SUPPLEMENTARY INFORMATION

Repeated Social Defeat Stress Induces an Inflammatory Gut Milieu by Altering the Mucosal Barrier Integrity and Gut Microbiota Homeostasis

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Supplementary Material and Methods

Animals and cell line

All animals were 8–12-week-old male mice of the C57BL/6J strain. All aggressive mice were 4–6-month-old retired breeder male mice of a CD-1 background (Charles River #022, Wilmington, MA, USA). The social defeat stress paradigm in related animal modeling precludes the use of female mice because of the difficulty of initiating attack behavior towards female mice. Some studies have utilized female mice in RSDS studies, however, these studies were associated with variabilities, and comparison between males and females was precluded [1-3]. Thus, the examination of the sex difference is not within the scope of the current study. Of interest, RSDS is a widely accepted model of psychological trauma and closely mimics human PTSD. All experimental mice were bred in-house to eliminate the potential of shipping-associated stress and/or stress caused by environmental changes. Littermate mice were group housed (≤ 5 mice per cage) before the stress induction protocol to eliminate social isolation stress. Mice cages were randomized before the start of all experiments and whenever possible experimenters were blinded to the control and stress groups of mice until the completion of the study. However, at all times of the study, mice from different groups were caged separately to avoid any cross-contamination of the gut microbiota. At the end of the experiment, mice were euthanized by CO_2 inhalation and cervical dislocation. All procedures were reviewed

and approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee.

Cell line -The Intestinal epithelial cell line, Caco2 cells, a widely used model of the polarized gut epithelium, was used as the *in vitro* model These cells were maintained in DMEM +10%FBS + Penicillin/Streptomycin antibiotics.

Mouse model of Repeated Social Defeat Stress (RSDS)

The social defeat stress was enacted by following the protocol described previously [4, 5]. Briefly, retired male breeder CD-1 mice were allowed three days before the start of the experiment to inhabit the standard cages outfitted with two sets of food, water, and bedding to allow territory establishment. On day one of the experiment, all elements of the cage (except corn bedding) were temporarily removed, and an experimental mouse was introduced into the cage for 5 min to allow for a physical confrontation (physical interaction). After 5 minutes of interaction, the mice were separated within the same cage by a transparent perforated barrier, and all housing elements were placed into the cage. The mice were then co-housed with physical separation for the remainder of the 24 hours (co-habitation).The physical interaction and co-habitation process were repeated by rotating the experimental mouse to a different CD-1 mouse cage for 10 consecutive days. Control mice were pair housed using identical separation and barrier housing techniques, but not allowing for any physical confrontation between mice during the 24-hour periods. We excluded any animal that had visual wounding greater than 0.5 cm in length or demonstrated any signs of lameness, hunching, or illness. At the end of the 10 days (day 11), all mice were assessed for behavioral changes using both social interactions and elevated zero tests to measure depression-like and anxiety-like behavior, respectively. After testing, control and experimental mice remained in their former co-housed barrier cage until the following day (day 12), when they were culled for blood and organ collection.

Measurement of behavioral parameters associated with RSDS mice:

Elevated Zero Maze

The Elevated Zero Maze test was performed to measure anxiety-like behavior described by Walf and Frye [5, 6]. In brief, an elevated circular maze consisting of 50% open and 50% closed quadrants was applied to these tests (50 cm diameter, 5 cm track width, 20 cm wall height, 61 cm stand height; Noldus Information Technology, Leesburg, VA, USA). Control and stressed mice were introduced into a closed arm of the maze and allowed to explore the novel environment for 5 min. Runs were performed with one mouse at a time, and the maze was thoroughly cleaned using water followed by 70% ethanol (allowing time for evaporation) to eliminate olfactory variables and possible gut microbiota contamination before the next mouse was tested. Sessions were recorded, tracked, and analyzed using Noldus Ethovision XT 13 software. Tests were performed within the housing room of mice during the light cycle using approximately 265 lux of ambient lighting at the testing arena.

