SUPPLEMENTAL MATERIAL

Hypertension-Linked Pathophysiological Alterations in the Gut

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Methods

Animal Models

All experimental procedures were approved by the University of Florida Institutional Animal Care and Use Committee and complied with the standards stated in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Rats were housed in a temperature-controlled room (22°C to 23°C) with a 12:12-hour light-dark cycle, in specific-pathogen free cages, and had access to standard rat chow and water *ad libidum*.

Twenty-week old Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR) (Charles River Laboratories) were used as the adult group, and four-week old WKY (young, WKY-y) and SHR (young, SHR-y) were used as the young group. In ACEi experiments, adult WKY and SHR animals received 85mg/day captopril in the drinking water for 4 weeks.

Eight-week-old Sprague Dawley (SD) rats were used for experiments involving angiotensin II (Ang II) infusion. Hypertension was established by chronic infusion of Ang II (200ng/kg/min) using mini osmotic pumps (ALZET 2004) implanted subcutaneously. Control animals received 0.9% saline in osmotic pumps.

Six-week-old SD (Charles River Laboratories) and SD-Tg (UBC-eGFP) (Rat Resource & Research Center strain 65; bred in-house) were used for bone marrow (BM) reconstitution experiments described below. This hemizygous transgenic strain contains a single enhanced green fluorescent protein gene under the control of human Ubiquitin-C promoter located at Chromosome 14.

Small intestine (medial ileum, ~3 cm) and proximal colon (~3 cm, most proximal to cecum) areas of the gut were isolated for all the studies.

Blood Pressure Measurement

Noninvasive blood pressure (BP) measurements were performed using the CODA System for Rats, following the manufacturer's protocol (Kent Scientific Corp). Additionally, Millar catheterization was performed to measure the direct BP as previously described [PMID: 26498282]. Briefly, rats were anesthetized with the 2% isoflurane-oxygen mixture. By cannulating the right carotid artery, an impedance-micromanometer catheter (Millar Instruments, Houston, Texas) was introduced into the carotid artery. The catheter was interfaced to a PowerLab (ADInstruments, Colorado Springs, CO, USA) signal transduction unit.

FITC-Dextran Permeability Assay

Following 6 hours of fasting, animals were orally gavaged with 4kDa FITC-dextran (44mg/100g, Sigma Aldrich, catalog #FD4). Four hours after feeding, blood was collected for plasma analysis of FITC fluorescence. Data were analyzed using a standard curve of FITC-dextran dilution, as previously described[1].

Multi-Scale Indentation Technique

Small intestine and proximal colon samples were tested from WKY-y, SHR-y, adult WKY, adult SHR, and ACEi treated WKY and SHR. Rats were euthanized and a 2-2.5 cm section of colon was excised under approved procedures from the University of Florida Institutional Animal Care and Use Committee. Freshly isolated tissue samples were placed in cold media solution consisting of Dulbecco's Modified Eagle Medium (DMEM) and 5% Fetal Bovine Serum (FBS) and tested within four hours of isolation.

Intestinal samples were cleaned initially by gently pushing feces out of the sample with a soft edge. Samples were then cut open longitudinally, rinsed in cold buffered saline, and placed flat on Petri

dishes, which served as the substrate during testing. Tissues were handled and placed to ensure that the inner lining of the intestine remained on top. Sample thickness was approximately 1.5 mm. All samples were prepared and tested at room temperature (approximately 22^{o}C). Small aliquots (less than 1 ml) of the DMEM solution were added periodically during testing to prevent dehydration of the samples.

