Supplemental Material

Post-stroke lung infection by opportunistic commensal bacteria is not mediated by their expansion in the gut microbiota

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The ARRIVE guidelines 2.0: author checklist

The ARRIVE Essential 10

These items are the basic minimum to include in a manuscript. Without this information, readers and reviewers cannot assess the reliability of the findings.

Item		Recommendation	Section/line number, or reason for not reporting
Study design	1	For each experiment, provide brief details of study design including: a. The groups being compared, including control groups. If no control group has been used, the rationale should be stated. b. The experimental unit (e.g. a single animal, litter, or cage of animals).	a) Legend to all figures b) Supp. Methods, page 2, section: Study design, line 6
Sample size	2	a. Specify the exact number of experimental units allocated to each group, and the total number in each experiment. Also indicate the total number of animals used. b. Explain how the sample size was decided. Provide details of any *a priori* sample size calculation, if done.	a) Exact number in each figure legend. Total number Supp. Methods page 2 / section: Study design, line 1. b) Supp. Methods page 2 / section: Study design, lines 1-6
Inclusion and exclusion criteria	3	a. Describe any criteria used for including and excluding animals (or experimental units) during the experiment, and data points during the analysis. Specify if these criteria were established *a priori.* If no criteria were set, state this explicitly. b. For each experimental group, report any animals, experimental units or data points not included in the analysis and explain why. If there were no exclusions, state so. c. For each analysis, report the exact value of *n* in each experimental group.	a) Supp. Methods page 2/ section: Study design, line 13-17 b) Supp. Methods page 2/ section: Study design, lines 17-26. c) In each figure legend
Randomisation	4	a. State whether randomisation was used to allocate experimental units to control and treatment groups. If done, provide the method used to generate the randomisation sequence. b. Describe the strategy used to minimise potential confounders such as the order of treatments and measurements, or animal/cage location. If confounders were not controlled, state this explicitly.	a) Supp. Methods, page 2/ section: Study design, line 9 b) Supp. Methods, page 2, section: Study design, line 9-10.
Blinding	5	Describe who was aware of the group allocation at the different stages of the experiment (during the allocation, the conduct of the experiment, the outcome assessment, and the data analysis).	Supp. Methods, Page 2 /section: Study design, lines 11-12
Outcome measures	6	a. Clearly define all outcome measures assessed (e.g. cell death, molecular markers, or behavioural changes). b. For hypothesis-testing studies, specify the primary outcome measure, i.e. the outcome measure that was used to determine the sample size.	a) It is all stated in the results section and figures b) Supp. Methods, Page 2, section: Study design, line 4-5
Statistical methods	7	a. Provide details of the statistical methods used for each analysis, including software used. b. Describe any methods used to assess whether the data met the assumptions of the statistical approach, and what was done if the assumptions were not met.	a) Supp. Methods, Page 9. Heading: Statistics b) Supp. Methods, Page 9. Heading: Statistics
Experimental animals	8	a. Provide species-appropriate details of the animals used, including species, strain and substrain, sex, age or developmental stage, and, if relevant, weight. b. Provide further relevant information on the provenance of animals, health/immune status, genetic modification status, genotype, and any previous procedures.	a)b) Supp. Methods, page 2 Section: Animals, lines 1-5
Experimental procedures	9	For each experimental group, including controls, describe the procedures in enough detail to allow others to replicate them, including: a. What was done, how it was done and what was used. b. When and how often. c. Where (including detail of any acclimatisation periods). d. Why (provide rationale for procedures).	a-d) All details are provided in methods section and results.
Results	10	For each experiment conducted, including independent replications, report: a. Summary/descriptive statistics for each experimental group, with a measure of variability where applicable (e.g. mean and SD, or median and range). b. If applicable, the effect size with a confidence interval.	a) Figure legends b) Figure legends

