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

Supplemental Information

Supplementary Methods

Antibodies used for FACS separation of blood leukocytes

Ly6C (clone HK1.4, eBiosciences, San Diego, CA), B220 (clone RA3-6B2, eBiosciences), CD11b (clone M1/70, eBiosciences), Ly6g (clone 1A8, BioLegend), CD90.2 (clone 53-2.1, eBiosciences), and CD45 (clone 30-F11, eBiosciences). Antibodies used for flow confirmation of MC-21 depletion were as follows: Ly6c (clone HK1.4, eBiosciences), B220 (clone RA3-6B2, eBiosciences), CD11b (clone M1/70, eBiosciences), CCR2 (clone FAB5538P, R&D, Minneapolis, MN), CD115 (clone AFS98, eBiosciences), CD45 (clone 30-F11, eBiosciences), Ly6g (clone 1A8, BioLegend), and CD3 (clone 17A2, BioLegend). Antibodies used for flow chimera analysis included: Ly6c (clone HK1.4, eBiosciences), B220 (clone RA3-6B2, eBiosciences), CD11b (clone M1/70, eBiosciences), CCR2 (clone FAB5538P, eBiosciences), CD115 (clone AFS98, eBiosciences), CD45.1 (clone A20, eBiosciences), CD45.2 (clone 104, eBiosciences), Ly6g (clone 1A8, BioLegend), and CD3 (clone 17A2, BioLegend). Beads were included for absolute quantification of cells in flow cytometry experiments, and 4,6-diamidino-2-phenylindole (DAPI) staining was used to exclude dead cells.

Calculation of social interaction ratio

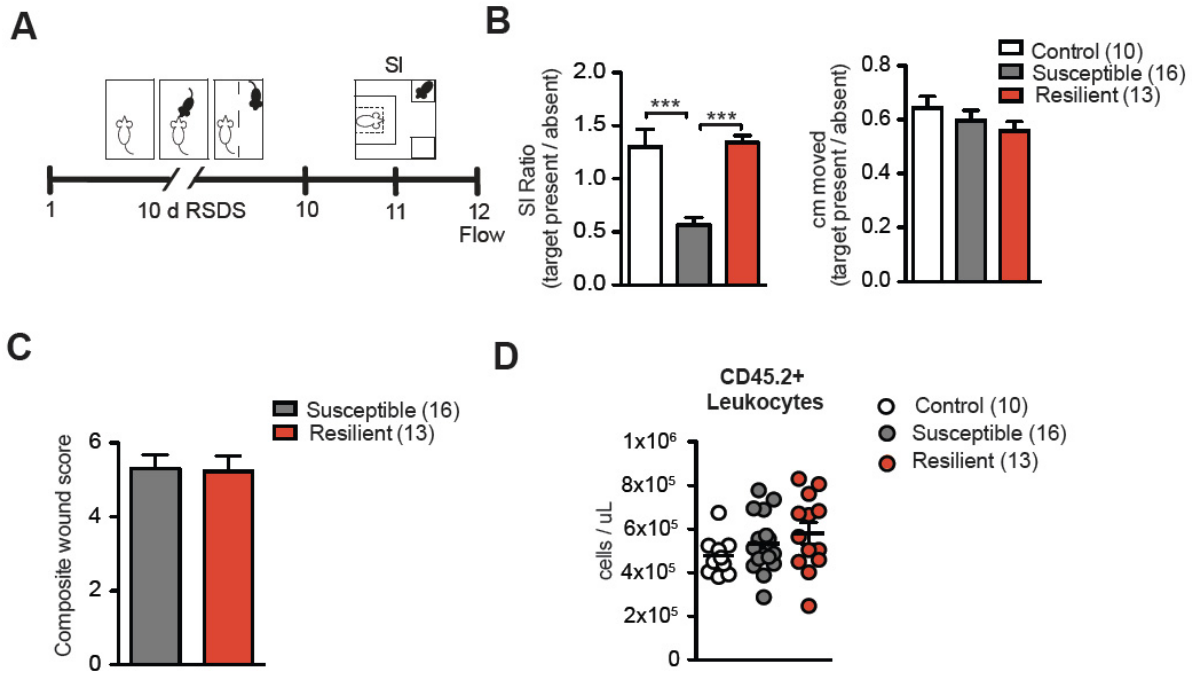
Phenotype was determined by calculation of SI ratio. SI ratio = time spent in the vicinity of the wire enclosure when it contained a novel CD-1 mouse / time spent in the vicinity of

