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The Influence of Microglial Elimination and Repopulation on Stress Sensitization Induced by Repeated Social Defeat

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ABSTRACT

BACKGROUND: Stress is associated with an increased prevalence of anxiety and depression. Repeated social defeat (RSD) stress in mice increases the release of monocytes from the bone marrow that are recruited to the brain by microglia. These monocytes enhance inflammatory signaling and augment anxiety. Moreover, RSD promotes stress sensitization, in which exposure to acute stress 24 days after cessation of RSD causes anxiety recurrence. The purpose of this study was to determine whether microglia were critical to stress sensitization and exhibited increased reactivity to subsequent acute stress or immune challenge.

METHODS: Mice were exposed to RSD, microglia were eliminated by colony-stimulating factor 1 receptor antagonism (PLX5622) and allowed to repopulate, and responses to acute stress or immune challenge (lipopolysaccharide) were determined 24 days after RSD sensitization.

RESULTS: Microglia maintained a unique messenger RNA signature 24 days after RSD. Moreover, elimination of RSD-sensitized microglia prevented monocyte accumulation in the brain and blocked anxiety recurrence following acute stress (24 days). When microglia were eliminated prior to RSD and repopulated and mice were subjected to acute stress, there was monocyte accumulation in the brain and anxiety in RSD-sensitized mice. These responses were unaffected by microglial elimination/repopulation. This may be related to neuronal sensitization that persisted 24 days after RSD. Following immune challenge, there was robust microglial reactivity in RSD-sensitized mice associated with prolonged sickness behavior. Here, microglial elimination/repopulation prevented the amplified immune reactivity ex vivo and in vivo in RSD-sensitized mice.

CONCLUSIONS: Microglia and neurons remain sensitized weeks after RSD, and only the immune reactivity component of RSD-sensitized microglia was prevented by elimination/repopulation.

Keywords: Anxiety, CSF1R antagonist, Microglia, Monocytes, Repeated social defeat, Stress

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Psychosocial stressors are associated with an increased prevalence of anxiety and depression (1,2). Moreover, individuals exposed to chronic stressors are vulnerable to subsequent adversity, known as stress sensitization (3,4). The immune system contributes to chronic stress responses and is implicated in poor mental health outcomes (5). Mounting clinical evidence indicates that chronic stress increases circulating inflammatory ($CD14^+/CD16^-$) monocytes in humans (6–10). $CD14^+/CD16^-$ monocytes have a higher inflammatory capacity and display increased ability to traffic into tissue (9,11–13). These immune alterations may be maladaptive, increase inflammation, and contribute to psychiatric complications associated with stress (14,15).

Repeated social defeat (RSD) is a preclinical model of stress that drives the sympathetic-mediated production and release of inflammatory $Ly6C^{hi}$ monocytes into circulation (6,16,17). Notably, rodent $Ly6C^{hi}$ monocytes are the functional counterpart to human $CD14^+/CD16^-$ monocytes (6,18). These monocytes have a primed profile characterized by glucocorticoid insensitivity, elevated expression of receptors for pathogen-associated molecular patterns, and higher expression of proinflammatory cytokines (interleukin-1β [IL-1β]) (6,19–23). RSD causes prolonged anxiety-like behavior that persists for 8 days and is dependent on recruitment of inflammatory monocytes to brain regions associated with fear circuitry (24,25). Notably, monocyte recruitment is microglial and chemokine dependent. Furthermore, recruited monocytes produce IL-1β, which is required for induction of anxiety-like behavior (26). Overall, inflammatory monocytes augment IL-1β signaling to endothelial cells, thereby causing anxiety following RSD (26).

RSD also promotes stress sensitization, in which subsequent exposure to acute (subthreshold) stress caused anxiety recurrence (25,27). This acute stress is one cycle of RSD and is

SEE COMMENTARY ON PAGE 619

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defined as subthreshold stress because it does not cause monocyte trafficking or anxiety-like behavior in undisturbed (naïve) mice (25,27). In RSD sensitization, we reported that the spleen served as a unique reservoir of inflammatory monocytes that were released and trafficked to the brain following acute stress (25). There were also longer-lasting changes in the central nervous system (CNS) 24 days after RSD. For example, isolated microglia from RSD-sensitized mice had elevated cluster of differentiation 14 (CD14) messenger RNA (mRNA) and were more reactive to ex vivo lipopolysaccharide (LPS) stimulation 24 days after RSD (17,28). A persistent alteration in resident microglia after RSD is relevant because microglia are involved with the recruitment of monocytes to the brain (26). In addition, primed or sensitized profiles of microglia with stress, injury, or age conferred hyperreactivity to peripheral immune challenges (29–33). Therefore, the purpose of this study was to determine whether microglia were a critical component of stress sensitization and increased reactivity to acute stress or innate immune challenge.

METHODS AND MATERIALS

Mice

Male C57BL/6 mice (6–8 weeks old) and CD-1 mice (12 months old) were used (Charles River Laboratories, Wilmington, MA). CX_3CR1^{CreER}/ROSA26-$STOP^{flfl}$-tdTomato (tdTom) mice were generated by crossing CX_3CR1^{CreER} and Ai9-ROSA26-$STOP^{flfl}$-tdTom mice (Jackson Laboratories, Bar Harbor, ME). All procedures were performed in accordance with the National Institutes of Health and Ohio State University guidelines.

RSD and Stress Sensitization

Mice were subjected to RSD as described (16) and outlined in the Supplement. An aggressive CD-1 mouse was introduced to an established cohort of three resident mice 2 hours daily for 6 days. Naïve control mice were left undisturbed in their home cages. For stress sensitization, mice were exposed to control (naïve) or RSD and exposed to acute social defeat 24 days later (25,27).

RNA Sequencing of Fluorescence-Activated Cell–Sorted Microglia

Microglia were enriched using Percoll separation, labeled with anti-CD11b and CD45 antibodies, and fluorescence-activated cell sorted. RNA was extracted and synthesized to complementary DNA, and 20 million 75-bp reads were sequenced on an Illumina NextSeq 500 (Illumina, San Diego, CA). Sequences were aligned to the mm10 mouse reference genome using STAR (Spliced Transcripts Alignment to a Reference) Aligner (34). Factors of unwanted variance were controlled with RUVSeq (35), and normalization and differential expression was determined using DESeq2 in R (36). Genes with $p < .05$ and fold changes greater than 1.5 ($\log_2 FC > 0.585$) were considered differentially expressed.

Plexxikon Administration

PLX5622 was provided by Plexxikon Inc. (Berkeley, CA) and formulated in standard AIN-76A rodent chow at a concentration of 1200 mg/kg and provided ad libitum.

Tamoxifen Injections

Postnatal day 21 mice received 20 mg/kg tamoxifen per day for 4 consecutive days by intraperitoneal injection as described (37).

Flow Cytometry

$CD11b^+$ cells were isolated from brain homogenates as reported (16) and described in the Supplement. In brief, brains were mechanically dissociated and $CD11b^+$ cells were enriched by Percoll separation. Cells from the blood, spleen, and brain were labeled with appropriate antibodies. Cell surface antigen expression was determined using a DxP9 cytometer (Cytek, Fremont, CA). Data were analyzed using FlowJo software, and positive labeling for each antibody was determined based on isotype-labeled controls.

