauri et al., 2003; Yoshizaki et al., 2012). We therefore directly assessed B cell IL10 production following stimulation ex vivo. We confirmed a significant increase in the percentage of IL10+ regulatory B cells in the SD group, both at baseline and following ex vivo stimulation, and an associated increase in secreted IL10 in the supernatants from these stimulations (Fig. 1D, 1E). It is striking that the effect of stress on intracellular IL10 is 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 and CD80 on B cells in SD animals (Fig. 1F, S3A). There were no differences in T cell number or activation in the SD group compared with controls (Fig. 1E, S1D).

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 IL10-producing B cells and plasmablasts/plasma cells (see Graphical Abstract).

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

In addition to allowing peripheral immune profiling to be extended to the spleen, the use of a murine model of psychosocial stress also allowed us to investigate its effects on meningeal immune cells. Analysis of healthy mouse meninges by flow cytometry revealed heterogeneous immune cell populations, including innate and adaptive immune cells (Fig. 2A-B, S2A), as described by other investigators (Ajami et al., 2018; Korin et al., 2017). To ensure 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, making up around 25% of all immune cells (Fig. 2B), consistent with recently published data (Brioschi et al., 2021). Following SD, only two meningeal immune populations showed significant changes from their counts in homeostatic B cells, which were significantly decreased, and Ly6Chi monocytes, which were increased (Fig. 2C, S2B). There were trends towards increases in neutrophils and macrophages but no significant change in meningeal T cell counts (Fig. 2C, S2B). Cell counts estimated derived from bulk meningeal transcriptomic analysis (microarray data) confirmed a reduction in naïve B cells and an increase in monocytes and neutrophils in meninges obtained from stressed mice (Fig. 2D, S2C).

An increased proportion of residual meningeal B cells were activated in SD compared with control, as evidenced by expression of CD69 (Fig. 2E, S2D), but there were insufficient cell numbers to perform ex vivo stimulation assays to measure IL10 production. Meningeal Ly6Chi and Ly6Cint monocytes, and macrophages had higher expression of CD11b, consistent with an activated phenotype (Fig. 2F). Bulk transcriptomic analysis of stressed meninges confirmed that innate immune response pathway genes associated with myeloid cell activation, including ‘TNF alpha 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 (Fig. 2G, S5A).

Within the stressed group, we observed a significant positive correlation between meningeal B cell numbers and the number of light–dark crosses (Fig. 2H), showing that lower meningeal B cell numbers were associated with a more severe behavioral phenotype. Likewise, higher numbers of meningeal Ly6Cint monocytes were associated with reduced distance travelled in a novel open-field arena (Fig. 2H), indicative of reduced exploratory drive. Correlations between splenic and meningeal cell counts (assayed in the same animals) were minimal (Fig. 5B), arguing against the hypothesis that the meningeal cell counts simply reflect immune activity in the periphery. B cell numbers in the meninges do not simply reflect those in the blood: while there is an early (at 2 h) decrease in both blood B cells and extravascular meningeal B cells following CSD, blood B cell numbers recover rapidly and have normalized by 16 h, while extravascular meningeal B cells remain decreased at 16 h (Fig. 2I). 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.

3.1. Meningeal B cell response to stress

To better characterize B cell responses to stress, we used droplet encapsulation high throughput single-cell RNA sequencing (scRNA seq) (10x Genomics platform) to profile meningeal immune cells from non-stressed and stressed mice (Fig. S6A). Of the 21 clusters that were annotated based on their expression of canonical marker genes, we found two mature B cell clusters – an MHCIIhi cluster (N = 150 cells) and an MHCIIlo cluster (N = 369 cells) (Figs. S6B, S6C), that were present in similar proportions in control and stressed meninges (Fig. 3B, S7A).