A custom cantilever-based indenter[2, 3] was used to indent tissue samples and record forced relaxation over time. A piezoelectric stage (P-628.1CD, Physik Instrumente) displaced a soft titanium cantilever with a 3 mm-diameter rigid tip. A custom program in LabVIEW (National Instruments) was used to control indentation profile and to read deflection of cantilever tip with capacitive sensor (C8S-3.2-2.0 and compact driver CD1-CD6, Lion Precision) through a data acquisition card system (NI 9220 and cDAQ-9171, National Instruments). Samples were indented at a depth of 150 micrometers at an indentation rate of 10 micrometers/s and then the probe was kept in place for 120 seconds to allow for the tissue to fully relax. Each sample was indented at four different locations along the length of the tissue. Force-displacement data from the tests were then fitted to the SLS model.

Western Blot

Segments of small intestine and proximal colon were homogenized in radioimmunoprecipitation assay buffer. 50 µg protein was fractionated in 12%SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat milk solution in Tris-buffered saline (TBS) with 0.1 % Tween-20 for 1 h. Then the membranes were incubated either with primary antibody for multiple proteins including occluding (Ocln), tight junction protein 1 (Tjp1), claudin 4 (Cldn4), and cingulin (Cgn) overnight at 4 °C [Ocln rabbit monoclonal antibody (1:5000; Abcam), Tjp1 rabbit polyclonal antibody (1:100; Novus Biologicals), Cldn4 rabbit polyclonal antibody (1:100; Abcam), and Cgn rabbit polyclonal antibody (1:100; Novus Biologicals)]. Mouse monoclonal anti-beta actin antibody (1:10000; Abcam) was used to confirm equal loading. The membranes were then washed three times for 5 min in TBS-T and incubated with secondary antibody conjugated with horseradish peroxidase (anti-goat, anti-rabbit, and anti-mouse IgG 1:2500; GE Healthcare) for 1 h. Finally, the membranes were subjected to a chemiluminescence detection system and exposed to a photographic film.

Histological Analysis

Paraffin-embedded sections were stained with hematoxylin-eosin and Masson-trichrome to evaluate general morphology and collagen formation. Specifically, villus length, fibrosis, the number of goblet cells and thickness of tunica muscularis externa were quantified to assess intestinal pathology. To determine the expression and localization of eGFP^{+} and inflammatory cells (CD3^{+}, CD68^{+} and Iba1^{+}) in the gut, paraformaldehyde-fixed intestines were protected with sucrose, embedded in OCT, and serially sectioned on a cryostat. We performed antigen retrieval via Citrate Buffer Antigen Retrieval Protocol on the OCT sections and blocked sections with normal goat serum. The slides were incubated with the primary antibodies against eGFP (Aves Labs, GFP-1020) with CD3 (Abcam, ab16669), CD68 (Abcam, ab31630), Iba1 (Wako, 019-19741), or TH (Millipore, AB152) followed by incubation with Alexa Fluor conjugated secondary antibodies (Invitrogen).

RT-PCR

RNA was isolated from ileum and proximal colon using TRIzol Reagent (Ambion) per manufacturer's protocols. Purity of RNA was evaluated spectrophotometrically by 260/280 ratio. Reverse transcription was accomplished using High Capacity Reverse Transcription kit (Applied Biosystems) and 500 ng

RNA. Real time RT-PCR was performed using Taqman Universal PCR Master Mix and Taqman Gene Expression Assay primers (Applied Biosystems): GAPDH (Rn01775763_g1), Il1b (Rn00580432_m1), Tnf (Rn01525859_g1), HMGB1 (Rn02377062_g1), TLR2 (Rn02133647_s1), TLR4 (Rn00569848_m1), RAGE (Rn01525753_g1). RT-PCR was run using StepOnePlus (Applied Biosystems) sequence detection system. All cDNA samples were assayed in duplicate. Data were normalized to GAPDH.

Intestinal Blood Flow Measurements

In six rats (three from each group), the blood flow in the mesenteric blood vessels in a section of small intestine was measured. Animals were anesthetized with isoflurane, placed in a supine position, and a short length of the small intestine was surgically isolated via laparotomy and placed on a platform warmed by circulating water. Measurements of blood flow were taken over a three minute period using a laser speckle contrast imager (LSCI; PeriCamPSI, PeriMed, Inc.) interfaced with a dedicated computer as described previously[4]. The laser generator probe was positioned 15.0 cm above the intestinal surface. The LSCI sample rate was 53 samples/sec and the digitized blood flow values were stored in a data file. Blood flow (perfusion) values were expressed in arbitrary “perfusion units”. The blood flow data was averaged over the three-minute record.

