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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 if 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 CSF1R-antagonism (PLX5622), allowed to repopulate, and responses to acute stress or immune challenge (lipopolysaccharide) were determined 24 days after RSD-sensitization.

Results: Microglia maintained a unique mRNA signature 24 d after RSD. Moreover, elimination of RSD-sensitized microglia prevented monocyte accumulation in the brain and blocked anxiety recurrence following acute stress (24 d). When microglia were eliminated prior to RSD, 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 d 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.

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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

Repeated Social Defeat; Stress; Anxiety; Microglia; Monocytes; CSF1R antagonist

Introduction

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 PAMPS and higher expression of pro-inflammatory cytokines (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 (sub-threshold) stress caused anxiety recurrence (25, 27). This acute stress is one cycle of RSD and is defined as a "subthreshold stress" because it does not cause monocyte trafficking or anxiety-like behavior in 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 CNS 24 days after RSD. For example, isolated microglia from RSD-sensitized mice had elevated CD14 mRNA and were more reactive to *ex vivo* 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 hyper-reactivity to peripheral immune challenges (29–33). Therefore, the purpose of this study was to determine if microglia were a critical component of stress-sensitization and increased reactivity to acute stress or innate immune challenge.

Methods

Mice:

Male C57BL/6 (6–8 weeks old) and CD-1 (12 months) mice were used (Charles River Laboratory). CX_3CR1^{CreER}/ROSA26-$STOP^{flfl}$-tdTomato were generated by crossing CX_3CR1^{CreER} and Ai9-ROSA26-$STOP^{flfl}$-tdTomato mice (Jackson Laboratories). All procedures were performed in accordance with the NIH and OSU Guidelines.

Repeated Social Defeat (RSD) and Stress-Sensitization:

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

RNA-sequencing of FAC-sorted Microglia:

Microglia were enriched using Percoll separation, labeled with anti-CD11b and CD45 antibodies, and FAC-sorted. RNA was extracted, synthesized to cDNA, and 20 million 75bp reads were sequenced on an Illumina NextSeq 500. Sequences were aligned to the mm10 mouse reference genome using STAR 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 $p<0.05$ and fold changes over 1.5 ($\log_2 FC>0.585$) were considered differentially expressed.

Plexxikon Administration:

PLX5622 was provided by Plexxikon Inc., 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 (i.p.) as described (37).

Flow Cytometry:

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

RNA Isolation and RT-PCR:

RNA was isolated from Percoll-enriched $CD11b^+$ cells and coronal brain sections. Real-time qPCR was performed using the Applied Biosystems Assay-on-Demand Gene Expression

protocol. mRNA expression was determined on an ABI PRISM 7300-sequence detection system, converted to CT, 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 post-fixed and cryosectioned. Labeling for Iba1, c-Fos, or Phospho-CREB was performed as described in the supplemental methods.

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 h after LPS injection as described (25, 26, 33). Both behaviors are detailed in the supplemental methods.

Statistical Analysis:

To determine significant main effects and interactions between main factors, data were analyzed using one-, two-, or three-way ANOVA using the General Linear Model procedures of SPSS statistical software (IBM). In the event of a main effect of treatment, differences between group-means were evaluated by *Post hoc* analyses (Fisher's LSD) and are graphically presented in figures ($p<0.05$)

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 hypothesize that microglia are a critical cellular component of stress-sensitization and are hyper-reactive to subsequent stressors and immune challenges.

First, we determined the microglial mRNA profile 24 days after RSD-sensitization. Microglia were FAC-sorted and RNA was sequenced (Fig. 1A). Volcano plots show genes in microglia that were increased or decreased by 1.5 fold ($p<0.05$) 24 d after RSD compared to controls (Fig. 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 over 1.5 fold ($p<0.05$) in microglia. Several other genes including *Tgfbr3*, *Il1r2*, *Aqp1*, and *Cadm2*, were decreased in microglia ($p<0.05$). 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 controls ($p<0.05$) were IL-1β, Receptor tyrosine

for glial derived neurotrophic factor ligands (Ret), Interferon−γ, MyD88, IL-4, and TLR4 (Fig. 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 d 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 by RSD (SS) or undisturbed (Naïve) and microglia were depleted using a CSF1R antagonist (PLX5622). All mice were exposed to acute defeat 24 d after RSD (Fig. 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 for these experiments (25, 27). Consistent with a subthreshold stressor, acute defeat increased $Ly6C^{hi}$ monocytes in circulation and in the spleen of stress-sensitized (SS) mice compared to naïve mice (Fig.2B&C, $p<0.002$ for each). Monocyte release and accumulation in the spleen following acute defeat was dependent on stress-sensitization (Fig.2B&C). PLX5622 neither affected the number of circulating monocytes nor monocyte accumulation in the spleen (Fig.2B&C). Acute defeat also increased IL-1β mRNA levels in the brain of stress-sensitized mice compared to controls ($p<0.001$) and this effect was abrogated by microglial elimination (Fig. 2D, intervention x SS, $p<0.04$). Acute defeat increased monocyte accumulation in the brain of stress-sensitized (SS) mice (Fig.2E&F, $p<0.05$), and this was prevented by microglial elimination (Fig.2E&F, intervention x SS, $p<0.004$). 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 stress-sensitized (SS) mice compared to controls (Fig. 2G). This was associated with increased time to enter the center of the open field (Fig. 2H, $p<0.03$) and decreased time spent in the center for stress-sensitized mice (Fig. 2I, $p<0.05$) compared to controls. Moreover, acute defeat-associated anxiety in stress-sensitized (SS) mice was prevented by microglial elimination (Fig. 2H, SS x intervention, $p<0.02$ and Fig. 2I, SS x intervention, $p<0.01$). Notably, there were no differences in total distance traveled between groups (Fig. 2J). Taken together, the recurrence of anxiety in RSD-sensitized mice with acute stress was blocked by microglial elimination.

