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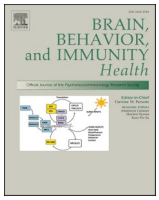

Repeated social defeat stress leads to immunometabolic shifts in innate immune cells of the spleen

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A B S T R A C T

Psychosocial stress has been shown to prime peripheral innate immune cells, which take on hyper-inflammatory phenotypes and are implicated in depressive-like behavior in mouse models. However, the impact of stress on cellular metabolic states that are thought to fuel inflammatory phenotypes in immune cells are unknown. Using single cell RNA-sequencing, we investigated mRNA enrichment of immunometabolic pathways in innate immune cells of the spleen in mice subjected to repeated social defeat stress (RSDS) or no stress (NS). RSDS mice displayed a significant increase in the number of splenic macrophages and granulocytes ($p < 0.05$) compared to NS littermates. RSDS-upregulated genes in macrophages, monocytes, and granulocytes significantly enriched immunometabolic pathways thought to play a role in myeloid-driven inflammation (glycolysis, HIF-1 signaling, MTORC1 signaling) as well as pathways related to oxidative phosphorylation (OXPHOS) and oxidative stress ($p < 0.05$ and FDR<0.1). These results suggest that the metabolic enhancement reflected by upregulation of glycolytic and OXPHOS pathways may be important for cellular proliferation of splenic macrophages and granulocytes following repeated stress exposure. A better understanding of these intracellular metabolic mechanisms may ultimately help develop novel strategies to reverse the impact of stress and associated peripheral immune changes on the brain and behavior.

1. Introduction

Psychosocial stress has been shown to elicit pro-inflammatory dysregulation including the priming of innate immune cells toward hyper-inflammatory phenotypes that are implicated in behavioral changes in several mouse models (Stark et al., 2001; McKim et al., 2016; Wohleb et al., 2013). For example, exposure to repeated social defeat stress (RSDS) leads to proliferation of myeloid cells in the spleen, which acts as a reservoir for primed monocytes (Swirski et al., 2009; McKim et al., 2018a). RSDS-primed splenic monocytes release more cytokines, display glucocorticoid resistance, and show increased recruitment to the brain that is necessary for the development of anxiety-like behavior (McKim et al., 2016; Wohleb et al., 2014). It is becoming increasingly appreciated that immune cell activation and effector functions require energy and depend on intracellular metabolic reprogramming. However, the impact of RSDS on cellular metabolic states that are thought to fuel inflammatory phenotypes are not well understood.

Monocytes, macrophages, and other innate immune cells primarily rely upon glucose among other nutrients to produce energy (i.e., adenosine triphosphate [ATP]) that is required to maintain their cellular functions. Activation by pro-inflammatory stimuli causes myeloid cells to rapidly undergo profound shifts in glucose metabolism (Viola et al., 2019). Generally, these shifts involve movement away from slow, energy-maximizing oxidative phosphorylation (OXPHOS) via mitochondrial respiration, to rapid but less energy-producing glycolysis (Ganeshan and Chawla, 2014). Increased glycolysis enables, and is required for, cytokine production and phagocytosis in monocytes/macrophages (Freemerman et al., 2014; Michl et al., 1976; Pavlou et al., 2017; Lee et al., 2019; Codo et al., 2020; Dietl et al., 2010; Otto et al., 2021) and provides fast energy and building blocks necessary for rapid cellular proliferation (Pearce and Pearce, 2013; Lunt and Vander Heiden, 2011; Tong et al., 2009). However, emerging reports suggest that an increase in OXPHOS is also observed and facilitates the survival, proliferation, and effector functions of immune cells in a stimulus- and context-dependent manner (Lachmandas et al., 2016; Keating et al., 2016; Kumar et al., 2019; Janzic et al., 2023; Boutens et al., 2018; Hong et al., 2022). Thus, changes to these immunometabolic pathways are a plausible contributor to the impact of, and possibly adaptation to,

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chronic stress within innate immune cells.