Social Interaction Test

The Social Interaction test was performed to measure depression-like behavior as described previously [4, 5]. Briefly, an open field chamber (40 cm wide, 40 cm long, 30 cm walls; Noldus Information Technology, Leesburg, VA, USA) was outfitted with a small wire mesh enclosure (6.5 cm wide, 10 cm long, 30 cm height; Noldus Information Technology, Leesburg, VA, USA) on one side. Control and stressed mice were introduced into the open field and allowed to explore their environment with an empty mesh enclosure for 2.5 min. Interaction of experimental mice was done with CD-1 mouse for 2.5 min whereas control mice were introduced in the open field of the cage without CD-1 mouse. Sessions were recorded, tracked, and analyzed using Noldus Ethovision XT 13 software. Social interaction and corner zone ratios were calculated by the amount of time spent in the respective zones in the presence and absence of a CD-1 mouse within the housing room of mice during the light cycle using a 265-lux light source.

Measurement of the gut barrier functions

The gut barrier functions including trans-epithelial resistance (TEER), conductance, and permeability for FITC-Dextran (4KDa) were measured by the Ussing Chamber system

(Physiologic Instrument, San Diego, CA) as described previously [7]. Briefly, following the humane euthanasia, the mouse colon was removed and cleaned by flushing with oxygenated ice-cold Krebs-Ringer bicarbonate (KBR) buffer and then opened longitudinally. The distal colonic mucosal tissue section was pinned into the slider (aperture area=0.3 Cm2), and then the slider fitted into the two halves of the Ussing Chamber. For equilibration of the tissue, serosal chamber halves were filled with 5 ml glucose KBR buffer (10 mM) to provide as an energy source to the tissue. Mucosal halves were filled with 5 ml cold mannitol KBR buffer (10 mM) to maintain osmotic balance without influencing glucose-mediated Na^+ transport. Throughout the experimental procedure, the chamber's temperature was maintained at 37^{o}C via the circulating water bath and continuous oxygenation with 95% O2 and 5% CO2. After equilibration of 10 min, transepithelial resistance (Rt) and conductance (Gt) were measured in real-time using a voltage/current clamp device. For permeability assay, 4-kDa FITC-dextran (stock 20mg/ml) was added to the mucosal side in the mannitol-KBR buffer to achieve a final concentration of 0.02 mM. 200 µl sample was taken at different time points from the serosal side. Equal volume (200 µl) of KBR buffer was added to the serosal side to maintain the same volume in both halves of the Ussing chamber. The presence of dye in the serosal compartment was determined using a microplate fluorescence reader (FL-500; BIO-TEK; Ex/Em wavelength at 485/528 nm). Acquire & analyze software was used to acquire and calculate transepithelial resistance and conductance.

Immunoblotting

Immunoblotting was done as described previously [8]. Briefly, lysates prepared in the RIPA lysis buffer were separated onto an SDS-PAGE gel. The PVDF membrane containing the immobilized proteins was probed with respective primary antibodies overnight at 4ºC. After incubation with secondary antibodies, blots were washed and developed with enhanced chemiluminescence (Millipore). Densitometric analysis of protein bands was performed using Image Lab software (Bio-Rad). Antibodies used for WB are listed in supplementary table S1.

Immunofluorescence and Immunohistochemistry

Immunostaining was done as previously described [7]. Briefly, tissue sections (4 µM) were deparaffinized, hydrated, and incubated with specific primary antibodies overnight at 4^0C in a humidified chamber. The next day, slides were washed with TBST and incubated with the secondary antibody at RT for 1 hr. After washing, the slides were mounted with a mounting medium containing DAPI. Image acquisition was done using the Nikon-ECLIPSE Ti-S.

For the immunohistochemistry (IHC), we used mouse and rabbit-specific VECTASTAIN Peroxidase (HRP) kits to probe mouse or rabbit-specific antigens in the gut tissue sections. Antigen unmasking was done using Tris-EDTA buffer (10 mM Tris Base and 1 mM EDTA at pH 9) in a pressure cooker. Sections were quenched with 3% H_2O_2 solution and blocked with 5% normal goat or horse serum. The color was developed using ABC kit reagents. Image acquisition was done using Nikon's ECLIPSE Ti-S microscope. Antibodies used for immunofluorescence (IF) and immunohistochemistry (IHC) staining are listed in supplementary table S1. For the quantitative analysis of the IHC staining intensity, we chose 6-8 random area /Swiss role/animal and measured the staining intensity using ImageJ software (https://imagej.nih.gov/ij/). An average of 6-8 selected areas was taken to determine the intensity.