Microglia Repopulated from Non-Progenitor 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 (Fig. 3A). As expected, Iba-1^+ microglia repopulated after elimination in a time-dependent manner (Fig. 3B, $p<0.0001$). $CD11b^+/CD45^{lo}$ microglia were reduced below baseline 0 d, 7 d, and 14 d after removal of the CSF1R antagonist (Fig.3B&C, $p<0.05$, for each). Microglia returned to baseline within 21 d, 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 tdTomato expression (Fig. 3E). In this experiment, $CX_3CR1^{CreER\text{-}YFP}/R26^{tdTOM}$ mice (3-week) were injected with tamoxifen, such that all CX_3CR1^+ cells became $YFP^+/tdTom^+$. After 28 d, 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 (Fig.3F&G, $p<0.0001$).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 d after tamoxifen injection (data not shown). This loss of tdTom with time in BM-derived monocyte/macrophages is consistent with turnover from the bone marrow (43–45).Also, 60% of the microglia from control and repopulated mice (Tam^{neg}) were also $YFP^+/tdTom^+$ in the absence of tamoxifen (Fig. 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 (Fig. 3H). These data indicate self-renew of microglia following elimination. The $YFP^+/tdTom^+$ microglia after elimination/repopulation indicates that these originate from the 3–5% 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 if microglia elimination/repopulation prevented the recurrence of anxiety after acute defeat in RSD-sensitized mice. Microglia were eliminated using a CSF1R antagonist prior to RSD (Fig. 4A). Microglial elimination prevented anxiety-like behavior 14 h after RSD (Fig.4B&C) in the time to enter the center of the open field (Fig. 4 B, intervention x SS, $p=0.06$) and time spent in the center (Fig. 4C, intervention x SS, $p<0.003$). Next PLX5622 diet was removed to allow for microglia repopulation. After 24 d repopulation, naive and stress-sensitized (SS) mice were subjected to acute defeat (Fig. 4A). Acute defeat increased monocytes in the blood of SS mice, but not in naïve mice (Fig. 4D, $p<0.0002$). This monocyte induction in SS mice with acute defeat, however, was independent of microglial elimination/repopulation (Fig. 4D). In addition, acute defeat increased monocyte accumulation in the brain of stress-sensitized mice (Fig.4E&F, $p<0.0002$) and was associated with increased IL-1β mRNA levels (Fig. 4G, $p<0.001$). Again these events induced by acute stress in SS mice were independent of microglial elimination/repopulation. Similar effects were evident in anxiety-like behavior (Fig.4H&I). While the time to enter the center of the open field was unaffected by acute stress (Fig. 4H), total time spent in the center was decreased by acute defeat (Fig. 4G, $p<0.001$). This behavior was independent of microglia elimination/repopulation. Thus, stress-induced monocyte trafficking to the brain, IL-1β induction, and anxiety were dependent on the presence of microglia but was 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 stress-sensitized 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 stress-sensitized (SS) mice immediately following acute defeat (46). As expected, there was no c-Fos activation 24 d after RSD (Control-SS group). After acute stress, there was a robust increase in c-Fos$^+$ neurons of prelimbic cortex (Fig. 5A&B, $p<0.001$) and dentate gyrus (Fig. 5A&C, $p<0.001$) in both naïve and stress-sensitized mice. There was no difference after acute defeat in the number of c-Fos$^+$ cells between naïve and SS groups (Fig.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 d after RSD (Fig. 5D). There tended to be an interaction between stress-sensitization and acute defeat for pCREB in the prelimbic cortex (interaction, $p=0.1$). Only the SS mice exposed to acute defeat had increased pCREB activity in the prelimbic cortex ($p<0.04$, Fig. 5D&E). Similar interactions were detected in the dentate gyrus (Fig. 5D&F, $p<0.001$). Again, the SS mice exposed to acute defeat had the most pCREB$^+$ neurons compared to all other groups ($p<0.03$). These data provide evidence of neuronal sensitization after RSD within fear and threat appraisal regions.

Microglial Hyperactivity to Innate Immune Challenge in Stress-Sensitized 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 lipopolysaccharide (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 (SS) and then allowed to repopulate for 24 d before assessing sensitization to LPS challenge. Microglia were collected,cultured *ex vivo* with saline or LPS and mRNA levels of TLR-4, CD14, IL-6, and IL-1β were determined (Fig. 6A). There was no effect of SS or LPS on TLR-4 expression *ex vivo* (Fig. 6B). CD14 mRNA was higher at baseline in SS-Repop mice compared to controls (Fig. 6C, $p<0.05$). LPS increased CD14 mRNA in microglia ($p<0.05$). The SS-Control-LPS group had the highest levels of CD14 mRNA ($p<0.05$) and these levels were attenuated by repopulation (Fig. 6C, $p<0.03$). LPS also increased IL-6 mRNA in microglia ($p<0.06$) and the SS-Control-LPS group had the highest levels of IL-6 mRNA compared to all other groups (Fig. 6D, $p<0.03$). LPS increased IL-1β mRNA in microglia. Again, the SS-Control-LPS group had the highest IL-1β mRNA expression compared to controls ($p<0.05$) and these levels were attenuated by elimination/repopulation (Fig. 6E, $p<0.05$). Taken together

the higher microglial reactivity to *ex vivo* LPS challenge in stress-sensitized mice was attenuated by microglial elimination/repopulation.

Using a similar design, we next investigated if elimination/repopulation of microglia reduced the reactivity of stress-sensitized 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 h (29, 33). Following microglial elimination/repopulation, mice were injected with LPS and social exploratory behavior was determined at baseline and 4, 8, and 24 h after LPS (Fig. 7A). LPS caused a significant reduction in social exploratory behavior ($p<0.03$) that tended to be dependent on time (Fig. 7B, $p=0.07$). LPS-injected naïve-control mice returned to baseline social interaction by 24 h after LPS, but the Con-SS mice had reduced social behavior 24 h after LPS (Fig.7B&C, $p<0.05$). This protracted sickness behavior after LPS in stress-sensitized mice was prevented by microglia elimination/repopulation. SS-Repop-LPS returned to baseline behavior by 24 h and were not different from control groups (Fig. 7B).

Next, the mRNA expression of several inflammatory genes (IL-1β, IL-6, CD14, and TLR4) was determined in enriched microglia/macrophages (Fig. 7D). LPS increased IL-1β mRNA levels in microglia/macrophages ($p<0.05$, for each) with the highest expression in the microglia of stress-sensitized (SS) mice injected with LPS (SS-Con-LPS) ($p<0.05$). Moreover, this exaggerated IL-1β mRNA response to LPS in SS mice was attenuated by elimination/repopulation (SS x Repop, $p<0.001$). This exaggerated response to LPS in microglia of stress-sensitized (SS-Con-LPS) was also evident in IL-6, CD14, and TLR4. Each mRNA level was highest in the Control-SS-LPS group ($p<0.05$) and was attenuated by elimination/repopulation (SS x Repop, $p<0.03$, for each). Collectively, removal of microglia prior to stress-sensitization and subsequent repopulation ablated the amplified immune reactivity to peripheral LPS challenge at 24 d.

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 recurrence 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 were that microglia from RSD-sensitized mice were primed and were critical for recruitment of monocytes to the brain after acute stress 24 d

later. The RNA signature of microglia at 24 d after RSD had 137 differentially expressed genes which were associated with pathways consistent with inflammatory and microglial priming. Also, our previous studies demonstrated that the recurrence of anxiety-like behavior in stress-sensitized 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, and 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 only measured using the open field test. Anxiety-like behavior in the open field (fully automated analyses) is the most consistent and reproducible of the 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 that monocyte recruitment to the brain was dependent on presence of microglia. Moreover, the increased accumulation of inflammatory monocytes within the brain vasculature of the stress-sensitized 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 d. 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 PLX6522 removal originated from the 3–4% of the microglia that remained throughout PLX5622 administration.

A critical question was to determine 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 recurrence 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 stress-sensitized mice was independent of microglial priming and corresponded with increased monocyte accumulation in the brain. In addition, acute defeat increased pCREB expression in neurons within two key threat appraisal areas, the prelimbic cortex and dentate gyrus, only in the stress-sensitized 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 stress-sensitized mice have a sensitized neuronal interpretation of acute stress compared to 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 stress-sensitized 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 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 stress-sensitized 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 co-receptor 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 RNASeq and *ex vivo* experiments support the conclusion of “microglial priming” 24 d 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. Parallel with the *ex vivo* data, LPS injection was associated with prolonged sickness behavior and exaggerated pro-inflammatory cytokine mRNA expression in RSD-sensitized mice compared to controls. 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 response 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, or injured) have

prolonged sickness behavior following LPS compared to controls (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 d 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 to 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 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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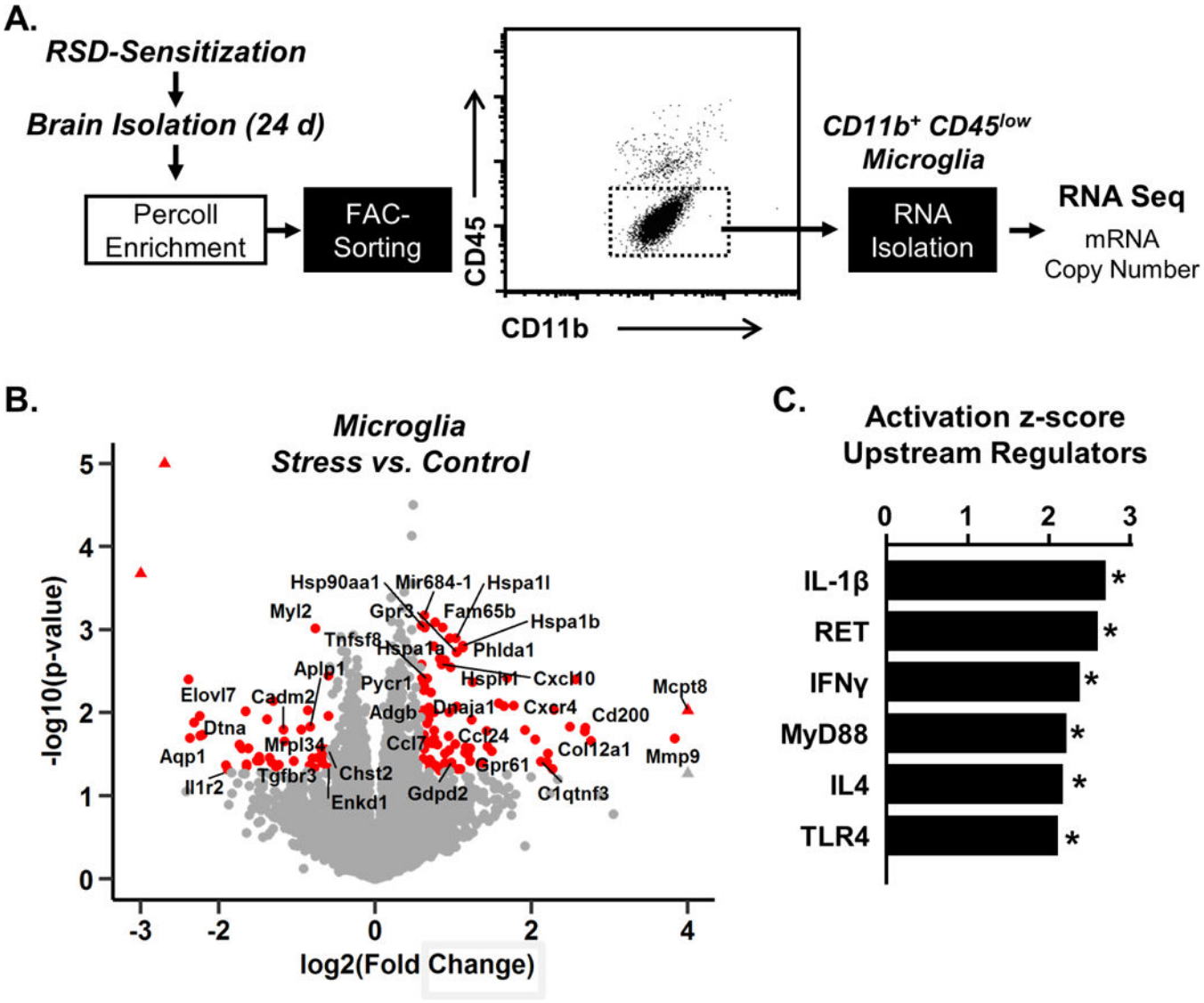


Figure 1: Evidence for Primed Microglial profile 24 days after RSD.

