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CCR2 monocytes repair cerebrovascular damage caused by chronic social defeat stress

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ABSTRACT

Immune surveillance of the brain plays an important role in health and disease. Peripheral leukocytes patrol blood–brain barrier interfaces, and after injury, monocytes cross the cerebrovasculature and follow a pattern of pro- and anti-inflammatory activity leading to tissue repair. We have shown that chronic social defeat (CSD) causes scattered vasculature disruptions. Here, we assessed CCR2 monocyte trafficking to the vascular injury sites in Ccr2 wt/rfp reporter mice both during CSD and one week following CSD cessation. We found that CSD for 14 days induced microhemorrhages where plasma fibrinogen leaked into perivascular spaces, but it did not affect the distribution or density of CCR2 monocytes in the brain. However, after recovery from CSD, many vascularly adhered CCR2 cells were detected, and gene expression of the CCR2 chemokine receptor ligands CCL7 and CCL12, but not CCL2, was elevated in endothelial cells. Adhered CCR2 cells were mostly the non-classical, anti-inflammatory Ly6C hi type, and they phagocytosed fibrinogen in perivascular spaces. In CCR2-deficient Ccr2 lo/rfp mice, fibrinogen levels remained elevated in recovery. Fibrinogen infused intracerebroventricularly induced CCR2 cells to adhere to the vasculature and phagocyte perivascular fibrinogen in Ccr2 wt/rfp but not Ccr2 lo/rfp mice. Depletion of monocytes with clodronate liposomes during CSD recovery prevented fibrinogen clearance and blocked behavioral recovery. We hypothesize that peripheral CCR2 monocytes are not elevated in the brain on day 14 at the end of CSD and do not contribute to its behavioral effects at that time, but in recovery following cessation of stress, they enter the brain and exert restorative functions mediating vascular repair and normalization of behavior.

1. Introduction

Mental health disorders such as major depressive disorder (MDD) have a complex etiology. Genetic, biological, and environmental factors contribute to their manifestation. The great disease burden of MDD inspires attention to factors that might be studied to achieve basic understanding and potential therapeutic treatments. A well-studied contributing environmental factor is psychosocial stress (Kendler et al., 1999; Tafet and Nemeroff, 2016). This form of stress predisposes not only to depression but also to other comorbid conditions such as hypertension and vascular disease (Finnell and Wood, 2016; Hare et al., 2014). In animals, psychosocial stress can be studied using a paradigm called chronic social defeat (CSD), which has validity (Belzung and Lemoine, 2011) in assessing the effects of similar kinds of stressors in humans (Dudek et al., 2021; Golden et al., 2011; Nestler and Hyman, 2010; Pryce and Fuchs, 2017). Mice subjected to CSD show depressive-like, anxiety-like, and asocial behaviors concordant with CNS changes in neural activity (Abe et al., 2019; Laine et al., 2017), hippocampal neurogenesis (Lagace et al., 2010; Schloesser et al., 2010), and activity of microglia (Lehmann et al., 2016), the immune cells of the brain. The basis for these cellular changes is not well understood, but CNS inflammation and involvement of the peripheral immune system may be contributing factors. For example, CSD causes elevations in levels of peripheral inflammatory cytokines (Azzinnari et al., 2014; Brachman et al., 2015; Lynall et al., 2021; Menard et al., 2017) and central reactive oxygen species (ROS) activity (Ibi et al., 2017; Lehmann et al., 2019). Gene profiling and ontology studies of CSD effects on microglia implicated pathways involved in breakdown of the extracellular matrix and blood–brain barrier (BBB) (Lehmann et al., 2018). Indeed, CSD was found to affect the cerebrovasculature, producing scattered cerebral microbleeds with associated local BBB breakdown and leakage of blood products into the brain parenchyma (Giannarelli et al., 2017; Lehmann

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et al., 2020; Menard et al., 2017). These blood products include fibrinogen, which is converted to fibrin by thrombin at blood clots. Fibrinogen is highly inflammatory (Languino et al., 1993) and may contribute to the inflammatory gene expression profile seen in the vascular compartment during CSD (Lehmann et al., 2020). During the recovery period following the cessation of CSD (CSDrec), profiling of cerebrovasculature-associated cells suggested activity associated with vascular repair and attraction of peripheral leukocytes to injury sites (Lehmann et al., 2020).

Such events at the BBB lie at the core of many brain disorders with cerebrovascular injury, such as ischemia (Dimitrijevic et al., 2007; Miro Mur et al., 2016) or traumatic brain injury (TBI) (Russo et al., 2018), in which vascular insults or blunt trauma damage the barrier elements. In such events, the innate peripheral immune system plays an important role. In early stages of injury, neutrophils and monocytes aggregate at sites of vascular injury to promote inflammation and phagocytosis of debris (Garcia-Bonilla et al., 2016). In later stages, restorative monocytes contribute to wound healing (Kraofoil et al., 2017). The sequelae of events is thus worked out for ischemia and TBI, but comparative analysis of monocytes in CSD and CSDrec is lacking.

Monocytes are hematogenous cells of the innate immune system activated by injury, infection, and other immune challenges throughout the body (Ousman and Kubes, 2012; Wynn and Vannella, 2016; Zhao et al., 2020). They express the chemokine receptor CCR2, which is important for monocyte infiltration induced under inflammatory conditions (Auffray et al., 2007; Nahrendorf et al., 2007). Monocytes are not the only cell type that is CCR2+; dendritic cells, natural killer cells, and other leukocytes express the marker in all tissues (Fujimura et al., 2015), but CCR2 is specific for directing monocytes into inflamed tissue (Saderup et al., 2010). In response to mobilization signals from the monocyte chemoattractant protein (MCP) family of chemokines including CCL2 (MCP-1), CCL7 (MCP-3) and CCL12 (MCP-5) (Chu et al., 2014), activated monocytes, which express CCR2 (Auffray et al., 2007; Chu et al., 2014), egress from bone marrow, enter blood, and traffic to injury sites (Cai et al., 2019; Tsou et al., 2007). Such CCR2+ monocytes contribute to elaboration and resolution of numerous CNS disorders, including autoimmune and neurodegenerative diseases, TBI, seizure, and stroke. Although there is a large literature on the linkage between psychiatric disorders and the cellular and molecular components of the immune system, there is scant evidence for involvement of classical monocytes in moderating affective states. Under normal conditions, very few bone marrow-derived immune cells reside in the brain (Korin et al., 2017), and monocytes do not enter the brain unless the blood–brain barrier (BBB) is compromised (Mildner et al., 2011; Prinz and Priller, 2017). Then, they largely act at brain-vascular interfaces. In this study, we tracked the presence of activated myelomonocytes at these interfaces in the Ccr2^<rt/or> reporter mouse during and after CSD to assess their roles in the production and resolution of cerebrovascular injury.

2. Materials and methods

2.1. Animals

All procedures were approved by the National Institute of Mental Health Institutional Animal Care and Use Committee and conducted in accordance with the National Institutes of Health guidelines. Experiments were performed using male CD-1 retired breeder mice and 8-10 week-old male Ccr2^<rt/or>, Ccr2^<rt/> and Ccr2^<rt/or> mice (Jackson model B6.129(Cg)-Ccr2tm2.1Ifc/J) backcrossed onto a C57BL/6N background (Charles River Laboratories). All test animals were group-housed in pathogen-free conditions in a 12 h light/dark cycle with lights off at 9:00 AM. Food and water were provided ad libitum. Behavioral testing was performed in the dark phase under dim lighting.

2.2. Chronic social defeat (CSD) and CSD recovery (CSDrec)

CSD was used to model the effects of chronic psychosocial stress in mice. As previously described (Lehmann et al., 2016; Lehmann et al., 2018), an experimental C57BL/6N mouse was housed for 14 days in the home cage (HC) of an aggressive male CD-1 mouse with a perforated transparent polycarbonate partition separating the mice. The partition was removed for 5 min each day to allow agonistic encounters between the mice. The mandibular incisors of the CD-1 mouse were trimmed to minimize wounding during the encounters. Defeat sessions were monitored to ensure that defeats reliably occurred (Schloesser et al., 2010). If defeats did not occur, the experimental mouse was moved to the cage of another, unfamiliar CD-1 mouse that had been previously determined to be aggressive. In the CSD group, mice were euthanized for tissue processing 2 h after the last CSD.

The recovery period (CSDrec) was for seven days immediately following CSD. Unstressed HC mice and CSDrec mice were housed 2/cage with a perforated partition permanently separating them.