EXPANDED MATERIALS & METHODS

Animals

We used wild-type male C57BL/6 mice aged 11-12 weeks. Mice were from the same supplier (Janvier, France), except for one experiment designed to validate the results in mice from another supplier (Harlan, Envigo, Spain). For the immunofluorescence study we used reporter CX3CR1$^{eGFP/+}$ male and female mice in the C57BL/6 background (12-month old) that were originally obtained from Jackson. Mice were maintained at the animal house of the School of Medicine at the University of Barcelona. Animal work was conducted according to the Spanish law (Real Decreto 53/2013) in compliance with the EU Directive 2010/63/EU for animal experiments, and with approval of the ethics committees: *Comité Ètic d'Experimentació Animal de la Universitat de Barcelona* (CEEA) (226/19; 227/19; 117/18, 9987; 472/18 P1), *Comité de Ética del Consejo Superior de Investigaciones Científicas* (831/2019, 10656), and *Comissió d'Ètica en l'Experimentació Animal i Humana de la Universitat Autònoma de Barcelona* (CEEAH) (4655,10710); all of them with approval of the local competent organs of the *Generalitat de Catalunya*. We report the study following the ARRIVE guidelines.

Study design

The total number of mice used in this study was 495. The number of mice per group used in each experiment is reported in each figure legend. For most experiments we estimated an effect size d=1.25, α error=0.05 and power of 0.90, yielding around 12 mice per group. However, for the bacterial CFUs we observed high variability and decided to use an effect size d=1. In exploratory experiments, e.g. mRNA, we used a minimum reasonable number of mice based on previous studies. Mice are the experimental units of this study given that the number of mice that could be processed each day was limited for most of the experiments and we processed mice of the different groups on the same day to minimize day-to-day variation. We did not randomize the allocation of mice. However, we assigned at least one mouse to different surgeries and/or treatments in the same day, varying the order every day. Treatments were blindly administered to mice since drugs and vehicles were prepared by a different researcher who did not reveal the identity of the treatments until the end of the analysis. Exclusion criteria were set a priori. Mice subjected to ischemia were excluded if they showed no brain lesion (14.7%; n=43 out of 292 mice subjected to MCAo surgery). We excluded the mice that died before the endpoint (7.2%; n=21 out of 292 mice subjected to MCAo). We did not observe differences between groups excepting for higher mortality in the RU486 treatment, as shown in Suppl. Fig. 7C. In addition, we excluded 16 data points in the different experiments as follows: two mice were excluded due to erroneous treatment with non-freshly prepared PDTC product; in the bacteria CFU study, three mice (1 control, 1 ischemic vehicle, and 1 ischemic PDTC) could not be processed due to unexpected problems, and one control sample was excluded due to contamination; in the organ and body weight group there is a missing value because one sham mouse was not weighted; one sham sample was excluded in the *ex vivo* intestinal Ussing chamber experiment for technical reasons; six samples (1 ischemic, 3 sham, and 2 control) could not be included in the metabolomics study for technical reasons; one control sample of the RNA study was excluded due to bad quality; and one ischemic sample in the flow cytometry study was excluded for technical problems.

Brain ischemia

Mice were anesthetized with isoflurane and received analgesia (buprenorphine, 140 µl of a 0.015 mg/mL solution, i.p.). We occluded the middle cerebral artery (MCA) with an

intraluminal filament (#701912PK5Re, Doccol Corporation). After the MCA occlusion, the skin wound was temporarily sutured and anesthesia was withdrawn to let the animal wake up. After 40 min of the arterial occlusion, mice were anesthetized again, the filament was removed at min 45, the wound was sutured, anesthesia was withdrawn, and mice were kept on a thermal blanket at 37ºC for 1 hour to facilitate recovery. Corresponding sham-operated mice received all the interventions except the filament MCA occlusion. At day 2, mice were anesthetized with 5% isoflurane and euthanized either by intracardiac perfusion with 60 mL heparinized phosphate-buffered saline (PBS) or by cervical dislocation for organ collection. For mice subjected to ischemia, we assessed the presence of brain infarction by staining the tissue with triphenyl tetrazolium chloride (TTC). In brief, we obtained 1-mm thick coronal brain sections that were immersed for 5 minutes in 0.5% triphenyl tetrazolium chloride solution, previously heated at 37º C and protected from light. Slices were fixed in 4% PFA for 24 hours and washed three times in phosphate buffer.