the wire enclosure when it was empty. Animals with an SI ratio above 1 showed a preference for interacting with the novel CD-1 mouse and were classified as resilient. Animals with an SI ratio below 1 displayed avoidance of the social target and were classified as susceptible. Mice were removed as outliers due to abnormal locomotion and SI ratio.

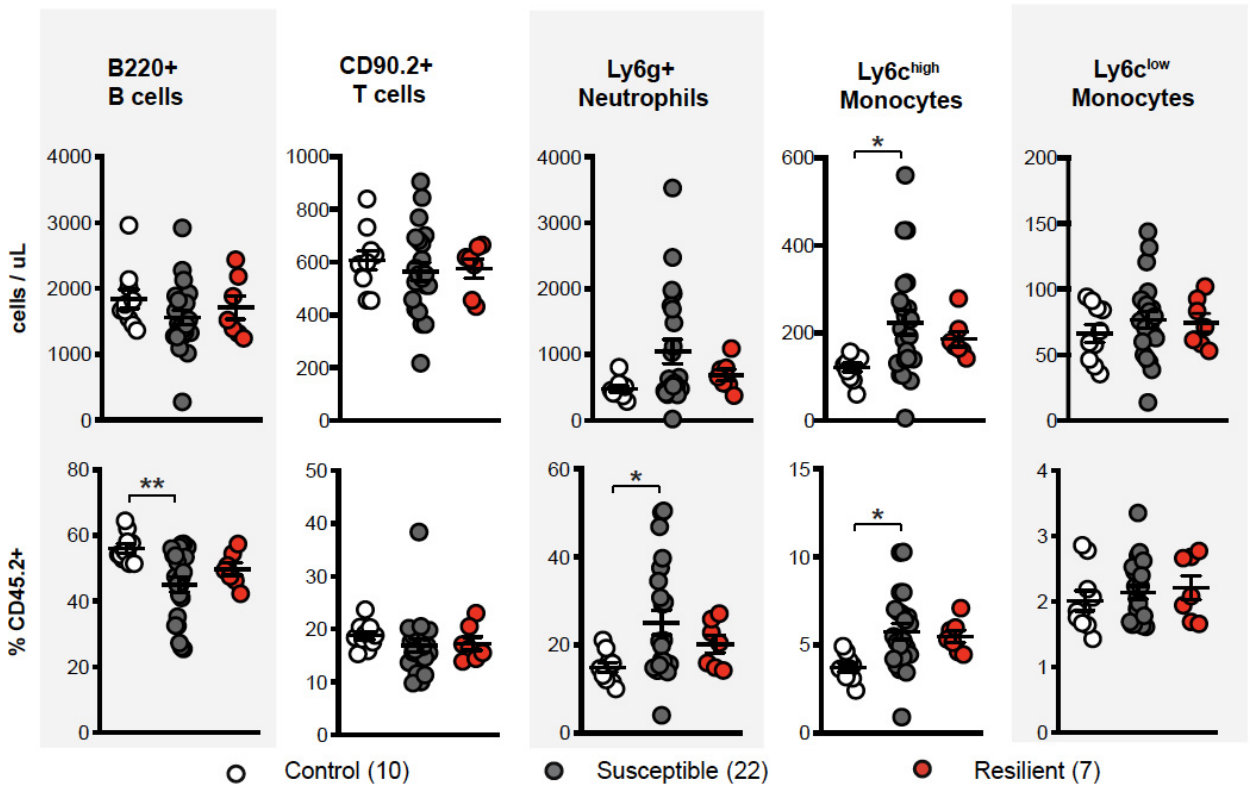
Analysis of PCR data

Raw data was processed using GenEx qPCR analysis software (Exiqon) with input from technical support staff. Briefly, raw data was imported into GenEx, interplate calibration was performed, and outlier tests were conducted against technical replicates and artificial Spike-Ins. Values less than 5 Ct from the no template control were removed. A Ct cut-off was set to remove values >38 and replace with blank values. Unamplified wells were replaced with blank values. Assays with only out-of-range data or with very low amplification were excluded from further analysis. Missing values were imputed based upon replicate values. Expression values were then normalized to four stably expressed reference assays identified using the NormFinder tool (mmu-miR-223-3p, 5S rRNA, Snord68, and miR-21a-5p). Still missing values were imputed based upon biological group, technical replicates were averaged, and expression data were normalized to the control group. Data were then log transformed for subsequent statistical analysis. Visual representations of gene expression changes were performed using MATLAB software. Individual values were used to compute correlation matrices and p values were also determined using MATLAB software.

