Archival Report

Role of Monocyte-Derived MicroRNA106b~25 in Resilience to Social Stress

Madeline L. Pfau, Caroline Menard, Flurin Cathomas, Fiona Desland, Veronika Kana, Kenny L. Chan, Yusuke Shimo, Katherine LeClair, Meghan E. Flanigan, Hossein Aleyasin, Deena M. Walker, Sylvain Bouchard, Matthias Mack, Georgia E. Hodes, Miriam M. Merad, and Scott J. Russo

ABSTRACT

BACKGROUND: Clinical studies suggest that heightened peripheral inflammation contributes to the pathogenesis of stress-related disorders, including major depressive disorder. However, the molecular mechanisms within peripheral immune cells that mediate enhanced stress vulnerability are not well known. Because microRNAs (miRs) are important regulators of immune response, we sought to examine their role in mediating inflammatory and behavioral responses to repeated social defeat stress (RSDS), a mouse model of stress vulnerability that produces susceptible and resilient phenotypes.

METHODS: We isolated Ly6c^{high} monocytes via fluorescence-activated cell sorting in the blood of susceptible and resilient mice following RSDS and profiled miR expression via quantitative real-time polymerase chain reaction. Bone marrow chimeric mice were generated to confirm a causal role of the miR-106b~25 cluster in bone marrow–derived leukocytes in mediating stress resilience versus susceptibility.

RESULTS: We found that RSDS produces an increase in circulating Ly6c^{high} inflammatory monocytes in both susceptible and resilient mice. We next investigated whether intrinsic leukocyte posttranscriptional mechanisms contribute to individual differences in stress response and the resilient phenotype. Of the miRs profiled in our panel, eight were significantly regulated by RSDS within Ly6c^{high} monocytes, including miR-25-3p, a member of the miR-106b~25 cluster. Selective knockout of the miR-106b~25 cluster in peripheral leukocytes promoted behavioral resilience to RSDS.

CONCLUSIONS: Our results identify the miR-106b~25 cluster as a key regulator of stress-induced inflammation and depression that may represent a novel therapeutic target for drug development.

Keywords: Immune system, Leukocyte, Major depressive disorder, microRNA, Resilience, Stress

https://doi.org/10.1016/j.biopsych.2019.02.023

While one in five Americans will suffer from major depressive disorder (MDD) in their lifetime (1), approved treatments are ineffective in an estimated 30% to 50% of patients (2). This high rate of treatment resistance suggests prominent pathophysiological mechanisms that are unresolved by current antidepressant medications (2). Increasing evidence suggests that one such mechanism is heightened peripheral and central inflammation (2,3). Strikingly, MDD is often comorbid with chronic inflammatory illness, and patients with preexisting diseases such as multiple sclerosis (4,5), type 2 diabetes (6,7), and cardiovascular diseases (8) are at increased risk for developing MDD and can be several times more likely to develop depression than healthy individuals (3). Even in the absence of chronic physical disease, subsets of patients with MDD display a dysregulation of inflammatory status (2). Some of the most consistently reported alterations of the immune system in patients with MDD include leukocytosis, or an increase in circulating white blood cells that is specific to inflammatory monocytes and neutrophils (9), and

elevations in serum proinflammatory cytokines, including tumor necrosis factor alpha (10), interleukin (IL)-1β (11), IL-6 (10,12), and interferon gamma (11).

Recent studies have replicated these symptoms in preclinical models and yielded important insights into the role of inflammation in stress-related disorders. Mice exposed to repeated social defeat, a chronic psychosocial stress paradigm that produces anxiety-like behavior, display a β-adrenergic signaling–dependent increase in Ly6c^{high} inflammatory monocytes and neutrophils in blood, bone marrow, and spleen (13). Ly6c^{high} monocytes are immature classic inflammatory monocytes that are recruited to sites of inflammation, where they can further differentiate into macrophages or complement the activities of macrophages and dendritic cells (14). A similar pattern of stress-induced myelopoiesis has been reported in mice exposed to chronic variable stress (15). We recently demonstrated that psychosocial stress in the form of repeated social defeat stress (RSDS) elevates peripheral levels of the cytokine IL-6 (16). In

contrast to the repeated social defeat paradigm, RSDS allows for the separation of resilient mice that behave similarly to unstressed control mice from susceptible mice that display depression-like behaviors following RSDS (17). Our previous studies indicate that susceptible mice exhibit elevated serum IL-6 levels compared with both control and resilient mice 48 hours after RSDS (16,18,19). Interestingly, prior to behavioral exposure to RSDS, isolated leukocytes from mice that will go on to become susceptible release more IL-6 in response to in vitro stimulation with lipopolysaccharide (LPS), a bacterial endotoxin and toll-like receptor 4 agonist, than do those from mice that will go on to become resilient (16). This finding suggests that intrinsic mechanisms at the level of the leukocyte likely contribute to increased inflammatory potential in susceptible mice and possible protection from inflammation in resilient mice.

In the current study, we aimed to characterize the effect of RSDS on the circulating leukocyte pool and to identify intrinsic leukocyte posttranscriptional mechanisms contributing to individual differences in stress-induced inflammation and resilience. We focused specifically on epigenetic regulation by microRNAs (miRs) as a potential mediator of the individual differences we observe in C57BL/6J mice—an inbred, highly genetically similar mouse strain. miRs are important regulators of immune processes, including immune cell differentiation and proliferation, and transcription factor, chemokine, and cytokine signaling (20). Recent studies in humans have reported dysregulated miR expression in whole blood (21,22) and peripheral blood mononuclear cells (23,24) of patients with depression compared with healthy control subjects. Human findings also indicate regulation of several blood miRs by antidepressant medication (23,25,26) and cognitive behavioral therapy (22) in treatment-responsive patients.