A) Male C57BL/6 mice were stress-sensitized (SS) by RSD or left undisturbed as controls (Naïve). At 24 d after stress, microglia were Percoll-enriched, FAC-sorted, and RNA was collected for RNASeq (n=6). B) Volcano plots of RSD vs control of differentially expressed genes. Red points indicate differentially expressed genes (p<0.05, absolute fold change>1.5). C) Ingenuity Pathway Analysis of significantly altered upstream regulators in microglia. Graphs with (*) are significantly different from controls.

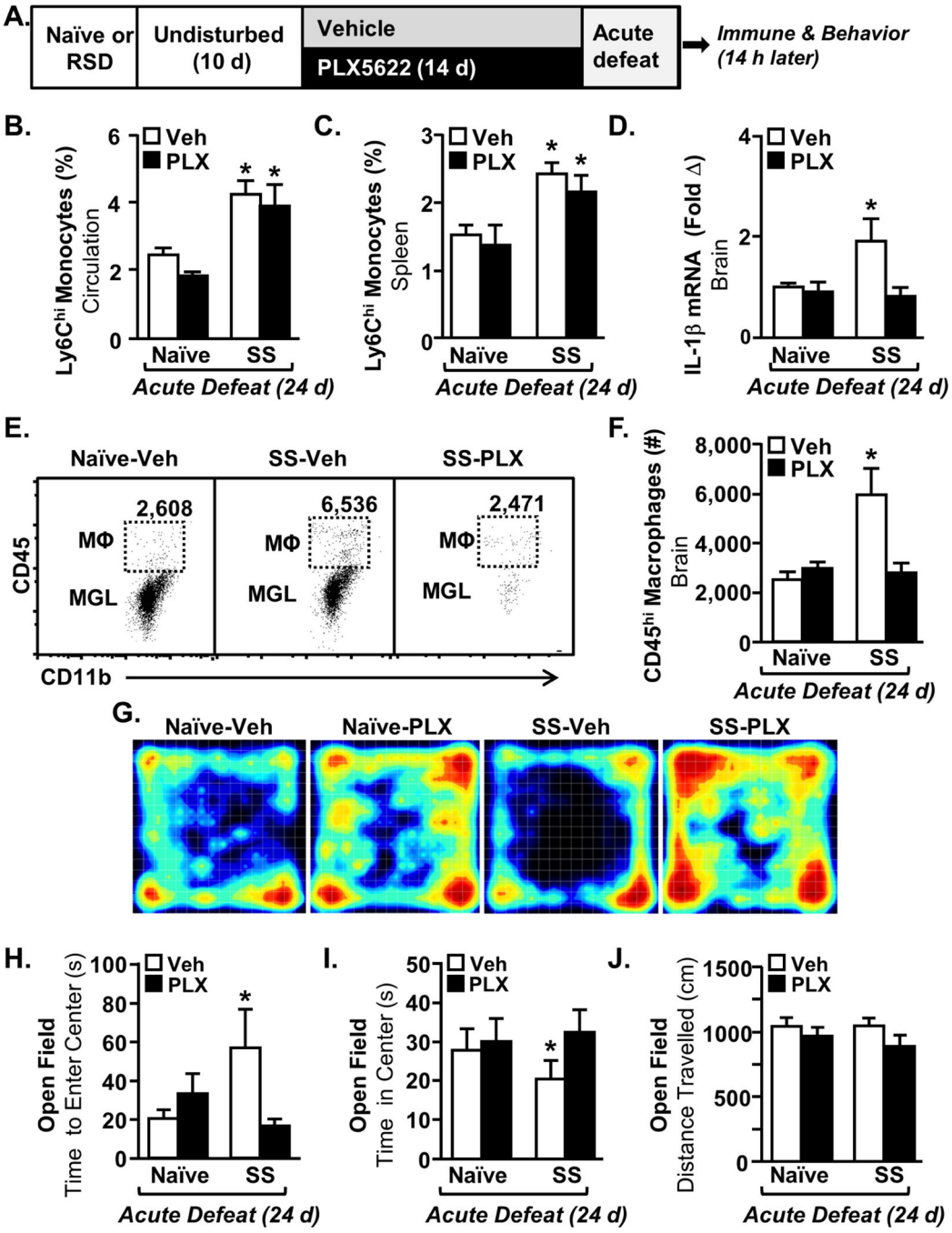


Figure 2: Monocyte Recruitment to the Brain and Recurrence of Anxiety in Stress-Sensitized Mice was Abrogated by the Elimination of Microglia.

A) Male C57BL/6 mice were stress-sensitized (SS) by RSD or left undisturbed as controls (Naïve). Ten days later, mice were provided diets formulated with vehicle (Veh) or a CSF1R antagonist (PLX5622). 24 days after stress-sensitization (SS), all mice were subjected to one cycle of social defeat (Acute Defeat) and blood, spleen, and brain samples were collected 14 h later (n=9–10). The percentage of monocytes ($CD11b^{+}/Ly6C^{hi}$) in the B) blood (main effect of SS, $F_{1,39}=21.45$, $p<0.0001$) and spleen (main effect of SS, $F_{1,39}=28.63$, $p<0.002$) were determined 14 h after acute defeat. D) IL-1β mRNA expression in a coronal brain

section was determined after acute defeat (SS x intervention, $F_{1,38}=4.35$, $p<0.04$). E) Representative bivariate dot plots of CD11b and CD45 labeling of Percoll-enriched microglia ($CD11b^{+}/CD45^{low}$) and macrophages ($CD11b^{+}/CD45^{high}$) in the brain after acute defeat. F) Number of $CD45^{+}$ macrophages in the brain (SS x intervention, $F_{1,36}=10.68$, $p<0.003$). G) Representative heat maps of activity during open-field testing. H) Time to enter center of the open field (SS x intervention, $F_{1,40}=5.20$, $p<0.02$). I) Time spent in center in the open field after acute defeat (SS x intervention, $F_{1,40}=7.03$, $p<0.01$). J) Total distance traveled in the open field (not significant). Bars represent the mean ± SEM. Means with (*) are significantly different from Naïve-Vehicle controls.

