Splenic Denervation Attenuates Repeated Social Defeat Stress-Induced T-lymphocyte Inflammation

SUPPLEMENTAL INFORMATION

Supplemental Methods

Splenic artery ultrasound

Mice were anesthetized using 1-3% isoflurane with appropriate oxygen balance. Mice were placed on a heated stage in a lateral decubitus position with their left side facing the probe. Hair was removed using a depilatory cream (Nair & Co., Nair, Bristol, UK). B-mode and color Doppler imaging were used in combination to find the splenic artery using the high frequency Vevo 3100 (FujiFilm VisualSonics Inc., Toronto, ON, Canada) ultrasound machine and the MX550D transducer (center frequency 40MHz, axial resolution 40 μm). Pulsed-wave Doppler was used to measure blood velocity by placing the Doppler gate at the site of maximum velocity. Ultrasound operator was blindly to sham or Dnx status of animals. VevoLab (Fujifilm VisualSonics Inc.) was used for post-imaging analyses and measurements.

Immunoblotting

Briefly, whole spleen lysate (50 μg) was first separated by SDS-PAGE, then wet transferred to a nitrocellulose membrane. Membranes were probed with primary antibodies targeted against tyrosine hydroxylase (TH, 1:1000 dilution, EMD Millipore #AB152, Burlington, MA, USA) or β-Actin (loading control; 1:1000 dilution, Sigma Aldrich #A2066, St. Louis, MO, USA), and secondary anti-mouse antibodies conjugated to horseradish-peroxidase (1:10,000, Thermo Fisher #31460, Waltham, MA, USA). Quantification was performed by densiometric analysis using ImageJ analysis software.

Catecholamine assessment

Total catecholamines within splenic lysate, renal lysate, and plasma were assessed using 3-CAT research ELISA (Rocky Mountain Diagnostics, BAE-5600, Colorado Springs, CO, USA). All assays were completed according to the manufacturer's protocol, with splenic and renal lysate catecholamine concentration normalized to wet tissue weight.

Circulating cytokine analysis

Blood was obtained by cardiac puncture immediately following sacrifice with anti-coagulation maintained through addition of ethylenediaminetetraacetic acid (EDTA). Plasma was separated from whole blood by centrifugation and stored at -80°C until assay. Cytokine concentration was assessed by Meso Scale Discovery V-Plex Mouse Cytokine 29-plex kit (#K15267D, Rockville, MD, USA). All experiments were conducted per manufacturer's instructions and quantified on a Meso Scale Discovery Quickplex SQ 120, with analyses conducted using Mesoscale Discovery Workbench software (Rockville, MD, USA).

Splenocyte isolation

Spleens isolated from mice were physically disrupted into a single cell suspension and passed through a 70 μm filter to remove cellular debris. Erythrocyte lysis buffer (15.5 mM NH_4Cl, 1mM $KHCO_3$, 10 μM EDTA) was added to deplete erythrocytes, followed by an additional 70 μm filter pass. Cells were then counted (Bio-Rad, TC20 Automated Cell counter, Hercules, CA, USA), validated for >90% viability by trypan blue exclusion, and re-suspended in supplemented RPMI media (RPMI media + 10% fetal bovine serum, 1% GlutaMAX™ (Gibco, #35050061, Grand Island, NY, USA), 1% HEPES, 1% penicillin and streptomycin, and 0.1% 50 μM β-mercaptoethanol).

T-lymphocyte isolation

Splenic T-lymphocytes were negatively selected from total splenocytes through use of EasySep Mouse T-cell Isolation Kit (Stem Cell Technologies #19851, Vancouver, BC, Canada). Samples were validated for >90% viability and purity before subsequent use.

RNA extraction, cDNA production, and real-time RT-qPCR

Total RNA was extracted from purified splenic T-lymphocytes utilizing the RNAeasy mini kit (Qiagen #74104, Valencia, CA, USA) prior to conversion to cDNA (Applied Biosystems, #4374966, Grand Island, NY, USA) per the manufacturer's instructions. Generated cDNA was assessed for transcript levels by qPCR using intron-spanning gene-specific oligonucleotides (**Table S1**). Gene specific PCR products were validated by thermal dissociation curves. Thresholds were set objectively to determine cycle thresholds (CT), with 18s rRNA utilized as a loading control to determine ΔCT. All values were normalized to sham-operated control samples to determine $\Delta\Delta$CT values, which were then transformed to generate fold changes by the $2^{-\Delta\Delta CT}$ method.