Differential gene expression analysis showed an increase in a number of innate immune response genes in the MHCIIhi B cell cluster in stressed meninges, including Ccnb1, Ccl2, and Il10 (Fig. 3C, S7B). 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, Nkg encodes a cysteine protease
Fig. 2. Chronic stress is associated with an increase in meningeal myelomonocytic cells but a reduction in meningeal B lymphocytes. (A) Automated clustering of meningeal flow cytometry data, visualized by tSNE; downsampled data from N = 53 mouse meninges. Intravascular immune cells were identified by intravenous CD45 labelling prior to harvest and excluded from analysis. Annotations were determined by surface marker expression profiles (Fig. S10C); clusters map closely to manual gating (Fig. S2A). (B) Relative proportions (median ± IQR) of extravascular immune cells identified in healthy meninges (manual gating, see Fig. S10B). Pie chart indicates median proportion of each subset. N = 13 HC mice (subset of mice in Fig. 2A). (C) 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); same mice as Fig. 2A. The 13 cell subsets tested were Ly6C+ 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. 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). (D) Cell count enrichment scores (median ± IQR) from cellular deconvolution of meningeal microarray data support the flow cytometry results (N = 7 SD, N = 7 HC, biological replicates). Mann-Whitney U tests (unadjusted): *P < 0.05, **P < 0.01. See Fig. S2C for all enrichment scores. (E) Effects of chronic stress on meningeal immune cell activation (manual gating for percent positivity). Rank-based linear models including condition and cohort. Subset of 7 comparisons shown, full results in Fig. S2D. FDR P* < 0.05, ** < 0.01, wild-type mice from Fig. 2A. (F) Effects of chronic stress on meningeal median functional marker expression (for Fig. 2A clusters). Tile colors indicate linear model estimates (coefficients) for the effect of stress (model includes condition and cohort). Tile size indicates significance following FDR adjustment (102 comparisons). Same mice as Fig. 2A. Full list of enriched pathways shown in Fig. S5A. (H) Spearman correlations between behavioral susceptibility to stress and immunophenotype in stressed wild-type animals. Correlations significant at FDR P < 0.05 are shown in inset. Same mice as Fig. 2A. All replicates are biological. (I) Meningeal and blood B cells (as a percentage of live CD45+ cells) at 2 h and 16 h post-defeat (CSD), compared to matched homecage (HC) animals. Animal numbers: meninges 2 h (HC = 21, CSD = 15), meninges 16 h (HC = 8, CSD = 4), blood 2 h (HC = 21, CSD = 14), blood 16 h (HC = 8, CSD = 4). Controls for the 16 h timepoint are a subset of the controls for the 2 h timepoint. HC and CSD groups were compared using a Mann Whitney U test.
that inhibits cathepsin (Boutet et al., 2011), whilst Lipocalin 2, the protein encoded by Lcn2, promotes an anti-inflammatory M2 macrophage phenotype (Guo et al., 2014) and inhibits microglial activation (Jang et al., 2013). S100a9 is an alarmin that can promote the generation of anti-inflammatory myeloid suppressor cells (Dai et al., 2017).

Together, these data are consistent with the conclusion that meningeal B cells undergo significant transcriptional changes during stress, with induction of innate immune transcriptional programs and the production of AMPs 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 scRNA seq 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 (Fig. 3D), 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 MHCIIhi 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 methodology, see Fig. 3E).

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 a number of 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 nor of Tnfsf13b (the gene encoding BAFF); in fact the trend was towards an increased expression of these transcripts (P < 0.05 uncorrected for multiple comparisons, Fig. 3A).

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 MHCIIhi B cell cluster in SD (Uncorrected P = 0.04, Fig. 3E). B cell CCR7 normally drives homing of B cells to lymph nodes, so this result goes against the
hypothesis that the reduced B cell number was due to enhanced migration away from the meninges to draining lymph nodes.

We also interrogated the scRNAseq data for evidence that meningeal B cells may be undergoing increased cell death during SD, resulting in the reduced number observed. However, there was no significant increase in cell stress-associated genes (Fig. S7E), apoptosis or necrosis Reactome pathways in meningeal B cells in SD, nor an increased proportion of mitochondrial reads per cell, as might be expected in dying cells (Fig. S7C). Stress was also not associated with changes in the proportion of B cells estimated to be in each cell cycle phase (Fig. S7D), suggesting the altered number of B cells is not due to suppression of local proliferation.

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

In order 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, with B1a cells, which include regulatory B cells, particularly affected (Engel et al., 1995; Engel et al., 2001).