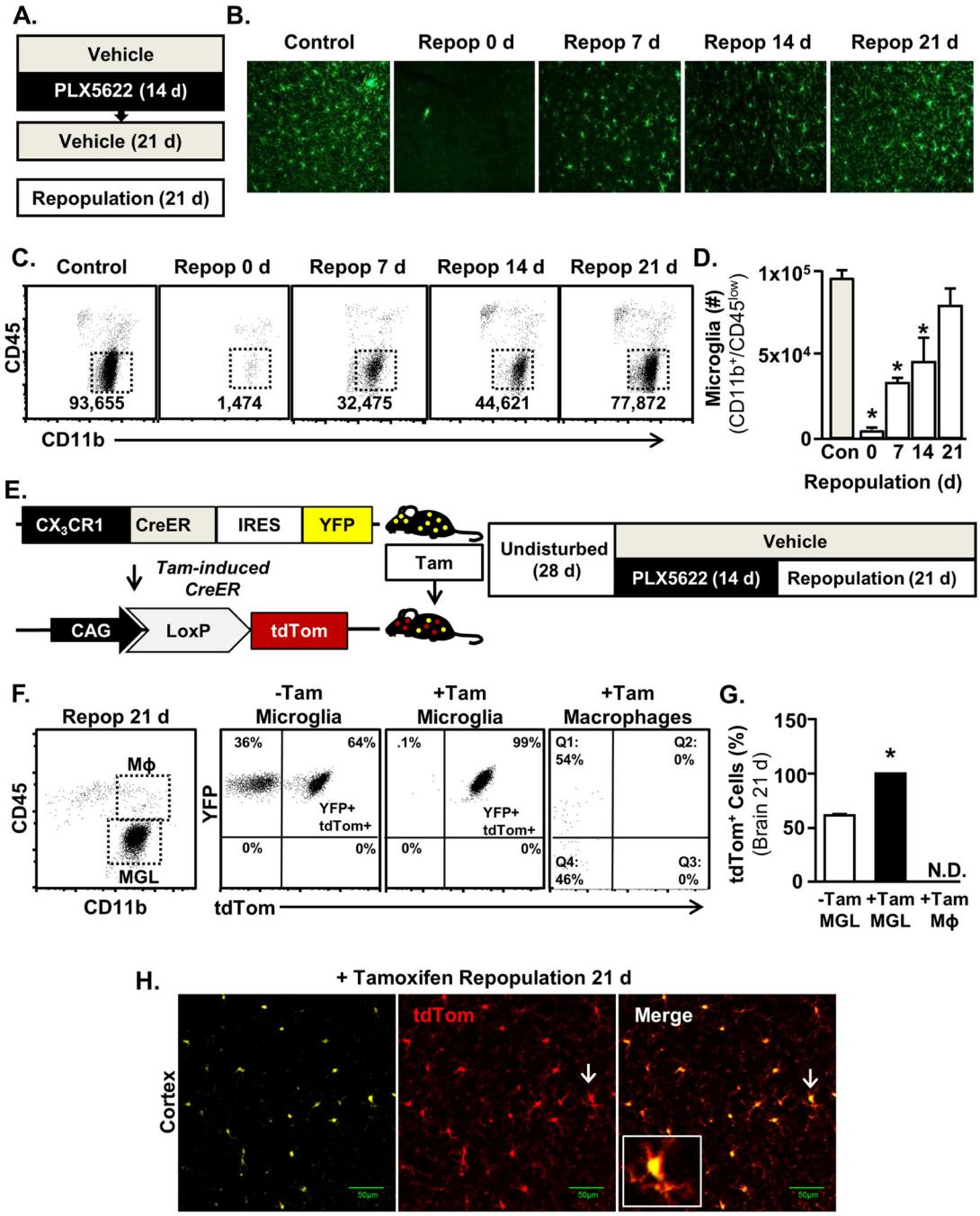


Figure 3: Microglia Repopulated from Non-Progenitor CX_3CR1^+ cells after CSF1R Antagonist-Mediated Elimination.

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 after brain 1, 7, 14, or 21 days of repopulation (main effect of time, $F_{4,16}= 8.57$, $p<0.001$).

E) Schematic representation of the experimental design using $CX_3CR1^{CreER/+}/R26^{tdTOM/+}$ mice, which were administered 4 daily injections of control or tamoxifen (20 mg/kg, i.p.) at 3 weeks of age. Mice were left undisturbed for 28 d, provided diets formulated with a CSF1R antagonist (PLX5622) for 14 d and then provided vehicle diets for an additional 21 days to allow for repopulation of microglia. F) Representative bivariate dot plots of 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 ($CD11b^+/CD45^{low}$) and macrophages ($CD11b^+/CD45^{high}$) 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 (*) are significantly different from control ($p<0.05$).