METHODS AND MATERIALS

Animals

Male CD45.2+ C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) were shipped to the Icahn School of Medicine at Mount Sinai (ISMMS) animal facility at 6 to 9 weeks of age and allowed at least 1 week of acclimation to the facility prior to the start of experiments. Male CD45.1+ C57BL/6J bone marrow transplant hosts (The Jackson Laboratory) were shipped to the ISMMS facility at 3 weeks of age and allowed 1 week of acclimation prior to irradiation. Homozygous male Mirc3tm1.1Tyj/J mice were bred on a CD45.2+ C57BL/6J background at ISMMS. Male CD-1 mice (Charles River Laboratories, Raleigh, NC) used as aggressors were retired breeders more than 4 months of age. C57BL/6J mice were group housed prior to social defeat and singly housed after social defeat until sacrifice. CD-1 mice were singly housed before and after social defeat. Animals were maintained on a 12-hour light/dark cycle with ad libitum access to food and water. Mouse procedures were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and the ISMMS Institutional Animal Care and Use Committee.

Repeated Social Defeat Stress

RSDS was performed as described previously (16,17). Briefly, CD-1 mice were selected as RSDS aggressors following repeated screening for intruder-elicited aggressive responses in the home cage. A full day (24 hours) prior to the start of RSDS, CD-1 aggressor mice were singly housed in static hamster cages with woodchip bedding on one side of a clear perforated Plexiglas partition. Each day for 10 consecutive days, experimental mice were exposed to a novel CD-1 aggressor mouse in its home cage for a 5- to 10-minute bout of physical aggression. After the defeat bout, experimental mice were moved to the opposite side of the perforated partition for 24 hours, allowing for physical separation but continuous sensory contact. Control mice were housed in mouse static cages with woodchip bedding, 2 mice to a cage, on opposite sides of a perforated Plexiglas divider. Control mice were rotated daily to simulate the rotation of the defeat group but were never exposed to CD-1 aggressors. Next, 24 hours after the final defeat bout, experimental mice were singly housed in static mouse cages with woodchip bedding. Animals were visually inspected for wounding during the course of RSDS. Mice with excessive wounding and moribund mice were immediately sacrificed. To quantify sickness behavior, food or water consumption was assessed by measuring the difference between the weight of the food pellets or water bottles immediately after the defeat and 16 hours later.
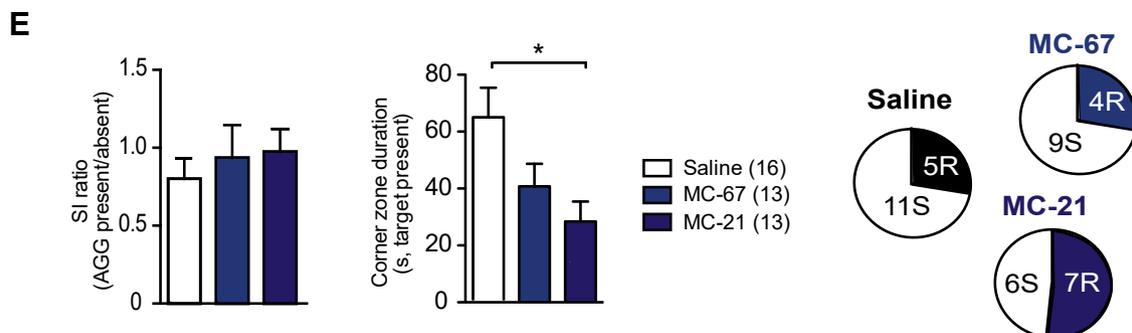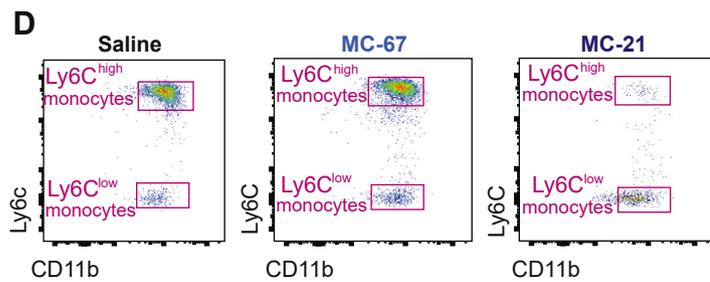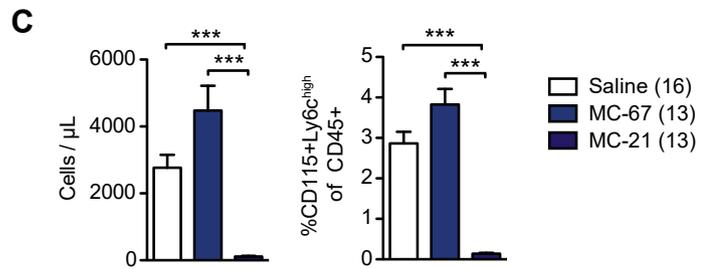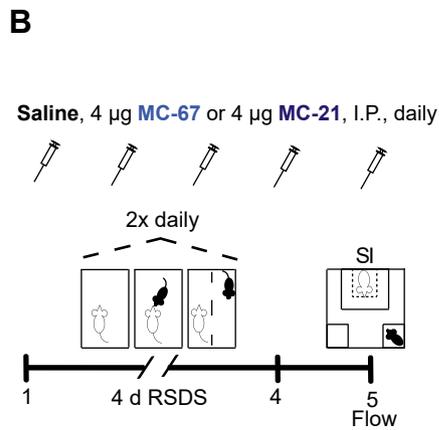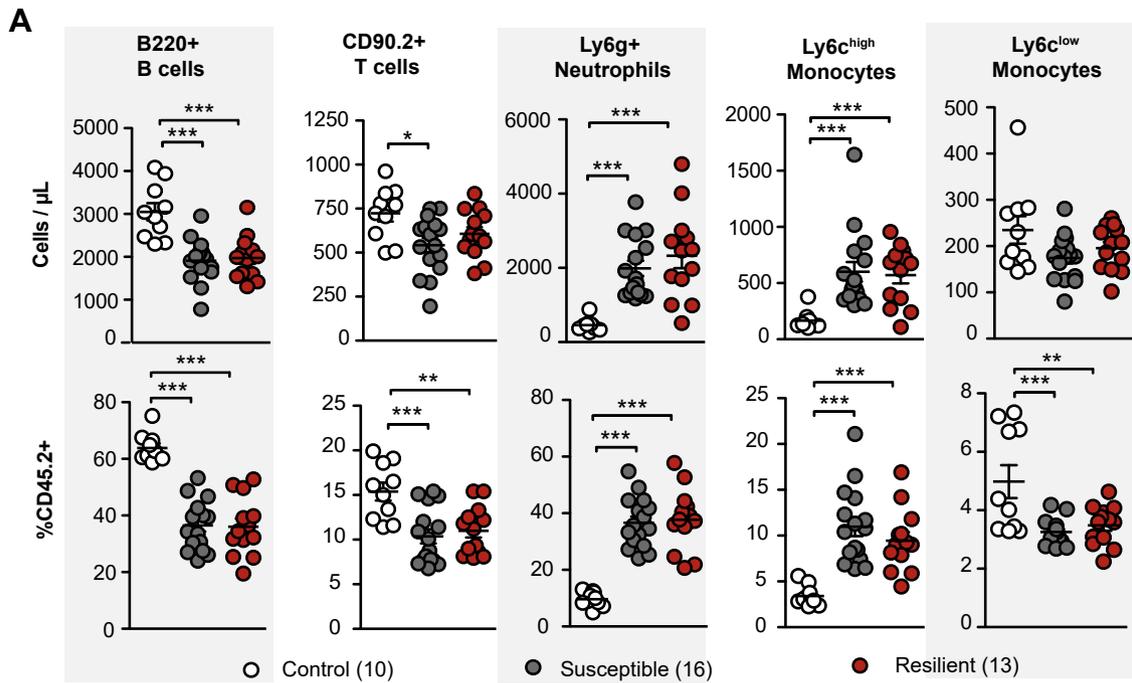
Social Avoidance Testing (Social Interaction Test)