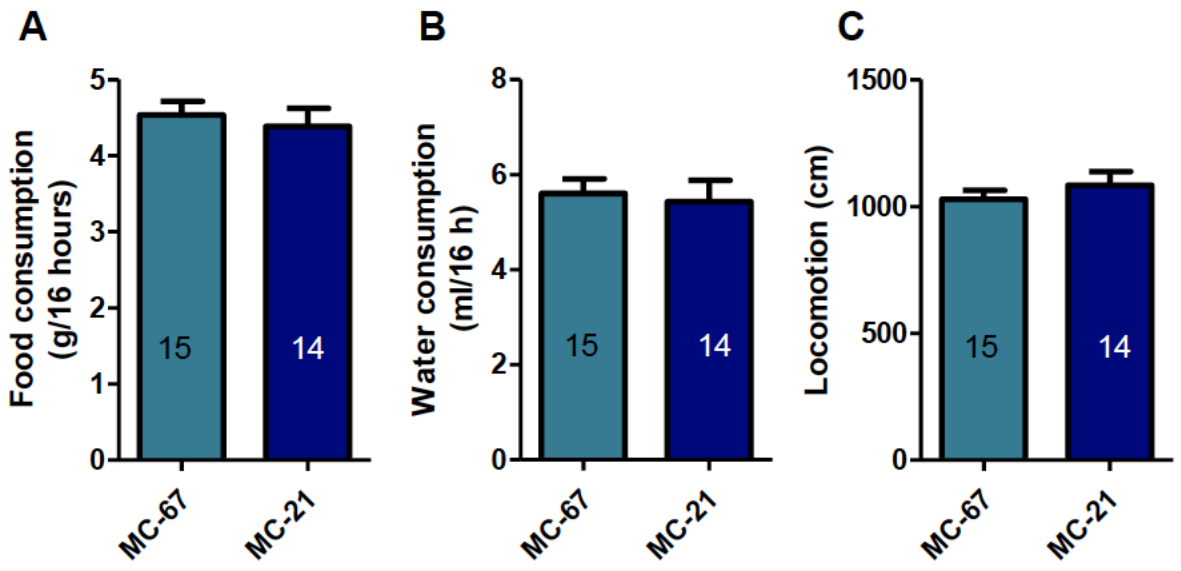
Supplementary Figures and Tables



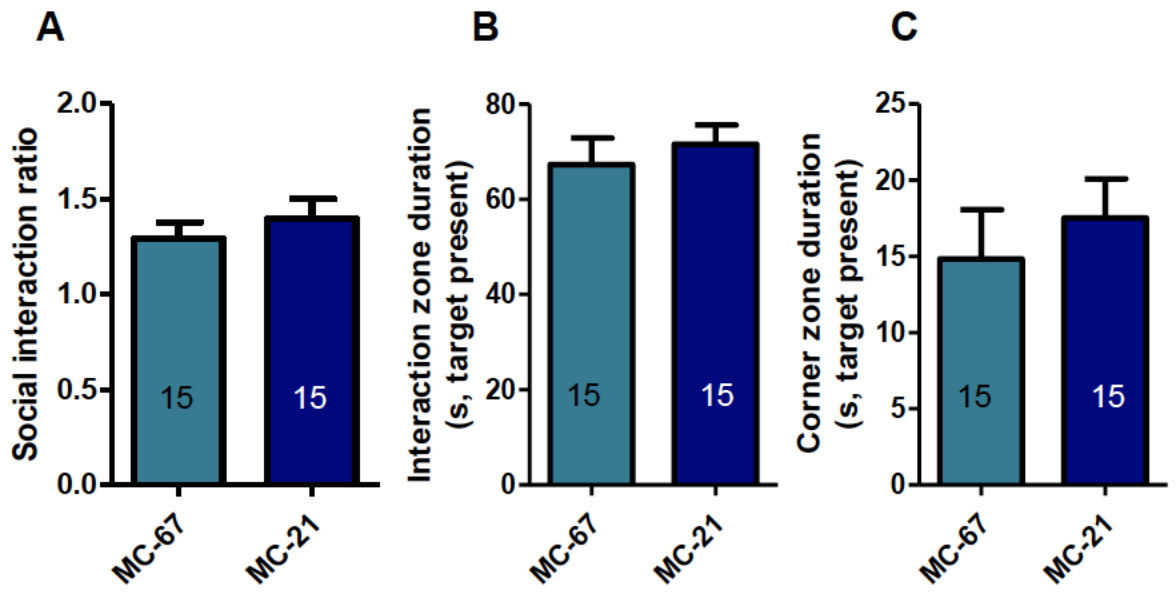
Supplementary Figure S1. Repeated social defeat stress for flow cytometry. (A) Experimental timeline of 10-day repeated social defeat stress (RSDS), social interaction (SI) testing, and flow cytometry analysis (flow). (B) Susceptible mice exhibited lower SI ratios than control and resilient mice (left). The three groups did not exhibit differences in distance traveled ratios (right). (C) Composite wound scores did not differ between susceptible and resilient groups 48 hours after RSDS. (D) Overall leukocyte count did not differ between susceptible, resilient, and control groups. For bar graphs, data represent mean ± SEM. For dot plots, data represent mean ± SEM. The number of animals (*n*) within each group is indicated beside the graphs. ***p<0.0001 (one-way ANOVA with Tukey's post hoc tests).



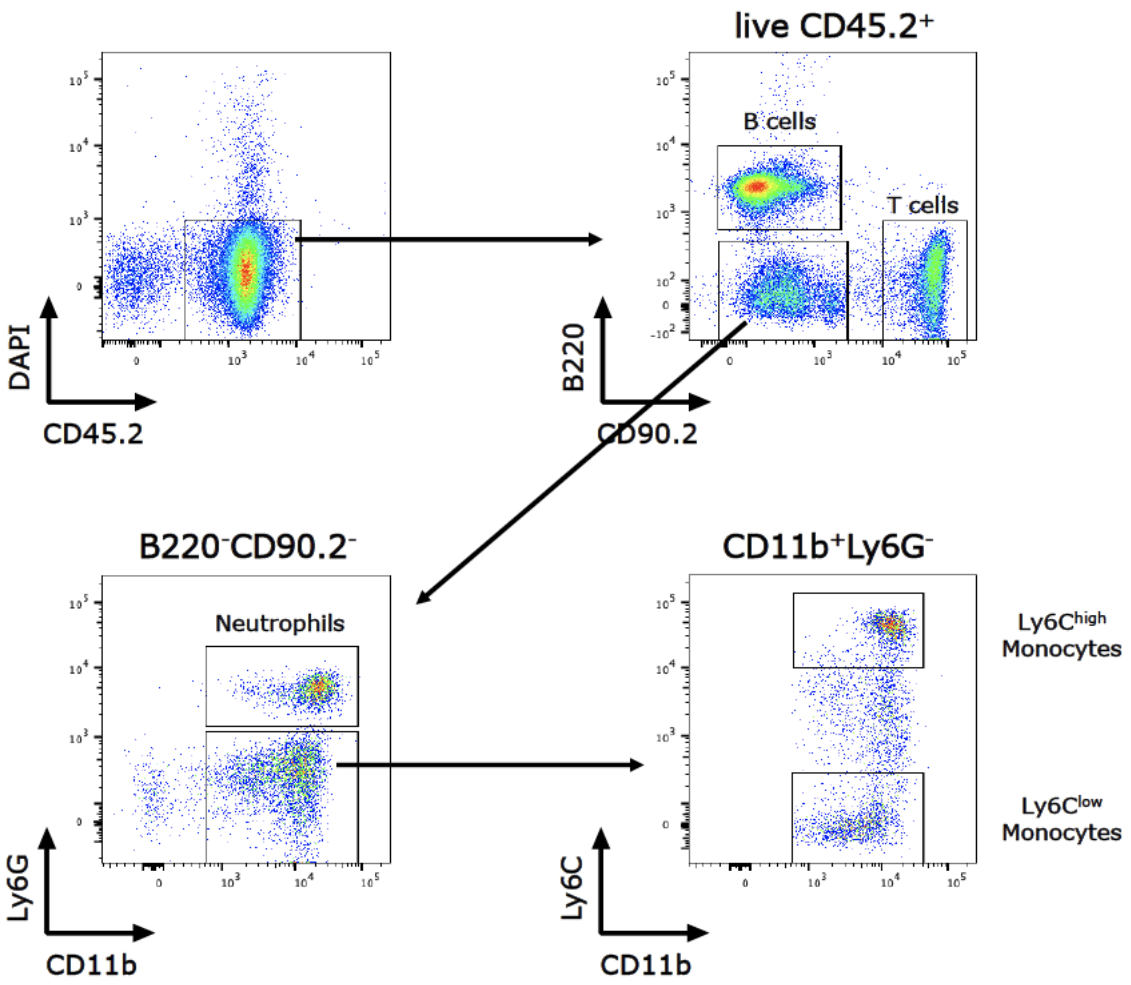
Supplementary Figure S2. Flow cytometry of leukocytes following RSDS. Absolute counts (top) and relative frequencies (bottom) of B cells, T cells, neutrophils, Ly6c^{high} monocytes, and Ly6c^{low} monocytes in peripheral blood 9 days post RSDS. Data represent mean ± SEM. *p<0.05, **p<0.01 (one-way ANOVA with Tukey's post hoc tests).