2.3. Clodronate liposome administration

Clodronate encapsulated in liposomes was used to deplete peripheral monocyte/macrophages (Biewenga et al., 1995). Long-term depletion was achieved by injecting 150 µl of clodronate-filled liposomes (FormuMax Scientific, Sunnyvale, CA) retro-orbitally 48 h prior to experimental start and 100 µl every 7 h until experimental endpoints were reached. Mice receiving liposomes only during CSDrec received their first injection 2 h after the last agonist exposure on CSD day 14 and every 72 h thereafter. Control mice received similar regiments of control plain liposomes (FormuMax).

2.4. Behavioral analysis

Mice were phenotyped two days prior to experimental endpoints/tissue harvest. In CSD groups, phenotyping occurred 20 h after the previous CSD exposure. Mice were acclimated to the behavioral testing room for at least 60 min prior to testing. Testing was run under red light conditions, lux < 5.

2.4.1. Urine scent marking (USM). Briefly, and described in greater detail previously (Lehmann et al., 2013b), 0.1 ml of estrous female mouse urine was blotted onto a 45.7 cm × 45.7 cm acid-free paper sheet (Strathmore Sketch Paper 400 series, SLS Arts) that was previously placed into an open field box (46 cm × 46 cm). A 2-cm-diameter spot of female urine was deposited in one corner of the arena, 10 cm from two adjacent edges of the paper. After the female urine was absorbed by the paper and dried (~1 min), male mice were placed in the center of the arena and allowed to freely explore and scent mark for 10 min. Sheets were air-dried at least 1 h and then sprayed with ninhydrin (Triitech Forensics), which stains amines in urine purple, and dried overnight. Dried sheets were digitally photographed and analyzed using ImageJ software (http://rsb.info.nih.gov/ij/). Images were converted to binary and areas measured. Male urine marks deposited within 10 cm of the female urine spot (20-cm-dia circle centered on female urine) were compared to the total area of marks within the arena. Preference of urine marking was calculated by dividing the area of urine marks within this 20-cm circle by the total area of urine marks within the arena and then multiplied by 100.

2.5. Immunohistochemistry

Mice were anesthetized with isoflurane, injected retro-orbitally with 100 µl of tomato lectin 488 (Toml; Vector Labs, USA) to label the luminal surfaces of vasculature structures (Robertson et al., 2015), then 10 min later perfused transcardially with 0.9% saline followed by ice-cold phosphate-buffered 4% paraformaldehyde. Brains were removed and postfixed in the same fixative overnight followed by 25% sucrose in
PBS for 24 h. Coronal brain slices (30 μm thick) were collected on a freezing microtome. For fibrinogen labeling, free-floating sections were washed, blocked in 5% bovine serum albumin (IGF-free, protease-free; Jackson ImmunoResearch, USA) and 0.3% Triton X-100 (TX; Sigma, USA) for 1 h, incubated overnight at room temperature in polyclonal rabbit anti-human fibrinogen (1:1000; catalog #a0080, DAKO). Sections were rinsed with 0.1% TX-PBS and incubated for 1 h in donkey anti-rabbit antiserum labeled with Alexa Fluor-647 (1:500; Life Technologies cat. #31573). Sections were washed again with 0.1% TX in 0.1 M PBS (5 min, three times), counterstained with DAPI for 1 min, washed with PBS, and cover slipped with polyvinyl alcohol (PVA)-DABCO (Sigma-Aldrich). All microscopy was captured on a Zeiss 780 confocal microscope with 10x, 20x, 40x, or 63x objectives. For aquaporin-4 (Aq4) staining, similar procedures were followed as above except tissue was blocked with 4% normal goat serum, incubated with polyclonal rabbit anti mouse Aquaporin-4 (1:1000; Sigma # HPA014784), and revealed with goat anti-rabbit antiserum labeled with Alexa Fluor-647 (1:500; Life Technologies #A21071).

2.6. Multiplex fluorescent in situ hybridization (FISH)

Mice were anesthetized with isoflurane and rapidly decapitated; brains were removed and stored at −80 °C, then sectioned on a cryostat. Two coronal brain slices (10-μm thick) were collected onto slides (Trubond 380, Matsunami) corresponding to areas +1.53 and −0.71 mm from Bregma. Ten slides were collected per brain. Fluorescent multiplex in situ hybridization (FISH) was performed according to the manufacturer’s instructions using RNAscope v2 fluorescent multiplex assay for fresh-frozen tissue (ACD/Bio-Techne, Newark, CA). Sections were fixed for 15 min at 4 °C in 10% neutral-buffered formalin, then dehydrated and digested with protease III (all reagents from ACD) for 30 min at room temperature. Sections were incubated for 2 h at 40 °C in a hybridization oven (HybEZ) using a combination of ACD probes for Mus musculus. Combinations included one probe for either Ccl2, Ccl7, or Ccl12 plus a probe for Pecam1, an endothelial cell-specific marker. Sections were then incubated with preamplifiers, amplifiers, and fluorescent dyes assigned to specific probes: Cy3 for Pecam1 and Cy5 for Ccl2, Ccl7, and Ccl12. After counterstaining with 1:10,000 DAPI (Life Technologies, Grand Island, NY) for 1 min, slides were cover-slipped with polyvinyl alcohol mounting medium with DABCO (1,4-diazabicyclo[2.2.2]octane, Sigma, St. Louis, MO) to prevent fading.

Slides were scanned first with a 10x objective on a Nikon A1R confocal microscope to determine if chemokine expression mapped onto specific anatomical regions. A blind observer digitally marked the location of each instance of chemokine mRNA expression. Chemokine expression was further quantitated with a 40x oil objective. Twelve images per sample were collected, randomly across two sections, containing >3500 cells. All image analysis steps were performed using Velocity 6.3 (PerkinElmer, Shelton, CT). The analysis was carried out in three main steps: nuclear segmentation, FISH spot identification, and cell classification/scoring. First, individual nuclei were segmented using the DAPI channel. To correct for under-segmentation caused by cell clustering, large clusters of nuclei were first identified and then processed through a shape-based watershed to divide individual nuclei. DAPI masks >20 μm² were excluded, as were all edge objects. Individual masks were then expanded by 2 μm from the edge to include peripheral labeling. The resulting region of interest for each cell nucleus was used as a search region for spot counting in the Cy3 and Cy5 channels. A threshold (three standard deviations higher than the median value) was then applied to each channel to detect bright spots in the DAPI mask. These were then further filtered based on size criteria to prevent false detection caused by background noise. Spots that overlapped spatially across two channels were counted as auto fluorescence and excluded from the analysis. Individual cells were counted as expressing chemokine or Pecam1 mRNA if two or more fluorescent dots were present in the DAPI mask. All acquired single-spot level data were exported and further analyzed in GraphPad Prism ver. 7 and Microsoft Excel.

2.7. CCR2<sup>rfp<sup>−</sup>−</sup> cell mapping

In determining the number and location of CCR2<sup>rfp<sup>−</sup>−</sup> cells in Ccr2<sup>wt/wt</sup> mice, whole brain sections labeled with TomL, prior to perfusion were captured on a Zeiss 780 microscope with a 10x plan aplanochromat objective. A blind observer analyzed the images on Zen Blue (Zeiss) and digitally marked the location of RFP “DAPI” cells. Six equidistant sections from each brain were examined spanning Bregma +1.9 to Bregma −3.1. Three anatomical compartments—brain, leptomeninges, and choroid plexus—were used to classify the location of each cell. The area of brain and choroid plexus was measured, and length of leptomeninges determined to normalize values across samples (Zen Blue). The presence of meninges on the peripheral surface of the brain was inconsistent across tissue sections, and cells in this region were not counted. Leptomeninges were present in the interhemispheric fissure and internal cisterns. A blind observer scanned sections stained with aquaporin-4 with a 20x objective to determine the location of CCR2<sup>rfp<sup>−</sup>−</sup> cells within the neurovascular structure. Cells nested between TomL labeling of the vessel lumen and aquaporin-4 immunostaining of the pial membrane were considered perivascular. For figure generation, marks from a representative sample were transposed onto a brain atlas maps (Paxinos and Franklin, 2001) corresponding to the appropriate coronal slice.