Chronic stress in mice and rats has been shown to lead to increased glycolysis and related changes in pathways such as hypoxia-inducible factor (HIF)-1α, mechanistic target of rapamycin (mTOR) and phosphoinositide 3-kinase (PI3K), and changes to OXPHOS in immune cells. For example, monocytes from mice subjected to chronic variable stress displayed increased expression of genes in glycolysis-promoting Akt-PI3K, mTOR, and HIF-1α pathways and decreased OXPHOS (Barrett et al., 2021). Moreover, behavioral susceptibility to stress has been shown to be associated with both enrichment of myeloid cells (Ambree et al., 2018; Hodes et al., 2014; Lee et al., 2022) as well as pro-glycolytic shifts (Lee et al., 2022; Li et al., 2017). Similar to stress-susceptible mice in the RSDS paradigm (Ambree et al., 2018; Hodes et al., 2014), subordinate mice living in social hierarchies were found to have higher levels of circulating monocytes and macrophages in the spleen (Lee et al., 2022). Interestingly, transcriptomic analysis of splenocytes showed that genes involved in both glycolysis and OXPHOS were more highly expressed in subordinate mice compared to alpha mice. Together, these findings led to our hypothesis that myeloid cells of RSDS mice would display immunometabolic shifts involving glycolysis and OXPHOS pathways as well as increased inflammatory signaling.

Using single cell (sc)RNA-Seq and bioenergetic assays we have previously shown that RSDS leads to profound immunometabolic changes in T cells of the spleen that included enhanced OXPHOS (Moshfegh et al., 2022). To examine the immunometabolic impact of RSDS on myeloid cells, herein we analyzed scRNA-Seq data from a subset of non-stressed (NS) or RSDS mice that were part of a larger cohort in which mice displayed both behavioral and systemic inflammatory dysregulation following RSDS (Moshfegh et al., 2022).

2. Methods

2.1. Mice

C57BL/6J experimental mice were originally obtained from Jackson Laboratories. All experimental mice were bred in house to eliminate shipping stress and microbiome shifts and co-housed with their littermates (≤5 mice per cage) prior to the start of experimentation to eliminate social isolation stress. CD-1 male retired breeder mice were obtained from Charles River Laboratories (Moshfegh et al., 2022). Experimental mice were sacrificed by pentobarbital overdose (150 mg/kg, Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI, USA) administered intraperitoneally. All mice were sacrificed between 7:00 and 10:00 a.m. Central Time to eliminate circadian rhythm effects on immune function. All procedures were reviewed and approved by the Texas A&M University Institutional Animal Care and Use Committee.

2.2. Repeated social defeat stress

Mice studied for scRNA-sequencing were a subset of a large cohort of mice (Moshfegh et al., 2022) exposed to no stress (NS; n = 3 out of 33) or repeated social defeat stress (RSDS; n = 3 out of 32) conducted as previously published (Moshfegh et al., 2019, 2022; Elkhatib et al., 2020, 2022). Briefly, RSDS mice were subjected to physical interactions with an unfamiliar, resident CD-1 aggressor (Golden et al., 2011) for 5 min per day over 10 days. After each session, experimental mice were housed in the social defeat cage on the opposite side of a clear perforated divider, allowing sensory contact with the aggressor over the next 24 h. NS mice were housed on either side of a divider and rotated similarly without being exposed to CD-1 aggressors. On Day 11, behavioral assessments were conducted (results reported in Moshfegh et al., 2022). Of note, in the larger cohort of mice from which the current study sample were drawn, RSDS was shown to decrease social interaction and induce anxiety-like behavior in the elevated plus maze test (Moshfegh et al., 2022). Mice for the scRNA sequencing were chosen based on their performance on the social interaction test and elevated zero maze (Table S1A). While not all mice showed behavioral changes on both tests, we selected only socially defeated mice that demonstrated deficits on both tests and non-stressed mice that did not. For the social interaction test, we used the definition put forth by Krishnan et al. (2007) that susceptible RSDS mice show a social interaction ratio <1, while non-stressed mice needed to demonstrate a social interaction ratio of >1. For the elevated zero maze, we used the definition put forth by our own group (Elkhatib et al., 2020) that uses a combination of distance moved and time spent in open arms. No animals sustained injuries during RSDS and body weight was similar between the RSDS and NS groups ($p = 0.63$). Moreover, the chosen RSDS animals showed increased levels of plasma cytokines including IL-2, IL-17A, IL-22, and TNF-alpha compared to the NS animals ($p < 0.05$, Table S1A). On Day 12, two days after the end of RSDS (or NS) spleens were collected for scRNA-Seq.