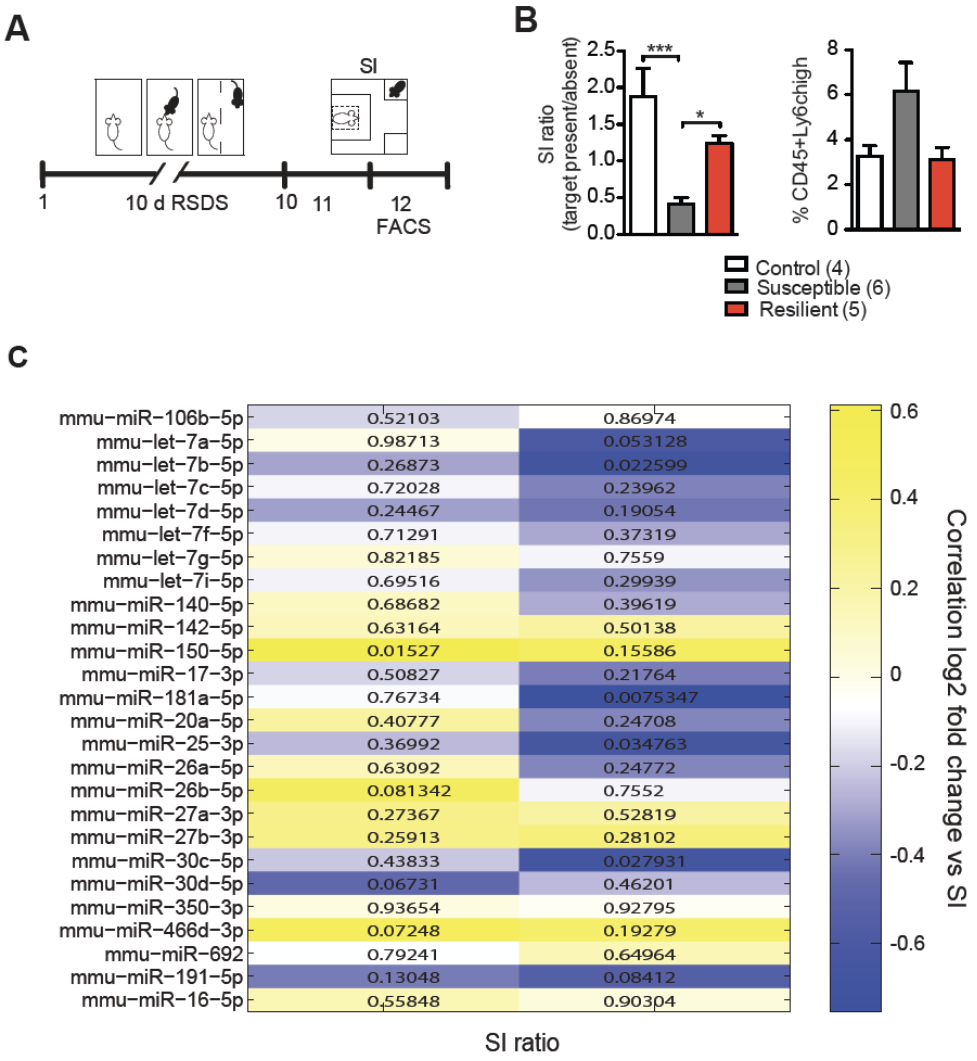
Supplementary Figure S3. Effects antibody treatment on sickness behavior. Antibody treatment during repeated social defeat stress did not induce sickness behavior as measured by A) food consumption, B) water consumption or C) locomotion. Data represent mean + SEM. All $p > 0.3$.