Fig. 4. 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 total B cells as per Fig. 4B, 4D models. Cd9 + B cells: Mann-Whitney U test \*\*P < 0.001. Splenic data: N = 10 wt, N = 8 Cd19−/−, WT mice same as for Fig. 1 cytometry. Meningeal data: N = 13 wt, N = 12 Cd19−/−, WT mice same as for Fig. 2 cytometry. (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 as in Fig. 1 B/4A. (C) Effects of Cd19 deficiency on splenic immune cell activation. Tile colors 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, same mice as Fig. 4 A. (D) Effects of Cd19 deficiency (negative binomial model) on absolute counts of meningeal extravascular immune cell subsets; 13 cell subsets tested as in Fig. 2 C, N = 53 mice in overall model, same mice as Fig. 2C/4A. (E) Effects of Cd19 deficiency on meningeal median functional marker expression (MFI) (bulk RNAseq data) at FDR \*P < 0.05 (N = 5 wt; N = 4 Cd19−/−). For full list of enriched pathways see Fig. S9. All replicates are biological.
Our data demonstrate that social stress in rodents is associated with secondary lymphoid organs, and these may differ from those in blood, changes in the peripheral B cell compartment. We further demonstrate meningeal interferon signaling. Whilst splenic B cell represent those in with an overlap in leading edge genes (Matsushita et al., 2008; Yanaba et al., 2008; Yoshizaki et al., 2012), without 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 (Fig. 4A), including IgD− (naïve) and IgD+ subsets, as well as CD95− B cells (a subset enriched for regulatory B cells) and plasma cells (Fig. 4A, 4B). 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 was variable, and did not reach statistical significance (Fig. 4A). Therefore, this model provided a means of examining the impact of a decrease in peripheral B cells, including regulatory B cells, on meningeal immunity in health and following social defeat. This observation 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 and Sorensen, 2019).

Normal 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 (Fig. 4B), and in MHCII expression on splenic DCs (Fig. 4C), as well as increased plasma G-CSF (Fig. S8A). The residual splenic B cells in Cd19−/− mice also showed a more activated phenotype, with increased expression of CD25 and MHCII (Fig. 4C, S8B).

In the meninges, we observed an increase in neutrophils and cytotoxic T cells in Cd19−/− mice (Fig. 4D) 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 (Fig. 4E, S8C).

We next sought to determine whether the Cd19−/− mice might have behavioral differences both at baseline and following exposure to SD. At baseline, Cd19−/− mice showed reduced exploration of a novel environment compared with WT controls (Fig. 4F, Fig. SBH). Following SD, there was no difference between Cd19−/− and WT animals on novel arena exploration or light dark testing (Fig. 4F). In the periphery, there was an increase in splenic neutrophils, Ly6C− monocytes, and macrophages in defeated Cd19−/− compared to WT mice (Figs. S8D, S8E). 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 significantly increase further (Fig. 4G).

Bulk RNA sequencing of meninges obtained from mice subjected to SD demonstrated an increase in ‘interferon response’ and ‘interferon γ response’ pathways in Cd19−/− mice compared to WT counterparts (Fig. 4H, S9A), suggesting convergent effects of psychological stress and CD19 deficiency on meningeal interferon signaling.Whilst splenic B cell represent those in secondary lymphoid organs, and these may differ from those in blood, our data demonstrate that social stress in rodents is associated with changes in the peripheral B cell compartment. We further demonstrate that B cells play a role in negatively regulating meningeal myeloid cell activation in homeostasis and in the context of social defeat.

4. Conclusions

Overall, our study shows that psychosocial stress is associated with activation of the peripheral B cell compartment, and an increase in regulatory (IL10-producing) B cells and terminally differentiated B cells (plasmablasts/plasma cells) in the spleen. Similarly, in the meninges following SD, we found that B cells became activated, and express genes encoding AMPs that have known immunomodulatory effects on myeloid cells; Lipocalin 2 can inhibit microglial activation (Jang et al., 2013). Camp inhibits macrophage pro-inflammatory cytokine production (Torres-Juarez et al., 2015) as well as IFNγ-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 (Ulás et al., 2017) and promote the generation of myeloid suppressor cells (Dai et al., 2017).