RNA Isolation and Reverse Transcription Polymerase Chain Reaction

RNA was isolated from Percoll-enriched $CD11b^+$ cells and coronal brain sections. Real-time quantitative polymerase chain reaction was performed using the Applied Biosystems (Foster City, CA) Assay-on-Demand Gene Expression protocol. mRNA expression was determined on an Applied Biosystems PRISM 7300 sequence detection system, converted to double delta cycle threshold, and results are expressed as fold change.

Ex Vivo Stimulation of Enriched $CD11b^+$ Cells From Brain

As reported (28,38), CD11b+ cells were isolated by Percoll separation, counted, plated, and stimulated with vehicle or LPS (100 ng/mL).

Immunohistochemistry

Mice were transcardially perfuse-fixed with paraformaldehyde. Brain samples were postfixed and cryosectioned. Labeling for Iba1, c-Fos, or phospho-CREB was performed as described in the Supplement.

Behavioral Analyses

Anxiety-like behavior in the open field was determined as described (16,25). Social exploratory behavior of a juvenile conspecific mouse was determined at baseline and again 4, 8, and 24 hours after LPS injection as described (25,26,33). Both behaviors are detailed in the Supplement.

Statistical Analysis

To determine significant main effects and interactions between main factors, data were analyzed using one-, two-, or three-way analysis of variance using the general linear model procedures of SPSS statistical software, version 25 (IBM Corp., Armonk, NY). In the event of a main effect of treatment, differences between group means were evaluated by post hoc analyses (Fisher's least significant difference) and are graphically presented in figures ($p < .05$).

RESULTS

Evidence for Primed Microglial Profile 24 Days After RSD

We reported that microglia were required for RSD-induced recruitment of monocytes to the brain and prolonged anxiety-like behavior (16,24,26). Moreover, anxiety reoccurred in RSD-sensitized mice following acute stress 24 days later and was dependent on the release and recruitment of splenic-derived monocytes to the brain (25,27). Thus, we hypothesized that microglia are a critical cellular component of stress sensitization and are hyperreactive to subsequent stressors and immune challenges.

First, we determined the microglial mRNA profile 24 days after RSD sensitization. Microglia were fluorescence-activated cell sorted and RNA was sequenced (Figure 1A). Volcano plots show genes in microglia that were increased or decreased by 1.5-fold ($p < .05$) 24 days after RSD compared with controls (Figure 1B). Of the 137 genes that were differentially expressed, 87 genes were increased and 50 were decreased. For instance, several immune-related genes, including *Mmp9, Cd200, Cxcl10, Cxcr4,* and *Ccl24,* were increased more than 1.5-fold ($p < .05$) in microglia. Several other genes, including *Tgfbr3, Il1r2, Aqp1,* and *Cadm2,* were decreased in microglia ($p < .05$). Next, significantly altered upstream regulators were determined by Ingenuity Pathway Analysis (39). The six pathways with the highest activation *z* scores between microglia from RSD mice versus control mice ($p < .05$) were IL-1β, receptor tyrosine for glial-derived neurotrophic factor ligands, interferon-γ, myeloid differentiation primary response 88, IL-4, and toll-like receptor 4 (TLR4) (Figure 1C). These findings confirm that significantly regulated genes are in pathways associated with microglial immune priming. Collectively, these data provide evidence that microglia develop a distinct immune profile that persists 24 days after RSD.

Monocyte Recruitment to the Brain and Recurrence of Anxiety in Stress-Sensitized Mice (24 Days Later) Was Abrogated by the Elimination of Microglia

The microglial mRNA signature remained altered 24 days after the cessation of RSD. Thus, we next eliminated microglia 10 days after RSD sensitization and maintained their absence throughout the exposure to acute stress 24 days later (26). Mice were stress-sensitized (SS) by RSD or naïve, and microglia were depleted using a colony-stimulating factor 1 receptor (CSF1R) antagonist (PLX5622). All mice were exposed to acute defeat 24 days after RSD (Figure 2A), and several immune and behavioral parameters were determined. Acute defeat does not cause monocyte trafficking or anxiety-like behavior in naïve mice; therefore, this group served as the control mice for these experiments (25,27). Consistent with a subthreshold stressor, acute defeat increased Ly6C^{hi} monocytes in circulation and in the spleen of SS mice compared with naïve mice (Figure 2B, C; $p < .002$ for each). Monocyte release and accumulation in the spleen following acute defeat was dependent on stress sensitization (Figure 2B, C). PLX5622 affected neither the number of circulating monocytes nor monocyte accumulation in the spleen (Figure 2B, C). Acute defeat also increased IL-1β mRNA levels in the brain of SS mice compared with control mice ($p < .001$), and this effect was abrogated by microglial elimination (Figure 2D) (intervention × SS, $p < .04$). Acute defeat increased monocyte accumulation in the brain of SS

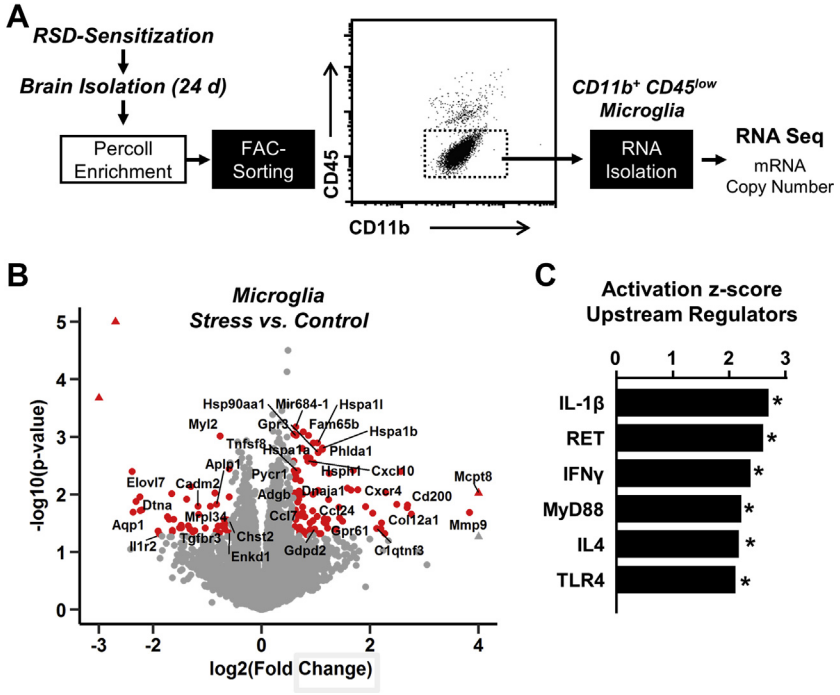


Figure 1. Evidence for primed microglial profile 24 days after repeated social defeat (RSD). **(A)** Male C57BL/6 mice were stress sensitized by RSD or left undisturbed as control mice. At 24 days after stress, microglia were Percoll enriched and fluorescence-activated cell (FAC) sorted, and RNA was collected for RNA sequencing (RNA Seq) ($n = 6$). **(B)** Volcano plots of differentially expressed genes between control and RSD. Red points indicate differentially expressed genes ($p < .05$, absolute fold change > 1.5). **(C)** Ingenuity Pathway Analysis of significantly altered upstream regulators in microglia. Asterisk (*) indicates significantly different from control. IFNγ, interferon-γ; IL-1β, interleukin-1β; IL4, interleukin-4; mRNA, messenger RNA; MyD88, myeloid differentiation primary response 88; RET, receptor tyrosine for glial-derived neurotrophic factor ligands; TLR4, toll-like receptor 4.