PAS and Alcian Blue staining

Periodic acid-Schiff (PAS) and Alcian Blue staining was performed to measure the neutral mucin secretion from the goblet cells by modifying a previously described protocol [9]. For the staining, colon tissue sections were deparaffinized, hydrated, and stained with PAS/ Alcian Blue /hematoxylin. We followed the manufacturer's instructions for both, PAS and Alcian staining. Slides were mounted with a toluene-based mounting medium. Images were captured using a Nikon microscope. The staining intensity for the PAS and Alcian blue staining was done using the ImageJ software (https://imagej.nih.gov/ij/). We chose 6-8 random area/Swiss roles/animals for this analysis.

Norepinephrine (NE) treatment and TEER measurement

Measurement of transepithelial electrical resistance (TEER) was done as previously described [10]. Caco-2 cells were seeded in a 12-well trans-well culture plate (0.4 µm). The culture medium in the apical and basolateral compartments was changed three times a week. Cells were regularly monitored under a microscope. TEER was measured using an epithelial volt/ohm (EOM) meter with an STX2 electrode set (World Precision Instruments). Confluent Caco-2 cells (TEER 500 Ω cm^2) were serum starved for 24 hours. Thereafter, cells were treated with NE (10 µM). Ascorbic acid was used as vehicle control. An effect on TEER was documented, which was calculated as a percentage of total vehicle control treated wells. Data are represented as the average of at least three independent experiments.

Isolation of mouse colon crypt and ex-vivo treatment with norepinephrine

The crypt isolation, 3d-culture, and treatments were done as described [11] with slight modification. In brief**,** the distal portion of the colon was cut and opened longitudinally and cleaned using ice-cold PBS. After washing (3–4-time), tissue was minced and pelleted down by centrifuging at 500 RPM for 5 min. After washing, crypt pellet was resuspended in DMEM depending upon experimental requirement and equal volume of matrigel was mixed properly with the freshly isolated colon crypts and seeded in a 24-well plate in a dome shape, avoiding the spread of matrigel as much as possible. The plate was kept at 37°C in a CO_2 incubator for 15-20 min. After the solidification of matrigel, serum-free DMEM medium was added to the well with vehicle control or norepinephrine (10 µM) for 24 hrs. The next day, at the end of the experiment, matrigel containing crypts were resuspended in 500 µL RIPA buffer containing protease and phosphatase inhibitors. Crypt cell lysates were prepared by mechanical lysis and sonication. Protein estimation was done using the Bradford reagent. SDS-PAGE and WB were done to determine the expression of different tight junction proteins.

RNA Isolation and qPCR analysis

RNA from the colon and ileum tissues was isolated using the ZYMO Research kit as per the manufacturer's instructions. cDNA was generated using the iScript master mix (Bio-

Rad). Prepared cDNA was used for quantification of gene expression using SYBR green master mix in the Thermocycler (Bio-Rad) and relative gene expression in stressed samples was determined compared to control mice samples. We analyzed the expression of chemokines CXCL9 and CXCL12 and antimicrobial peptides Reg3, Reg3, and α-defensin.

16S DNA sequencing and library generation

At the end of the behavior experiments stool samples were collected using established sterile technique before sacrificing the mice and stored at -80^0C until further processing . Genomic DNA was extracted from the mouse stool samples following the QIAamp DNA Stool mini kit protocol. After quantification of DNA, 12.5 ng of DNA was used to generate an Illumina library for 16S rRNA sequencing. An approximately 460 bp sized fragment of the V3/V4 region of the 16S rRNA gene was amplified for each of the DNA samples, beginning with 12.5ng of DNA as per Illumina's recommended protocol outlined in the 16S Metagenomic Sequencing Library Preparation protocol (Illumina, San Diego, CA) using 16S Forward and reversed primer listed in table 2. Following the generation of the amplicons, dual indices and Illumina sequencing adapters were added using the Nextera XT Index kit (Illumina catalog # FC-131-1001). Resultant libraries were multiplexed and 300 bp paired-end sequencing was performed on an Illumina MiSeq instrument using V3 chemistry per Illumina's recommendations at UNMC Genomic core facility.