2.8. Intracerebroventricular fibrinogen infusions

Male Ccr2<sup>wt/wt</sup> and Ccr2<sup>rfp<sup>−</sup>−</sup> mice were anesthetized with isoflurane and placed in a stereotactic frame. Fibrinogen conjugated with Alexa Fluor 647 (Fib647; ThermoFisher) was dissolved in endotoxin-free distilled water, diluted to 10 mg/ml with artificial cerebrospinal fluid (aCSF), and kept at 37 °C. Under stereotaxic guidance, 3 μl of Fib647 or aCSF vehicle was infused into the lateral ventricle at the following coordinates: AP −0.4 mm, ML 1.0 mm, DV −2.5 mm. Infusions were at 0.2 μl/min using a 10-μl Hamilton syringe attached to a 33-gauge blunt-tip needle (Hamilton, USA).

2.9. Flow cytometry

CCR2<sup>rfp<sup>−</sup>−</sup> cells were isolated and enumerated from brains of male Ccr2<sup>wt/wt</sup> mice with the following methods: mice were anesthetized and injected retro-orbitally with 8 μl of anti-CD45-conjugated FITC (Biolegend) in 92 μl sterile saline to label circulating leukocytes, then 30 min later perfused with cold saline. Brains were removed, placed in a C-tube (Miltenyi Biotec) with 2.5 ml of 2.5 mg/ml Collagenase D (0.29 μg/ml; Roche, USA) in HBSS plus 10 μl of Solution A and 20 μl Solution Y (Miltenyi Biotec) and minced with a gentleMACS Dissociator for 45 sec (brain protocol 1, Miltenyi Biotec). C-tubes were incubated for 30 min at 37 °C with rotation, triturated 100x with a 1000 pipet tip, then filtered through a 70 μm filter. The filter was washed with 20 ml HBSS, and the solution pelletted at 300 g for 5 min. The cell pellet was resuspended in 30% isotonic Percoll (Sigma, USA), then centrifuged at 800g for 30 min with no brake. After the myelin layer was removed, Percoll and cell pellet were diluted 4-fold with HBSS and resulting cell pellet was stained for flow cytometry. Cells were stained at 1:2400 dilution with Fixable Viability Dye eFluor<sup>780</sup> (65-0865; eBioscience) for 10 min at room temperature. Cells were washed, then blocked with 1 μl of normal goat serum (Sigma) and 1 μl of Fc block (Biolegend #101302) for 10 min on ice. 25 μl Brilliant Violet stain buffer (BD Horizon #563794) was then added, and the antibody master mix (5 μl Panel A was added [BD Biosciences; (#564279 CD45 - BUV395) (#612800 CD11b - BUV771]), [Biolegend; (#127106 PDCA1 - AF647) (#141716 CD206 - PerCP/Cy5.5) (#100228 CD3 - BV421) (#127633 Ly6G - BV510) (#117334 CD11c - BV605) (#115555 CD19 - BV711) (#128041 Ly6C - BV785), (ThermoFisher #56-5321-82 MHCI - AF700)]. The antibody reaction proceeded on ice for 25 min; cells to be analyzed were then washed with...
PBS, pelleted, and fixed in 2% PFA at room temperature for 15 min and analyzed within 24 h on a BD LSR Fortessa flow cytometer. A brain from one Ccr2<sup+w/+</sup> mouse was processed and stained identically for use as a fluorescence minus one (FMO) control. This sample was used to define RFP<sup+</sup> and RFP<sup−</sup> cells. Samples were run in their entirety to acquire absolute counts. Compensation was performed for each session using UltraComp eBeads (eBioscience #01-2222-42) conjugated to antibodies used in the sample panels, unstained blood cells from Ccr2<sup+w/+</sup> mice, and wild type splenocytes stained with the Fixable Viability Dye eFluor<sup+</sup>M 780 alone.

The efficacy of monocyte depletion with clodronate liposomes was determined with a second panel. Blood was collected from anesthetized mice by facial-mandibular punch into EDTA-coated tubes on the day of perfusion, and prior to vascular labeling with TomL. Erythrocytes were removed with ACK lysis buffer (Quality Biological, MD). Remaining cells were washed, pelleted and stained with Zombie Aqua (1:200; Biolegend #423101), washed again then blocked as above and stained with the following antibody master mix on ice for 25 min: Biolegend (#101228 CD11b – PerCP/Cy5.5, #103151 CD45 – BV650, # 127,614 Ly6G – APC). Cells were washed, pelleted then analyzed with a Beckman Coulter Cytoflex flow cytometer.

Cell subsets were defined by manual gating in FlowJo (TreeStar) (Supplemental Figs. 1 and 2). Data were analyzed with FlowJo software. Cell counts were normalized to live single cells for brain samples and live single CD45<sup+</sup> cells for blood samples. Statistics were run on these normalized counts.

2.10. Statistics

Behavioral data, cell counts, and cytometry data were summarized as the mean ± s.e.m., and differences among experimental conditions were considered statistically significant when the p value was < 0.05. One- and two-way ANOVAs, analyzed by SPSS (https://www.ibm.com/analytics/us/en/technology/spss/), were applied where appropriate to assess between-subject comparisons along the variables experimental condition versus, for example, behavioral or biological assay score. Cytometry data for brain samples were analyzed with Prism 7 and observed to have non-normal distribution. Thus, a Kruskal Wallis analysis of variance with Dunn’s test corrected for multiple comparisons was used. Post hoc tests were performed with Tukey’s post hoc or Sidak’s correction where appropriate, and the P level was adjusted for multiple comparisons.

3. Results

3.1. CSD causes scattered fibrinogen leakages that are partially resolved during recovery.

Our previous studies showed the accumulation of the blood products immunoglobulin, fibrinogen, and platelets localized in and around microbleeds scattered throughout the brain of susceptible CSD-exposed mice (Lehmann et al., 2020; Lehmann et al., 2018). Fibrinogen/fibrin (fibrinogen) is highly inflammatory (Languino et al., 1993), suggesting that the brain’s immune response to the adverse effects of psychological stress is driven in part by vascular damage and blood leakage across the BBB. Here we confirmed our previous findings that CSD produced scattered fibrinogen deposits in brain, and that these deposits were significantly reduced after mice were exposed to a nonstress one-week recovery period after CSD (CSDrec). Deposits were mapped in five equally spaced coronal brain sections of home cage (HC) control, CSD, and CSDrec mice (Fig. 1a). In CSD brains, fibrinogen was observed in perivascular spaces (Fig. 1b, c). The deposits were not seen in HC brains and were present in reduced numbers in CSDrec brains (Fig. 1d; F<sub>2,12</sub> = (section location from bregma; F value, P value): (1.98 mm; 7.22, 0.008), (1.1 mm; 6.07, 0.01), (0.02 mm; 6.74, 0.01), (−0.94 mm; 17.6, 0.003), (−1.98 mm; 9.39, 0.003)). Deposits were stochastically distributed across all sections; no specific anatomical organization was detected.

3.2. Monocyte entry into brain occurs in CSDrec but not in CSD

The decline of deposited fibrinogen during the recovery period was...
pronounced. In earlier studies, we documented the changes in brain vascular cell programming during CSD and during stress recovery and observed that biological processes related to leukocyte migration became dominant during CSDrec (Lehmann et al., 2020). In vascular injury models, extravascular fibrin(ogen) deposits are infiltrated by leukocytes, specifically CCR2$^+$ leukocytes (Forsyth et al., 2001; Languino et al., 1993; Motley et al., 2016). Therefore, we examined if a similar process occurs in CSD and CSDrec brains. In Ccr2$^{wt/rfp}$ reporter mice, we mapped the location of CCR2$^{rfp+}$ leukocytes across the three conditions and probed for changes in cell number associated with brain vasculature, leptomeninges, and choroid plexus (Fig. 2). CSDrec brains showed a significant increase in CCR2$^{rfp+}$ cell trafficking to brain vasculature compared to all other conditions (Brain; F$(2,21)=65.2, \ P<0.0001$) (Fig. 2a-c). CCR2$^{rfp+}$ cell density was not altered in CSD brains (Tukey’s HC vs. CSD; P > 0.05). No change in CCR2$^{rfp+}$ cell density was observed in choroid plexus (F$(2,21)=0.55, \ P=0.59$) or

![Fig. 2. CCR2$^{rfp+}$ cells tracked in Ccr2$^{wt/rfp}$ mice accumulate at the blood-brain barrier in CSDrec only. a, Experimental design and maps showing representative distribution of CCR2$^{rfp+}$ cells, marked by arrows, in brain sections across three conditions: home cage (HC), chronic social defeat (CSD), and post CSD recovery (CSDrec). Numbers adjacent to sections show distance from bregma in mm. b, Confocal images show the location and density of CCR2$^{rfp+}$ cells associated with brain vasculature. Vessels are labeled with TomL (green). c, Density of CCR2$^{rfp+}$ cells was determined for brain vasculature, leptomeninges, and choroid plexus. d, Confocal image with orthogonal view shows a CCR2$^{rfp+}$ cell nested between the pial layer stained with Aq4 (white) and endothelial lumen (TomL, green). e, Bar graph shows the anatomical location of CCR2$^{rfp+}$ cells across three conditions. One-way ANOVA with Tukey’s post hoc test (***, P < 0.001), mean ± s.e.m. N = 8/group. Aq4, aquaporin-4; TomL, tomato lectin; rfp, red fluorescent protein. Scale bars: b, 20 µm; d, 10 µm.](image-url)
leptomeninges ($F_{(2,21)} = 2.4, P = 0.12$) (Fig. 2c).