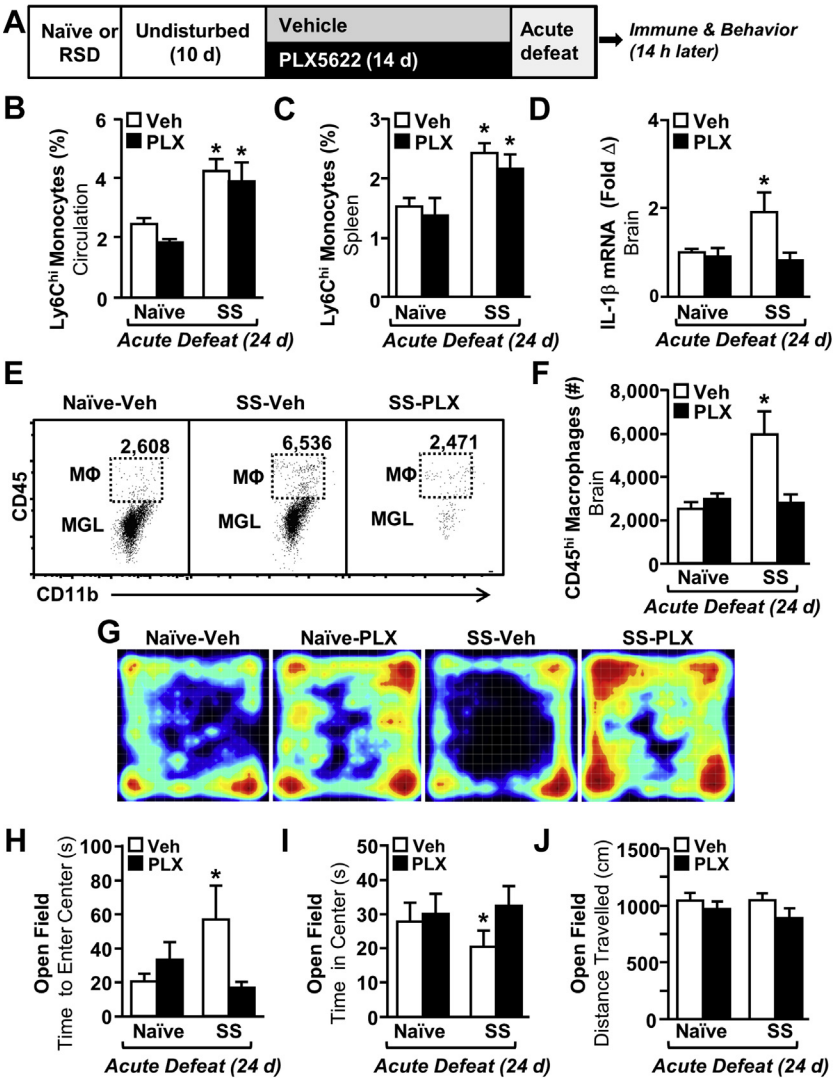


Figure 2. Monocyte recruitment to the brain and recurrence of anxiety in stress-sensitized (SS) mice were abrogated by the elimination of microglia. **(A)** Male C57BL/6 mice were SS by repeated social defeat (RSD) or left undisturbed as controls (naïve). Ten days later, mice were provided diets formulated with vehicle (Veh) or a colony-stimulating factor 1 receptor antagonist (PLX5622 [PLX]). Then, 24 days after stress sensitization, all mice were subjected to one cycle of social defeat (acute defeat), and blood, spleen, and brain samples were collected 14 hours later (n = 9–10). **(B, C)** The percentage of monocytes ($CD11b^+/Ly6C^{hi}$) in the blood **(B)** (main effect of SS, $F_{1,39} = 21.45$, $p < .0001$) and spleen **(C)** (main effect of SS, $F_{1,39} = 28.63$, $p < .002$) were determined 14 hours after acute defeat. **(D)** Interleukin-1β (IL-1β) messenger RNA (mRNA) expression in a coronal brain section was determined after acute defeat (SS × intervention, $F_{1,38} = 4.35$, $p < .04$). **(E)** Representative bivariate dot plots of CD11b and CD45 labeling of Percoll-enriched microglia (MGL) ($CD11b^+/CD45^{low}$) and macrophages (MΦ) ($CD11b^+/CD45^{high}$) in the brain after acute defeat. **(F)** Number of $CD45^+$ macrophages in the brain (SS × intervention, $F_{1,36} = 10.68$, $p < .003$). **(G)** Representative heat maps of activity during open-field testing. **(H)** Time to enter center of the open field (SS × intervention, $F_{1,40} = 5.20$, $p < .02$). **(I)** Time spent in center in the open field after acute defeat (SS × intervention, $F_{1,40} = 7.03$, $p < .01$). **(J)** Total distance traveled in the open field (not significant). Bars represent the mean ± SEM. Means with an asterisk (*) are significantly different from naïve Veh controls.

mice (Figure 2E, F; $p < .05$), and this was prevented by microglial elimination (Figure 2E, F; intervention × SS, $p < .004$). Thus, microglia were essential for monocyte accumulation in the brain of RSD-sensitized mice exposed to acute stress.

Acute defeat caused anxiety-like behavior in SS mice compared with control mice (Figure 2G). This was associated with increased time to enter the center of the open field (Figure 2H; $p < .03$) and decreased time spent in the center for SS mice (Figure 2I; $p < .05$) compared with control mice. Moreover, acute defeat-associated anxiety in SS mice was prevented by microglial elimination (Figure 2H [SS × intervention, $p < .02$] and Figure 2I [SS × intervention, $p < .01$]). Notably, there were no differences in total distance traveled between groups (Figure 2J). Taken together, the recurrence of anxiety in RSD-sensitized mice with acute stress was blocked by microglial elimination.

Microglia Repopulated From Nonprogenitor CX_3CR1^+ Cells After CSF1R Antagonist–Mediated Elimination

The time course of repopulation and the origin of microglia after CSF1R antagonist–mediated elimination was assessed (Figure 3A). As expected, Iba-1^+ microglia repopulated after elimination in a time-dependent manner (Figure 3B; $p < .0001$). $CD11b^+/CD45^{lo}$ microglia were reduced below baseline 0, 7, and 14 days after removal of the CSF1R antagonist (Figure 3B, C; $p < .05$ for each). Microglia returned to baseline within 21 days, which was in line with previous studies (26,40,41). Next, $CX_3CR1^{CreER/+}/R26^{tdTom}$ mice (42) were used to assess the origin of repopulated microglia. In these mice, CX_3CR1^+ microglia express yellow fluorescent protein (YFP) at baseline. Following tamoxifen injection, Cre recombination in CX_3CR1^+ cells induces tdTom expression (Figure 3E). In this experiment, $CX_3CR1^{CreER\text{-}YFP}/R26^{tdTom}$ mice (3 weeks old) were injected

