Sympathetic Release of Splenic Monocytes Promotes Recurring Anxiety Following Repeated Social Defeat

Supplemental Information

Supplementary Methods and Materials

Mice

Male C57BL/6 (6–8 weeks old) and CD-1 (12 months old) mice were purchased from Charles River Breeding Laboratories (Wilmington, MA) and allowed to acclimate to their surroundings for 7–10 d before initiation of any experimental procedures. C57BL/6 mice were housed in cohorts of three and CD-1 mice were singly housed. All mice were housed in 11.5 × 7.5 × 6 inch polypropylene cages. Rooms were maintained at 21°C under a 12 h light/dark cycle with ad libitum access to water and rodent chow. All procedures were in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee.

Repeated Social Defeat (RSD)

Mice were subjected to RSD as previously described [1]. In brief, an aggressive intruder male CD-1 mouse (retired breeder) was introduced into cages of established male cohorts (three per cage) of C57BL/6 mice for 6 consecutive nights between 1700 and 1900 (2 h). During each cycle, submissive behaviors including upright posture, fleeing, and crouching were observed to ensure that the resident mice showed subordinate behavior. If the intruder did not initiate a defeat within 5–10 min or was defeated by any of the resident mice, then a new intruder was introduced. At the end of the 2 h period, the intruder was removed and the residents were left

undisturbed until the following day when the paradigm was repeated. Different intruders were used on consecutive nights. The health status of the mice was examined throughout the paradigm and injured or moribund mice were removed from the study. Consistent with previous studies using RSD, <5% of mice met the early removal criteria [1]. Control mice were left undisturbed in their home cages. As previously described [2], to study the sensitizing effects of RSD, mice were either exposed to control (naïve) or RSD conditions (stress-sensitized, SS), and 24 days later naïve and SS mice were subjected to an additional cycle of social defeat. All behavior and biological measures were obtained 14 h after the final cycle. This time point was chosen because SNS and HPA activation returns to baseline by 14 hours after the final cycle [3].

Splenectomy

As previously described [2], mice were anesthetized with isoflurane and the spleen was removed through a small incision in the abdomen. Splenic nerves and vasculature were cauterized. Incisions were closed with sutures and surgical staples. Surgeries were performed in sterile conditions and tools were sterilized with hot bead sterilizer (Fine Science Tools). To relieve pain, mice received subcutaneous injection of buprenorphine (0.05 mg/ml) immediately after surgery and 24 hours later. Sham surgery involved abdominal incision without removal of the spleen. To allow time to heal, splenectomy was performed 14 days before the beginning of experiments.

Drug Treatments

Mice were injected subcutaneously with either vehicle (100 uL sterile H_2O), 50 mg/kg guanethidine (Santa Cruz Biotechnology), or 10 mg/kg propranolol (Sigma). For guanethidine, mice were injected 24 hours prior to acute social defeat. For propranolol, mice were injected 1 h

prior to acute social defeat. Dosage and injection regimen of guanethidine were selected based on a previous report [4]. Propranolol dose and regimen were selected based on our previous reports [3, 5].

Anxiety-Like Behavior

Anxiety-like behavior was determined using open-field activity as previously described [1, 2, 5]. The open field test was used to assess anxiety-like behavior in these studies because our previous work shows that it is a robust and reproducible behavioral test in the context of social defeat [1, 2, 5-8]. Furthermore, measures of thigmotaxis in the open field test demonstrate high degrees of validity for modeling anxiety-like behaviors (reviewed by [9]). For the open-field test, mice were placed in the corner of the test apparatus (40 × 40 × 25 cm Plexiglas box) and activity was recorded for 5 min. Mice with anxiety-like behavior enter the center of the open-field slower and spend less time in the open field. Behavior was recorded and analyzed using an automated system (VersaMax, AccuScan Instruments).

Isolation of Cells from BM, Spleen, and Blood

Tissues were collected immediately following CO_2 asphyxiation. Whole blood was collected with EDTA-lined syringes by cardiac puncture. Spleens were collected in ice-cold Hanks' balanced salt solution (HBSS) and mechanically disrupted to obtain single cell suspensions. To collect bone marrow, the epiphyses of femurs were cut off and the marrow was flushed out with ice-cold HBSS. Red blood cells were lysed in spleen and blood samples. Cell pellets were washed, filtered through a 70-μm nylon cell strainer, and then the total number of cells was determined with a BD Coulter Particle Count and Size Analyzer (Beckman Coulter).

Isolation of Brain CD11b$^+$ Cells

CD11b$^+$ cells were isolated from whole-brain homogenates as described previously [1, 2, 5]. In brief, brains were passed through a 70 μm cell strainer. Homogenates were centrifuged at 600 × *g* for 6 min. Supernatants were removed and cell pellets were resuspended in 70% isotonic Percoll (GE-Healthcare). A discontinuous Percoll density gradient was layered as follows: 50, 35, and 0% isotonic Percoll. The gradient was centrifuged for 20 min at 2000 × *g* and cells were collected from the interphase between the 70 and 50% Percoll layers. These cells were referred to as enriched brain CD11b$^+$ cells based on previous studies demonstrating that viable cells isolated by Percoll density gradient yields >90% CD11b$^+$ cells [1, 5].

Flow Cytometry

Staining of cell surface antigens was performed as previously described [1, 5]. In brief, Fc receptors were blocked with anti-CD16/CD32 antibody (eBioscience). Cells were washed and then incubated with the appropriate antibodies (CD45, CD11b, CD115, eBioscience; Ly6C, BD Biosciences) for 1 h at 4°C. Cells were washed and then resuspended in FACS buffer for analysis. Nonspecific binding was assessed using isotype-matched antibodies. Antigen expression was determined using a Becton-Dickinson FACSCalibur four-color cytometer (BD Biosciences). Data were analyzed using FlowJo software (Tree Star) and positive labeling for each antibody was determined based on isotype stained controls.