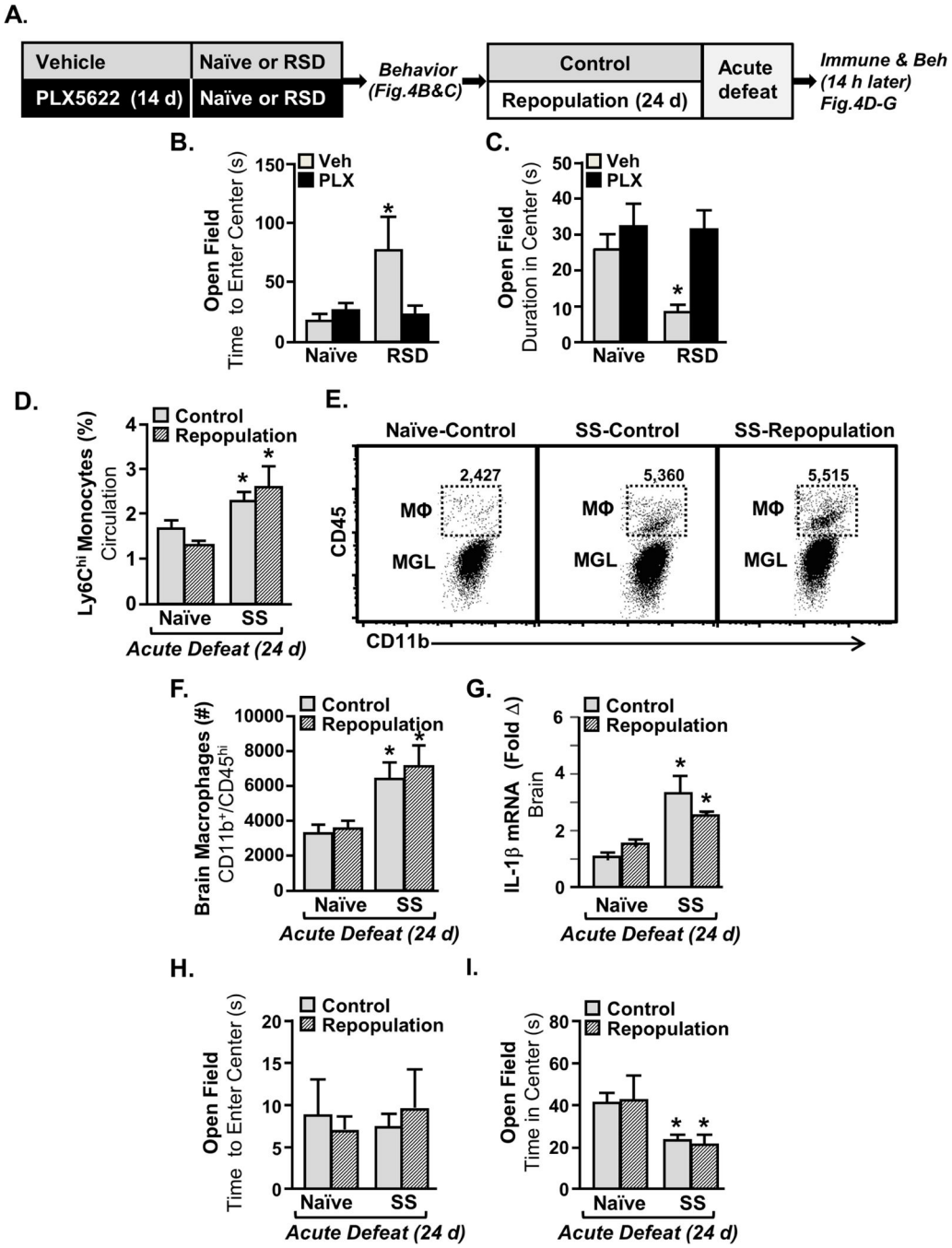


Figure 4: Microglia Repopulation in Stress-Sensitized Mice Re-established Monocyte Trafficking and Anxiety-Like Behavior Induced by Acute Defeat.

A) Male C57BL/6 mice were provided diets formulated with vehicle or CSF1R antagonist (PLX5622) for 14 days. Next, mice were stress-sensitized (SS) by RSD or left undisturbed as controls (Naïve). Anxiety-like behavior was determined 14 h after the last cycle of RSD in the open field (n=9–10) by B) time to enter the center (Intervention x SS interaction; $F_{1,38}$=10.36, p<0.003) and C) time spent in the center (Intervention x SS interaction; $F_{1,38}$=3.675, p=0.06). After RSD, all mice were provided vehicle diets for an additional 24 d 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 h after acute defeat (main effect of SS, $F_{1,37}=16.46$, $p<0.0002$). E) Representative bivariate dot plots of CD11b and CD45 labeling on enriched microglia and macrophages. F) Number of brain macrophages ($CD11b^{+}/CD45^{high}$) 14 h after acute defeat (main effect of SS, $F_{1,37}=16.46$, $p<0.0002$). G) mRNA levels of IL-1β were determined in a coronal brain section (n=4) collected 14 h after acute defeat (main effect of SS; $F_{1,14}=20.1$, $p<0.001$). Anxiety-like behavior (n=10) was determined by H) Time to enter (not significant) and I) time spent in the center of the open field 0.5 d after acute defeat (main effect of SS; $F_{1,39}=14.85$, $p<0.001$). Bars represent the mean ± SEM. Means with (*) are significantly different from Control-Naïve ($p<0.05$).