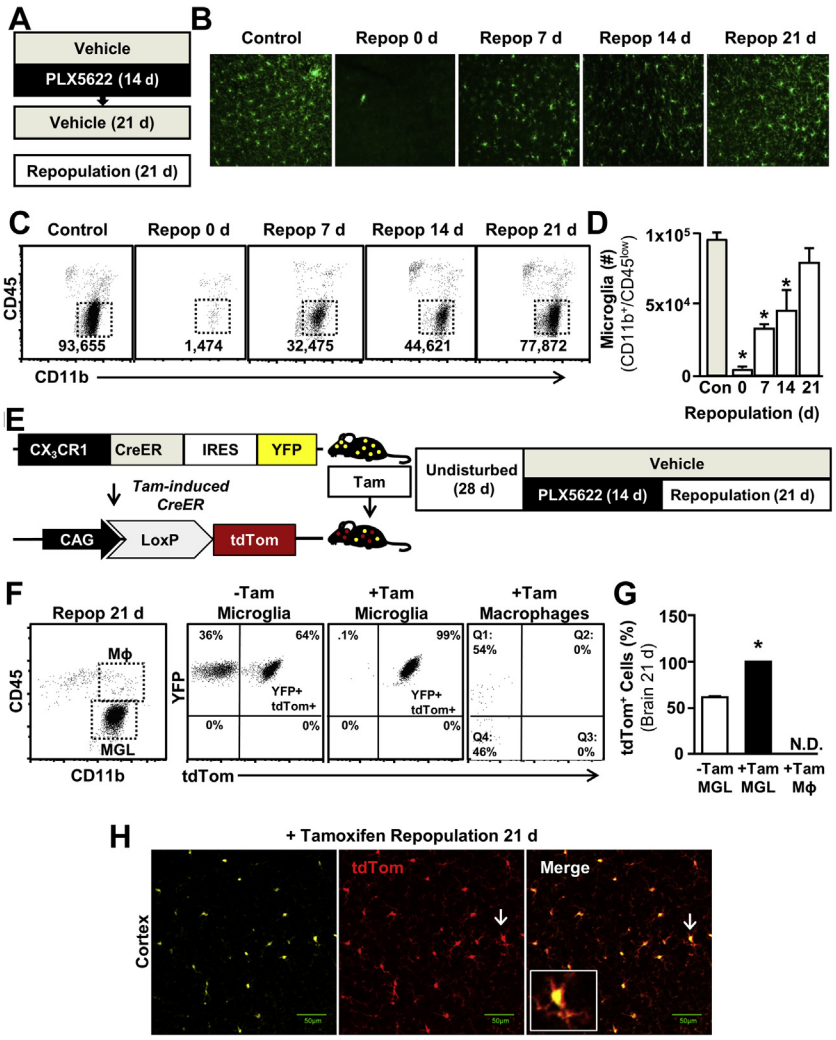


Figure 3. Microglia repopulated from non-progenitor CX_3CR1^+ cells after colony-stimulating factor 1 receptor (CSF1R) antagonist–mediated elimination. **(A)** Male C57BL/6 mice were provided diets formulated with vehicle or CSF1R antagonist (PLX5622) for 14 days. Next, the CSF1R antagonist diet was removed and all mice were provided vehicle diets for 1, 7, 14, or 21 days to allow for repopulation of microglia. **(B)** Representative images of Iba-1 labeling in the cortex 1, 7, 14, or 21 days after the cessation of the CSF1R antagonist. **(C)** Representative bivariate dot plots of CD11b/CD45 labeling of Percoll-enriched cells at each time point. **(D)** Number of microglia ($CD11b^+/CD45^{low}$) in the brain after 1, 7, 14, or 21 days of repopulation (main effect of time, $F_{4,16} = 8.57$, $p < .001$). **(E)** Schematic representation of the experimental design using $CX_3CR1^{CreER/+}/R26^{tdTom/+}$ ($tdTomato^+$) mice, which were administered four daily injections of control or tamoxifen (20 mg/kg, intraperitoneal) at 3 weeks of age. Mice were left undisturbed for 28 days, provided diets formulated with a CSF1R antagonist (PLX5622) for 14 days, and then provided vehicle diets for an additional 21 days to allow for repopulation of microglia. **(F)** Representative bivariate dot plots of yellow fluorescent protein (YFP) and tdTom expression in microglia ($CD11b^+/CD45^{low}$) and macrophages ($CD11b^+/CD45^{high}$) isolated from ± tamoxifen-injected $CX_3CR1^{CreER/+}/R26^{tdTOM/+}$ mice subjected to microglial elimination/repopulation. **(G)** Percentage of $tdTom^+$ microglia (MGL) ($CD11b^+/CD45^{low}$) and macrophages (MΦ) ($CD11b^+/CD45^{high}$) in the brain isolated from ± tamoxifen-injected $CX_3CR1^{CreER/+}/R26^{tdTOM/+}$ mice subjected to microglial elimination/repopulation. **(H)** Representative images of YFP and tdTom expression in tamoxifen-injected $CX_3CR1^{CreER/+}/R26^{tdTOM/+}$ mice subjected to microglial elimination/repopulation. Inset shows $YFP^+/tdTom^+$ microglia identified by white arrows. Bars represent the mean ± SEM. Means with an asterisk (*) are significantly different from control ($p < .05$). Repop, repopulation.

with tamoxifen, such that all CX_3CR1^+ cells became $YFP^+/tdTom^+$ cells. After 28 days, mice were subjected to microglial elimination and subsequent repopulation. Microglia that repopulated from a CX_3CR1^{neg} progenitor cell would be $YFP^+/tdTom^{neg}$, while microglia repopulating from CX_3CR1^+ microglia that remained following elimination would be $YFP^+/tdTom^+$. Indeed, the majority of microglia (99% of $CD11b^+/CD45^{lo}$) were $YFP^+/tdTom^+$ after tamoxifen induction, elimination, and subsequent repopulation (Figure 3F, G; $p < .0001$). While CNS macrophages ($CD11b^+/CD45^{hi}$) were approximately 50% YFP^+, none were $YFP^+/tdTom^+$ weeks after tamoxifen injection. Notably, 50% of circulating monocytes were $YFP^+/tdTom^+$ 7 days after tamoxifen injection (data not shown). This loss of tdTom over time in bone marrow–derived monocyte/macrophages is consistent with turnover from the bone marrow (43–45). In addition, 60% of the microglia from control and repopulated mice (Tam^{neg}) were also $YFP^+/tdTom^+$ in the absence of tamoxifen (Figure 3G). This may reflect a "leaky" Cre recombinase with spontaneous tdTom induction over time. Nonetheless, all of the microglia were $YFP^+/tdTom^+$ after tamoxifen induction, elimination, and subsequent repopulation (Figure 3H). These data indicate self-renewal of microglia following elimination. The $YFP^+/tdTom^+$ microglia after elimination/repopulation indicate that these originate from the 3% to 5% of microglia that remained after CSF1R antagonism.

Stress Sensitization to Acute Stress Was Maintained After Elimination and Repopulation of Microglia

The next objective was to determine whether microglial elimination/repopulation prevented the recurrence of anxiety after acute defeat in RSD-sensitized mice. Microglia were eliminated using a CSF1R antagonist prior to RSD (Figure 4A). Microglial elimination prevented anxiety-like behavior 14 hours after RSD (Figure 4B, C) in the time to enter the center of the open field (Figure 4B; intervention x SS, $p = .06$) and time spent in the center (Figure 4C; intervention × SS, $p < .003$). Next, PLX5622 diet was