Table S1. *Primer templates used for real-time RT-PCR*.

Gene name	Gene symbol	Forward sequence (5' – 3')	Reverse sequence (5' – 3')
18s rRNA (loading)	*18s*	gcccgaagcgtttactttga	tcatggcctcagttccgaa
Interleukin-2	*il2*	tctacagcggaagcacagc	cctggggagtttcaggttc
Interleukin-6	*il6*	gctaccaaactggatataatcagga	ccaggtagctatggtactccagaa
Interleukin-10	*il10*	cagagccacatgctcctaga	tgtccagctggtcctttgtt
Interleukin-17A	*il17a*	cagggagagcttcatctgtgt	gctgagctttgagggatgat
Interleukin-22	*il22*	tgacgaccagaacatccaga	aatcgccttgatctctccac
Tumor Necrosis Factor α	*tnf*	ctgtagcccacgtcgtagc	ttgagatccatgccgttg
Interferon gamma	*ifng*	atctggaggaactggcaaaa	ttcaagacttcaaagagtctgaggta
Interleukin-4	*il4*	agagatcatcggcattttgaac	gagctcactctctgtggtgttct

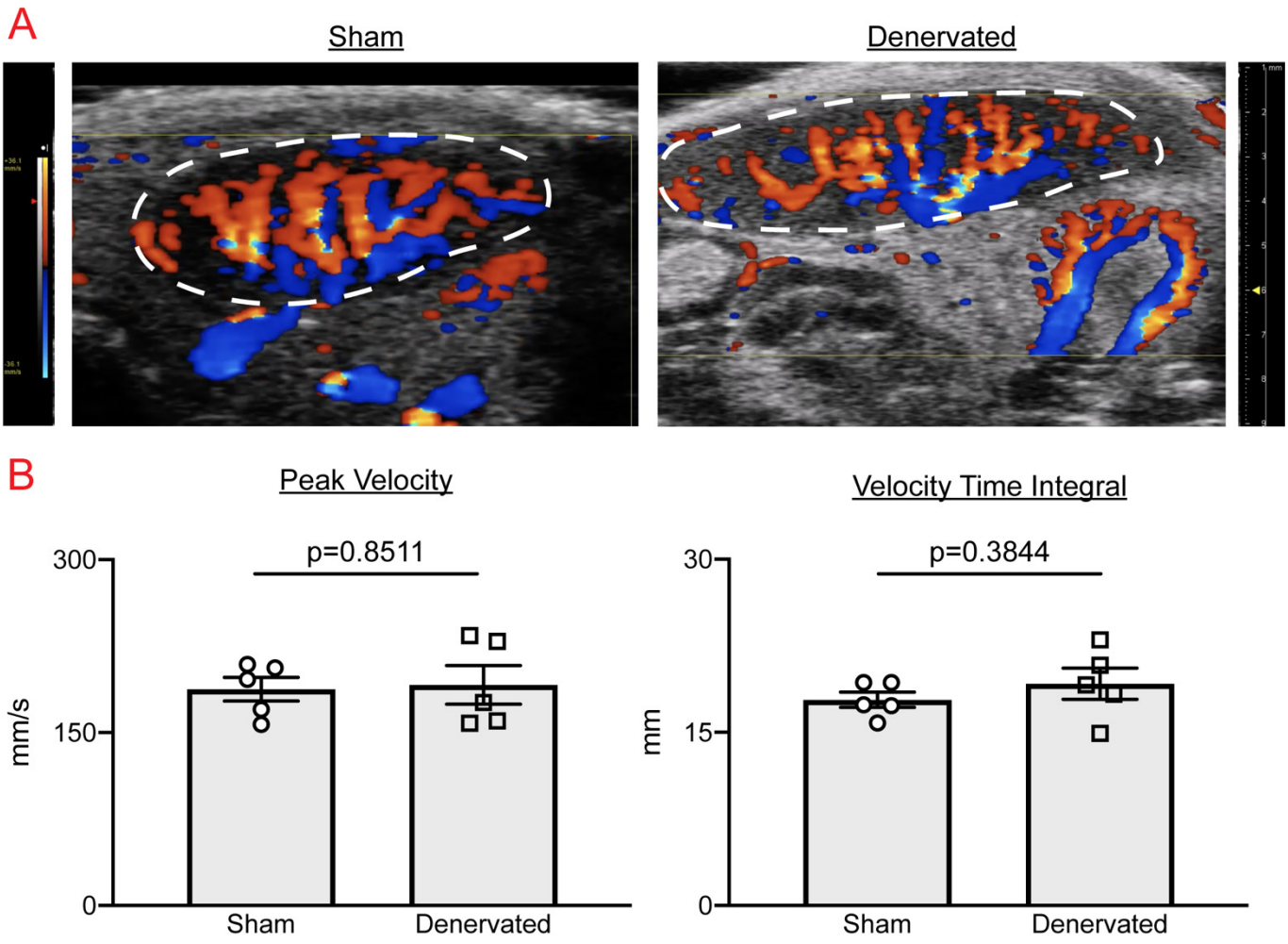

Figure S1. Dnx does not alter blood flow to the spleen. C57BL/6J wild-type mice were sham-operated or denervated, then assessed for blood flow by B-mode and color mode Doppler imaging under isoflurane anesthetic. **A)** Representative doppler ultrasound of spleen following sham or Dnx. **B)** *Left*, Peak velocity calculated by pulsed wave doppler, with gate placed at point of maximal velocity. *Right*, velocity time integral by pulsed wave doppler. Statistical analyses conducted by parametric t-test as appropriate.