Fecal Analysis

Rat fecal DNA was extracted by using ZR Fecal DNA MiniPrep (Zymo Research, Irvine, CA). Primers with adaptor sequences for Illumina Miseq (Illumina, Inc., San Diego, CA) were used to amplify the bacterial 16S ribosomal DNA V4-V5 region. PCR amplicons were purified (Qiagen, Madison, WI) and subsequently quantified by Qubit 2.0 Fluorometer (Invitrogen, Grand Island, NY) and KAPA SYBR FAST qPCR kit (Kapa Biosystems, Inc., Woburn, MA). DNA library was finalized with equal amount of amplicons for all data analysis.

Standard bioinformatics alignment comparison was utilized for data analysis[5]. Paired end reads were demultiplexed according to a combination of forward and reverse indices. Additional quality filtering included exact match to sequencing primers and an average quality score of 30 or higher on each read. Prior to further analysis, each paired end read was stitched into one contiguous read using the FLASh (Fast Length Adjustment of Short reads) software tool. Reads that could not be joined were excluded from downstream analysis. All sequences passing filters were aligned against a Silva non-redundant 16S reference database (v108) and assigned taxonomic classifications using USEARCH at a 97% identity threshold, and dereplication to unique reference sequence-based Operational Taxonomic Units (refOTU) was performed using UCLUST at a 97% clustering threshold and summarized in a refOTU table. Additional alpha diversity measures and normalized per level taxonomic abundances were created using custom scripts written in R. Differentially significant features at each level were identified using linear discriminant analysis (LDA) along with effect size measurements (LEfSe)[6] to generate the taxonomic cladogram.

Decerebrate artificially perfused rat (DAPR) preparation and splanchnic sympathetic nerve activity (sSNA) recording

SHR (HT) and Sprague Dawley (NT) rats were used for this preparation (male, 4-6 weeks old, 80-120 g). The rats were anesthetized, exsanguinated and decerebrated, and experimental set up was performed as previously described[7, 8]. Perfusion pressure, corresponding to the arterial pressure *in situ*, was measured using a pressure transducer connected to an amplifier. Simultaneous recordings of the phrenic nerve activity (PNA) and the lower splanchnic sympathetic nerve (sSNA) were obtained using glass suction electrodes. These were sampled at 5KHz (CED, Cambridge), amplified (20-50K), filtered

(3-30K) and monitored using Spike2 (CED). The perfusate flow (19-24 ml/min) was adjusted to produce a healthy eupneic pattern of PNA. Vasopressin (1.25-4 nM final concentration, Sigma-Aldrich, USA) was added to the perfusate to adjust perfusion pressure by increasing vascular resistance. In order to qualitatively characterize the sSNA, phrenic-triggered averaging was performed as previously described[8-10]. This allowed for the classification of averaged sSNA signal into two respiratory-related phases: inspiration (I) and expiration (E), determined based on PNA activity[8-10]. In this way, peak levels of sSNA during each respiratory phase can be compared across preparations. The immediate response of sSNA to peripheral chemoreceptor stimulation was tested by i.a. bolus injection of 100 µl of 0.03% potassium cyanide (KCN). This allowed for quantification of the maximum sSNA response, presented as percentage increase in sSNA during the 5s post-KCN injection, compared to the equivalent pre-KCN baseline timeline, as previously described[11].