Bioinformatic analysis of the 16S sequencing data

Raw sequencing reads (fastq files) were analyzed using QIIME2 (Quantitative Insights into Microbial Ecology version 2, 2020.2) software [12], a next-generation microbiome bioinformatics platform to determine the taxonomic diversity profiles of the microbiota in control and test (stressed mice) samples. A QIIME2 plugin and DADA2 algorithm were used for quality-score-based filtering of input sequences and the construction of the feature table, which contains the count of each unique sequence in each sample. To assign the taxonomy of the feature data (unique sequences), the pre-trained Naive Bayes and q2-feature classifiers were used. The sequences were clustered into Operational

Taxonomic Units (OTUs) using a closed-reference OTU picking workflow against the Silva 132 reference set.

Preparation of fecal contents and treatment

For preparation of fecal content, an equal amount of stool (0.5 mg) was taken and resuspended in sterile PBS. The fecal material was first mixed by vertexing and then sonication. The resultant fecal suspension was then centrifuged at 4000 rpm for 5 min. Supernatant was collected, aliquoted and either used immediately or stored at -80^{o}C. For the experiments, Caco2 cells (~70% confluent) were serum starved (overnight) and then treated with 10 µl of fecal contents from control or RSDS mice. Twenty-four hours after the treatment, protein lysates were prepared. Colonic crypts (isolation method as mentioned above) were also treated with 10 µl fecal contents however only for 3 hrs. Claudin-2 expression was determined by western blotting. For measurement of TEER, Caco2 cells cultured on trans-well filters were treated with 10 µl fecal contents from control and stressed mice and effect on TEER was documented using epithelial volt/ohm (EOM) meter with an STX2 electrode set (World Precision Instruments). Relative changes were calculated as a % of values from cells treated with control mice fecal contents.

Quantitative analysis of Catecholamines using LC-MS/MS

The quantitative analysis of Catecholamines was done using Liquid chromatographic and mass spectrometry (LS/MS). A Shimadzu Nexera UPLC system equipped with two pumps (LC - 30 AD), column oven (CTO - 30AS) along with an auto-sampler (SIL - 30AC) was used. Mass spectrometric detection was performed on an LC-MS/MS 8060NX system (Shimadzu Scientific Instruments, Columbia, MD), in positive atmospheric pressure chemical ionization (APCI) mode. All chromatographic separations were performed with a UPLC ACE Excel C18 PFP (1.7 µm, 100 X 2.1 mm, Advance Chromatography Technologies LTD., UK) as the analytical columns equipped with an Acquity UPLC C18 guard column (Waters, Inc. Milford MA). The mobile phase consisted of 2 mM ammonium formate containing formic acid (0.05% v/v) (mobile phase A) and 0.5 mM ammonium fluoride in methanol (mobile phase A), at a total flow rate of 0.25 mL/min. The chromatographic separation was achieved using a 9 min gradient elution for

Catecholamines (CAs). This method is sensitive with a limit of quantification of 0.2 ng/mL for all analytes, has a large dynamic range (200 ng/mL), and a run time of 9 min. Serum and colon concentrations of epinephrine, norepinephrine, and dopamine at a range of 0.2 – 500 ng/ml were quantified in bio-matrices samples.