Social interaction (SI) testing was performed as described previously (16,27). Under red light conditions, experimental mice were placed in a novel Plexiglas open-field arena with a small wire enclosure placed at one end. The SI test comprised two phases. In the first phase, mouse baseline exploratory behavior was tracked for 2.5 minutes in the absence of a novel CD-1 mouse using EthoVision 3.0 software (Noldus Information Technology, Leesburg, VA). In the second phase, a novel CD-1 mouse was placed in the wire enclosure, and exploratory behavior of the experimental mouse was recorded for 2.5 minutes. See Supplement for calculation of SI ratio.

Accelerated Defeat

To measure susceptibility and resilience to RSDS within the 5-day treatment constraints of the MC-21 antibody, we performed an adapted, accelerated RSDS protocol as described previously (28). Experimental mice received two 5-minute defeats per day for 4 days, totaling eight defeats. Defeats were administered in the morning and evening. Accelerated defeat is not a subthreshold stressor given that this protocol is sufficient to induce social avoidance behavior under control conditions (29). Data shown for accelerated defeat are combined from two separate cohorts. Animals were visually inspected for wounding during the course of accelerated RSDS. Mice with excessive wounding and moribund mice were immediately sacrificed.

MC-21/MC-67 Antibody Treatment

Male CD45.2+ C57BL/6J mice were given daily injections (intraperitoneal) of saline, rat anti-mouse (C-C motif chemokine receptor [CCR2] monoclonal antibody MC-21) (Lot No. AK1480/01), or IgG2b kappa isotype control MC-67 (Lot No.

A

B220+ B cells · CD90.2+ T cells · Ly6g+ Neutrophils · Ly6c^high Monocytes · Ly6c^low Monocytes (Cells / µL and %CD45.2+)

○ Control (10) ● Susceptible (16) ● Resilient (13)

B

Saline, 4 µg MC-67 or 4 µg MC-21, I.P., daily

2x daily

4 d RSDS

SI

Flow

C

Saline (16) MC-67 (13) MC-21 (13)

D

Saline MC-67 MC-21

Ly6C^high monocytes / Ly6C^low monocytes (Ly6c vs CD11b)

E

SI ratio (AGG present/absent) Corner zone duration (s, target present)

Saline (16) MC-67 (13) MC-21 (13)

Saline: 11S, 5R
MC-67: 9S, 4R
MC-21: 6S, 7R

AK1481/02). Antibodies were administered at a dose of 4 μg per mouse per day in 100 μL of sterile saline. Antibodies were administered at least 5 minutes prior to defeat or SI testing. Animals showing poor depletion in subsequent flow cytometry analysis were excluded.