Pseudorabies viral (PRV) retrograde tracing from the small intestine to PVN

Adult (male, 12 weeks old) WKY and SHR rats were used in this experiment. Some WKY were infused with Ang II (200 ng/kg/min) for seven days prior to the viral tracing injections. Pseudorabies Virus (PRV-152; Virus Center grant no. P40RR018604) tagged with GFP was a gift from Dr. J. Patrick Card, University of Pittsburgh, and was used as a retrograde tracer. The replication-competent virus was applied to the small intestine regions supplied by the mesenteric blood vessels (2 µl), and green fluorescence was examined in the autonomic brain regions four days later. Briefly, the surgical site was shaved and prepared with sterile scrub and the intestine was briefly exposed. PRV was applied onto the surface of the small intestine and spread by soft sterile paintbrush (PRV-152; 3 µl of 4.86 x 108 PFU/ml viral recombinants). The intestine was left exposed for one minute following the application, after which it was gently placed back into the abdominal cavity. The abdominal muscle was sutured, and the skin closed with surgical wound clips. Analgesics were administered prior to surgery and for 48 hours after, as needed. PRV-injected rats (SHR, WKY, and WKY+Ang II) were anesthetized with isoflurane and perfused with 200 ml heparinized saline, followed by 100 ml of 10% formaldehyde solution four days following the PRV injections. This time point was determined as optimal for green fluorescence expression by our previous study[8]. The brains were collected and processed as previously described[8].