The CAs were extracted from mouse serum and colons, Each colon sample was accurately weighed and then homogenized with triple distilled de-ionized water at a 9-fold dilution factor using a TissueLyserII (Qiagen Science, KY). The resultant colon homogenate or serum sample (100 µl) was spiked with IS (20 µl) and diluted with 200µL sodium citrate buffer (10 mM, pH 7) and vortexed for 30 sec, and then loaded onto SPE cartridges pre-conditioned with methanol (MeOH, 0.5mL), followed by 10 mM ammonium acetate pH 6 (0.5mL). The loaded cartridges were washed with 10 mM ammonium acetate pH 6 (0.5mL) then 80% MeOH (0.5mL) to elute neutral organic interferences then wash with dichloromethane (0.5mL) to elute lipophilic interferences. Finally, elute CAs analytes with 200 µl of water: propan-2-ol (85:15, v/v) containing formic acid (0.1 % v/v). For all standards and samples, eluates were evaporated under vacuum at room temperature and reconstituted with water: MeOH (95:5, v/v, 100µL) containing formic acid (0.1% v/v) and mixed thoroughly. The resultant supernatant was transferred into autosampler vials (~80 µL) and 10µL of the sample was injected into LC-MS/MS.

Supplementary References

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Supplementary Table 1

S.N	Antibody	Company	Catalog Number	Dilution of antibodies
1	Claudin-1 Rabbit polyclonal antibody	Invitrogen	51-900	WB 1:500, IF 1:500
2	Claudin 2 Mouse monoclonal antibody (12H12)	Invitrogen	32-5600	WB 1:500, IF 1:500
3	Claudin3 Rabbit Polyclonal Antibody	Invitrogen	34-1700	WB 1:500, IF 1:500
4	Claudin 7 Mouse monoclonal antibody	Invitrogen	37-4800	WB 1:500, IF 1:500
5	Occludin Rabbit polyclonal	Proteintech	27260-1-AP	WB 1:500
6	ZO-1 Rabbit polyclonal	Proteintech	21773-1-AP	WB 1:500
7	pSTAT3	Cell Signaling Technology	9145s	WB 1:1000, IHC 1:400
8	STAT3 Rabbit monoclonal antibody	Santa Cruz	sc8019	WB 1:500
9	pNFkB Mouse monoclonal	Santa Cruz	sc136548	WB 1:400, IHC 1:200
10	NFkB Mouse monoclonal antibody	Santa Cruz	sc-8008	WB 1:500
11	Tyrosine Hydroxylase (TH)	Millipore	AB152	WB 1:1000 IHC 1:500
12	Lysozyme Rabbit polyclonal antibody	Novus	NBP 2-61118	WB 1:500, IHC 1:400
13	ICAM-1 Rabbit polyclonal antibody	Proteintech	10020-1-AP	WB 1:500, IHC 1:400
14	CD45 Mouse monoclonal antibody	Proteintech	60287-1-AP	IHC 1:5000
15	CD68 Mouse monoclonal antibody	Invitrogen	MA 5-13324	IHC 1:500
16	CD3e Mouse monoclonal antibody	Invitrogen	190582	IHC 1:500
17	Periodic acid- Schiff (PAS) staining kit	Sigma	Procedure No. 395	As per kit
18	Alcian Blue Stain Kit	Abcam	150662	As per kit
19	VECTASTAIN Elite ABC Universal PLUS Kit	Vector Laboratory	PK-8200	As per kit

Supplementary Table 2: Primer sequences

S.N	Primer	Forward sequence (5'-3')	Reverse sequence (5'-3')
1	CXCL9	CCGAGGCACGATCCACTAC	AGGCAGGTTTGATCTCCGTT
2	CXCL12	TGCATCAGTGACGGTAAACCA	CACAGTTTGGAGTGTTGAGGA
3	RegIIIβ	AATGGAGGTGGATGGGAATG	CCACAGAAAGCACGGTCTAA
4	RegIIIγ	TTCTCAGGTGCAAGGTGAAG	GGCATAGCAATAGGAGCCATAG
5	α-defensin	GGCCGTATCTGTCTCCTTTG	CTCTTCCTTTGCAGCCTCTT
	16S	TCGTCGGCAGCGTCAGATG TGTATAAGAGACAGCCTACG GGNGGCWGCAG	GTCTCGTGGGCTCGGAGATGTGTATA AGAGACAGGACTACHVGGGTATCTAA TCC