Generation of Bone Marrow Chimeras

As described in Hodes *et al.* (16), 4-week-old CD45.1+ C57BL/6J host mice were irradiated with 1200 rad delivered in two doses of 600 rad, 10 to 11 hours apart, to ablate the peripheral immune system. During irradiation, animals were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and placed in lead tubes (Nuclead, West Bridgewater, MA) such that only the head was shielded. Donor bone marrow progenitor cells were lavaged from femurs of 4 male Mirc3$^{tm1.1Tyj}$/J$^{-/-}$ mice and 4 male Mirc3$^{tm1.1Tyj}$/J$^{+/+}$ mice with fluorescence-activated cell sorting (FACS) buffer (2% heat-inactivated fetal bovine serum, and 5 mM ethylenediamine tetraacetate in 1× Dulbecco's phosphate-buffered saline; ThermoFisher, Waltham, MA). Progenitor cells were next incubated with 1× red blood cell lysis solution (BioLegend, San Diego, CA), washed in FACS buffer, and counted with a hemocytometer. Shortly following the second bout of host irradiation, approximately 2 × 10^6 donor bone marrow progenitor cells were transplanted through retro-orbital injection. Host mice received 3 weeks of antibiotic (Sulfatrim) treatment, followed by 5 weeks of rest, for a total of 8 weeks of recovery and donor repopulation. At sacrifice, the level of chimerism was assayed via flow cytometry by comparing populations of CD45.1+ leukocytes (host) and CD45.2+ leukocytes (donor). One statistical outlier mouse was excluded owing to low chimerism.

Blood Sample Collection, Processing, and Antibody Staining

Trunk blood was collected following rapid decapitation. For FACS, 500 μL to 1 mL of blood was collected from each mouse into 3 mL of FACS buffer. For flow cytometry, 100 μL of blood was collected into 2 to 3 mL of FACS buffer. Cells were depleted of red blood cells with 1× red blood cell lysis solution (BioLegend), filtered and washed in FACS buffer, and surface stained in 50 μL of FACS buffer for 20 to 30 minutes on ice. See the Supplement for antibodies used for FACS.

Flow Cytometry and FACS

Multiparameter flow cytometry analysis was performed on an LSRII Fortessa cytometer (BD Biosciences, San Jose, CA). Flow data were analyzed with FlowJo software (Tree Star, Ashland, OR). FACS was performed on a FacsAria II sorter (BD

Biosciences), followed by analysis with FlowJo software. DAPI+ dead cells and doublets were excluded from analysis and collection. Cells were sorted directly into 500 μL of Trizol LS reagent (Ambion, Waltham, MA), lysed, and flash frozen on dry ice.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated using the DirectZol RNA MiniPrep and RNA Clean & Concentrator-5 kit (Zymo Research, Irvine, CA) according to the manufacturer's instructions. miR expression was assayed using the miRCURY LNA Universal real-time miR polymerase chain reaction (PCR) protocol with custom Pick-&-Mix panels (Exiqon, Woburn, MA) according to the manufacturer's instructions, with modifications for low input. Here, 1.35 ng of total RNA was reverse transcribed to complementary DNA for each sample (Universal cDNA Synthesis Kit II, Exiqon). The complementary DNA product was diluted 50× in nuclease-free water, combined 1:1 with PCR Master Mix (ExiLENT SYBR Green master mix, Exiqon), and dispensed into custom 384-well plates. Samples were run in duplicate, and two no-template controls were included. Real-time PCR amplification followed by melting curve analysis was performed on an Applied Biosystems 7900HT machine (Thermo-Fisher). Samples were heated to 95°C for 10 minutes, followed by 40 amplification cycles of 95°C for 10 seconds and 60°C for 1 minute at a ramp rate of 1.6°C/s. See the Supplement for detailed description of PCR analysis.

Statistical Analysis

Comparisons of three or more means were analyzed by one-way analysis of variance (ANOVA) with Tukey's post hoc tests. Comparisons of multiple factors were analyzed by two-way ANOVA with Bonferroni post hoc tests. Data were log transformed prior to ANOVA when variances were unequal. Statistical analysis was performed using Prism 5.0 software (GraphPad, La Jolla, CA), and statistical significance was set at $p < .05$. Outliers for behavioral testing were identified as being more than 2 SD from the mean and were excluded from statistical analysis.