Drug treatments

For each mouse, stool samples were collected before ischemia and treatments, and again at 48 hours after reperfusion before euthanasia.

Propranolol: Immediately before surgery, propranolol or vehicle (saline) was administered intraperitoneally at 30 mg/kg from a stock of 6 mg/ml.[3] Subsequent doses were administered at 4, 8, 12, 24, 28, 32 and 36 hours after reperfusion.

LED 209: Three hours before induction of stroke, mice received LED 209 (N-phenyl-4-benzenesulfamide, 1.6 mg/ml) or vehicle (70% sodium bicarbonate pH 9, 23% polyethylene glycol, 5% DMSO, 2% Tween-80) by gavage. A second dose was administered 24 hours after reperfusion. The dose (16 mg/kg), the vehicle, and the administration route were chosen following a previous report.[20]

RU-486: Animals were injected intraperitoneally with 25 mg/Kg of the glucocorticoid receptor antagonist RU486 (Mifepristone, #M8046, Merck) diluted at 6 µg/µl in ethanol/soybean oil solution (1:10) according to a previously published protocol.[3,6] The treatment and the relative control (ethanol/soybean oil solution only) were administered 12h and 2h before inducing cerebral ischemia.

PDTC: Animals were injected intraperitoneally with 25 mg/Kg of the NFκB inhibitor ammonium pyrrolidine dithiocarbamate (PDTC, #P8765, Merck) freshly diluted at 5 µg/µl in saline solution for each injection.[30] The treatment and the relative control (saline solution only) were administered 4h and 24h after inducing cerebral ischemia.

Fecal sample collection and processing

Fresh fecal pellets from each animal and cecum at the culling time were collected in aseptic conditions and kept at −80°C until samples processing. Fecal samples and cecal content were weighed, diluted 1:7 (w/v) in PBS and vigorously homogenized by vortexing. The fecal supernatant was separated by centrifugation (10,000 rpm, 15 min) and stored at −20°C until further analysis.

Determination of Short Chain Fatty Acids

SCFAs levels were determined in the fecal supernatants by gas chromatography (GC) in a system composed of a 6890NGC injection module (Agilent Technologies, Madrid, Spain)

connected to a flame injection detector and a mass spectrometry 5973N detector (Agilent). Briefly, 100 µl cell free-supernatants were mixed with 450 µl methanol (Merck, Darmstadt, Germany), 50 µl internal standard solution (2-ethylbutyric 1.05 mg/ml) (Sigma-Aldrich, St. Louis, MO, USA) and 50 µl 20% v/v formic acid (Sigma-Aldrich, St. Louis, MO, USA), and centrifuged. The supernatant obtained was used for injecting in the GC and quantification of SCFAs. Samples were analyzed in duplicate and molar concentrations for the main SCFAs were calculated.

Analysis of fecal microbial groups by 16S rRNA gene profiling and quantitative PCR

Bacterial genomic DNA was isolated from feces by using QIAamp DNA stool kit (Qiagen, GmbH, Germany) following manufacturer's instructions. The hypervariable V3-V4 region of the 16S rRNA gene was amplified using the pair primers 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNGGGTATCTAAT) on the Illumina platform (NovaSeq 6000) following in-house protocols (Novogene, Beijing, China) from the isolated genomic DNA. Raw 250bp paired-end reads were assigned to different samples, merged to raw tags by using FLASH (Version 1.2.7) and filtered into clean tags according to QIIME (Version 1.7.0) quality-controlled process. Clean tags were compared with the reference Gold database (Release 20110519) and chimera sequence was detected and deleted by using UCHIME Algorithm (Version 7.0.1001). The non-chimera clean effective tags were clustered into OTUs with ≥ 97% similarity by Uparse software (Version 7.0.1001) and the taxonomic information was annotated using SILVA database.