Ex Vivo Culture with LPS and Corticosterone

As previously described [3], bone marrow and spleen cells were suspended in RPMI 1640 medium (supplemented with 10% FBS, 10 mM HEPES, 1.5 mM l-glutamine, 8.9 mM NaHCO3,

100 U/ml penicillin G and 100 μg/ml streptomycin) and were cultured at a concentration of 10^6 cells per mL. Cells were either untreated or treated with combinations of 1 μg/ml lipopolysaccharide (LPS) from Escherichia coli (serotype 0127:B8, Sigma-Aldrich, St. Louis, Missouri) and various concentrations of corticosterone (Sigma-Aldrich) including both physiological and pharmacological doses. The corticosterone concentrations were 0.005, 0.05, 0.1, 0.5, and 5 μM, respectively. Triplicates of each treatment were incubated in a humidified incubator (37°C, 5% CO_2). Cells were incubated 18 hours for supernatant cytokine measurements or 48 hours for assessment of cell viability.

Cell Viability Assay

As previously described [3], the Cell Titer 96 aqueous nonradioactive proliferation assay (Promega; Madison, WI) was used to determine cell viability in cultures incubated for 48 hours. The tetrazolium substrate solution (20 μl) was added to each well of the 96-well plates. The plates were further incubated at 37°C in 5% CO_2 for 3 h, and the resulting color changes were quantified by obtaining optical density (OD) readings at 490 nm on an ELISA plate reader. To account for differences in background activity of cells, the mean OD of three control wells were subtracted for a given treatment from each of the corresponding LPS-stimulated values. Control wells contained untreated cells.

IL-6 ELISA

As previously described [3], supernatants were harvested at 18 h post culture and quantified for IL-6 by standard sandwich ELISA. For IL-6 determination, rat anti-mouse antibody was used and preformed per manufacturer's instructions (BD Pharmingen; San Diego, CA).

Norepinephrine ELISA

Similar to previously reported methods [10], spleen tissue was homogenized in 0.01 M HCl at 2% volume per spleen tissue weight. Norepinephrine was determined by ELISA (Rocky Mountain Diagnostics) according to manufacturer's instructions at dilution of 1:16.

RNA Isolation and Real-Time PCR

RNA was isolated from homogenized brain regions using tri-reagent/isopropanol precipitation, RNA was reverse transcribed to cDNA using an RT-RETROscript kit (Ambion) and RNA concentration was determined by spectrophotometry (Eppendorf). Quantitative PCR was performed using the Applied Biosystems Assay-on-Demand Gene Expression protocol as previously described [1, 2, 5, 7]. In brief, experimental cDNA was amplified by real-time PCR where a target cDNA (e.g., IL-1β, CCL2, IL-6) and a reference cDNA [glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] were amplified simultaneously using an oligonucleotide probe with a 5′ fluorescent reporter dye (6-FAM) and a 3′ quencher dye (nonfluorescent quencher). Fluorescence was determined on an ABI PRISM 7300-sequence detection system (Applied Biosystems). Data were analyzed using the comparative threshold cycle method and results are expressed as fold difference from GAPDH.

Statistical Analysis

To test for normal distribution, data were subjected to Shapiro–Wilk test using Statistical Analysis Systems (SAS) statistical software. Observations >3 interquartile ranges from the first and third quartile were considered outliers and were excluded in the subsequent analysis. To determine significant main effects and interactions between main factors, data were analyzed

using two-way (stress × treatment) ANOVA using the General Linear Model procedures of SAS. When there was a main effect of experimental treatment or a treatment interaction effect, differences between group means were evaluated by an F-protected *t* test using the Least-Significant Difference procedure of SAS. All data are expressed as treatment means ± SEM.

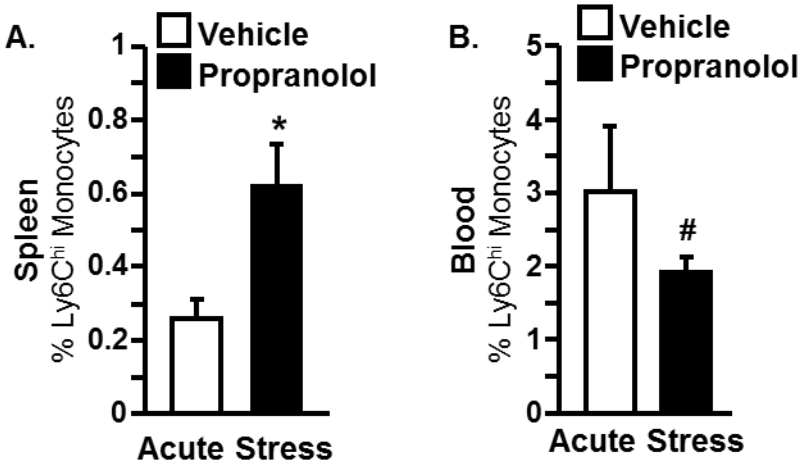

Figure S1. Propranolol prevents monocyte release in SS mice following acute stress. Male C57BL/6 mice were stress-sensitized (SS) by 6 repeated cycles of social defeat. Twenty four days later, mice were pretreated with vehicle or propranolol (10 mg/kg s.c.) 1 hour before acute social defeat. Flow cytometry was completed 14 h later**.** The percentage of $Ly6C^{hi}$ monocytes was determined in spleen **(A)** and blood **(B)**. Means with asterisk (*) are significantly different from CON ($p < 0.05$) and means with (#) tended to be different from CON ($p < 0.1$), according to *t*-test.

Supplemental References

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