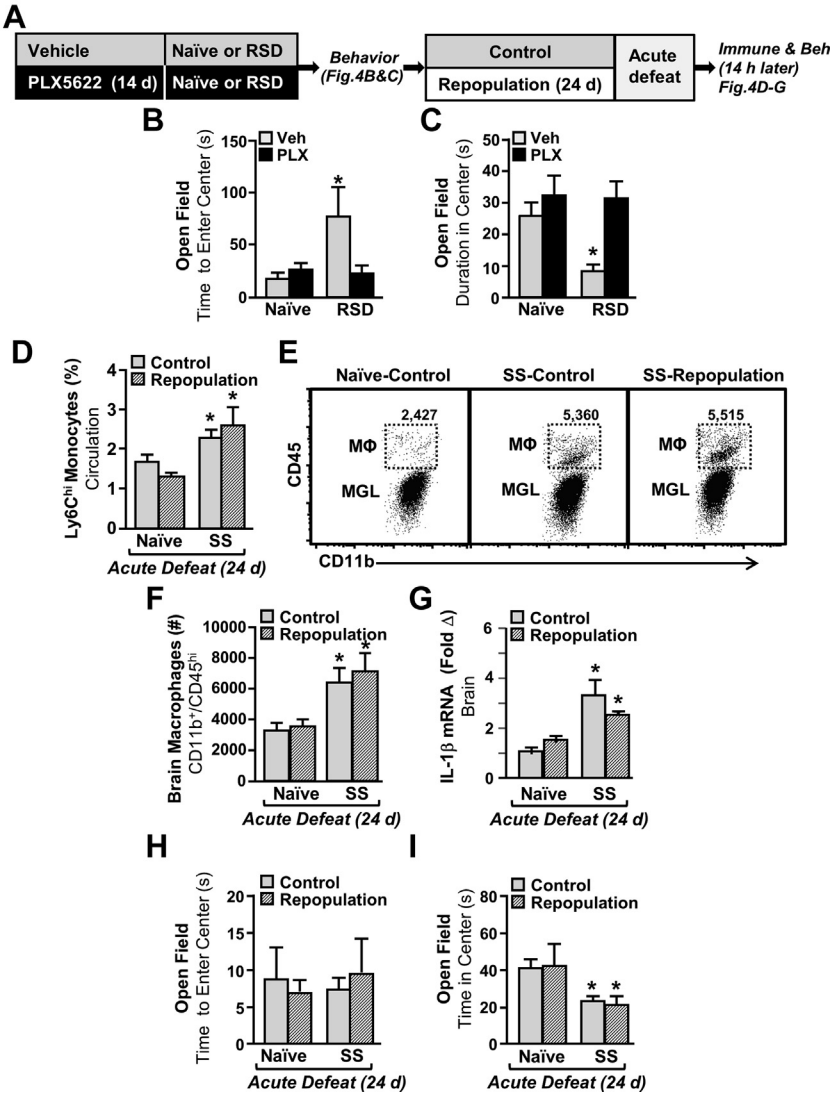


Figure 4. Microglia repopulation in stress-sensitized (SS) mice reestablished monocyte trafficking and anxiety-like behavior induced by acute defeat. **(A)** Male C57BL/6 mice were provided diets formulated with vehicle (Veh) or colony-stimulating factor 1 receptor antagonist (PLX5622 [PLX]) for 14 days. Next, mice were SS by repeated social defeat (RSD) or left undisturbed as control mice (naïve). **(B, C)** Anxiety-like behavior was determined 14 hour after the last cycle of RSD in the open field (n = 9–10) by time to enter the center **(B)** (intervention × SS interaction, $F_{1,38} = 10.36$, $p < .003$) and time spent in the center **(C)** (intervention × SS interaction, $F_{1,38} = 3.675$, $p = .06$). After RSD, all mice were provided Veh diets for an additional 24 days to allow for repopulation of microglia. After 24 days of repopulation, all mice were exposed to one cycle of social defeat (acute defeat). **(D)** Percentage of monocytes ($CD11b^{+}/CD45^{high}$) in circulation 14 hours after acute defeat (main effect of SS, $F_{1,37} = 16.46$, $p < .0002$). **(E)** Representative bivariate dot plots of CD11b and CD45 labeling on enriched microglia (MGL) and macrophages (MΦ). **(F)** Number of brain macrophages ($CD11b^{+}/CD45^{high}$) 14 hours after acute defeat (main effect of SS, $F_{1,37} = 16.46$, $p < .0002$). **(G)** Messenger RNA (mRNA) levels of interleukin-1β (IL-1β) were determined in a coronal brain section (n = 4) collected 14 hours after acute defeat (main effect of SS, $F_{1,14} = 20.1$, $p < .001$). **(H, I)** Anxiety-like behavior (n = 10) was determined by time to enter **(H)** (not significant) and time spent in the center of the open field 0.5 days after acute defeat **(I)** (main effect of SS, $F_{1,39} = 14.85$, $p < .001$). Bars represent the mean ± SEM. Means with an asterisk (*) are significantly different from naïve controls ($p < .05$). Beh, behavior.

removed to allow for microglia repopulation. After 24 days of repopulation, naïve and SS mice were subjected to acute defeat (Figure 4A). Acute defeat increased monocytes in the blood of SS mice but not in naïve mice (Figure 4D; $p < .0002$). This monocyte induction in SS mice with acute defeat, however, was independent of microglial elimination/repopulation (Figure 4D). In addition, acute defeat increased monocyte accumulation in the brain of SS mice (Figure 4E, F; $p < .0002$) and was associated with increased IL-1β mRNA levels (Figure 4G; $p < .001$). Again, these events induced by acute stress in SS mice were independent of microglial elimination/repopulation. Similar effects were evident in anxiety-like behavior (Figure 4H, I). While the time to enter the center of the open field was unaffected by acute stress (Figure 4H), total time spent in the center was decreased by acute defeat (Figure 4G; $p < .001$). This behavior was independent of microglial elimination/repopulation. Thus, stress-induced monocyte trafficking to the brain, IL-1β induction, and anxiety were dependent on the presence of microglia but were not prevented by microglial repopulation.

Evidence for Neuronal Sensitization With RSD

Microglial elimination prior to RSD and then subsequent repopulation did not prevent the exaggerated immune and behavioral responsiveness to acute defeat in the SS mice. Thus, there are likely other CNS components involved in stress sensitization besides microglia. To address this, neuronal activation (c-Fos) and reactivity (phospho-CREB) to acute defeat were determined in two relevant fear and threat appraisal areas (prelimbic cortex and hippocampus) of naïve and SS mice immediately following acute defeat (46). As expected, there was no c-Fos activation 24 days after RSD (control SS group). After acute stress, there was a robust increase in c-Fos^{+} neurons of the prelimbic cortex (Figure 5A, B; $p < .001$) and dentate gyrus (Figure 5A, C; $p < .001$) in both