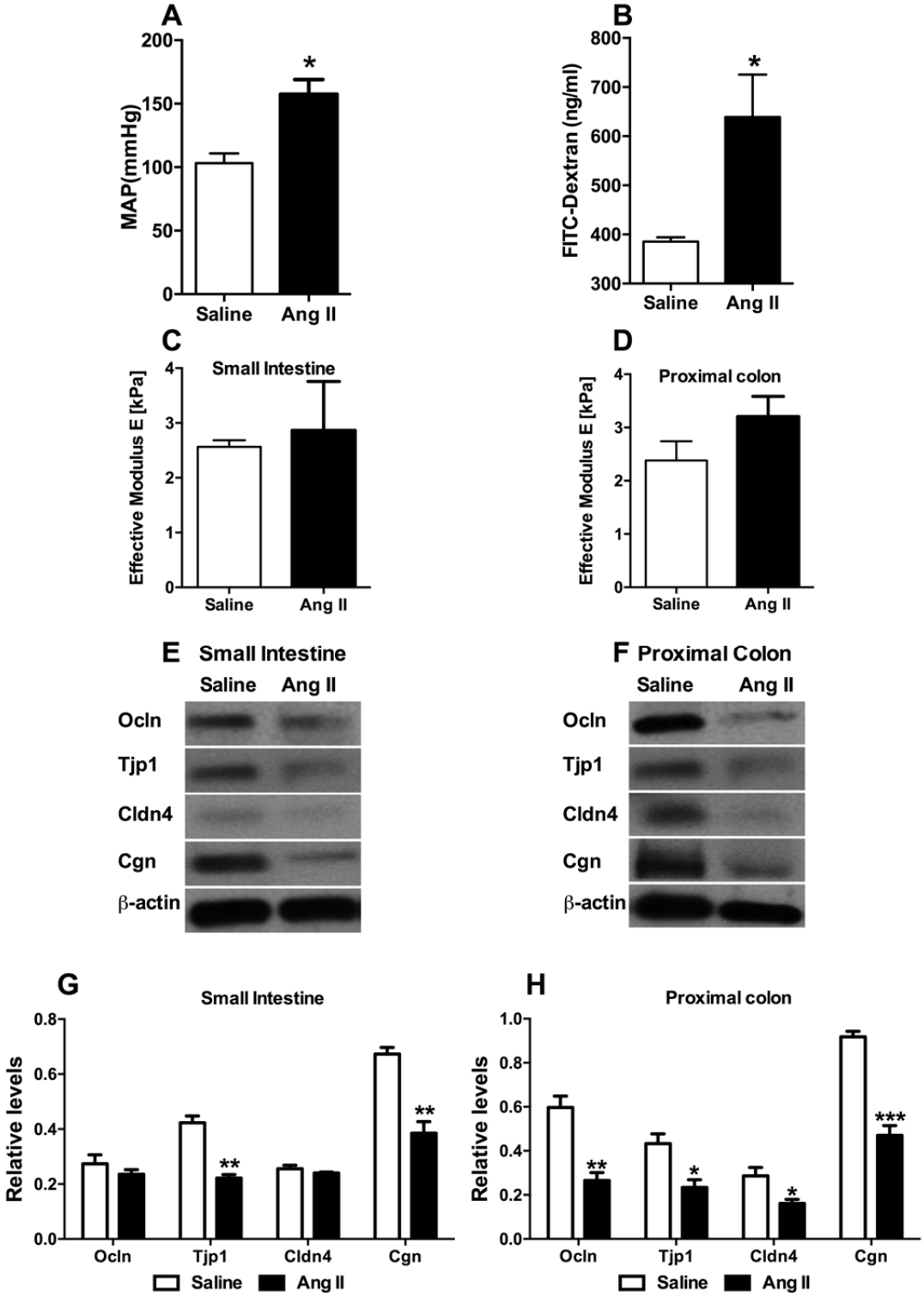
Bone Marrow Chimeras

Six-week-old SD rats were lethally irradiated (950 cGy of X- rays; service provided by University of Florida Animal Care Services) and reconstituted with age matched $1x10^7$ eGFP-SD whole BM cells. Following irradiation, animals were allowed to recover for 2.5 months before the initiation of experiments. During this time, they received 0.5168 mg/ml Baytril antibiotic (enrofloxacin; Bayer) in the drinking water, moist chow, and Nutri-Cal during the first 3 weeks. Subcutaneous injections of sterile 0.9% saline were performed as necessary to mildly dehydrated animals. BM reconstitution was confirmed by GFP+ FACS in blood mononuclear cells (MNCs).