We immunostained for aquaporin-4 (Aq4) in a subset of tissue from each condition to reveal BBB structure and determine the anatomical location of CCR2$^{rfp^+}$ cells. Brain CCR2$^{rfp^+}$ cells were primarily nested in the perivascular space between the vessel wall (TomL) and astrocytic end feet (Aq4). A much smaller number were found either in parenchyma or adhered to the vessel lumen (Fig. 2d, e). Stress treatments had no effect on the proportion of cells found in any compartment (ANOVA, $F_{(2,21)} = 0.29, 0.4, 0.35, P > 0.05$) for perivascular space, parenchymal space, and lumen, respectively.

3.3. Specific leukocyte subsets cross the vasculature in CSDrec

Changes to the brain immune landscape following CSD and CSDrec were mapped with flow cytometry. Vascular and non-vascular cells were segregated using an intravenous (i.v.) injection of FITC-conjugated CD45; circulating cells, including those adhered to the inner vessel lumen, were excluded from further analysis (and not shown in any of the flow plots). A full gating strategy and enumeration of all identifiable cell types is presented in Supplemental Fig. 1. For each sample, enumerated cells were normalized to doublet-excluded, live cells and further stratified into either CCR2$^{rfp^+}$ (Fig. 3a) or CCR2$^{rfp^-}$ fractions (Fig. 3b) by gating on a fluorescence minus one (FMO, or wildtype) control. Dendritic cells (DCs) were gated by CD11c and MHCII and further gated by CD11b and PDCA1 into conventional (cDCs) and plasmacytoid (pDCs) cells. Neutrophils (neuts), monocytes (Mo), macrophages (MAC), T cells (CD8) and B cells (CD19) were gated by appropriate antibody markers (Suppl Fig. 1). Bivariate dot plots in Fig. 3c demonstrate the increased numbers of myeloid cell populations isolated from CSDrec samples. The intensity spectrum of Ly6c labeling suggests a continuum (“waterfall”) of monocyte phenotypes rather than two circumscribed profiles (Ly6c$^{lo}$ vs. $^{hi}$).

Fig. 3. Mapping changes to the immune landscape in brain show that myeloid cell populations were recruited to perivascular spaces during stress recovery. Non-vascular (not labeled by intravenous FITC-CD45) extravasated brain immune cells from Ccr2$^{wt/rfp}$ mice were analyzed with flow cytometry in HC, CSD, and CSDrec conditions. Identifiable CCR2$^{rfp^-}$ and CCR2$^{rfp^+}$ cells, normalized as percent of recovered live cells, are shown in a and b, respectively. c, Bivariate dotplots showing identification of major leukocyte subtypes demonstrate elevated CCR2$^{rfp^+}$ myeloid populations in the three conditions. d, Median fluorescent intensity for RFP in CCR2$^{rfp^+}$ cells is shown for each enumerated cell population and compared across different conditions. e, Quantification of neutrophils and (f) cDCs isolated from CCR2$^{rfp^-}$ populations in each condition. g, Number of CCR2$^{rfp^-}$ cells isolated in each condition. h, i, j, Ccr2$^{rfp^+}$ cell populations identified as Ly6c$^{lo}$ monocyte (h), cDC (i), or Ly6c$^{hi}$ monocyte (j) found in each condition. MFI; median fluorescent intensity, RFP; red fluorescent protein, cDC; conventional dendritic cell, pDC; plasmacytoid dendritic cell, NP; neutrophil, Mo; monocyte, MAC; macrophage. Bar graphs depict the mean ± s.e.m. for the indicated groups, and asterisks denote statistical significance, $N = 5$/group. Kruskal-Wallis ANOVA with Dunn’s post hoc test corrected for multiple comparisons (*, $P < 0.05$; **, $P < 0.01$; n.s., post hoc test not significant).
As expected, in the CCR2rfp- subset (Fig. 3a), the majority of cells were resident immune cells (microglia and perivascular macrophages). There was also a smaller subset of cells unidentified by the markers in our antibody panel, which likely includes neurons, astrocytes, pericytes, and endothelial cells (Suppl Fig. 1b). Of the remaining CCR2rfp- cells, statistical variations were detected in neutrophils (Fig. 3e; H(3) = 7.3, P = 0.018), CD19+ B cells (H(3) = 8.1, P = 0.009), macrophages (H(3) = 6.54, P = 0.029), and conventional dendritic cells (cDCs, Fig. 3f; H(3) = 6.74, P = 0.024). Dunn’s post hoc test showed that B cells were lower in CSDrec samples compared to CSD, and macrophages and cDCs were elevated in CSDrec vs. HC samples. No significant post hoc difference was found for neutrophils.

Endogenous red fluorescent protein (RFP) median fluorescent intensity (MFI) has been measured as a proxy for CCR2 protein levels across different CCR2+ cell populations (Fujimura et al., 2015). We found heterogeneous RFP MFI across our cell populations (Fig. 3d). Higher MFI levels were observed in Ly6C+ monocytes, and lower levels were found in macrophage populations. No significant effect of treatment was observed, but CSDrec pDCs, Ly6C+, and Ly6c- populations tended to exhibit elevated RFP MFI compared to those cells from other groups, suggesting that CCR2 protein expression is not diminished during CSD recovery. We note however that RFP may have a longer half-

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**Fig. 4.** Increased CCR2rfp+ cell trafficking during post-stress recovery and the removal of fibrin deposits required functional CCR2. a, Experimental timeline is shown. Map shows the representative distribution of CCR2rfp+ cells at one brain level from a Ccr2wt/rfp (CCR2 intact) and a Ccr2rfp/rfp (deficient) mouse exposed to HC, CSD, or CSDrec. Additional levels from a CCR2-deficient mouse are shown in Supplemental Fig. 3. b, Increased CCR2rfp+ cell density associated with brain vasculature occurred during CSDrec in mice with functional CCR2; ***, significantly elevated vs. all other groups (Tukey’s post hoc tests, P < 0.001). c, Representative confocal images show CCR2rfp+ cells (red, appearing alongside the edge of the vessels, green) associated with brain vasculature in Ccr2rfp/rfp mice from HC, CSD, and CSDrec conditions, contrasted with an image showing increased CCR2rfp+ cell density from a Ccr2wt/+/rfp mouse during CSDrec. d, Graph shows percent of tomato lectin (TomL) labeled blood vessels that colocalize with fibrin(ogen) labeling; different letters above the bars indicate significant differences between groups (Tukey’s post hoc tests, P < 0.05). e, Representative confocal images show increased fibrin(ogen) (white) deposits during CSD and persistence of these deposits during CSDrec in Ccr2rfp/rfp mice. Last image with inset shows endocytic uptake of endogenous fibrin(ogen) by an RFP+ cell from a Ccr2wt/+/rfp mouse in CSDrec. TomL labeling is removed from the orthogonal view for clarity. Two-way ANOVA, mean ± s.e.m. N = 8 / group. Scale bar = 20 µm and 10 µm for inset.
life than membrane-associated CCR2 (perhaps CCR2 is internalized (Dal-Secco et al., 2015)).

Dynamic responses were observed in the CCR2rfp–/– fraction (Fig. 3b). CCR2rfp–/– cells were substantially elevated in CSDrec samples (Fig. 3g; H<sub>(3)</sub> = 7.58, P < 0.01), confirming our earlier histological analysis (Fig. 2a,c). Assessment of cell counts showed that Ly6c<sup>hi</sup> monocytes (Dal-Secco et al., 2015) were substantially elevated in CSDrec samples (Fig. 3h, cDCs (Fig. 3i), and plasmacytoid dendritic cells (pDCs) were also elevated in CSDrec samples (dCD; H<sub>(3)</sub> = 7.46, P = 0.01, pDC; H<sub>(3)</sub> = 9.75, P = 0.0001). Numbers of isolated pDCs were minimal compared to other cell populations (non-normalized means: HC, CSD, CSDrec = 1.2 , 4.8, 8.2 respectively). Significant variation was detected in classical Ly6c<sup>hi</sup> monocytes (H<sub>(3)</sub> = 6.62, P = 0.029); however, no significant difference was found (Dunn > 0.05). Significant alterations were not observed in other cell populations (Kruskal-Wallis; H<sub>(3)</sub> = (cell type: H value) neutrophils (NP): 3.5; macrophages (MAC): 4.9; T cells (CD3<sup>+</sup>): 0.38, and B cells (CD19<sup>+</sup>): 1.3, all p > 0.05.