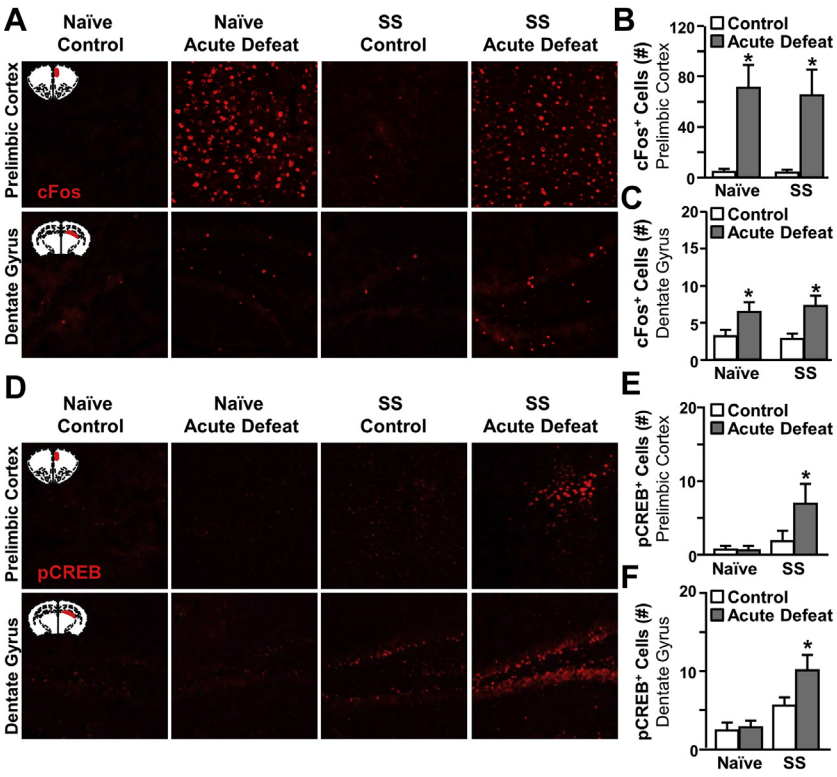


Figure 5. Evidence of neuronal sensitization with repeated social defeat. Male C57BL/6 mice were stress-sensitized (SS) by repeated social defeat or left undisturbed as control mice (naïve). At 24 days after stress, all mice were exposed to one cycle of social defeat (acute defeat). Immediately after acute defeat, brains were perfused, fixed, sectioned, and labeled for c-Fos or phospho-CREB (pCREB) ($n = 6$). **(A)** Representative images of c-Fos expression in the prelimbic cortex (top panel) and dentate gyrus (bottom panel). **(B, C)** The number of c-Fos$^+$ cells in the prelimbic cortex **(B)** ($F_{1,23} = 27.6$, $p < 0.001$) and dentate gyrus **(C)** of the hippocampus in control and SS mice 14 hours after acute defeat ($F_{1,23} = 75.8$, $p < .001$). **(D)** Representative images of pCREB expression in the prelimbic cortex (top panel) and dentate gyrus (bottom panel). **(E, F)** Number of pCREB$^+$ cells in the prelimbic cortex **(E)** (SS, $F_{1,24} = 4.7$, $p < .04$; SS × acute stress interaction, $F_{1,24} = 2.9$, $p = .10$), and dentate gyrus **(F)** of control and SS mice 0.5 days after acute defeat (SS, $F_{1,24} = 17.7$, $p < .001$). Bars represent the mean ± SEM. Means with an asterisk (*) are significantly different from naïve controls ($p < .05$).

naïve and SS mice. There was no difference after acute defeat in the number of c-Fos$^+$ cells between naïve and SS groups (Figure 5B, C).

Increased expression of phospho-CREB is implicated in learning-induced synaptic plasticity and therefore may indicate altered neuronal reactivity to threatening stimuli following stress sensitization (47–49). Similar to the c-Fos induction, there were few pCREB$^+$ cells present 24 days after RSD (Figure 5D). There tended to be an interaction between stress sensitization and acute defeat for pCREB in the prelimbic cortex (interaction, $p = .1$). Only the SS mice exposed to acute defeat had increased pCREB activity in the prelimbic cortex (Figure 5D, E; $p < .04$). Similar interactions were detected in the dentate gyrus (Figure 5D, F; $p < .001$). Again, the SS mice exposed to acute defeat had the most pCREB$^+$ neurons compared with all other groups ($p < .03$). These data provide evidence of neuronal sensitization after RSD within fear and threat appraisal regions.

Microglial Hyperactivity to Innate Immune Challenge in SS Mice Was Attenuated by Microglia Elimination and Repopulation

To further delineate the role of microglial sensitization after RSD, the response to an immune challenge with LPS was determined. LPS challenge activates microglia by a different pathway than the one elicited by acute stress. Acutely following RSD, microglia respond to LPS challenge with an exaggerated immune and neuroinflammatory response (33).

Here, microglia were eliminated prior to RSD sensitization and then were allowed to repopulate for 24 days before assessing sensitization to LPS challenge. Microglia were collected and cultured ex vivo with saline or LPS, and mRNA levels of TLR-4, CD14, IL-6, and IL-1β were determined (Figure 6A). There was no effect of SS or LPS on TLR-4 expression ex vivo (Figure 6B). CD14 mRNA was higher at baseline in SS repopulated mice compared with control mice (Figure 6C; $p < .05$). LPS increased CD14 mRNA in microglia ($p < .05$). The SS control LPS group had the highest levels of CD14 mRNA ($p < .05$), and these levels were attenuated by repopulation (Figure 6C; $p < .03$). LPS also increased IL-6 mRNA in microglia ($p < .06$), and the SS control LPS group had the highest levels of IL-6 mRNA compared with all other groups (Figure 6D; $p < .03$). LPS increased IL-1β mRNA in microglia. Again, the SS mice injected with LPS had the highest IL-1β mRNA expression compared with controls ($p < .05$), and these levels were attenuated by elimination/repopulation (Figure 6E; $p < .05$). Taken together, the higher microglial reactivity to ex vivo LPS challenge in SS mice was attenuated by microglial elimination/repopulation.

Using a similar design, we next investigated whether elimination/repopulation of microglia reduced the reactivity of SS mice to an in vivo challenge with LPS. Social exploratory behavior of a juvenile conspecific is a measure of sickness behavior following peripheral LPS challenge (29,33). For example, peripheral LPS injection elicits a transient sickness behavior response in control mice associated with reduced social exploratory behavior that returns to baseline within 24 hours (29,33). Following microglial elimination/repopulation,

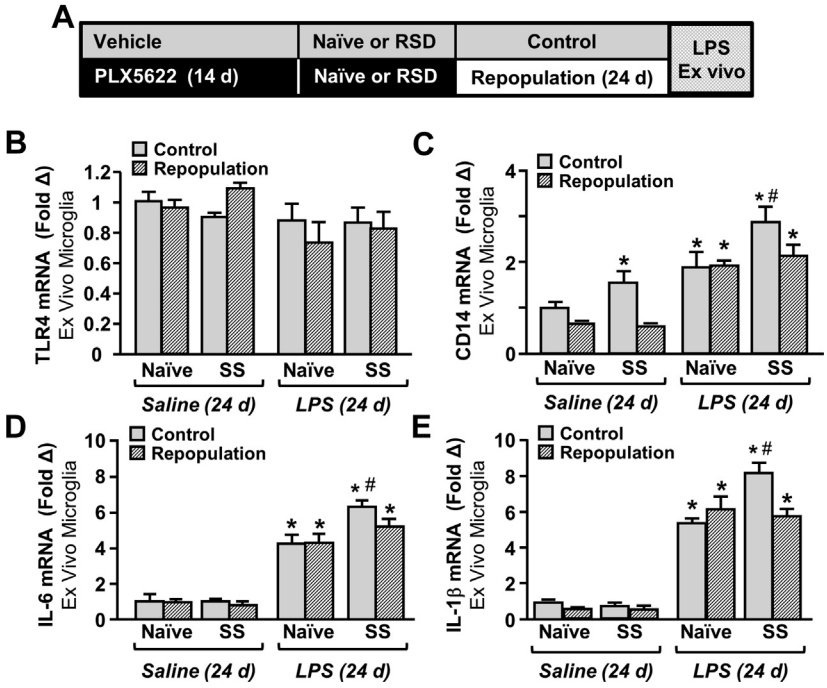


Figure 6. Microglial hyperactivity to ex vivo lipopolysaccharide (LPS) stimulation in stress-sensitized (SS) mice was attenuated by microglial elimination and repopulation. **(A)** Male C57BL/6 mice were provided diets formulated with vehicle or colony-stimulating factor 1 receptor antagonist (PLX5622) for 14 days. Next, mice were SS by repeated social defeat (RSD) or left undisturbed as control mice (naïve). After RSD sensitization, all mice were provided vehicle diets for an additional 24 days to allow for repopulation of microglia. After 24 days of repopulation, microglia were collected by Percoll-enrichment and were cultured ex vivo with saline or LPS (100 ng/mL). **(B–E)** Messenger RNA (mRNA) levels of toll-like receptor 4 (TLR4) **(B)**, cluster of differentiation 14 (CD14) **(C)**, interleukin-6 (IL-6) **(D)**, and interleukin-1β (IL-1β) **(E)** were determined in ex vivo microglia 4 hours after LPS stimulation. Means with an asterisk (*) are significantly different from naïve controls ($p < .05$), and means with a pound symbol (#) are significantly different from naïve SS ($p < .05$).