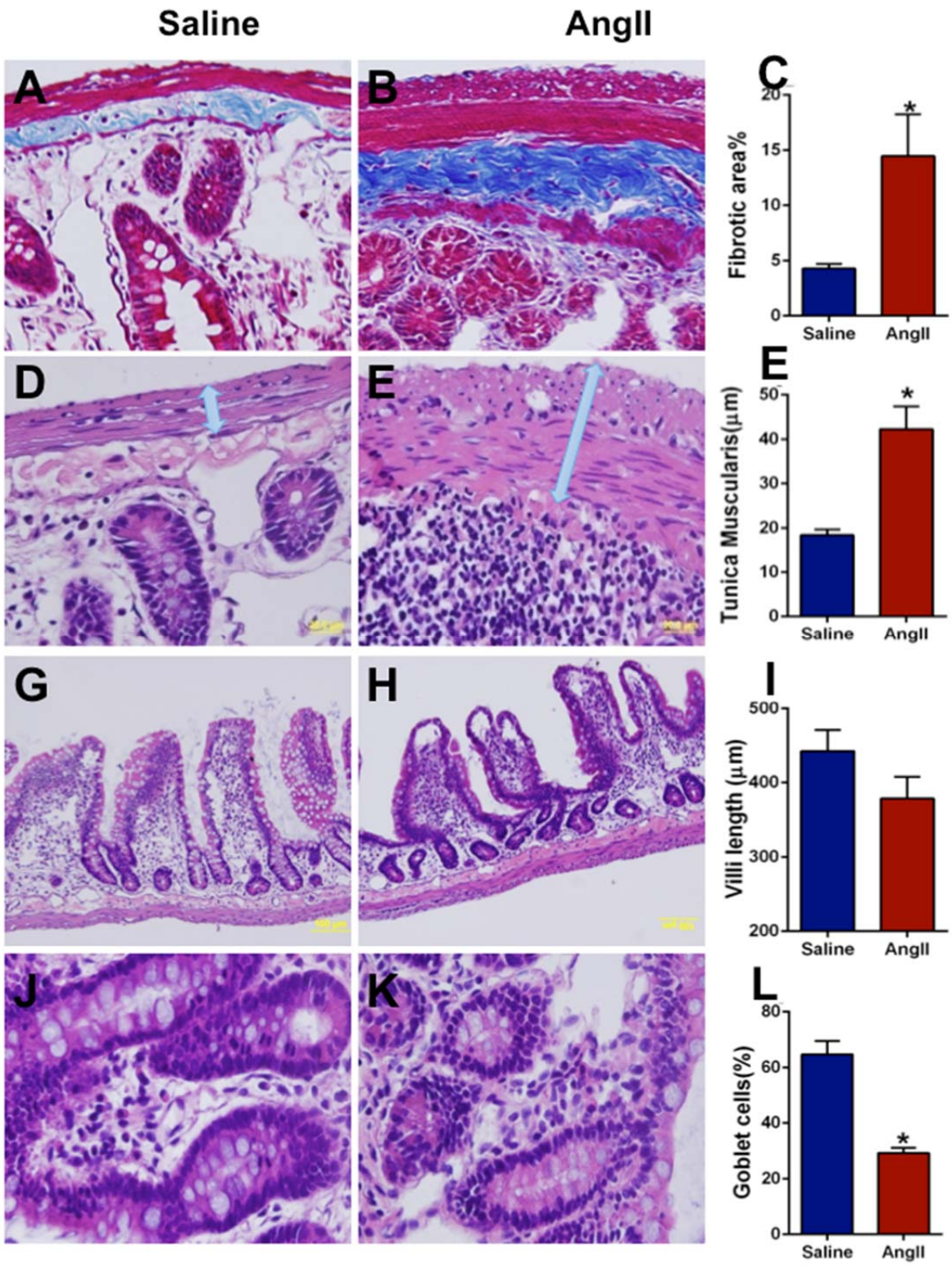
Data and Statistical Analysis

All data are expressed as mean±SEM. 1-way ANOVAs and Bonferroni post-tests were used to allow multiple comparisons between different groups, and non-parametric tests (Kruskal-Wallis and Dunnet's post-test) were used where necessary. Unpaired Student *t* tests were used for comparisons between 2 groups where applicable, with $p<0.05$ considered significant. GraphPad Prism 6 was used as the statistical software and for graph generation.