3.4. CCR2 is required for monocyte entry and fibrinogen removal in CSDrec

CCR2 plays an essential role in leukocyte recruitment and the progression of wound repair. We examined whether CCR2 deficiency altered the pattern of recruitment to the BBB during stress and stress recovery. CCR2-deficient (Ccr2<sup>rfp/rfp</sup>) mice exposed to CSD or to CSDrec showed no change in CCR2<sup>rfp+</sup> cell recruitment to brain vasculature (F<sub>(2,15)</sub> = 0.11, P = 0.89) compared to HC. The response to CSD in Ccr2<sup>rfp/rfp</sup> mice is contrasted to Ccr2<sup>wt/wt</sup> mice in Fig. 4. The substantial increase in CCR2 cell trafficking that occurred during CSDrec (as detailed in Fig. 2) was absent in Ccr2<sup>rfp/rfp</sup> mice (Fig. 4a-c and Suppl. Fig. 2) (Two-way ANOVA comparing CCR2-deficient vs. intact; Interaction effect F<sub>(2,36)</sub> = 41.6, P < 0.0001). Removal of fibrinogen (ogen) deposits was also impaired in Ccr2<sup>rfp/rfp</sup> mice; deposits were significantly elevated during CSD and CSDrec timepoints compared to HC (One-way ANOVA; F<sub>(2,15)</sub> = 31.9, P = 0.0001) (Fig. 4d, e). In contrast, intact mice showed significant fibrinogen (ogen) content during CSD. Diminished fibrinogen (ogen) deposits in CSDrec were comparable to HC levels (similar to the effect observed in Fig. 1) (F<sub>(2,21)</sub> = 15.27, P < 0.0001, post hoc CSD vs. CSDrec P < 0.05). Removal of fibrin during post stress recovery was dependent on functional CCR2; deposits were significantly reduced in CCR2 intact vs. deficient mice (Interaction; F<sub>(2,36)</sub> = 3.35, P = 0.041). Endocytic uptake of fibrinogen (ogen) was detected in CCR2<sup>rfp</sup> cells with confocal microscopy (Fig. 4e). These results suggest that CCR2 cell trafficking is integral to the removal of fibrin deposits.

3.5. Fibrinogen triggers monocyte entry into brain that is CCR2-dependent

We were interested to determine whether fibrin deposits would cause CCR2<sup>+</sup> cell migration to brain vasculature and whether that migration was dependent on functional CCR2. Fibrinogen conjugated with Alex647 (fib-A647) or vehicle was infused i.c.v. into CCR2-intact and -deficient mice and allowed to flow in cerebrospinal fluid through ventricles, cisterns, and subarachnoid and perivascular spaces for 24 h. Fib-A647 caused a significant influx of CCR2<sup>rfp</sup> cells adhered to the vasculature (Fig. 5) in both genotypes (two-way ANOVA infusion effect F<sub>(1,12)</sub> = 189.3, P < 0.0001). The response was significantly more robust in CCR2-intact vs. –deficient mice (genotype x infusion interaction; F<sub>(1,12)</sub> = 22.36, P = 0.0005) (Fig. 5a). The results show that fibrin(ogen) is a robust stimulus for CCR2 cell migration, and the effect is mediated in part by functioning CCR2. Endocytic uptake of fib-A647 was detected in CCR2<sup>rfp</sup> cells (Fig. 5b).

3.6. Chemokine mRNA expression is elevated during CSDrec

Monocyte recruitment to a wound follows chemokine gradients established by the signaling molecules and their receptors. We previously found that transcriptional expression of the CCR2 ligands CCL7 and CCL12 was elevated in brain endothelial cells during post-stress recovery (Lehmann et al., 2020). A multiplex in-situ hybridization study was performed to verify the prior bioinformatic findings and give support for a putative mechanism driving CCR2<sup>rfp</sup> cell recruitment. The transcriptional expression of three CCR2 ligands (Ccl2, Ccl7, and Ccl12) was mapped alongside the expression of the endothelial cell marker Pecam1 in mice exposed to HC, CSD, or CSDrec (Fig. 6). Transcriptional expression of Ccl7 and Ccl12 was substantially increased during CSDrec only (Ccl7; F<sub>(2,11)</sub> = 7.89, P = 0.012, Ccl12; F<sub>(2,11)</sub> = 52.98, P = 0.0001) (Fig. 6b, c). Ccl2 mRNA expression was not altered by CSD or CSDrec, and expression was minimal across all conditions (F<sub>(2,11)</sub> = 1.13, P = 0.3) (Fig. 6a). Pecam1 mRNA expression endothelial cells contained a notable portion of the chemokine signal in CSDrec brains—21.9% ± 4.3% of Ccl7<sup>hi</sup> cells and 32.5 ± 3.0% of Ccl12<sup>hi</sup> cells. The number of Pecam<sup>+</sup> cells expressing Ccl7 or Ccl12 was markedly increased in CSDrec brains versus all other conditions (Ccl7; F<sub>(2,11)</sub> = 9.34, P = 0.008, Ccl12; F<sub>(2,11)</sub> = 25.8, P = 0.0003) (Fig. 6a). Importantly, this expression pattern was not limited to any specific brain region.

3.7. Monocytes/Macrophages clear fibrin and restore behavior in CSDrec

Our data support a model in which CCR2<sup>+</sup> monocytes are recruited
to damaged cerebrovasculature upon the cessation of CSD and engage in wound healing. To test this hypothesis, we administered repeated i.v. doses of clodronate or control liposomes during CSD or during CSDrec (Fig. 7a) and used clearance of fibrin(ogen) as a surrogate for tissue remodeling during healing. Clodronate treatment led to a profound depletion of circulating CD11b<sup>+</sup>CD45<sup>+</sup>Ly6G<sup>+</sup> monocyte/macrophage in all conditions but had no effect on Ly6G<sup>+</sup> neutrophils (Fig. 7b-c) (vs. within group control liposome injected: HC: 64%; CSD: 79%; CSDrec: 77%) (Drug effect; F<sub>(2,24) </sub>= 273.8, P = 0.0001). A full gating strategy and enumeration of all identifiable cell types for each condition is presented in Supplemental Fig. 2. Substantial fibrin(ogen) accumulation occurred during CSD and was not altered by monocyte/macrophage depletion. Notably, these deposits persisted if depletion occurred during CSDrec (Fig. 7d, e), revealing a direct role for peripherally-derived monocytes/macrophages in cerebrovascular-associated fibrin deposit removal during CSDrec (Interaction; F<sub>(2,24) </sub>= 3.85, P = 0.035).

Inefficient fibrin removal can have an inhibitory effect on wound healing and promote inflammation (Opneja et al., 2019). We tested whether disruption of fibrin removal would alter urine scent marking (USM), a measure of anhedonia sensitive to CSD exposure (Lehmann et al., 2013b) and inflammation (Pitcher et al., 2019). The behavioral scores paralleled fibrin deposition in brain (Fig. 7f-g). Clodronate injections did not affect the high preference for scent marking in HC mice or the absence of preference in CSD mice. However, significant interactions occurred during CSDrec. Mice that received control liposomes during CSDrec showed preference scores similar to those of nonstressed HC mice. This behavioral recovery did not occur in mice receiving clodronate injections during CSDrec (Interaction; F<sub>(2,24) </sub>= 7.66, P = 0.003). The data show that monocyte/macrophages are engaged in the removal of neurovascular fibrin deposits during CSDrec. Disrupting this process prevents behavioral recovery after CSD.