RESULTS

Chronic Social Stress Promotes a Robust Induction of Ly6c^{high} Inflammatory Monocytes and Neutrophils in Peripheral Blood

To assess the effect of RSDS on the circulating leukocyte pool, we exposed mice to 10 days of RSDS and performed flow cytometry on blood collected 48 hours after the last defeat

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Figure 1. Role of Ly6c^{high} monocytes in behavioral response to repeated social defeat stress (RSDS). **(A)** Absolute counts (top) and relative frequencies (bottom) of B cells, T cells, neutrophils, Ly6c^{high} monocytes, and Ly6c^{low} monocytes in peripheral blood 48 hours post-RSDS. **(B)** Experimental timeline of 4-day accelerated RSDS, daily injection of saline, MC-21 anti-CCR2 antibody or MC-67 isotype control, social interaction (SI) testing, and flow cytometry analysis (flow). **(C)** Depletion of Ly6c^{high} monocytes in peripheral blood by daily injection of MC-21, shown as a decrease in absolute count (left) and frequency (right). **(D)** Representative flow scatter plots of blood from mice treated with saline (left), MC-67 (middle), and MC-21 (right). **(E)** MC-21-treated mice showed a nonsignificant increase in SI ratio compared with the saline-treated group (left). MC-21-treated mice spent less time in the corner zones than saline-treated mice when the target was present (middle). Whereas the saline and MC-67 groups showed an approximately 1:2 ratio of resilience (R) to susceptibility (S), MC-21-treated mice showed a roughly 1:1 ratio (right). For bar graphs, data represent mean + SEM. For dot plots, data represent mean ± SEM. The number of animals within each group is indicated below the graphs. *$p < .05$, **$p < .01$, ***$p < .0001$ [one-way analysis of variance with Tukey's post hoc tests **(A, C, E)**]. AGG, aggressor; I.P., intraperitoneal.
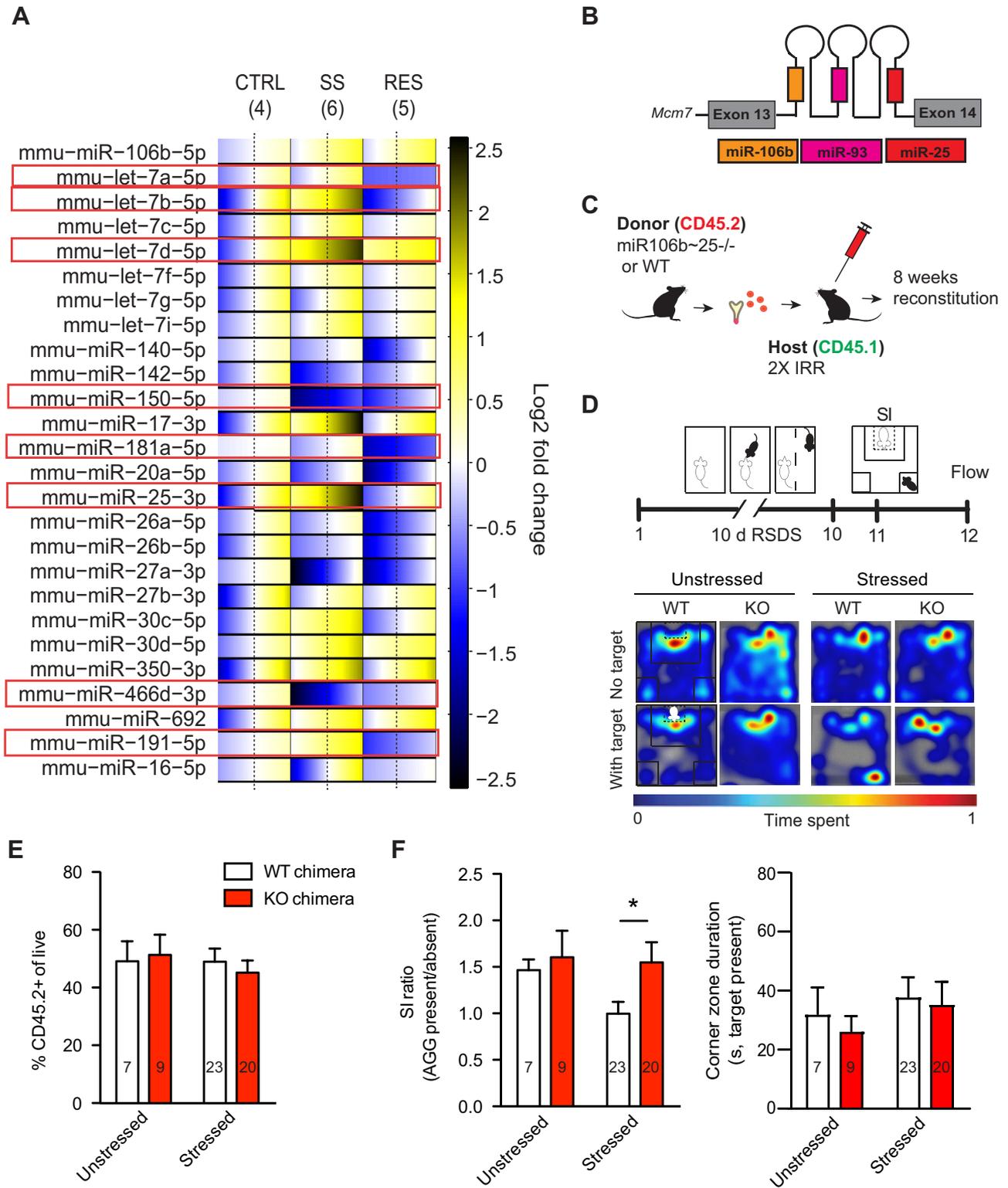
Figure 2. Peripheral knockdown of microRNA (miR)-106b~25 cluster promotes behavioral resilience to repeated social defeat stress (RSDS). **(A)** miR profiling in Ly6c^{high} monocytes 48 hours after RSDS. The heatmap illustrates normalized expression pattern of detectable miRs in resilient mice (RES) (right) and susceptible mice (SS) (middle) relative to unstressed control mice (CTRL) (left). The range of colors indicates individual differences within each group (SEM) from the average, which is represented by a dashed line. Blue indicates lower expression, yellow indicates increased expression, and white indicates no