The potential importance of the immunoregulatory effects of B cells on meningeal myeloid cells is evident in 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, Cd19−/− deficient meninges demonstrated an enrichment of IFNγ response genes following SD compared with their WT counterparts, confirming the importance of B cells in regulating the effects of IFNγ in the meninges, potentially via Camp expression (Nijnik et al., 2009). IFNγ is a potent activator of myeloid cells and has been implicated in the link between immunity and behavior, with evidence for both beneficial and pathogenic effects. Animal models have shown that IFNγ activates the choroid plexus to promote leucocyte recruitment into the CNS following injury (Kunis et al., 2013). It can also increase hippocampal neurogenesis, improve learning (Baron et al., 2008), and activate GABAergic neurotransmission to support normal social behavior (Filiano et al., 2016).

Conversely, IFNγ may mediate increased synaptic pruning in infection-associated inflammation (French et al., 2019). Furthermore, in a murine chronic stress model, IFNγ deficiency led to reduced corticosterone, cytokine, and behavioral responses to stress (Litteljohn et al., 2010). In humans, there is evidence that elevated plasma IFNγ is associated with stress-induced and generalized anxiety (Hou et al., 2017; Maes et al., 1998). Therefore, our data showing the ability of B cells to control the magnitude of IFNγ signaling in the meninges suggest an important, clinically relevant facet of their function.

Other mechanisms by which B cells might modulate myeloid cells include the production of the regulatory cytokine IL10. We observed an increase in splenic B cell IL10 production in defeated animals, however Il10 transcript expression in meningeal B cells was not sufficient to allow its detection using scRNA seq using the 10x Genomics platform, which provides relatively shallow sequencing depth. We were not able to robustly interrogate meningeal B cell IL10 protein production due to the limited number of cells present. However, it may be that these cells also produce IL10, in addition to immunoregulatory AMPs. The phenotype and contribution of crawling/intravascular meningeal B cells also remains an interesting question.

Our study did not definitively identify the cause of the reduced meningeal B cell number we observed in the context of stress, nor the increase in peripheral regulatory B cells or plasmablasts/plasma cells. There are a number of potential explanations for these observations. Firstly, if there is continuous re-circulation between the meningeal and peripheral B cell compartments, it may be that psychosocial stress leads to an increase in B cell migration from the meninges into the circulation or deep cervical lymph nodes, without a corresponding uplift in egress of B cells out of 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 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 scRNA seq data set, the chronic nature of the stress is such that 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.

In summary, our study shows that psychosocial stress is 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, our data suggest that B cells may affect behavior by regulating meningeal myeloid cell activation and meningeal interferon responses, shedding light on the cellular networks at play in the immunological response to stress.

5. Materials and Methods

5.1. Experimental animals

C57BL/6J mice (Jackson Laboratories) or (for Fig. 2D) and 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) (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 in Fig. 1A-C, E, F, Fig. 2A-C, E, F, H and Fig. 4A-E were performed over 7 cohorts of animals balanced as far as possible for all four conditions (C57BL6 social defeat, C57BL6 home cage, Cd19<−/− social defeat and Cd19<−/− homecage; animals aged 7–11 weeks at baseline). For comparison of splenic IL-10 secretion (Fig. 1D), animals were aged 12–13 weeks. For comparison of behavior in WT vs. Cd19<−/− mice (Fig. 4F and 58H), behavioral testing of littermate animals was performed over 9 cohorts (animals aged 7–28 weeks). For meningeal microarray data in Fig. 2D, 2G, 3A and SSA animals were aged 11–12 weeks at baseline. For meningeal bulk RNAseq data in Fig. 4H, animals were aged 7–9 weeks. For meningeal single cell RNA sequencing (Fig. 3B-E, S6, S7), animals were aged 9–10 weeks at baseline. The procedures described were conducted in accordance with the National Institutes of Health guidelines and were approved by the National Institute of Mental Health Institutional Animal Care and Use Committee.

5.2. Chronic social defeat (SD) paradigm

As previously described (Lehmann and 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.

5.3. Behavioral phenotyping

At baseline and on day 11 following SD or HC housing, mice underwent behavioral 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 acclimate 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 behavior 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 × 25 × 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 × 50 × 50 cm open-field arena. The proportion of time spent in the central 50% of the arena during 15 min was measured (except for mice used in Fig. 4F and 58H, in which mice underwent testing for 10 min).

6. 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 11th 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 min 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 (RPML +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 RPML +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 (Tables S2, S3) and then PBS were added 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).