2.3. scRNA-seq, cell clustering and annotation

scRNA sequencing and data preprocessing were conducted as previously described (Moshfegh et al., 2022). Splenocytes from three NS and three RSDS male mice were analyzed. Approximately 2500 cells per animal were captured, lysed, and RNA was reverse transcribed and barcoded using Chromium Single Cell 3' Reagent Kits (v2 chemistry) on a 10x Genomics Chromium instrument (10x Genomics, Pleasanton, CA, USA). Libraries were prepared and quantified using the KAPA Library Quantification Kit (KAPA Biosystems, Wilmington, MA). Raw sequence quality was checked using FastQC v0.11.9, and bioinformatics was conducted on an AWS R4 instance type utilizing Ubuntu 18.04. Alignment was performed by STAR 2.7.10a (Dobin et al., 2013) using the GRCm39 reference genome to generate the count data, which were then imported into Seurat (Satija et al., 2015). Quality control was performed to filter to cells with > 200 and < 2500 detectable genes and mitochondrial content < 10%. Seurat default parameters were used to log-normalize, scale, and integrate samples (variable features = 2000, reduction method for integration = reciprocal PCA).

To achieve a more granular annotation of the innate immune cells of the spleen (which comprised 17.5% of all cells), previously identified T and B lymphocytes expressing CD8a or CD4 (Moshfegh et al., 2022) were excluded from the scRNA-Seq data. The remaining cells were re-clustered into 11 distinct populations, which were then annotated using canonical markers (Fig. S1).

2.4. Differential expression and pathway analysis

Genes differentially expressed between RSDS and NS groups were identified using the Wilcoxon rank sum test implemented in Seurat 4.1.1 with the default parameters (including $|\log\text{-fold change}| > 0.25$ and $p < 0.05$, except min.pct was set to 0 to include low-frequency genes). Functional annotation of the differentially expressed genes within curated pathways was assessed using WikiPathways, KEGG (Kyoto Encyclopedia Gene and Genome), and MSigDB databases as implemented in clusterProfiler (Yu et al., 2012). A significance threshold of FDR-adjusted $p < 0.1$ was used for pathway analysis to ensure biologically meaningful pathway results (Hulsegge et al., 2009; Yang et al., 2008, 2014; Jansen et al., 2016; de Kluiver et al., 2019; Mostafavi et al., 2014; Zhou et al., 2018; Storey and Tibshirani, 2003).

2.5. Statistical analysis

Bodyweight, behavioral, cytokine, and cellular abundance data with normal distribution (per Shapiro's test) and equal variance (per F-test if normal and Levene's test if non-normal) were compared between NS and RSDS groups via two-tailed Student's t-tests with $p < 0.05$. Non-normal data (NK cell percent and IL-6 levels) were analyzed using the Mann-Whitney *U* test.

3. Results

The non-lymphoid cells of the spleen were clustered into 11 distinct populations (Table S1B, Fig. S1) based on canonical marker expression shown in Fig. S2. Positively enriched genes (i.e., the "transcriptomic signature") in each of the 11 unique clusters were determined and reported in Table S1C. Of the 11 clusters, seven were annotated as innate immune cells as follows:

Two populations of myeloid cells (Csf1r) (Alshetaiwi et al., 2020) were identified as macrophages (Borges da Silva et al., 2015) (Cluster 0; Cd11b^{lo} [Itgam], F4/80^{hi} [Adgre1], Ly6C^{-} [Ly6c2], Vcam1^{+}) and monocytes (Cluster 6; Cd11b^{hi}, Ly6C^{+}) (Fig. 2). The monocytes displayed mixed expression of classical (Ly6C^{+}, Ccr2^{+}) and non-classical (Cx3cr1^{+}, Cd43^{+} [Spn]) markers (Wolf et al., 2019). Two populations of neutrophils (Clusters 4 and 8) were identified based on Cd11b [Itgam], Cxcr2, Csf3r, and Ly6g markers (Alshetaiwi et al., 2020; Combes et al., 2021; Lee et al., 2013) along with one population of NKs (Cluster 2; NK1.1 [Klrb1c], Ncr1). In addition, two populations of dendritic cells (DCs; Cd11c [Itgax], MHC-II [H2-Ab1, H2-Eb1]) (Pizzolato et al., 2019) were identified as conventional DCs (Cluster 1, cDCs; Xcr1) and plasmacytoid DCs (Cluster 10, pDCs; Siglec-H^{+}, Bst2^{+}).

Other clusters which were not a focus of analysis herein included Cluster 4, which displayed markers of B cells (Cd19, Cd79a) along with low expression of other innate cell markers; erythroid cells (Clusters 3 and 7; Hbb-bs), and a small, unassigned cluster which comprised less than 2.5% of innate immune cells (Cluster 9).

3.1. RSDS leads to myelopoiesis and increased glycolytic genes in immune cells of the spleen

Consistent with previous literature (Wohleb et al., 2013; Ambree et al., 2018; Hodes et al., 2014; Ishikawa et al., 2021), RSDS led to increases in the proportion of splenic innate immune cells ($t(4) = 5.24$, $p = 0.007$), which was in large part driven by an increase in the percent of macrophages/monocytes ($t(4) = 5.2$, $p = 0.006$) and granulocytes ($t(4) = 4.16$, $p = 0.01$) (Fig. 1A, Table S1B). In RSDS mice compared to NS littermates, a total of 263 genes were upregulated in macrophages. These genes significantly enriched a number of pathways related to immunometabolic shifts thought to play a role in myeloid-driven inflammation (glycolysis, HIF-1 signaling, MTORC1 signaling) as well as a pathway related to reactive oxygen species (Fig. 1B, Table S2B; all $p < 0.05$ and FDR-adjusted $p < 0.1$). In addition, the 308 genes upregulated in monocytes of RSDS mice were enriched for pathways related to inflammation (interferon gamma response) and mitophagy (Fig. 1B, Table S3B; all $p < 0.05$ and FDR-adjusted <0.1). Similar to macrophages/monocytes, the 272 genes upregulated in granulocytes of RSDS mice also enriched immunometabolic (glycolysis, HIF-1), inflammatory (neutrophil extracellular trap formation, complement), and oxidative stress pathways (Fig. 1B, Table S4; all $p < 0.05$ and FDR-adjusted <0.1).

Exploratory analyses suggested that RSDS macrophages/monocytes show indices of glucocorticoid resistance such as higher expression of FKBP5, (Menke et al., 2012) a negative regulator of the GR, lower expression of the glucocorticoid receptor (GR; NR3C1) (Cattaneo et al., 2020; Hasselmann et al., 2018) as well as glucocorticoid-inducible anti-inflammatory genes (dual-specificity phosphatases [DUSP] and Kruppel-like factor 2 [KLF2]) (Menke et al., 2012; Hasselmann et al., 2018; Das et al., 2006) (Fig. 2 and Table S8) compared to macrophages/monocytes of NS controls.

In addition to enrichment of glycolytic pathways discussed above, all innate immune cell types of RSDS mice also showed upregulation of genes enriched in OXPHOS and electron transport chain pathways (Tables S2B–7B). Unexpectedly, RSDS-downregulated genes in all immune cell types enriched various biological pathways including several that are involved in inflammatory signaling (Tables S2C–7C). Full pathway results are shown in Tables S2–7.