Absolute levels of Enterobacteriaceae family, *Bacteroides group*, *Akkermasia*, *Bifidobacterium*, former *Lactobacillus* genera, *Clostridium* cluster IV and cluster XIVa groups, and total bacteria were determined by quantitative PCR in a 7500 Fast Real Time PCR System (Applied Biosystems) by using sealed 96 well plates (Applied Biosystems, Foster City, CA). Each reaction was run in a volume of 25 µL with 1 µL of fecal DNA as template, 0.2 µM of each primer and 2X SYBR-Green PCR Master MIX (Applied Biosystems). Thermal cycling conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 1 min at the appropriate primer-pair temperature previously described.[23] Data are presented as a logarithm with base 10 of colony-forming units (CFUs) per gram of feces.

Microbiological analysis of mouse tissues

For the analysis of bacterial presence in organs, tissues were obtained under sterile conditions two days after ischemia or sham-operation, and from naïve controls. Mice were deeply anesthetized with 5% isoflurane, the skin was cleaned with 70% alcohol, and organs were extracted using surgical material sterilized in a dry sterilizer (Germinator 500, Cell Point Scientific). Mesenteric lymph nodes (mLN), liver, spleen and lung were extracted, weighted and homogenized in a Polytron (#PT1200E, Kinematica) that was cleaned with chloroform, 100% ethanol, 70% ethanol and twice with sterile PBS prior to processing each tissue sample. The homogenates were filtered through 35 µm filters (#352235, Falcon), and three dilutions were prepared for each tissue, ranging from 1:2 to 1:1,000, depending on the tissue. One-hundred µl of each dilution were placed on a sterile 90-mm Petri dish and 25 mL of 2% agar in brain-heart infusion (BHI) medium at 46-48 ºC was added to the plate. In experiments designed to selectively grow gram negative bacterial species, we spread tissues on the surface of plates containing agar with MacConkey medium instead of BHI. As internal controls, in each experiment we also plated samples from the incubation medium or liquid of various washing steps to monitor possible contaminations. After gently shaking, the mix of inoculum and

medium was allowed to solidify, plates were inverted and kept at 37ºC for 24h. We obtained pictures of the plates and colony forming units (CFUs) were counted using ImageJ Software (https://imagej.nih.gov/ij/) by two independent observers blind to the treatments and conditions.

Bacteria were picked up from agar plates, and kept in BHI broth medium plus 30% glycerol and kept frozen at -80ºC until further identification. The identity of these strains was performed by partial sequence analysis of the 16S rRNA gene. Briefly, the bacterial genomic DNA was isolated from 1mL of pure cultures by using the Gen Elute TM Bacterial Genomic DNA Kit (Sigma), following the manufacturer's instructions. The 16S rRNA gene was partially amplified using the universal primers plb 16 and mlb 16, by PCR performed in a total volume of 25 µL containing 2 µL of genomic DNA as template. The reaction mixture was composed of 10x buffer (EurX, Gdansk, Poland), dNTP 0.2mM (Amersham) and using 0.2 mM of each primer. The amplification was carried out in a Simplio Amp Thermal Cycler (Applied Biosystems) and the PCR conditions used were 5 min at 95ºC, 30 cycles of 45 s at 94ºC, 60 s at 55ºC and 35 s at 72ºC, followed by 10 min at 72ºC. Amplicons were purified using the Gen Elute PCR Clean-Up kit (SIMGA, USA) and sequenced at Macrogen Inc. (Madrid, Spain). The sequences obtained were compared to those held in GenBank using BLAST algorithm at NCBI.