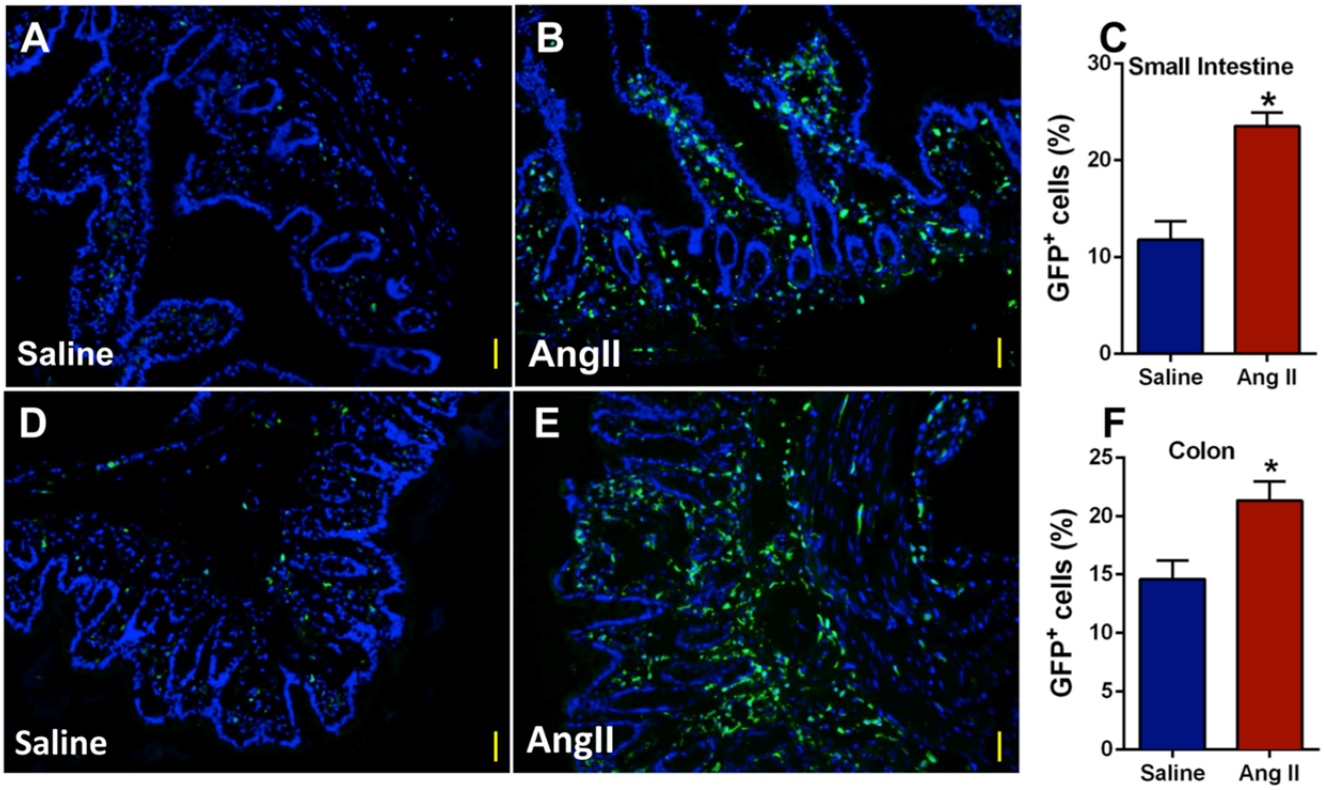
Figures



Online Figure I: Permeability and stiffness of small intestine and colon are increased in Ang II hypertension. A, MAP is increased in Ang II hypertension. **B,** FITC-dextran concentration is also increased in the plasma of Ang II-infused rats (n=4-8/group). **C-D,** Elastic modulus is not altered in the small intestine and proximal colon of Ang II-infused rats (n=4-8/group). **E-H,** Protein analysis of tight junction proteins in small intestine (E, G) and proximal colon (F, H) in saline and Ang II-infused rats. *p<0.05 vs. Saline.