4. Discussion

Our findings here suggest that repeated social defeat stress in mice led to cellular immunometabolic changes in immune cells of the spleen. These immunometabolic phenotypes included enrichment of glycolysis and related signaling pathways (HIF-1, MTORC1 signaling) in macrophages and granulocytes. These transcriptomic results complement our previous findings from depressed patients with high inflammation suggesting that human CD14^{+} and CD16^{+} monocytes expressing activation and trafficking markers (e.g., CCR2) exhibit evidence of glycolytic transcriptional shifts in association with depressive symptom severity (Bekhbat et al., 2022).

Generally, glycolysis is thought to enable rapid cell proliferation as well as effector functions of innate immune cells, including cytokine production, phagocytosis, and trafficking (Ganeshan and Chawla, 2014). Although several inflammatory pathways such as interferon gamma, complement, and neutrophil antimicrobial defense were

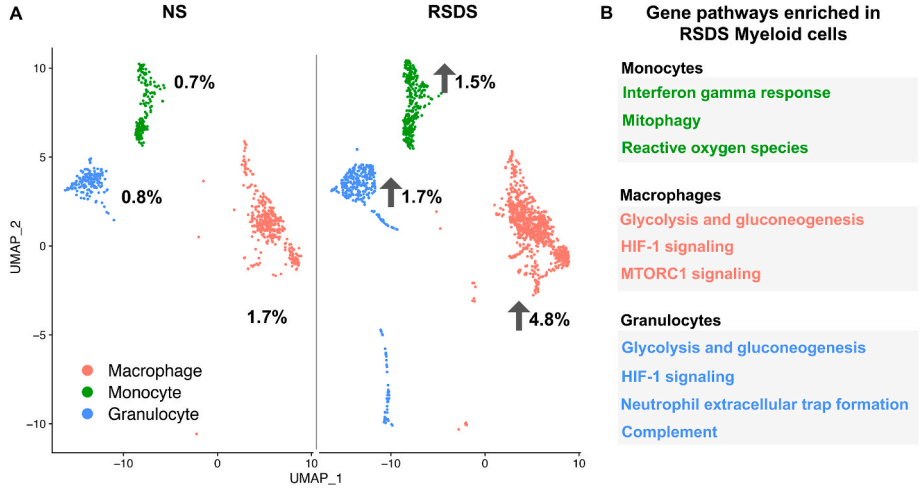

Fig. 1. RSDS increased the relative abundance of macrophages, monocytes, and granulocytes in the spleen and promoted the enrichment of immunometabolic and inflammatory pathways. Two-dimensional uniform manifold approximation and projection (UMAP) plots show changes in cell abundance expressed as % of total splenocytes **(A)**. Representative gene pathways that are enriched in RSDS monocytes, macrophages, and granulocytes are shown **(B)**. RSDS, repeated social defeat stress; NS, non-stressed; HIF-1, hypoxia-inducible factor-1; MTORC1, mechanistic target of rapamycin complex 1. N = 3 mice/group; % shown are of total splenocytes.

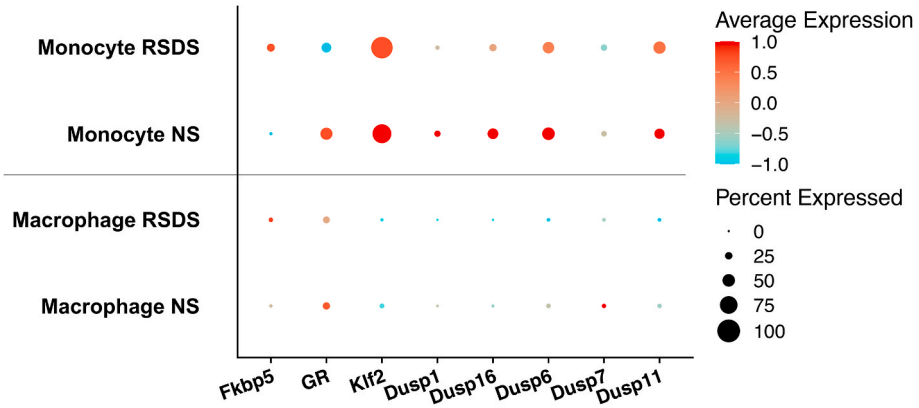

Fig. 2. Differential expression of genes involved in glucocorticoid resistance in macrophages and monocytes of RSDS and NS mice. Dot plots depict the average expression level and % of cells expressing each marker.