4. Discussion

Mental disorders like MDD are biologically linked both with elevated immune system activity (Haapakoski et al., 2016) and with risk for vascular disease (Meng et al., 2012; Seligman and Nemeroff, 2015), but the links are not well understood. Chronic psychosocial stress is an environmental condition that contributes to MDD (Kendler et al., 1999; Tafet and Nemeroff, 2016), dysregulated immune system activity (Bergamini et al., 2018), and endothelial dysfunction (Sher et al., 2020). Our studies of chronic social defeat (CSD) in mice revealed that this form of psychosocial stress caused BBB breakdown, scattered microbleeds, and leakage of inflammatory blood products like fibrinogen into the brain (Lehmann et al., 2020; Lehmann et al., 2018). This finding called attention to the possibility that peripheral immune system cellular responses to cerebrovascular disruption in CSD are similar to those in other brain injury disorders. In particular, in stroke (Dimitrijevic et al., 2007; Miro-Mur et al., 2016), seizures (Varvel et al., 2016), TBI (Mas et al., 2018), intracerebral hemorrhage (Hammond et al., 2014; Matorakos et al., 2021), classical and non-classical monocyte responses have been well characterized. Therefore, in the CSD model, we focused on these innate immune cells by using Ccr2<sup>lo</sup>Ccr2<sup>hi</sup> myelomonocytic reporter mice. Monocytes are rare in the normal brain (Korin et al., 2017), residing mainly at BBB interfaces such as the meninges, choroid plexus, and perivascular spaces. Histochemical tracking of CCR2<sup>lo</sup>C<sup>hi</sup> cells confirmed this distribution. CSD for 2 weeks led to scattered microbleeds that were associated with fibrinogen leakage into perivascular and parenchymal spaces, and these measures declined at the end of the one-week recovery period after stress cessation (CSDrec). Strikingly, CCR2<sup>lo</sup>C<sup>hi</sup> cell numbers were unchanged during CSD, but in CSDrec, they trafficked to brain, occupied perivascular spaces, and phagocytosed fibrin(ogen). A significant proportion of these cells were Ly6G<sup>+</sup> non-classical monocytes. These cells participate in vascular repair and fibrin endocytosis after traumatic brain injury (Russo et al., 2018); they physically remodel vessels after vascular
We hypothesize that peripheral leukocytes similarly serve to repair vascular damage and mitigate inflammation following recovery from CSD.

In CSD, red blood cells and plasma proteins leak into the brain at sites of BBB breakdown (Lehmann et al., 2018; Lehmann et al., 2019). Fibrinogen appears to be an essential component of the leakage. Fibrinogen deposition and conversion to fibrin is a key process of blood coagulation, stabilizing the hemostatic plug and providing an extracellular matrix for wound and vessel repair. Although intrinsic to the repair of injured tissue, excessive or prolonged fibrin deposition is deleterious to surrounding tissue environments due to its property as a potent inflammmogen (Petersen et al., 2018). We showed that intracerebroventricular fibrinogen infusion triggered an influx of CCR2$^+$ cells into the brain. We hypothesize that fibrin accumulation supports the transcriptional programming we found in brain endothelial cells (Lehmann et al., 2020) and microglia (Lehmann et al., 2018). Fibrinogen stimulates microglia into an activated destructive phenotype that may promote demyelination in experimental allergic encephalomyelitis (EAE) (Adams et al., 2007). Similar structural mal-adaptations are observed in CSD mice (Lehmann et al., 2017).

Aside from its important role in hemostasis, fibrinogen functions as a bridging molecule for many types of cell–cell interactions. Fibrin deposited during the early wounding period forms a scaffold for migrating and sprouting endothelial cells (Hadjipanayi et al., 2015; Tonnisen et al., 2000) where they align to become new vascular structures during tissue repair. These reports mirror our findings showing the spatial overlap between angiogenesis markers and fibrin deposits in CSD-exposed brain (Lehmann et al., 2020). After the process of wound healing has started, extravascular fibrin must be removed to restore vascular patency and reduce the risk of organ damage and chronic inflammation (Lin et al., 2020). The initial extracellular degradation occurs through proteolysis via plasminogen activation. Fibrin degradation products are then removed at the cellular level by CCR2$^+$ cells that infiltrate fibrin deposits (Motley et al., 2016). The current findings, detailing internalization of extravascular fibrin in vivo, suggest an analogous process occurs here as well.

Monocytes often play dual roles in injury—both perpetuating inflammation and promoting subsequent processes involved in wound resolution. Driving the recruitment of different monocyte populations, CCR2 appears essential for proper healing of injured tissues; CCR2 deficiency results in dysregulation of inflammation and altered tissue repair in numerous models of injury and disease (Conterras-Shannon et al., 2007; Lu et al., 2011; Mitchell et al., 2009). In ischemic stroke models, selective deletion of CCR2 in monocytes delays inflammatory response, reduces angiogenesis, and impairs neurological function (Pedragosa et al., 2020). In CSD, Ccr2$^+$ mice lacking CCR2 had
reduced monocyte trafficking and elevated fibrinogen, indicating that CCR2 deficiency impairs wound healing in the CSD model as well. Remaining trafficking was likely due to other chemokines and respective receptors, as reviewed for instances of ischemia/reperfusion (Dimitrijevic et al., 2007).

Macrophage action after injury typically involves two phases. In early phases, the cells may exacerbate the injury by expressing inflammatory cytokines and phagocytosing debris and dead cells, but in later phases, they aid in resolving the injury (Chu et al., 2014). Monocytes are dynamic and can convert from a pro-inflammatory type, characterized as Ly6C<sup>hi</sup> early in infection or injury, to an anti-inflammatory Ly6C<sup>lo</sup> type (Geissmann et al., 2006) during progression of the injury (Garcia-Bonilla et al., 2016; Kratofil et al., 2017; Miro-Mur et al., 2016). Importantly, the polarization of later appearing monocytes is M2-like (Chu et al., 2015; Wattananit et al., 2016), and these cells promote recovery. In injury models, they are specialized for vascular remodeling within the perivascular niche (Olingy et al., 2017).

We were surprised that there was no evidence for the presence of Ly6C<sup>hi</sup> monocytes in the brain after 14 days of CSD. Monocyte recruitment is generally thought not to occur unless there is infection, injury, or damage to the BBB (reviewed in (Lehmann et al., 2016) and (Prinz and Priller, 2017)). However, a form of psychosocial stress termed repeated social defeat (RSD) caused monocytes to enter the brain (Wohleb et al., 2014a; Wohleb et al., 2013). In the RSD paradigm, agonist encounters last for two hours. RSD is characterized by strong peripheral immune activation (Weber et al., 2017) and splenomegaly (Engler et al., 2004).

In general, chronic stress dysregulates the hypothalamic-pituitary-adrenal (HPA) axis, manifested as abnormal adrenal glucocorticoid hormone secretion and disrupted feedback loops (Lehmann et al., 2013a; Lehmann et al., 2013c; van der Kooij et al., 2018). Importantly, high hormone levels lead to glucocorticoid resistance (Foertsch et al., 2017; Niraula et al., 2018), which can free monocytes from inhibitory influences. In addition, physical injury is required for splenomegaly and glucocorticoid resistance in a similar social conflict model (Foertsch et al., 2017). We previously proposed that wounding-associated peripheral endocrine effects allowed glucocorticoid-resistant monocytes to enter the brain (Lehmann et al., 2016).

However, another study employed CSD, similar to our paradigm, with minimal wounding during the ten-minute agonist interaction period, and monocytes were present in the brain as assessed by flow cytometry (Ambree et al., 2018). However, monocytes can adhere to the inner lumen of blood vessels and not be washed out by perfusion, a point also made by Witcher et al. (2019). Such non-extravasated cells would be counted by flow cytometry. For flow cytometry, we exclude these cells by marking them with prior i.v. injection of tagged CD45 antibody. The procedural difference may account for the different findings.

Alternatively, we propose that temporal factors can explain the difference in outcomes. That study (Ambree et al., 2018) examined for monocyte presence in the brain after 10 days of CSD. Similarly, the RSD studies employed only six days of defeat (Wohleb et al., 2013). In the RSD paradigm, monocytes were not found in the brain after 8–10 days of defeats (Witcher et al., 2019). It is thus likely that there is a dynamic temporal landscape of monocyte activity over the course of the procedure. Perhaps the stress-induced elevated glucocorticoid resistance at early times rendered monocytes insensitive to inhibition and permitted their appearance in the brain, but as the animals adapted and lost the corticosterone surge after defeats (Lehmann et al., 2013a), the cells retreated. Our data on endothelial cell biological pathways show that there is a transition between 7 and 14 days from metabolic processes towards angiogenesis, suggesting that vascular repair is the most active function at day 14, perhaps contributing to the later exclusion of monocytes.