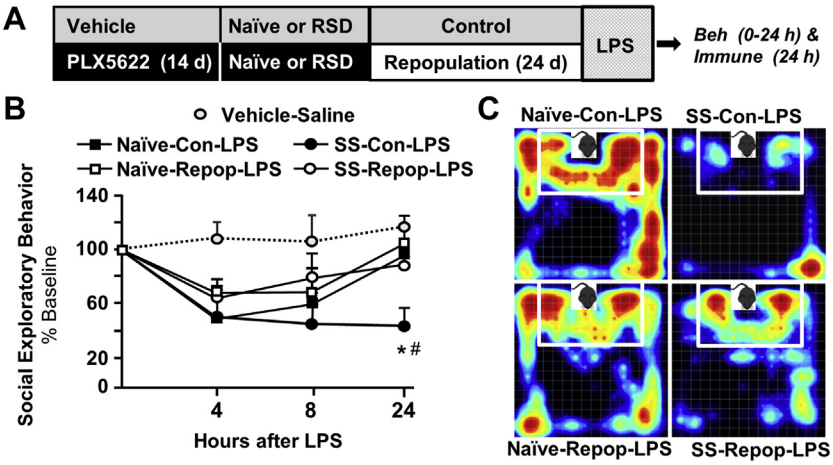
mice were injected with LPS and social exploratory behavior was determined at baseline and 4, 8, and 24 hours after LPS (Figure 7A). LPS caused a significant reduction in social exploratory behavior ($p < .03$) that tended to be dependent on time (Figure 7B; $p = .07$). LPS-injected naïve control mice returned to baseline social interaction by 24 hours after LPS, but the control SS mice had reduced social behavior 24 hours after LPS (Figure 7B, C; $p < .05$). This protracted sickness behavior after LPS in SS mice was prevented by microglia elimination/repopulation. SS repopulated LPS mice returned to baseline behavior by 24 hours and were not different from control groups (Figure 7B).

Next, the mRNA expression of several inflammatory genes (IL-1β, IL-6, CD14, and TLR4) was determined in enriched microglia/macrophages (Figure 7D). LPS increased IL-1β mRNA levels in microglia/macrophages ($p < .05$ for each), with the highest expression being in the microglia of SS mice injected with LPS ($p < .05$). Moreover, this exaggerated IL-1β mRNA response to LPS in SS mice was attenuated by elimination/repopulation (SS × repopulation, $p < .001$). This exaggerated response to LPS in microglia of SS mice was also evident in IL-6, CD14, and TLR4. Expression of each gene was highest in the SS mice injected with LPS ($p < .05$) and was attenuated by elimination/repopulation (SS × repopulation, $p < .03$, for each). Collectively, removal of microglia prior to stress sensitization and subsequent repopulation ablated the amplified immune reactivity to peripheral LPS challenge at 24 days.

DISCUSSION

Chronic stress may elicit stress sensitization, in which individuals become more vulnerable to subsequent stressful stimuli (3,4). We provide mRNA and functional evidence that microglia remain primed or sensitized weeks after RSD. For instance, acute defeat in RSD-sensitized mice caused significant monocyte accumulation in the brain (microglia dependent) and promoted the reoccurrence of anxiety. Immune challenge (LPS) also elicited microglial reactivity in RSD-sensitized mice that corresponded with prolonged sickness behavior. Stress reactivity to acute defeat, however, remained when microglia were eliminated and repopulated after RSD. Immune reactivity to LPS (ex vivo and in vivo) was prevented when microglia were eliminated and repopulated after RSD. Collectively, RSD sensitization is a complex process in which microglia play a role in the recurrence of anxiety with acute defeat and are essential for the increased reactivity to immune challenge.

Two important aspects of this study are that microglia from RSD-sensitized mice were primed and were critical for recruitment of monocytes to the brain after acute stress 24 days later. The RNA signature of microglia at 24 days after RSD had 137 differentially expressed genes that were associated with pathways consistent with inflammatory and microglial priming. In addition, our previous studies demonstrated that the recurrence of anxiety-like behavior in SS mice was dependent on recruitment of splenic monocytes to the brain (25,27). Notably, anxiety-like behavior following RSD is well validated and is evident in exploratory-based measures [e.g., open field, light/dark preference, elevated plus maze (16,24,50)]. Moreover, anxiety after RSD is also evident in other tests, including the Morris water maze (51,52) and fear conditioning paradigms (53). A potential limitation of these studies is that anxiety-like behavior was measured only using the open field test. Anxiety-like behavior in the open field (fully automated analyses) is the most consistent and reproducible of the



D

Enriched CD11b+ Cell mRNA Analysis (24 h)					
	Saline	Control-LPS		Repop-LPS	
Gene	Naive	Naive	SS	Naive	SS
IL-1β	1.1 ± 0.2	3.7 ± 0.3*	6.7 ± 0.7*#	5.1 ± 0.4 *	4.4 ± 0.5*
IL-6	1.0 ± 0.1	1.2 ± 0.2	3.7 ± 0.3*	1.4 ± 0.4	1.6 ± 0.3
CD14	1.0 ± 0.1	1.4 ± 0.1*	3.4 ± 0.1*#	0.9 ± 0.1	0.9 ± 0.2
TLR4	1.0 ± 0.1	1.6 ± 0.1	2.7 ± 0.3*#	0.5 ± 0.2 *	0.7 ± 0.1 *

Figure 7. Microglial hyperactivity to in vivo lipopolysaccharide (LPS) challenge in stress-sensitized (SS) mice was attenuated by microglial elimination and repopulation. **(A)** Male C57BL/6 mice were provided diets formulated with vehicle or colony-stimulating factor 1 receptor antagonist (PLX5622) for 14 days. Next, mice were SS by repeated social defeat or left undisturbed as control mice (naïve). After RSD sensitization, all mice were provided vehicle diets for an additional 24 days to allow for repopulation of microglia. After 24 days of repopulation, mice were injected with LPS (0.5 mg/kg, intraperitoneal), and **(B)** social exploratory behavior (percentage of baseline) was determined at baseline and 4, 8, and 24 hours after LPS challenge (main effect of SS, $F_{1,\ 83} = 18.59$, $p < .03$; SS × time, $F_{3,83} = 3.42$, $p = .066$). **(C)** Representative heat maps of social exploratory behavior 24 hours after LPS. **(D)** Messenger RNA (mRNA) levels of interleukin-1β (IL-1β) (SS × Repop interaction, $F_{1,18} = 14.6$, $p < .001$), interleukin 6 (IL-6) (SS × Repop interaction, $F_{1,18} = 14.2$, $p < .001$, cluster of differentiation 14 (CD14) (SS × Repop interaction, $F_{1,18} = 10.1$, $p < .005$), and toll-like receptor 4 (TLR4) (SS × Repop interaction, $F_{1,18} = 5.715$, $p < .03$) were determined in Percoll-enriched microglia collected 24 hours after LPS challenge. Bars represent the mean ± SEM. Means with an asterisk (*) are significantly different from naïve controls ($p < .05$), and means with a pound symbol (#) are significantly different from saline controls ($p < .05$). Con, control; Repop, repopulation.