6.1. 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
Fig. S10 and S11. The following meningeal immune populations were identified: B cells, CD11b+ DCs, CD11b+ DCS, pDCs, Ly6C– 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, CD95+ B cells, cytotoxic T cells, helper T cells, NK cells, NKT cells, CD11b– cDCs, CD11b– cDCs, pDCs, neutrophils, F4/80– macrophages, Ly6C–CD11b– cells and Ly6C+monocytes. 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.

6.2. Automated cluster detection

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 Gasen et al., 2015) and ConsensusClusterPlus to perform clustering of meningeal extravascular immune cells (Wilkinson and Hayes, 2010) (see schematic in Fig. S10A and Methods for further details).

6.3. Statistics: Comparison of cell counts

DESeq2 was used to compare the effects of strain and condition on meningeal absolute cell counts, with library size correction used to account for the effects of differing dissection efficacy. Absolute splenic counts were estimated by multiplying percentage flow cytometry counts and splenic mass and compared using DESeq2, but without library size correction, as a normalization for dissection efficacy is not required (see further details below). The model used to compare cell counts (for both meninges, N = 53 mice, and spleen, N = 40 mice) was: count = cohort + strain + condition + strain:condition. Volcano plots show the effects of the relevant contrast (e.g., SD vs. HC or Cd19–/– vs. WT).

7. Analysis of meningeal cell count data

Previous publications have assessed the effects of experimental manipulations by comparing percentages of live CD45 + between groups. This method is subject to bias because, for example, if the manipulation causes a decrease in one cell type, this can falsely lead to an apparent increase in another cell type if only percentages are compared. However, it is difficult to compare absolute cell numbers in the meninges because the efficacy 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 balanced (as far as possible) cohorts. The same experimenter performed all dissections for which the data were pooled for flow cytometry analysis, avoiding any systematic bias in dissection by mouse strain or experimental condition. Data were combined across all cohorts by including cohort as a variable in the models used to compare cellular abundance and marker expression. 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. We thus adjusted the meningeal cell subtype counts for the median cell subtype count of all cell counts obtained for each meningeal sample by using the library size correction function in [R] package DESeq2. This approach would risk introducing bias if there were overall group- or strain-related differences in the median cell subset count. We confirmed that in our data, there was no significant effect of experimental group or strain on median cell subtype count by ANOVA. We note that results were not substantially different if library size correction was not used.

7.1. Calculation of total splenic counts

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. As such, to compare two conditions or groups, it is preferable to assess changes in absolute counts. However, it is not practical to immunophenotype an entire spleen for each animal. We thus combined proportional flow counts with splenic mass to obtain estimated absolute cell counts using the following formula:

\[ c_{ij} = 22.9 \times 10^3 \times m_i \times p_j \]

where \( c \) = estimated absolute count, \( i \) = mouse identity, \( j \) = cell subtype, \( m \) = splenic mass (in milligrams) and \( p \) = the proportion of live cells for celltype \( j \) in mouse \( i \) splenic cells, derived from flow cytometry data. The constant multiplier 22.9 \times 10^3 was taken from a previous report of the number of cellular nuclei per milligram of mouse spleen (Mizen and Petermann, 1952). Cell counts were then compared using package Deseq2 (Love et al., 2014) as for meningeal cell counts, but without the need for library size correction because splenic weight - unlike total meningeal weight - can be measured, so a correction for dissection efficiency is not required.

7.2. Statistics: Functional marker expression

Expression of functional markers was assessed from flow cytometry data in two ways. 1) For functional markers of interest in a given cell subset (e.g., % CD69+ of B cells), percent positive cells was manually gated. This was followed by (because of non-normality of the data) rank-based linear models (using [R] package Rfit (Kloke and McKean, 2012)) for the effect of animal cohort and condition or strain on these percentages. P-values for the effect of strain or condition were FDR-corrected across the markers tested (see figure legends). 2) 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, including animal cohort in the model. See Figure S10A for schematic of analysis.

7.3. 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 > 1600g, and stored at –80°C. 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 using [R] glm(plate ~ strain + cohort + condition + strain:condition) showed no significant relationship between plate and any aspect of experimental design. Plasma cytokines were analyzed in duplicate according to manufacturer’s instructions on a MAGPIXTM using Bio-Plex ProTM Mouse Cyto- kine 23-plex Assay M60009RFDP, MAGPIXTM 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, IPN-1b, IL-1alpha, IL-1beta, 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-1alpha), CCL4 (MIP-1beta), CCL5 (RANTES) and TNF-alpha. 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

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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 and McKean, 2012) to account for the non-parametric distribution of data and 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.