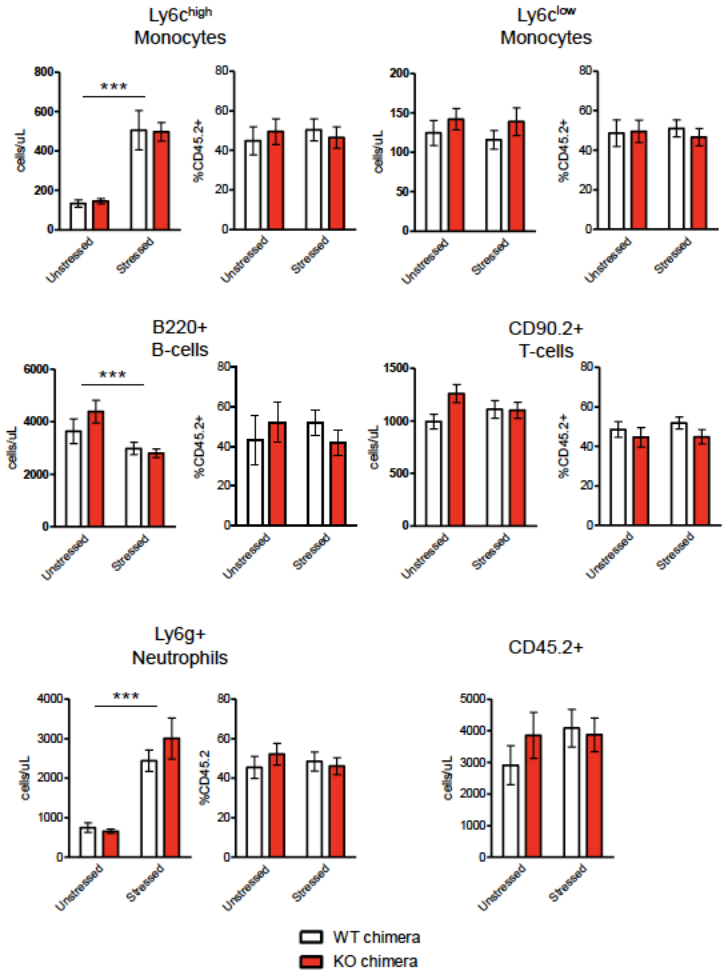
Supplementary Figure S4. Effects of antibody treatment on social interactions. In unstressed mice, 5 days of MC-21 or MC-67 administration had no effect on social interaction behavior. Data represent mean + SEM. All $p > 0.4$.