tests we have used. Furthermore, exploratory-based tests can confound one another when used in the same mice. Thus, we selectively used the open field test for these experiments. Here, we confirmed our previous work (26) that monocyte recruitment to the brain was dependent on the presence of microglia. Moreover, the increased accumulation of inflammatory monocytes within the brain vasculature of the SS mice was associated with the recurrence of anxiety. It is important to note that CSF1R antagonism does not influence circulating monocyte numbers (26). Collectively, we interpreted these data to indicate that monocytes were recruited to the brain by microglia, and these monocytes augmented neuroinflammatory signaling that reinforced the recurrence of anxiety in RSD-sensitized mice.

Another relevant finding was that microglia repopulated after removal of PLX5622, returning to baseline by 21 days. Several other studies show similar kinetics of microglial repopulation (40,41,54,55). Here, elimination/repopulation resulted in 99% of repopulated microglia that were double-positive for YFP and tdTom. Notably, if microglia from $CX_3CR1^{CreER/+}/R26^{tdTom/+}$ mice repopulated from CNS myeloid progenitor cells, then these cells would be YFP^+ and $tdTom^{neg}$. This finding is consistent with several recent studies into the origin of the repopulated microglia (42,56). Thus, it is likely that repopulation of microglia after PLX5622 removal originated from the 3% to 4% of the microglia that remained throughout PLX5622 administration.

A critical question was the extent to which microglia underlie maintenance of stress sensitization and the recurrence of anxiety with acute stress. We hypothesized that preventing the microglial sensitization by eliminating prior to RSD and allowing for repopulation would prevent recurrence of anxiety following subsequent acute stress. Nonetheless, microglia elimination/repopulation did not affect hypersensitivity of RSD-sensitized mice to acute stress.

We show that the presence of microglia at the time of acute stress was essential for the expression of stress sensitization, but depletion and repopulation did not prevent the sensitized stress response. Thus, these data indicate that microglia are not essential for the sensitization per se but are essential for the expression of that sensitization.

One explanation for the above finding is that the priming of microglia represents only one component of RSD sensitization. Our previous and current data implicate RSD sensitization of neurons and myeloid cells in the spleen. For example, we reported a splenic population of myeloid cells that persisted in the spleen 24 days after RSD (27). Indeed, acute stress increased trafficking of $Ly6C^{hi}$ monocytes from the spleen to the brain in SS mice (25,27). Furthermore, removal of the spleen blocked the acute stress-induced reoccurrence of anxiety (26,57). Thus, the spleen acts as a unique reservoir for maintaining inflammatory monocytes that are readily releasable into circulation after acute stress in SS mice (27). Here, microglial elimination/repopulation did not diminish the number of $Ly6C^{hi}$ monocytes in circulation in SS mice. Notably, PLX5622 does not affect circulating monocyte survival (26). Thus, the splenic release of monocytes in SS mice was independent of microglial priming and corresponded with increased monocyte accumulation in the brain. In addition, acute defeat increased pCREB expression in neurons in two key threat appraisal areas, the prelimbic cortex and dentate gyrus, only in the SS mice. pCREB activity in the hippocampus

has been implicated in associative learning (58), suggesting a mechanism for altered neuronal reactivity following stress sensitization. We interpret these findings to indicate that SS mice have a sensitized neuronal interpretation of acute stress compared with otherwise naïve mice. These sensitized neurons may in turn cause microglial activation (24,26). Moreover, other murine stressors elicited microglial activation that was dependent on neuronal activity (59–62). Thus, heightened fear and threat appraisal in SS mice may explain the higher reactivity to a subsequent acute stressor. In addition, monocyte release is dependent on the sympathetic nervous system (6,21,24,27), so neuronal sensitization may also persist weeks after RSD. These data indicate that there are multiple CNS cell types that contribute to RSD sensitization.

A critical finding was that the elimination/repopulation of microglia after RSD attenuated the amplified response to LPS. For example, microglia from SS mice had increased inflammatory cytokine expression following direct ex vivo stimulation with LPS. This response was associated with a higher level of CD14 mRNA, a coreceptor for LPS (33), which persisted in microglia 24 days after RSD. This direct responsiveness of microglia to LPS and the higher CD14 mRNA from SS mice was consistent with our previous reports (24,33). The results from the RNA sequencing and ex vivo experiments support the conclusion of microglial priming 24 days after RSD (25). Here, we extend our previous work and show that this reactivity to LPS ex vivo was prevented when microglia were absent during RSD and then repopulated. In parallel with the ex vivo data, LPS injection was associated with prolonged sickness behavior and exaggerated proinflammatory cytokine mRNA expression in RSD-sensitized mice compared with control mice. Importantly, the elimination/repopulation of microglia in RSD-sensitized mice prevented the prolonged sickness behavior induced by LPS and attenuated the cytokine responses in enriched microglia/macrophages. Despite renewing from microglia present at the time of stress, the repopulated microglia were no longer primed to peripheral immune challenge. These findings indicate that microglial elimination/repopulation was a successful strategy to prevent microglial priming to immune challenge. Therefore, microglial priming in response to RSD can be prevented by elimination/repopulation with PLX5622.

Stress sensitization differentially affected microglia-mediated immune responses to acute stress and LPS challenge because of intrinsic differences in the responses to these stimuli. For instance, LPS challenge activates the peripheral innate immune response first prior to microglial activation (63). In this context, peripheral cytokine production initiates a transient sickness response characterized by lethargy, anorexia, and reduced social interaction. Notably, mice with evidence of primed microglia (e.g., aged, stressed, injured) have prolonged sickness behavior following LPS compared with control mice (29,31–33,64). Thus, microglial priming has a role in exacerbating social withdrawal following peripheral LPS challenge. The mechanism of behavioral perturbation differs following acute stress. In acute stress, there is rapid increase in neuronal activation in brain regions associated with fear and threat appraisal, followed by subsequent activation of microglia (26,62,65,66). Thus, stress causes brain-to-immune communication. Microglial repopulation did not affect neuronal sensitization to acute stress, evidenced by persistent increases in pCREB 24 days following RSD. Neuronal activation in regions associated with fear and threat appraisal is likely not a critical modulator of the inflammatory response to LPS (31,63,67). Therefore, microglia play a more central role in initiating the behavioral and immune responses to LPS challenge compared with acute defeat.

In summary, we highlight the complex immune and CNS cellular interactions that occur during stress sensitization. Our findings reinforce the idea of long-lasting priming of microglia after exposure to RSD (68). Moreover, our findings continue to show a critical role of inflammatory monocytes, which are actively recruited to the brain by microglia, in the augmentation and recurrence of anxiety. While microglial elimination/repopulation in RSD-sensitized mice did not affect hyperactivity to acute stress, it was effective in reversing microglial reactivity to LPS challenge (ex vivo and in vivo). Taken together, microglia and neurons remain sensitized weeks after RSD, and only the immune reactivity component of primed microglia was prevented by elimination/repopulation.

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