7.4. Debatching variables for the effect of cohort

For visualizations, the following features were debatched for the effects of cohort using limma (Ritchie et al., 2015): plasma cytokine levels; meningeal percentage positive and absolute counts; behavioral outcomes. Prior to debatching, all data (apart from count data, which were transformed using DESeq2::logTransformation) were Box-Cox transformed using [R] package caret (Kuhn, 2008) using commands caret::preProcess and caret::predict with method = "BoxCox" to improve the normality of the data. Data were then debatched for the effect of cohort using limma’s removeBatchEffect function. Transformed data were then inverse Box-Cox transformed to return the data to their original scales. These debatched counts were used for graphical representation and for correlating immunophenotypes with behavior but were not used for statistical analysis of group differences (cohort was simply included as a factor in the statistical models).

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

Meningeal flow cytometry data were processed for automated cluster detection broadly following the method and code outlined in (Nowicka et al., 2017) as follows:

1) FCS files and manual FlowJo workspaces and gates (for comparison) were imported and manipulated using [R] flowWorkspace (Finak and Jiang, 2018) and flowCore (Hahne et al., 2009).
2) Extravascular immune cells (intravascular CD45-negative microglia-like macrophages and leucocytes) were selected for further processing (see gating strategy Fig. S1OB).
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).
4) Prior to clustering, each of the 53 meningeal datasets were 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).
5) Downsampling 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 and 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 and clusters were manually annotated by inspection of lineage marker expression in each cluster (Fig. S1OC).
6) A linear model ([R] lm) was used to test the effects of condition or strain on the biexponentially transformed median fluorescence intensity (MFI) of functional markers (CD69, MHCII, CX3CR1, Ly6C, CD45 and CD11b) in each automatically detected cluster as shown on the tSNE plots (Fig. 1G, 2F, Fig. 4E). For the effects of strain (tested in homecage animals only, wildtype vs. Cd119\(^{−/−}\)) the model used was MFI ∼ strain + cohort. For the effects of condition (tested in wild type animals only, SD vs. HC), the model used was MFI ∼ condition + cohort. P-values for the effect of interest were Benjamini-Hochberg FDR-corrected across all 102 tested comparisons (all functional markers on all clusters). Tile plots show the linear model coefficients for the effect of interest.

7) Cells were further downsampled to 1000 cells per sample to produce a tSNE representation using [R] Rtsne (Krijthe, 2015), onto which manual and automated clustering labels were overlaid for visualization (Fig. 2A, S2A).

Automated clustering was not performed for splenic flow cytometry data as the markers were split across two multicolour panels (lymphoid and myeloid panels), which precludes joint clustering analysis across all markers. The effects of stress and mouse strain on biexponentially transformed MFI of functional markers on manually gated splenic subsets was compared as described above for meningeal clusters i.e. QC using flowAI, then comparison of biexponentially transformed MFI using a linear model, either: MFI ∼ strain + cohort (in homecage animals, wildtype vs. Cd119\(^{−/−}\)) or MFI ∼ condition + cohort (in wildtype animals; homecage vs. stress).

7.6. Intracellular IL10 staining

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 IL10 production and intracellular IL10 staining were performed similarly to as described elsewhere (Matsushita and 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 IL10 prior to acquisition of flow cytometry data. Antibodies used are shown in Table S4. B cells were defined as live CD45^CD11b^CD3^+^CD19^−^ cells. B cells and % IL10 expression were manually gated as per Fig. S4. The effect of stress on B cell IL10 production was tested by linear model (IL10 ∼ condition + cohort). Supernatants from these experiments were tested in duplicate or triplicate for IL10 protein levels by ELISA (mouse IL-10 DuoSet #DY417), performed as per manufacturer’s instructions, with lower limit of detection 31.2 pg/mL.