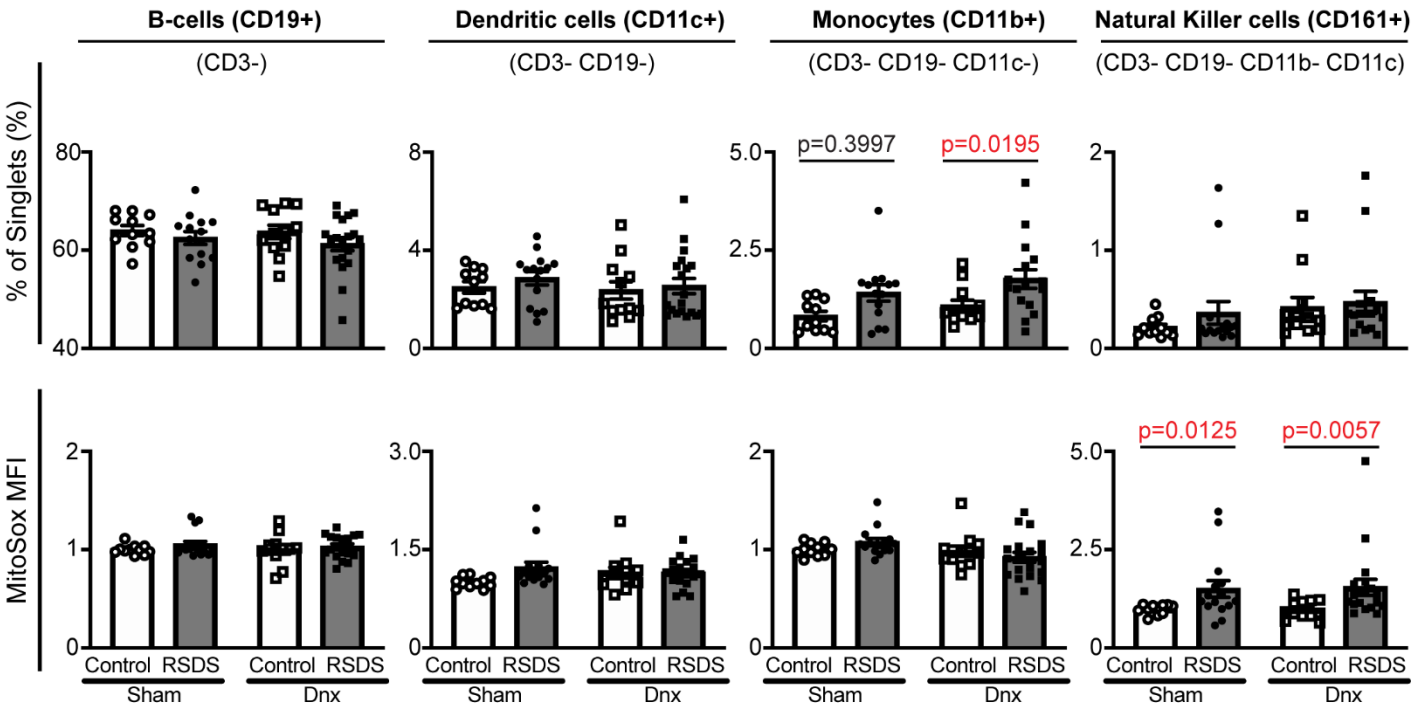

Figure S2. Dnx interacts to alter select splenocyte percentages and respective mitochondrial superoxide. Mice were assigned to ±Dnx and ±RSDS cohorts followed by splenocyte isolation, extracellular marker and MitoSox Red staining for analysis by flow cytometry. *Upper,* splenocyte populations expressed as frequency of singlet cells, with gating strategy listed above respective graphs. *Lower,* respective cell population quantification of MitoSOX Red mean fluorescence intensity (MFI) normalized to intra-experiment sham-operated control animals. 2-way ANOVA group results: **B-cell %** Stress p=0.1437, Dnx p=0.5735, Interaction p=0.7122; **B-cell MitoSox** Stress p=0.2842, Dnx p=0.7843, Interaction p=0.7241; **Dendritic Cell %** Stress p=0.3883, Dnx p=0.4782, Interaction p=0.7660; **Dendritic Cell MitoSox** Stress p=0.1014, Dnx p=0.6192, Interaction