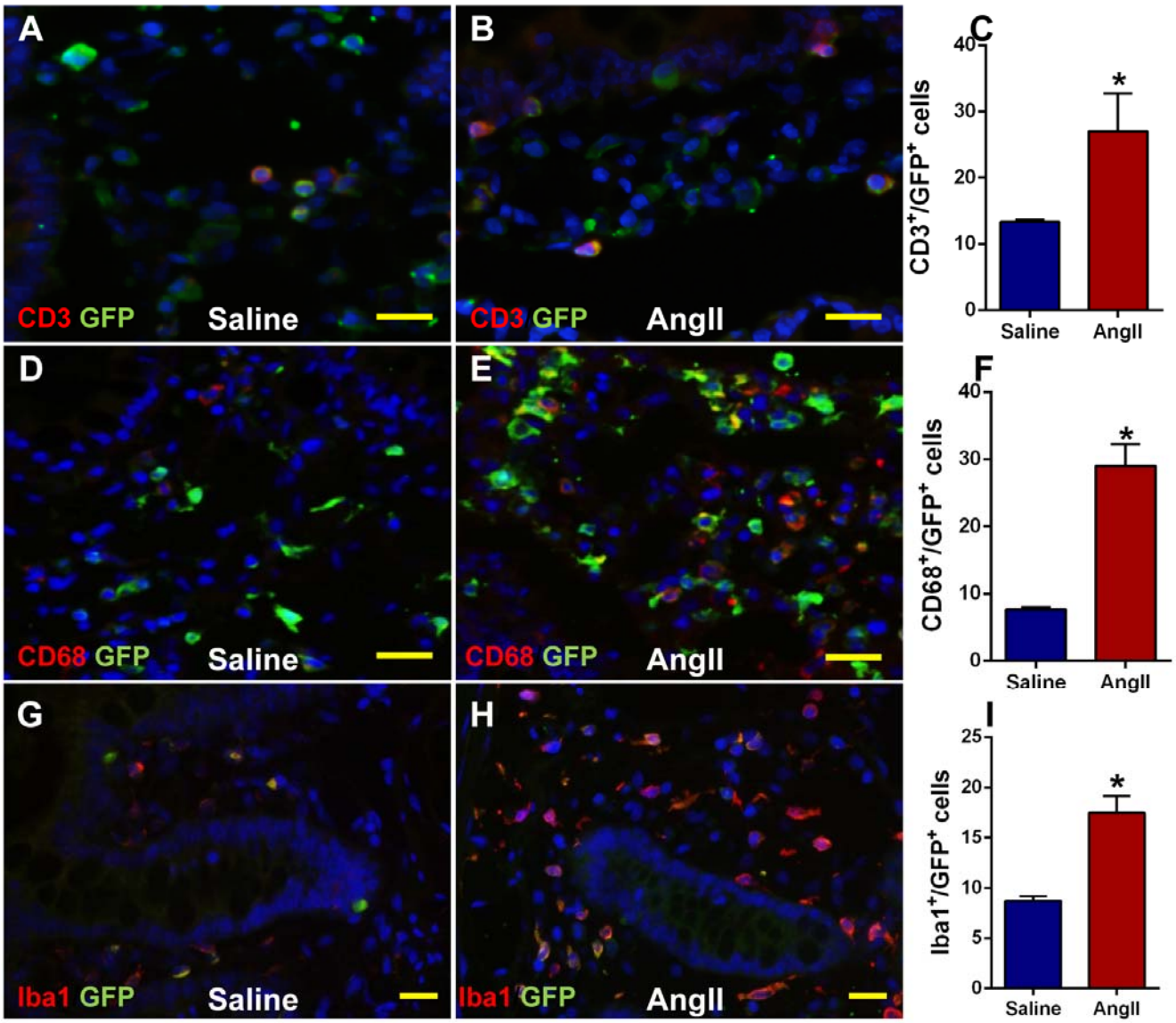


Online Figure II: Intestinal pathology in Ang II infusion rat model of hypertension.
A-C, Cross sections of small intestine of saline and Ang II rats were stained with Masson's trichrome to quantify the fibrosis (n=4/group) and **D-F,** stained with H&E to measure the thickness of tunica muscularis layer (n=4/group). **G-I,** Villi of the Ang II rats were shortened (n=4/group) and **J-L,** the number of goblet cells per 100 epithelial cells was decreased in Ang II rats (n=4/group). *p<0.05 vs. saline.



<u>Online Figure III</u>: Chronic Ang II infusion increases BM-derived GFP$^+$ cells in small intestine and colon.

Representative images and quantification of GFP$^+$ cells in small intestine **(A-C)** and proximal colon **(D-F)**. *p<0.05 vs. WKY (n=4/group).



<u>Online Figure IV</u>: Chronic Ang II infusion increases BM-derived CD3$^+$, CD68$^+$, and Iba1$^+$ cells in small intestine.

A-C, Representative images and quantification of CD3$^+$/GFP$^+$ BM-derived T-cells in the small intestine of Ang II-infused rats. **D-F,** Representative images and quantification of CD68+/GFP+ bone marrow-derived macrophages in small intestine of Ang II-infused rats. **G-I,** Representative images and quantification of Iba1$^+$/GFP$^+$ BM-derived myeloid cells in small intestine of Ang II-infused rats. *p<0.05 vs. WKY (n=3-6/group).

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