bout (Supplemental Figure S1A, B). Lymphocytes, monocytes, and neutrophils have been implicated in response to (30,31) or regulated by (13,15) other stress paradigms. Flow cytometry analysis revealed stress-induced changes in the absolute counts and frequencies of several leukocyte subtype populations 48 hours after defeat (Figure 1A). In both susceptible and resilient mice compared with controls, RSDS exposure induced an increase in absolute count and frequency of circulating neutrophils ($F_{2,36} = 37.55$, $p < .0001$ [count]; $F_{2,36} = 86.90$, $p < .0001$ [frequency]) and Ly6c^{high} monocytes ($F_{2,36} = 21.04$, $p < .0001$ [count]; $F_{2,36} = 37.91$, $p < .0001$ [frequency]). RSDS exposure induced a robust decrease in absolute count ($F_{2,36} = 15.97$, $p < .0001$) and frequency ($F_{2,36} = 38.69$, $p < .0001$) of circulating B cells in susceptible and resilient groups compared with control mice. RSDS led to a decrease in absolute count of T cells ($F_{2,36} = 4.51$, $p < .05$) only in susceptible mice. While we observed no group differences in absolute count of Ly6c^{low} monocytes, total frequency was decreased ($F_{2,36} = 9.026$, $p < .001$) in both susceptible and resilient mice compared with control mice. These differences in leukocyte cell populations were not related to wounding (Supplemental Figure S1C) or to changes in the total number of circulating leukocytes (Supplemental Figure S1D). To characterize longer term changes in the leukocyte pool, we next performed flow cytometry 9 days after defeat in a second cohort of mice. We observed sustained changes in leukocytes following RSDS only in susceptible mice at this time point. For example, Ly6c^{high} monocytes remained increased in both absolute count and frequency ($F_{2,36} = 3.45$, $p < .05$ [count]; $F_{2,36} = 4.80$, $p < .05$ [frequency]), neutrophil frequencies were increased ($F_{2,36} = 3.33$, $p < .05$), and B-cell frequencies were decreased ($F_{2,36} = 5.42$, $p < .01$) (Supplemental Figure S2).

Depletion of Ly6c^{high} Monocytes Is Mildly Antidepressant

Because we observed an RSDS-induced increase in Ly6c^{high} monocytes in susceptible and resilient mice, and owing to prior publications attributing the leukocyte transcriptional response to stress to the monocyte compartment (13), we next sought to determine the functional role of Ly6c^{high} monocytes in behavioral responses to RSDS. We administered daily systemic injections of the rat anti-mouse CCR2 antibody MC-21, its IgG2b isotype control MC-67, or saline and performed an accelerated RSDS protocol (Figure 1B). CCR2, a receptor for the chemokine CCL2 necessary for Ly6c^{high} monocyte recruitment from bone marrow, is expressed by Ly6c^{high} monocytes but not by Ly6c^{low} monocytes (32). Daily injection of MC-21 has been shown to deplete Ly6c^{high} monocytes in blood for 5 days, after which the antibody becomes ineffective owing to humoral immune response and the generation of mouse anti-rat antibodies (32), necessitating our use of the accelerated defeat protocol (Figure 1B). We observed a robust

depletion of Ly6c^{high} monocytes in blood in both relative frequency ($F_{2,39} = 192.0$, $p < .0001$) and absolute count ($F_{2,39} = 129.4$, $p < .0001$) in mice treated with MC-21 antibody injections (Figure 1C, D). Compared with saline-treated mice, MC-21-treated mice showed a higher SI ratio; however, this trend did not reach statistical significance (Figure 1E). When the aggressor was present, MC-21-treated mice spent significantly less time in the corners than saline-treated mice ($F_{2,39} = 4.345$, $p < .05$) (Figure 1E). Overall, more mice were classified as resilient in the MC-21-treated group, as indicated by the pie charts in Figure 1E, suggesting that Ly6c^{high} depletion has a mild antidepressant effect. There were no differences between stressed mice receiving MC-21 and those receiving MC-67 in measures of sickness behavior (Supplemental Figure S3). Lastly, in unstressed mice, there was no difference between mice receiving MC-21 and those receiving MC-67 in SI behavior (Supplemental Figure S4).

RSDS Regulates miR Expression in Ly6c^{high} Monocytes

Given that RSDS similarly increased Ly6c^{high} monocytes in susceptible and resilient mice and that Ly6c^{high} monocyte depletion had only mild effects on social avoidance, we next sought to profile molecular signatures within Ly6c^{high} monocytes that may contribute to individual differences in behavioral and inflammatory responses to RSDS. We collected Ly6c^{high} monocytes via FACS 48 hours post-RSDS and isolated total RNA (Supplemental Figures S5 and 6A, B). We profiled 44 miRs using a custom-designed quantitative PCR panel (Supplemental Table S1). miRs were chosen based on fulfillment of at least three of the following criteria: 1) predicted to target cytokines (IL-6, IL-10, or IL-1β) dysregulated in social defeat (16), 2) highly expressed in mouse Ly6c^{high} monocytes or human peripheral blood mononuclear cells (33,34), 3) regulated in human depression or antidepressant response (24–26,35), 4) regulated by LPS (36–38), and 5) dysregulated in chronic inflammatory disorders. Of the 44 miRs assayed, 30 were detectable in our samples (see Figure 2A for an expression heat map of assays not used for reference normalization). We identified eight miRs that were significantly regulated by RSDS by univariate ANOVA, with significant or strongly trending post hoc differences between groups (Figure 2A [highlighted in red boxes] and Supplemental Table S2).