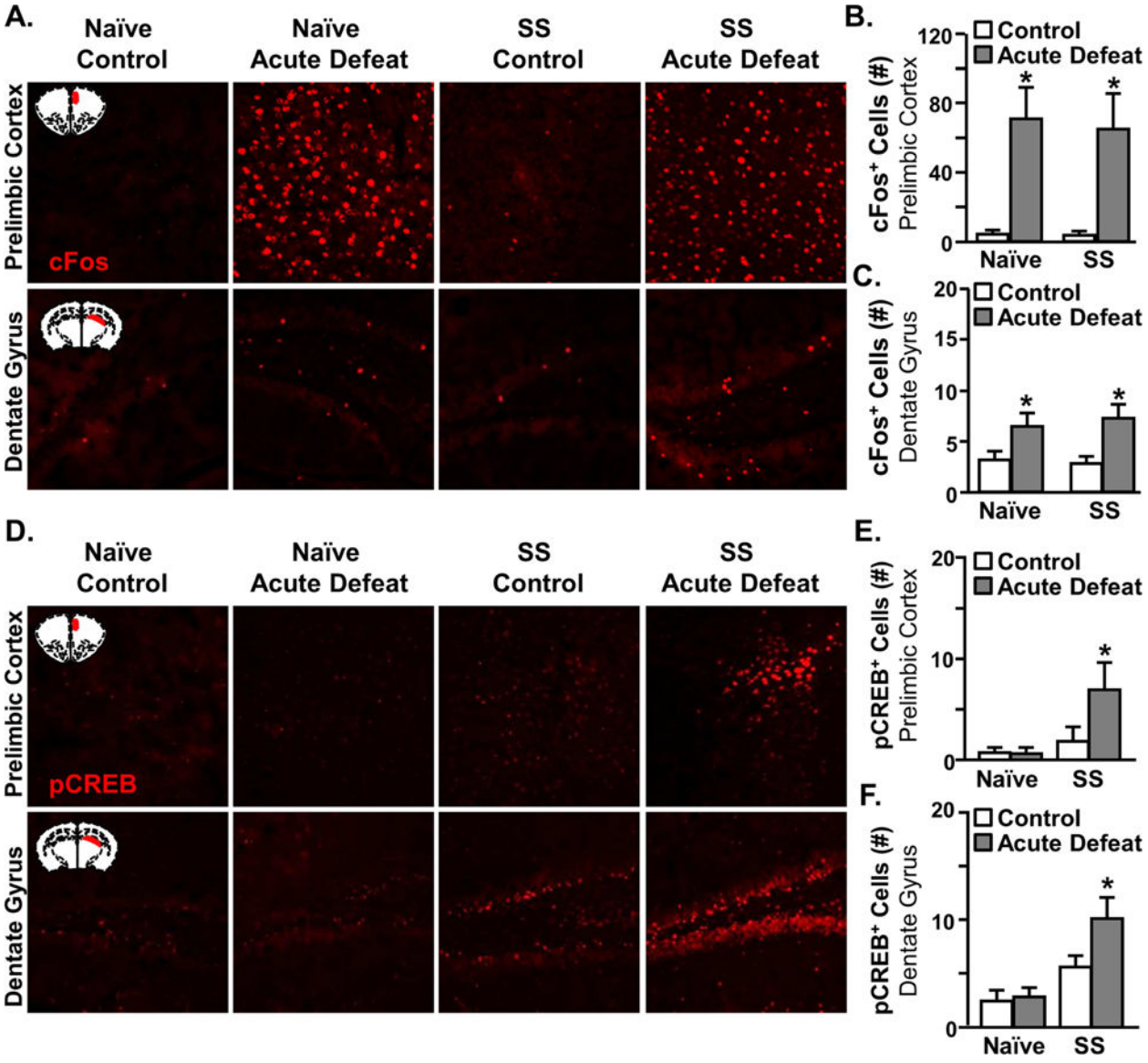


Figure 5: Evidence of Neuronal Sensitization with RSD.

Male C57BL/6 mice were stress-sensitized (SS) by RSD or left undisturbed as controls (Naïve). At 24 d 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 pCREB (n=6). A) Representative images of c-Fos expression in the prelimbic cortex (top panel) and dentate gyrus (bottom panel). The number of c-Fos$^+$ cells in the B) prelimbic cortex ($F_{1,23}$=27.6,p<0.001) and C) dentate gyrus of the hippocampus in control and SS mice 14 h after acute defeat ($F_{1,23}$=75.8,p<0.001). D) Representative images of pCREB expression in the prelimbic cortex (top panel) and dentate gyrus (bottom panel). Number of pCREB$^+$ cells in the E) prelimbic cortex (SS, $F_{1,24}$=4.7 p<0.04), SS x acute stress interaction ($F_{1,24}$=2.9, p=0.1) and F) dentate gyrus of control and SS mice 0.5 d after acute defeat (SS, $F_{1,24}$=17.7, p<0.001). Bars represent the mean ± SEM. Means with (*) are significantly different from Control-Naïve (p<0.05).