upregulated in RSDS cells, various inflammatory and immune pathways were downregulated compared to non-stressed littermates. Related, RSDS models have been shown to induce monocyte trafficking to stress-sensitive regions of the brain (McKim et al., 2016, 2018b; Wohleb et al., 2013, 2014; Sawicki et al., 2015; Menard et al., 2017; Dudek et al., 2020; Dion-Albert et al., 2022; Bekhbat et al., 2021a) which is required for the full expression of depressive and anxiety-like behavior in these models (Menard et al., 2017; McKim et al., 2018b; Weber et al., 2017). While glycolysis has been shown to be required for monocyte migration in multiple contexts, (Hara et al., 2017; Semba et al., 2016; Kaushik et al., 2019; Cramer et al., 2003) and pro-glycolytic metabolic shifts in monocytes have been demonstrated in similar stress models, (Barrett et al., 2021; Lee et al., 2022) trafficking-related markers were not upregulated in macrophages/monocytes of RSDS mice. Together, these data hint that at two days post-RSDS, stress-primed, hyper-inflammatory cells may have already egressed from the spleen into circulation, thus driving the "downregulation" of inflammatory pathways in the spleen observed herein. This interpretation is further supported by data from the parent study from which the current sample was drawn, where RSDS increased plasma cytokines including TNF-α, IL-2, and IL-22 (Moshfegh et al., 2022). Further time course studies can elucidate this result.

Unlike the Warburg effect, which is a shift to aerobic glycolysis at the expense of OXPHOS, here we observed that both glycolysis and OXPHOS pathways were significantly enriched in RSDS innate immune cells. These findings are consistent with a previous report utilizing a different form of psychosocial stress in mice (Lee et al., 2022) and transcriptomic signatures associated with symptom severity in depression (Bekhbat et al., 2021b). Our results suggest a metabolic enhancement in RSDS immune cells that may support various cellular functions such as regulating redox pathways (Muri and Kopf, 2021) (which were upregulated in RSDS splenocytes) or maintaining metabolic flexibility (Lachmandas et al., 2016) that may be needed to meet demands of any RSDS-related change in cellular function and the spleen microenvironment. Moreover, as actively dividing cells often enhance both glycolysis and oxidative metabolism to meet the energetic and biosynthetic demands for proliferation (Yao et al., 2019), upregulation of these pathways may have enabled the cellular proliferation seen in macrophages and granulocytes. These reports suggest that metabolic enhancement could be a new phenotype that is associated with priming of the innate immune system by psychosocial stress. Further bioenergetic or mitochondrial redox studies are needed to confirm whether stress can promote a hypermetabolic phenotype similar to what has been demonstrated in inflammatory disease (McGarry et al., 2021).

The small sample size per group, while compatible with the scRNA-Seq method and consistent with sample sizes in similar scRNA-Seq publications in recent years (Alshetaiwi et al., 2020; Kimmel et al., 2019; Grieshaber-Bouyer et al., 2021), is nonetheless a limitation of the current study. The cell type-specific transcriptomic profiling conducted here complements and extends existing bulk-seq findings (Barrett et al., 2021; Lee et al., 2022) by showing that chronic stress-induced immunometabolic shifts occur across multiple immune cell types in the spleen including macrophages and granulocytes.

In sum, our data indicate that RSDS, a chronic stress paradigm commonly used in studies of immune cell trafficking into the brain, leads to immunometabolic changes in several innate immune cell types in the mouse spleen, which may facilitate myelopoiesis. Better understanding these intracellular mechanisms can ultimately help develop more nuanced and specific immune cell- or metabolism-based strategies to reverse the impact of repeated psychosocial stress and associated peripheral immune activation on the brain and behavior.

Declaration of competing interest

None

Data availability

Data were reanalyzed from https://doi.org/10.1016/j.bpsgos.2022.12.002.

Acknowledgements/Disclosures

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2023.100690.

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