Supplementary Figure S5. Gating strategy for Fluorescence-Activated Cell Sorting (FACS). FACS was performed 48 hours after the last defeat, and gated for live (DAPI-) CD45.2+B220-CD90.2-Ly6G-CD11b+Ly6C^{high} monocytes to isolate small RNAs for microRNA profiling.



Supplementary Figure S6. microRNA profiling in sorted Ly6c^{high} monocytes 48 hours after repeated social defeat stress (RSDS). (A) Experimental timeline of 10-day RSDS, social interaction (SI) testing, and collection of blood leukocytes by Fluorescence-Activated Cell Sorting (FACS). (B) SI ratio (left) and frequency of circulating Ly6c^{high} monocytes (right) of mice included in FACS analysis. (C) Heat map showing correlation of miR expression with SI ratio. Blue color is associated with a negative correlation, white with no change and yellow with a positive correlation. P values for individual miR expression vs SI ratio correlation is shown within the box with all mice included on the left and only stressed – susceptible and resilient – mice on the right.



Supplementary Figure S7. Cell type specific chimerism data for miR-106b~25$^{-/-}$ and WT (miR-106b~25$^{+/+}$) chimeras. Level of chimerism for each leukocyte population is shown as proportion of CD45.2+ cells as well as absolute cell counts for each cell population. WT and miR-106b~25$^{-/-}$ chimeras show no differences in leukocyte subpopulation frequencies or in absolute counts. On the other hand, stress effects are observed for absolute cell counts of neutrophils, B-cells and Ly6c^{high} monocytes. For bar graphs, data represent mean ± SEM.***p<0.0001.

Supplementary Table S1. Monocyte microRNA (miR) panel. miRs and reference assays included in the qPCR panel and their target sequences when applicable.

microRNA	Target Sequence
mmu-miR-106b-5p	UAAAGUGCUGACAGUGCAGAU
mmu-let-7a-5p	UGAGGUAGUAGGUUGUAUAGUU
mmu-let-7b-5p	UGAGGUAGUAGGUUGUGUGGUU
mmu-let-7c-5p	UGAGGUAGUAGGUUGUAUGGUU
mmu-let-7d-5p	AGAGGUAGUAGGUUGCAUAGUU
mmu-let-7e-5p	UGAGGUAGGAGGUUGUAUAGUU
mmu-let-7f-5p	UGAGGUAGUAGAUUGUAUAGUU
mmu-let-7g-5p	UGAGGUAGUAGUUUGUACAGUU
mmu-let-7i-5p	UGAGGUAGUAGUUUGUGCUGUU
mmu-miR-10a-5p	UACCCUGUAGAUCCGAAUUUGUG
mmu-miR-140-5p	CAGUGGUUUUACCCUAUGGUAG
mmu-miR-142-5p	CAUAAAGUAGAAAGCACUACU
mmu-miR-146a-5p	UGAGAACUGAAUUCCAUGGGUU
mmu-miR-146b-5p	UGAGAACUGAAUUCCAUAGGCU
mmu-miR-148a-3p	UCAGUGCACUACAGAACUUUGU
mmu-miR-150-5p	UCUCCCAACCCUUGUACCAGUG
mmu-miR-17-3p	ACUGCAGUGAGGGCACUUGUAG
mmu-miR-181a-5p	AACAUUCAACGCUGUCGGUGAGU
mmu-miR-181c-5p	AACAUUCAACCUGUCGGUGAGU
mmu-miR-181d-5p	AACAUUCAUUGUUGUCGGUGGGU
mmu-miR-200c-3p	UAAUACUGCCGGGUAAUGAUGGA
mmu-miR-20a-5p	UAAAGUGCUUAUAGUGCAGGUAG
mmu-miR-20b-5p	CAAAGUGCUCAUAGUGCAGGUAG
mmu-miR-21a-5p	UAGCUUAUCAGACUGAUGUUGA
mmu-miR-223-3p	UGUCAGUUUGUCAAAUACCCCA
mmu-miR-25-3p	CAUUGCACUUGUCUCGGUCUGA
mmu-miR-26a-5p	UUCAAGUAAUCCAGGAUAGGCU
mmu-miR-26b-5p	UUCAAGUAAUUCAGGAUAGGU
mmu-miR-27a-3p	UUCACAGUGGCUAAGUUCCGC
mmu-miR-27b-3p	UUCACAGUGGCUAAGUUCUGC
mmu-miR-30c-5p	UGUAAACAUCCUACACUCUCAGC
mmu-miR-30d-5p	UGUAAACAUCCCCGACUGGAAG
mmu-miR-330-3p	GCAAAGCACAGGGCCUGCAGAGA
mmu-miR-350-3p	UUCACAAAGCCCAUACACUUUC
mmu-miR-455-5p	UAUGUGCCUUUGGACUACAUCG
mmu-miR-466d-3p	UAUACAUACACGCACACAUAG
mmu-miR-683	CCUGCUGUAAGCUGUGUCCUC
mmu-miR-692	AUCUCUUUGAGCGCCUCACUC
mmu-miR-693-3p	GCAGCUUUCAGAUGUGGCUGUAA
mmu-miR-98-5p	UGAGGUAGUAAGUUGUAUUGUU
mmu-miR-155-5p	UUAAUGCUAAUUGUGAUAGGGGU
mmu-miR-191-5p	CAACGGAAUCCCAAAAGCAGCUG
mmu-miR-16-5p	UAGCAGCACGUAAAUAUUGGCG
mmu-miR-701-5p	UUAGCCGCUGAAAUAGAUGGA
5S rRNA	
SNORD68	