Downregulation of the miR-106b∼25 Cluster Promotes Behavioral Resilience to RSDS

Of the eight miRs we identified to be regulated by RSDS, we chose to focus on miR-25-3p because its expression was oppositely regulated in stressed mice and because it was negatively correlated with SI ($p < .05$) (Supplemental Figure S6C). miR-25-3p is a member of the miR-106b∼25 polycistronic cluster encoded within the 13th intron of the

change. **(B)** Schematic of the polycistronic miR-106b∼25 cluster. **(C)** Schematic illustrating the generation of miR-106b∼25$^{-/-}$ bone marrow chimeras. **(D)** Experimental timeline of 10-day RSDS, social interaction (SI) testing, and flow cytometry. **(E)** Percentage CD45.2+ cells in wild-type (WT) and miR-106b∼25$^{-/-}$ chimeras showing no differences in level of chimerism. **(F)** Social interaction ratios in stressed and unstressed WT and miR-106b∼25$^{-/-}$ chimeras. The 106b∼25$^{-/-}$ chimeras exhibited significantly higher social interaction ratios than the stressed WT chimeras and no differences in corner zone duration. Representative heat maps of SI tests are shown on the top right. For bar graphs, data represent mean + SEM. The number of animals within each group is indicated on the graphs. *$p < .05$ (two-way analysis of variance with Bonferroni post hoc test). AGG, aggressor; IRR, irradiation; KO, knockout.

minichromosome maintenance complex component 7 (*Mcm7*) DNA replication gene (Figure 2B) (39). The cluster is widely expressed and has been primarily studied for its oncogenic properties (40). To functionally interrogate the role of the miR-106b~25 cluster in behavioral susceptibility and resilience, we generated bone marrow chimeric mice, using either homozygous miR-106b~25 cluster knockout mice (Mirc3$^{tm1.1Tyj}$/J$^{+/-}$, henceforth referred to as miR-106b~25$^{-/-}$) or littermate wild-type (WT) control mice (Mirc3$^{tm1.1Tyj}$/J$^{+/+}$, henceforth referred to as WT) as bone marrow donors (Figure 2C). Based on our miR profiling results, we hypothesized that miR-106b~25$^{-/-}$ chimeras would display enhanced behavioral resilience. We exposed chimeric mice to RSDS followed by behavioral analysis and blood flow cytometry to confirm chimerism (Figure 2D). In both groups, we observed ~50% chimerism within leukocytes, a partial chimerism likely attributable to our use of lead head shielding to prevent radiation-induced changes in hippocampal neurogenesis and blood-brain barrier permeability (Figure 2E). We observed no group differences in chimerism within leukocyte subtypes (Supplemental Figure S7). Consistent with our hypothesis, our behavioral results indicated that downregulation of the cluster promotes resilience to RSDS. miR-106b~25$^{-/-}$ chimeras exposed to RSDS displayed higher SI ratios than stressed WT chimeras (chimerism effect; $F_{1,57} = 2.70$, Bonferroni post hoc test $p < .05$ for stressed mice) (Figure 2F) but no effect on corner time.

DISCUSSION

We have reported that RSDS induces changes in leukocyte subtype frequency in peripheral blood, including a robust induction of Ly6c^{high} monocytes in both susceptible and resilient mice. Depletion of peripheral Ly6c^{high} monocytes is mildly antidepressant, leading us to explore whether intrinsic posttranscriptional mechanisms are involved in resilience. Within Ly6c^{high} monocytes of mice exposed to RSDS, we identified several miRs regulated by RSDS 48 hours after defeat, including miR-25-3p, a member of the miR-106b~25 cluster. Peripheral downregulation of this cluster through generation of bone marrow chimeras promotes behavioral resilience to RSDS.

Our findings of stress induction of monocytes and neutrophils in blood support previous findings in mice (13,15,41) and humans (13,15) exposed to chronic stress. Interestingly, we also observed a decrease in absolute counts of circulating T cells in susceptible animals versus control animals. Recent studies have highlighted a potential role for T cells in stress resilience. Cohen *et al.* (30) reported that mice deficient in mature T cells are more susceptible to predator odor stress than WT mice. This phenomenon may be related to behavioral "immunization" to stress that is mediated by T cells (42). Indeed, Brachman *et al.* (31) found that infusion of lymphocytes (which include both T and B cells) from mice exposed to RSDS into mice deficient in mature lymphocytes confers resilience to developing depression and anxiety-like behaviors. Further study of T cells in the RSDS model, as well as of potential miR-mediated mechanisms for establishing immunological memory of stress, may yield additional insights into the biology of resilience.