7.7. IL10 stimulation conditions

Stimulation medium composition (cells plated in triplicate):

| Constituent                  | Product number | Final concentration |
|------------------------------|----------------|---------------------|
| RPMI + Glutamax              | GIBCO # 61870-036 |                     |
| Fetal Bovine Serum           | Sigma # F4135   | 10%                 |
| Sodium Pyruvate              | GIBCO # 11,360  | 1 mM                |
| Heps Buffer                  | Corning/Celgro #25-060-CI | 10 mM              |
| MEM Nonessential amino acids | Corning/Celgro #25-025-CI | 1X                  |
| Penicillin-Streptomycin      | Gibco # 15140-148 | Penicillin: 100U/mL, Streptomycin: 100 μg/ml |
| Beta-Mercaptoethanol         | Sigma # M-7522  | 50 μM               |

Compounds used for stimulation:

| Constituent                | Product number | Final concentration |
|----------------------------|----------------|---------------------|
|                            |                | 10 ng/ml            |

(continued on next page)
Mark pathways (Liberzon et al., 2015) in stress, with Benjamini-Profiler (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.

7.8. Meningeal microarray data

Meninges were dissected as described above, then centrifuged and stored in Trizol. Samples were triturated using syringe needles, then total RNA was extracted using a Qiagen miRNAeasy Mini kit (Cat:217004). Labelled probes were run on an Affymetrix GeneChip® Mouse Gene 2.0 ST Array (Cat:90211B) 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 Benjami-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.

7.9. Meningeal bulk RNA sequencing data

Meningeal samples were prepared as above. 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™. 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. Salmon, run in mapping-based mode (Patro et al., 2017) was used to generate per-gene counts, initially aligning to the Gencode transcriptome, taking advantage of transcript-resolution estimates to improve differential 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 (msigdb v7.0.1) (Liberzon et al., 2015) in stress, with FDR-correction of P-values across all tested pathways.

7.10. Meningeal single cell RNA sequencing

10x Genomics’ Drop-seq platform (Chromium v2) for single cell gene expression data was performed on 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 (DAP1::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. Data were then processed following the strategy shown in Figure S6A. In brief, to obtain N = 6694 quality-controlled single cells, we performed the following steps: cell calling using DropletUtils::emptyDrops (Lun et al., 2019); exclusion of outlier cells based on mitochondrial reads (<8.3% of total) or total features per cell (range for included cells = 174 – 4548); exclusion of genes which were not expressed with > 1 count in > 1 cell; doublet detection and removal using scrublet (Wolok et al., 2019), where doublet rates in the three samples were estimated as 6.9%, 5.1% and 3.4%. Samples were normalised using scan deconvolution-based normalization (Lun et al., 2016); highly-variable genes (3599 genes) were selected by choosing those genes where biological variation across samples was > 0 (using scan::decompose). Batch correction across the three 10x lanes was performed using batchelor::multiBatchNorm and fastMNN (default 50 components used for dimensionality reduction) (Haghverdi et al., 2018). Clustering of MNNG-corrected PCA components across all single cells was performed using the leidenalg clustering algorithm (Traag et al., 2019). Clusters were manually annotated (Fig. S6A) by comparing marker genes expressed with existing single cell datasets. Differential gene expression between SD and HC cells within B cell clusters was performed as follows: counts were renormalized within the cluster; genes differentially expressed in pseudobulk of empty droplets (i.e., likely representing ambient RNA) were removed as described elsewhere (Ernst et al., 2019); genes expressed in ≤ 15% of cell in the cluster were removed; then gene expression in SD vs. HC cells was compared using a Mann-Whitney U test, with Benjami-Hochberg FDR correction of p-values across all tested genes. 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). Cell cycle stage of each cell was estimated using scan::cycleone (Scialdone et al., 2015).

8. Cellular stress-related genes

A list of cellular stress related genes (Fig. S7E) was generated by choosing the top 20 genes found to be upregulated in murine single cells following incubation at 37 °C for 60 min (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 using a Mann Whitney U test.

9. Study approval

Animal experiments were approved by the National Institute of Mental Health Institutional Animal Care and Use Committee (LCMR-06).

10. Data and materials availability

Data to support this study are available from the authors on request. For [R] code used to perform these analyses, see https://github.com/maryellenlynall/2019_bcell_stress.

11. One sentence Summary

Peripheral and meningeal B cells are dysregulated in psychosocial stress and B cell deficiency leads to behavioral abnormalities and meningeal myeloid cell activation.

12. Competing interests

E.T.B. is a consultant for Sosei Heptares. The other authors have no conflicts to declare.

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