In CSDrec, elevated HPA activity rapidly returns to homeostatic levels upon the end of CSD (Lehmann et al., 2013c). Changes in levels of glucocorticoid secretion may drive shifts in chemokine expression patterns, thereby affecting CCR2<sup>−</sup> monocyte trafficking. The CCR2 receptor has several chemokine ligands, and transcriptional elevations in its ligands CCL7 and CCL12 but not CCL2 in vascular endothelia were selectively observed in CSDrec. In culture, the glucocorticoid dexamethasone suppressed CCL7 and CCL12 expression and secretion in brain endothelial cell lines (Burek et al., 2014). The lack of elevation of CCL2 transcription in our model was unexpected because it is a feature of injury (Miro-Mur et al., 2016) and RSD (Wohleb et al., 2014b) paradigms. It is possible that an injury component is needed to trigger this response. Additional studies of chemokine protein levels may help to resolve the differences.

When monocytes enter the brain after RSD, they are primarily restricted to stress-responsive brain regions (Wohleb et al., 2013) where they locally elevate immune activity in microglia that may promote anxiety-like behavior (Reader et al., 2015). In contrast, after 14 days of CSD, there is limited or no leukocyte entry into the brain parenchyma, with no evidence of trafficking to specific anatomical or circuit-specific regions (Lehmann et al., 2020; Lehmann et al., 2018). The absence of elevated CCL2 signaling in brain during or after CSD indicates that microglial CCL2 does not attract leukocyte migration towards specific brain regions, as reported in these studies. Predictably, therefore, knocking out CCR2 signaling did not impart protective effects on behavior in the CSD paradigm (Lehmann et al., 2016), whereas it did in the RSD paradigm (Wohleb et al., 2013).

In addition, in the clodronate administration experiment, monocyte absence during CSD had no effect on USM scores. If monocytes were involved with decline in affective behavior during CSD, we would expect a rescue response from clodronate during CSD; none was observed. If monocytes were removed in CSDrec, then the disrupted behaviors acquired during CSD remained disrupted during recovery. This suggests a beneficial effect of monocyte trafficking on behavioral maintenance. Further time-course studies are needed to determine what role monocytes might play during different durations of psychosocial stress.

In CSDrec, the appearance of Ly6C<sup>hi</sup> monocytes in the brain may represent a de novo appearance of this cell type from the blood. For example, patrolling Ly6C<sup>hi</sup> monocytes attach to veins and clear amyloid in a mouse model of Alzheimer’s disease (Michaud et al., 2013). Alternatively, perhaps during the two weeks of CSD, the cerebrovasculature exists in different stages of inflammation and resolution occurring continuously. Typically, in cases of injury such as ischemia or TBI, there is a conversion of Ly6C<sup>hi</sup> monocytes to Ly6C<sup>lo</sup> profiles (Kratofil et al., 2017) during wounding and wound healing. The microenvironments that bleeds create should exist in different phases of wound resolution that depend on whether the wound occurred early or late in 14-day CSD exposure period. The reparatory process during CSD could be hampered by the repeated cycles of inflammation caused by each successive CSD exposure (Azzinari et al., 2014; Giannarelli et al., 2017; Menard et al., 2017) and may explain why a “monocyte waterfall” is not present at the end of CSD. It is also possible that wounding resolution doesn’t start until late stages of CSD (days 10–14). This would explain why cell expression profiles in the cerebrovasculature at CSD day-14 presents like the initial stages of wounding and angiogenesis (Lehmann et al., 2020).

In our prior studies (Lehmann et al., 2020), the degree of fibrinogen deposition accrued during CSD correlated with the extent of behavioral decline, suggesting that the two are intertwined. Ongoing studies are examining how fibrin deposition leads to inflammatory microenvironments in brain and how these signals may alter neurocircuitry involved with behavioral affect.

In summary, our data show that CSD induces microbleeds and fibrinogen leakage into the brain. During recovery from CSD, these deposits are cleared by peripheral CCR2<sup>−</sup> monocytes that cross the BBB to lodge in perivascular spaces and engage in clearance of debris. The critical role for monocytes in recovery from the effects of CSD was demonstrated by clodronate-induced removal of peripheral monocytes during CSDrec, resulting in diminished fibrinogen clearance and blocked behavioral recovery from the chronic stress effects. Together these findings support a key role for monocytes in behavioral protection.
and vascular repair after stress-induced injury of the cerebrovasculature.

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Appendix A. Supplementary data

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References

Cai, R., Pan, C., Ghasemigharagoz, A., Todorov, M.I., Forstera, B., Zhao, S., Bhatia, H.S., Brachman, R.A., Lehmann, M.L., Maric, D., Herkenham, M., 2015. Lymphocytes from Biewenga, J., van der Ende, M.B., Krist, L.F., Borst, A., Ghufron, M., van Rooijen, N., Chu, H.X., Arumugam, T.V., Gelderblom, M., Magnus, T., Drummond, G.R., Sobey, C.G., Azzinnari, D., Sigrist, H., Staehli, S., Palme, R., Hildebrandt, T., Leparc, G., Hengerer, B., Ambree, O., Ruland, C., Scheu, S., Arolt, V., Alferink, J., 2018. Alterations of the innate immune system in susceptibility and resilience after social stress. Front. Behav. Neurosci. 12, 141.

Auffray, C., Fogg, D., Garfa, M., Elain, G., Join-Lambert, O., Kayal, S., Sarnacki, S., Cumano, A., Lauvau, G., Geissmann, F., 2007. Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. Science 317, 666–670.

Azzinnari, D., Sighist, H., Staelhi, S., Palme, R., Hildebrandt, T., Leparc, G., Geissmann, F., 2007. Macrophage depletion in the rat after intraperitoneal administration of liposome-encapsulated clodronate: depletion kinetics and accelerated repopulation of peritoneal and omental macrophages by administration of Freund’s adjuvant. Cell Tissue Res. 280, 189–196.

Bruchman, R.A., Lehmann, M.L., Marcic, D., Herkenham, M., 2015. Lymphocytes from chronically stressed mice confer antidepressant-like effects to naive mice. J. Neurosci. 35, 1530–1538.

Burek, M., Haghkia, A., Gold, R., Roewer, N., Chan, A., Förster, C.Y., 2014. Differential cytokine release from brain macrovascular endothelial cells treated with dexamethasone and multiple sclerosis patient sera. J. Steroids Horm. Sci. 8, 42–56.

Biewenga, J., van der Ende, M.B., Krist, L.F., Borst, A., Ghufron, M., van Rooijen, N., 1995. Macrophage depletion in the rat after intraperitoneal administration of liposome-encapsulated clodronate: depletion kinetics and accelerated repopulation of peritoneal and omental macrophages by administration of Freund’s adjuvant. Cell Tissue Res. 280, 189–196.

Bruchman, R.A., Lehmann, M.L., Marcic, D., Herkenham, M., 2015. Lymphocytes from chronically stressed mice confer antidepressant-like effects to naive mice. J. Neurosci. 35, 1530–1538.

Ibid., M., Liu, J., Arakawa, N., Kato, M., Tsuda, K., Iwata, K., Matsumoto, M., Katsuyama, M., Zhu, K., Teramukai, S., Furuyushiki, T., Yabe-Hiromasa, C., 2017. Depression results from bilateral intraperitoneal NADPH oxidase by redox modification of NMDA receptor 1. J. Neurosci. 37, 4200–4212.

Kendler, K.S., Karkowski, L.M., Prescott, C.A., 1999. Causal relationship between stressful life events and the onset of major depression. Am. J. Psychiatry 156, 837–841.

Korin, B., Ben-Shaanan, T.L., Schiller, M., Dovnikov, A., Avital, A., Shalit, N., Hoshnat, N.T., Koren, T., Rolls, A., 2017. High-dimensional, single-cell characterization of the brain’s immune compartment. Nat. Neurosci. 20, 1399–1405.

Kratofil, R.M., Kubek, P., Deniset, J.F., 2017. Monocyte conversion during inflammation and injury. Arterioscler. Thromb. Vasc. Biol. 37, 35–42.

Lagade, D.C., Donovan, M.H., de Carlos, N.A., Barnauch, L.A., Malhotra, S., Berton, O., Nishikawa, E.I., Krishnan, V., Nishikawa, V., 2010. Adult hippocampal neurogenesis is functionally important for stress-induced social avoidance. Proc. Natl Acad. Sci. U.S.A. 107, 4436–4441.

Laine, M.A., Sokolovskaya, E., Dudek, M., Callan, S.A., Hyytia, P., Hovatta, I., 2017. Brain monocytes induced by chronic social stress in mice. Sci. Rep. 7, 15601.