We observed that peripheral antibody depletion of CCR2$^+$Ly6c^{high} monocytes is mildly antidepressant,

supporting and expanding on recent studies indicating anxiolytic effects of whole body CCR2 knockout (41) and antidepressant effects of Ly6c monoclonal antibody treatment (43). We did not, however, find that depletion of blood monocytes prevents susceptibility to RSDS. Zheng *et al.* (43) used similar monocyte depletion approaches, reporting that peripheral depletion of Ly6c^{high} monocytes with an Ly6c monoclonal antibody prevents LPS-induced depression-like behavior in the sucrose preference and forced swim tests and attenuates LPS-induced proinflammatory transcription in brain. They also reported results from enriching for Ly6c^{high} monocytes, in which they took advantage of the recovery of peripheral monocytes heavily enriched for the Ly6c^{high} subtype that follows clodronate liposome depletion. Mice with enriched Ly6c^{high} monocyte populations in blood displayed enhanced LPS-induced depression-like behavior in the sucrose preference and forced swim tests. While this group reported antidepressant findings in the RSDS behavioral model, for these studies they administered ginsenoside Rg1, which does not deplete Ly6c^{high} monocytes in blood but rather reduces Ly6c^{high} monocyte recruitment to the central nervous system through its inhibition of cerebral CCL2. Collectively, these results indicate that the antidepressant effects of monocyte depletion may require both reduced peripheral circulation and reduced signaling with the central nervous system.

We generated bone marrow chimeras using miR-106b~25$^{-/-}$ mice in order to investigate the functional importance of the miR-106b~25 cluster in behavioral response to stress. miR-25-3p, a member of the cluster, was regulated by RSDS in monocytes 48 hours after defeat, and its expression was signficantly correlated with individual SI ratios of stressed mice. We also included miR-106b-5p in our panel, observing nonsignificant increased expression in both susceptible and resilient mice. The miR-106b~25 cluster has been shown to mediate transforming growth factor beta signaling (39), which can potentiate inflammatory signaling in monocytes (44), but this is the first demonstration linking this specific cluster to depression or mood disorders. One study reported elevated miR-106b-5p expression in the cerebrospinal fluid of patients with MDD versus control subjects (45). While it is still unclear what inflammatory targets miR106b~25 may regulate to promote resilience, recent studies have shown IL-10 to be posttranscriptionally regulated by miR106b~25 (46,47). Thus, it is possible that depletion of miR106b~25 results in increased levels of the anti-inflammatory cytokine IL-10. Future studies are required to investigate the potential downstream mechanisms of miR-106b and their roles in promoting resilience versus susceptibility.

miR profiling in monocytes revealed several additional targets of interest. For instance, we observed a reduction of miR-150-5p in both susceptible and resilient mice. miR-150-5p is enriched in monocytes (48,49) and has been shown to regulate monocytosis in acute myocardial infarction (MI), a condition associated with depressive symptoms in 65% of patients (50). MI results in an inflammatory response that triggers the recruitment of neutrophils and monocytes to the heart (49). miR-150-5p is downregulated in human and mouse monocytes after MI, and in vitro overexpression of miR-150-5p in THP-1 (human monocytic leukemia) cells inhibits migration and

proinflammatory cytokine release, whereas blockade produces the opposite effect (49). miR-150-5p$^{-/-}$ bone marrow chimeras exhibit a prominent post-MI infiltration of Ly6c^{high} monocytes in blood and spleen (49). Unexpectedly, we observed a pattern of downregulation of monocyte-dervived let-7 miRs in resilient mice and upregulation in susceptible mice. This was surprising because the ubiquitous let-7 family has anti-inflammatory activities (51) and is a known regulator of IL-6 (52,53). However, these results may be related to our postdefeat time point and may indicate that the let-7 family serves as a homeostatic mechanism to control excessive inflammation in susceptible mice. miR-181a-5p, which was selectively downregulated in monocytes of resilient mice, has been shown to be an important regulator of T cell receptor sensitivity and age-related changes in adaptive immunity (54,55). miR-191-5p showed a similar expression pattern to miR-25-3p and, like miR-25-3p, is a member of an oncogenic miR cluster (56). It has also been shown to be dysregulated in plasma of patients with chronic conditions, including type 2 diabetes (57) and Alzheimer's disease (58).

In conclusion, we found that RSDS induces changes in circulating leukocyte subpopulations and monocyte miR expression profiles. Inflammatory monocyte depletion is mildly antidepressant, whereas downregulation of the miR-106b~25 cluster promotes behavioral resilience. These findings shed light on the role of peripheral inflammation in depression and identify novel targets for further research and potential therapeutic intervention.

ACKNOWLEDGMENTS AND DISCLOSURES

ARTICLE INFORMATION

From the Nash Family Department of Neuroscience (MLP, CM, FC, FD, KLC, YS, KL, MEF, HA, DMW, SB, GEH, SJR), Center for Affective Neuroscience and Friedman Brain Institute (MLP, FC, KLC, YS, KL, MEF, HA, DMW, SJR), and Department of Oncological Science (FD, VK, MMM), Tisch Cancer Institute and Precision Immunology Institute, Icahn School of Medicine at Mount Sinai, New York, New York; School of Neuroscience (GEH), Virginia Polytechnic Institute and State University, Blacksburg, Virginia; Department of Psychiatry and Neuroscience (CM), Faculty of Medicine and Cervo Brain Research Center, Université Laval, Quebec City, Quebec, Canada; and Department of Internal Medicine II (MM), University Hospital Regensburg, Regensburg, Germany.

Address correspondence to Scott J. Russo, Ph.D., Icahn School of Medicine at Mount Sinai, Nash Family Department of Neuroscience, Center for Affective Neuroscience and Friedman Brain Institute, 1425 Madison Avenue, New York, NY 10029; E-mail: scott.russo@mssm.edu.

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