p=0.1011; **Monocyte %** Stress p=0.0088, Dnx p=0.1055, Interaction p=0.3612; **Monocyte MitoSox** Stress p=0.8744, Dnx p=0.0720, Interaction p=0.1415; **NK %** Stress p=0.3546, Dnx p=0.1498, Interaction p=0.6544; **NK MitoSox** Stress p=0.0053, Dnx p=0.9283, Interaction p=0.8858. Statistical analyses by 2-way ANOVA with Tukey post-hoc correction p-values listed on each figure if respective effects were found significant.

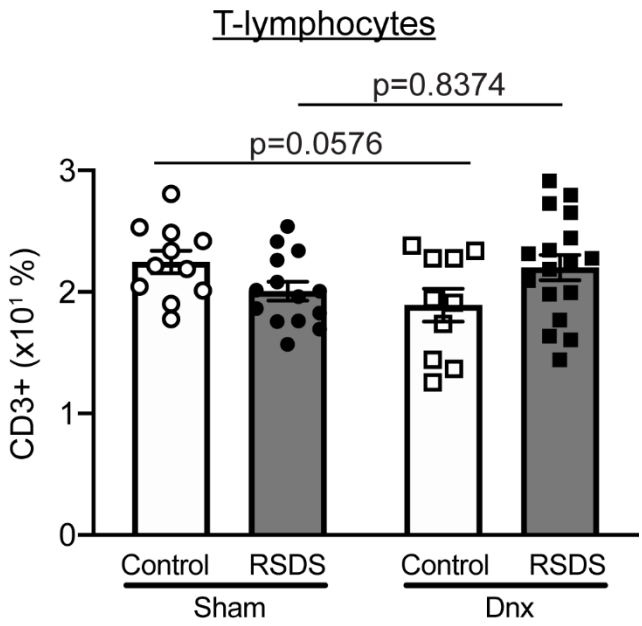

Figure S3. Dnx or RSDS do not alter Splenic T-lymphocytes. Mice were assigned to ±Dnx and ±RSDS cohorts followed by live splenocyte isolation and assessment by flow cytometry for CD3+ T-lymphocytes as a percentage of singlets. 2-way ANOVA results; Stress p=0.7474, Dnx p=0.0173, Interaction p=0.1885. Statistical analyses by 2-way ANOVA with Tukey post-hoc correction p-values listed if respective effects were found significant.