Languino, L.R., Plescia, J., Dsuparry, A., Bryan, A.A., Flow, E.F., Gotlaksy, J.E., Albrecht, D.C., 1993. Fibrinogen mediates leukocyte adhesion to vascular endothelium through an ICAM-1-dependent pathway. Cell 73, 1423–1434.

Lehmann, M.L., Bruchman, R.A., Martinowich, K., Schloeser, R.J., Herkenham, M., 2013a. Glucocorticoids orchestrate divergent effects on mood through adult neurogenesis. J. Neurosci. 33, 2961–2972.

Lehmann, M.L., Geddes, C.E., Lee, J.L., Herkenham, M., 2013b. Urine scent marking (USM): a novel test for depressive-like behavior and a predictor of stress resiliency in mice. PLoS ONE 8, e69822.

Lehmann, M.L., Mustafa, T., Eiden, A.M., Herkenham, M., Eiden, L.E., 2013c. PACAP-deficient mice show attenuated corticosterone secretion and fail to develop depressive behavior during chronic social stress. Psychoneuroendocrinology 38, 702–715.

Lehmann, M.L., Cooper, H.A., Marcic, D., Herkenham, M., 2016. Social defeat induces depressive-like states and microglial activation without involvement of peripheral macrophages. J. Neuroinflamm. 13, 224.

Lehmann, M.L., Weigel, T.K., Elkahlon, A.G., Herkenham, M., 2017. Chronic social defeat reduces myelination in the mouse medial prefrontal cortex. Sci. Rep. 7, 6565.

Lehmann, M.L., Weigel, T.K., Cooper, H.A., Elkahlon, A.G., Kigar, S.L., Herkenham, M., 2018. Decoding microglia responses to psychosocial stress reveals blood-brain barrier breakdown that may drive stress susceptibility. Sci. Rep. 8, 11240.

Lehmann, M.L., Weigel, T.K., Poffenberger, C., Herkenham, M., 2019. The behavioral sequelae of social defeat are microglia and are driven by oxidative stress in mice. J. Neurosci. 39, 5954–5960.

Lehmann, M.L., Poffenberger, C.N., Elkahlon, A.G., Herkenham, M., 2020. Analysis of cerebrovascular dysfunction caused by chronic social defeat in mice. Brain Behav. Immun. 88, 735–747.
Lin, H., Xu, L., Yu, S., Hong, W., Huang, M., Xu, P., 2020. Therapeutics targeting the fibrinolytic system. Exp. Mol. Med. 52, 367–379.
Lin, C., Wu, C., Yang, Q., Gao, J., Li, L., Yang, D., Luo, L., 2016. Macrophages mediate the repair of brain vascular rupture through direct physical adhesion and mechanical traction. Immunity 44, 1162–1176.
Lu, H., Huang, D., Saederup, N., Charo, I.F., Ransohoff, R.M., Zhou, L., 2011. Macrophages recruited via CCR2 produce insulin-like growth factor-1 to repair acute skeletal muscle injury. FASEB J. 25, 358–369.
Lynall, M.E., Kigar, S.L., Lehmann, M.L., DePuyt, A.E., Tsu, K., Listwak, S.J., Elkahab, A.G., Bullmore, E.T., Herkenham, M., Catzow, M.R., 2021. B-cells are abnormal in psychosocial stress and regulate meningeal myeloid cell activation. Brain Behav. Immun. 97, 226–238.
Mastorakos, P., Mihelis, N., Luby, M., Burke, S.R., Johnson, K., Hsieh, A.W., Witko, J., Frank, J.A., Latour, L., McGavern, D.B., 2021. Temporally distinct myeloid cell responses mediate damage and repair after cerebralischemic injury. Nat. Neurosci. 24, 245–258.
Menard, C., Pfu, M.L., Hodes, G.E., Kana, V., Wang, V.X., Bouchard, S., Takahashi, A., Flanigan, M.E., Aleyasin, H., LeClair, K.B., Janssen, W.G., Labonte, B., Parise, E.M., Luby, M., Chen, D., Yang, Y., Zheng, Y., Hui, R., 2012. Depression increases the risk of neurovascular pathology promoting depression. Nat. Neurosci. 20, 1752–1760.
Meng, L., Chen, D., Yang, Y., Zheng, Y., Hui, R., 2012. Depression increases the risk of hypertension incidence: a meta-analysis of prospective cohort studies. J. Hypertens. 30, 842–851.
Michaud, J.P., Bellavance, M.A., Prefontaine, P., Rivest, S., 2013. Real-time in vivo imaging reveals the ability of monocytes to clear vascular amyloid beta. Cell Rep. 5, 646–653.
Mildner, A., Schlevogt, B., Kierdorf, K., Bottcher, C., Erny, D., Kummer, M.P., Quinn, M., Bruck, W., Rehmann, I., Heneka, M.T., Friller, J., Frinz, M., 2011. Distinct and non-redundant roles of microglia and myeloid subsets in mice models of Alzheimer’s disease. J. Neurosci. 31, 11159–11171.
Miro-Mur, F., Perez-de-Puig, I., Ferrer-Ferrero, M., Urra, X., Justicia, C., Chamorro, A., Planas, A.M., 2016. Immature monocytes recruited to the ischemic mouse brain differentiate into macrophages with features of alternative activation. Brain Behav. Immun. 53, 18–33.
Mitchell, C., Couton, D., Couty, J.P., Amon, M., Crain, A.M., Bizet, V., Renia, L., Pol, S., Matley, A., Olingy, C.E., San Emeterio, C.L., Ogle, M.E., Krieger, J.R., Bruce, A.C., Pfau, D.D., M.L. Lehmann et al.
Mildner, A., Schlevogt, B., Kierdorf, K., Bottcher, C., Erny, D., Kummer, M.P., Quinn, M., Bruck, W., Rehmann, I., Heneka, M.T., Friller, J., Frinz, M., 2011. Distinct and non-redundant roles of microglia and myeloid subsets in mice models of Alzheimer’s disease. J. Neurosci. 31, 11159–11171.
Miro-Mur, F., Perez-de-Puig, I., Ferrer-Ferrero, M., Urra, X., Justicia, C., Chamorro, A., Planas, A.M., 2016. Immature monocytes recruited to the ischemic mouse brain differentiate into macrophages with features of alternative activation. Brain Behav. Immun. 53, 18–33.
Mitchell, C., Couton, D., Couty, J.P., Amon, M., Crain, A.M., Bizet, V., Renia, L., Pol, S., Matley, A., Olingy, C.E., San Emeterio, C.L., Ogle, M.E., Krieger, J.R., Bruce, A.C., Pfau, D.D., M.L. Lehmann et al.
Mildner, A., Schlevogt, B., Kierdorf, K., Bottcher, C., Erny, D., Kummer, M.P., Quinn, M., Bruck, W., Rehmann, I., Heneka, M.T., Friller, J., Frinz, M., 2011. Distinct and non-redundant roles of microglia and myeloid subsets in mice models of Alzheimer’s disease. J. Neurosci. 31, 11159–11171.
Miro-Mur, F., Perez-de-Puig, I., Ferrer-Ferrero, M., Urra, X., Justicia, C., Chamorro, A., Planas, A.M., 2016. Immature monocytes recruited to the ischemic mouse brain differentiate into macrophages with features of alternative activation. Brain Behav. Immun. 53, 18–33.
Mitchell, C., Couton, D., Couty, J.P., Amon, M., Crain, A.M., Bizet, V., Renia, L., Pol, S., Matley, A., Olingy, C.E., San Emeterio, C.L., Ogle, M.E., Krieger, J.R., Bruce, A.C., Pfau, D.D., M.L. Lehmann et al.
Mildner, A., Schlevogt, B., Kierdorf, K., Bottcher, C., Erny, D., Kummer, M.P., Quinn, M., Bruck, W., Rehmann, I., Heneka, M.T., Friller, J., Frinz, M., 2011. Distinct and non-redundant roles of microglia and myeloid subsets in mice models of Alzheimer’s disease. J. Neurosci. 31, 11159–11171.
Miro-Mur, F., Perez-de-Puig, I., Ferrer-Ferrero, M., Urra, X., Justicia, C., Chamorro, A., Planas, A.M., 2016. Immature monocytes recruited to the ischemic mouse brain differentiate into macrophages with features of alternative activation. Brain Behav. Immun. 53, 18–33.
Mitchell, C., Couton, D., Couty, J.P., Amon, M., Crain, A.M., Bizet, V., Renia, L., Pol, S., Matley, A., Olingy, C.E., San Emeterio, C.L., Ogle, M.E., Krieger, J.R., Bruce, A.C., Pfau, D.D., M.L. Lehmann et al.
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