Supplementary Table S2. microRNA profiling in sorted Ly6c^{high} monocytes 48 hours after repeated social defeat stress. miRs regulated by Repeated Social Defeat Stress (RSDS) as determined by univariate ANOVA analysis.

mmu-miR	Susceptible		Resilient		F ratio	N
	fold change	log$_2$(fold change)	fold change	log$_2$(fold change)		
let-7a-5p	1.29 ± 0.23	0.24 ± 0.27[b]	0.61 ± 0.02	-0.71 ± 0.05[c]	$F_{2,12} = 4.38$ $p < 0.05$	C (4) R (5) S (6)
let-7b-5p	2.74 ± 0.62	1.25 ± 0.35[b]	0.73 ± 0.16	-0.62 ± 0.38[c]	$F_{2,12} = 4.37$ $p < 0.05$	C (4) R (5) S (6)
let-7d-5p	3.50 ± 0.70	1.66 ± 0.29[a]	1.89 ± 0.25	0.87 ± 0.20	$F_{2,12} = 5.67$ $p < 0.05$	C (4) R (5) S (6)
150-5p	0.41 ± 0.08	-1.4 ± 0.24[a]	0.70 ± 0.12	-0.63 ± 0.27[d]	$F_{2,12} = 8.40$ $p < 0.01$	C (4) R (5) S (6)
181a-5p	0.97 ± 0.12	-0.10 ± 0.18[b]	0.46 ± 0.07	-1.20 ± 0.21[a,c]	$F_{2,12} = 13.27$ $p < 0.001$	C (4) R (5) S (6)
25-3p	3.51 ± 0.90	1.54 ± 0.41[e]	1.04 ± 0.26	-0.12 ± 0.35[f]	$F_{2,12} = 4.18$ $p < 0.05$	C (4) R (5) S (6)
466d-3p	0.49 ± 0.13	-1.35 ± 0.45[a]	0.82 ± 0.08	-0.32 ± 0.14	$F_{2,12} = 4.21$ $p < 0.05$	C (4) R (5) S (6)
191-5p	1.74 ± 0.25	0.69 ± 0.28[b]	0.67 ± 0.09	-0.64 ± 0.20[c]	$F_{2,12} = 8.05$ $p < 0.01$	C (4) R (5) S (6)

Data are displayed as mean fold change ± SEM and log$_2$(fold change) ± SEM. Sample numbers are listed in column N. Superscripts indicate post hoc significance (Tukey's post hoc tests) as follows: [a]Significant difference from control, [b]Significant difference from resilient, [c]Significant difference from susceptible, [d]Trending difference from control ($p<0.1$), [e]Trending difference from resilient ($p<0.06$), [f]Trending difference from susceptible ($p<0.06$).