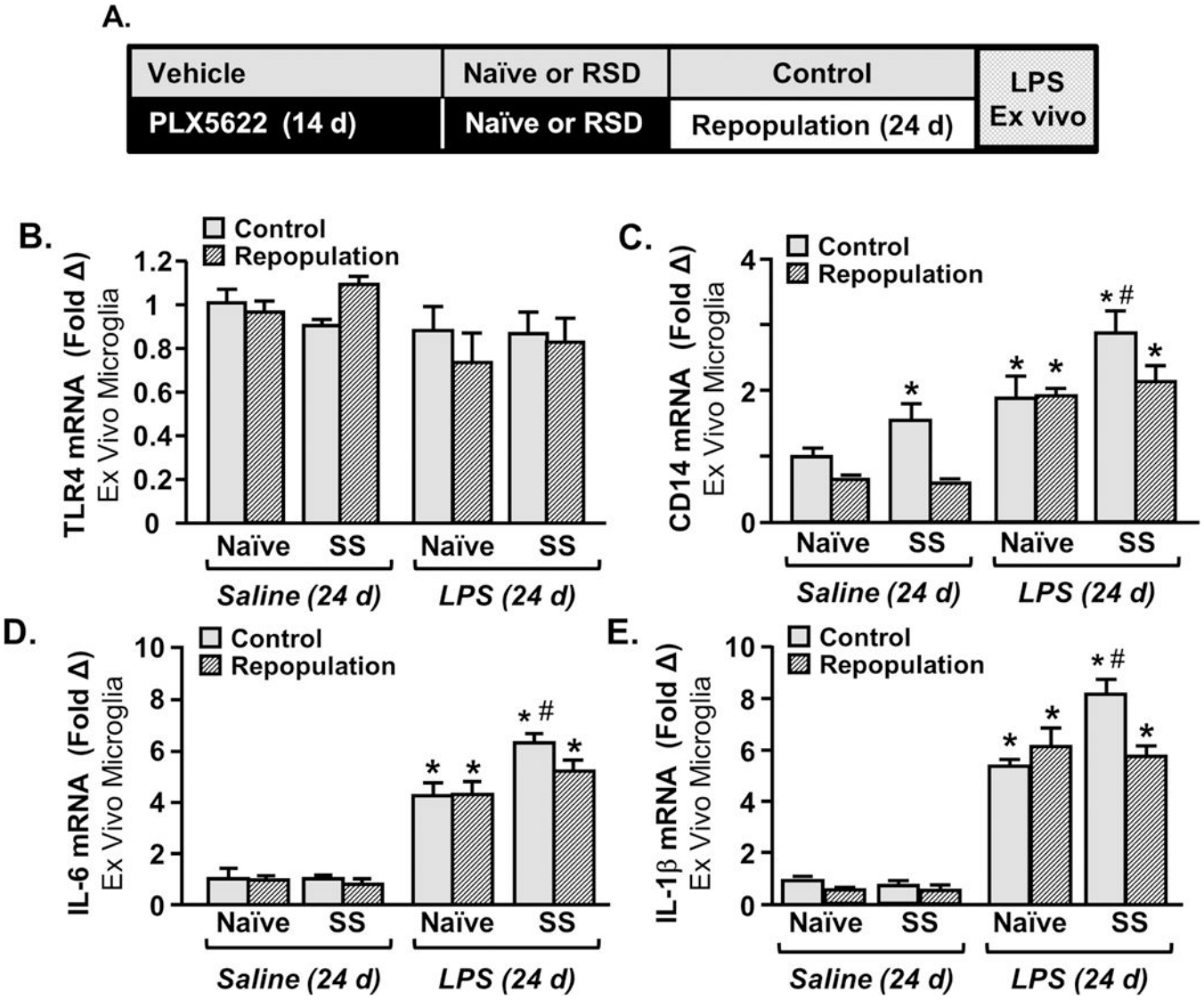
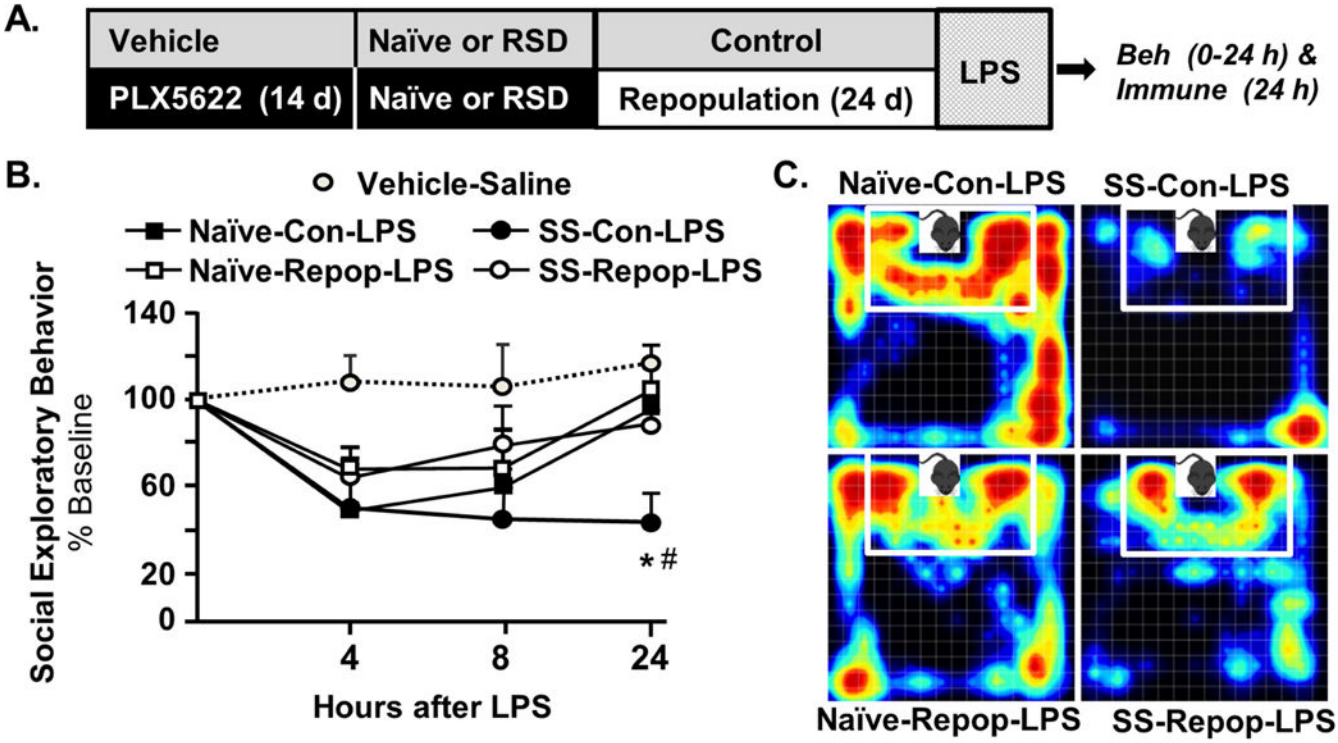


Figure 6: Microglial Hyperactivity to *ex vivo* LPS Stimulation in Stress-Sensitized Mice was Attenuated by Microglial Elimination and Repopulation.

A) Male C57BL/6 mice were provided diets formulated with vehicle or CSF1R antagonist (PLX5622) for 14 days. Next, mice were stress-sensitized (SS) by RSD or left undisturbed as controls (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). mRNA levels of B) TLR4, CD14, D) IL-6, and E) IL-1β were determined in *ex vivo* microglia 4 h after LPS stimulation. Means with (*) are significantly different from Control-Naïve ($p<0.05$) and means with ($^{\#}$) are significantly different from SS-Naïve ($p<0.05$).



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* LPS Challenge in Stress-Sensitized Mice was Attenuated by Microglial Elimination and Repopulation.

A) Male C57BL/6 mice were provided diets formulated with vehicle or CSF1R antagonist (PLX5622) for 14 days. Next, mice were stress-sensitized (SS) by RSD or left undisturbed as controls (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; i.p.) and B) social exploratory behavior (percent of baseline) was determined at baseline and 4, 8, and 24 h after LPS challenge (main effect of SS: $F_{1, 83}$=18.59, p<0.03, SS x Time $F_{3,83}$=3.42, p=.066). C) Representative heat maps of social exploratory behavior 24 h after LPS.D) mRNA levels of IL-1β (SS x Repop interaction; $F_{1,18}$=14.6, p<0.001), IL-6 (SS x Repop interaction; $F_{1,18}$=14.2, p<0.001, CD14 (SS x Repop interaction; $F_{1,18}$=10.1, p<0.005), and TLR4 SS x Repop interaction; $F_{1,18}$=5.715, p<0.03) were determined in Percoll-enriched microglia collected 24 h after LPS challenge. Bars represent the mean ± SEM. Means with (*) are significantly different

from Control-Naive ($p<0.05$) and means with ($^{\#}$) are significantly different from saline control ($p<0.05$).