MALDI-TOF MS for bacterial identification

In certain experiments we analyzed the bacteria from CFUs by MALDI-TOF mass spectrometry (MS). To this end, approximately 0.1 mg of cell material was directly transferred from a bacterial colony (if possible) or smear of colonies to a MALDI target spot. After drying at laboratory temperature, sample spots were overlaid with 1 µL of matrix solution. MS analysis was performed on an Autoflex MALDI-TOF mass spectrometer (Bruker Daltonics, Germany) using Flex Control 3.4 software (Bruker Daltonics). Calibration was carried out with the use of the Bacterial Test Standard (Bruker Daltonics). All MS spectra were measured automatically using Flex Control software according to the standard measurement method for microbial identification. Specifically, our set-up values in linear positive mode were as follows: ion source 1 voltage, 20 kV; ion source 2 voltage, 19 kV; lens voltage, 6.5 kV; mass range, 2–20 kDa; the final spectrum was the sum of 10 single spectra, each obtained by 200 laser shots on random target spot positions. With regards to the functioning of MALDI-TOF MS, by which +1 ions are predominantly generated and detected, Da is used as a unit of m/z throughout the study. We occasionally detected *Staphylococcus epidermis* and *Staphylococcus sciuri* in BHI tissue plates but also among internal controls, suggesting they were contaminants.

In vivo bacterial imaging

Mice received antibiotic treatment (ampicillin 1 mg/mL, metronidazole 1 mg/mL, neomycin 1 mg/mL, and vancomycin 0.5 mg/mL) in the drinking water for 1 week. After the last day of treatment, food was removed for 24 hours before bacterial oral gavage. In parallel, bacterial cultures of *Escherichia coli* K-12 MG1655 were grown in lysogeny broth (LB) medium with chloramphenicol (25 µg/mL) in aerobic conditions at 37ºC with shaking for 12-16 hours (OD: 600 nm). E. coli K-12 MG1655, kindly provided by Dr. Cormac Gahan (Cork University), expresses PhelpLuxABCDE operon,[21] which allows constitutive luminescence activity thanks to the complex machinery provided by the genes in the operon. Bacteria was then pelleted at 6,000g for 5 min and diluted with drinking water up to $5x10^6$ CFUs/mL for oral administration. Mice were subjected to stroke or sham surgery 24 hours after bacteria oral gavage. Image acquisition was carried out using a high-efficiency ORCA-2BT Imaging System (Hamamatsu

Photonics) provided with a C4742-98-LWG-MOD-CCD digital camera cooled at -80ºC. Mice were euthanized and organs were imaged for photon detection 48 hours after stroke or sham surgery.

In vivo intestinal permeability

After 4 hours of fasting, mice were orally administered 4KDa FITC-Dextran diluted in PBS (#46944-100 Sigma-Aldrich), 500 mg/kg, either 3 or 24 hours after surgery. One hour later, blood samples were collected from the vena cava and whole intestines from the stomach to the anus were preserved in 50 ml tubes with cold PBS protected from light. Blood samples were centrifuged at 1,500 x*g* for 10 minutes at room temperature to obtain plasma. Fluorescent measurements were completed in a fluorescent plate reader (SynergyTM 2, BioTek) at 485 nm excitation and 528 nm emission. Concentrations were calculated by interpolating emission values into a standard curve of known concentrations of FITC-Dextran after prior subtraction of blank controls. Intestines were imaged using a chemiluminescence detection system (The Image Quant LAS 4000, GE Healthcare), with an excitation filter of 312 nm and 605 nm for emission. The length of the small intestine was measured (from the pylorus to the Ileocecal valve) with ImageJ software and the relative FITC dextran distance was calculated.

Ex vivo measurement of intestinal permeability and electrical parameters

Measurement of intestinal permeability and electrical parameters, i.e. transepithelial resistance (TER), conductance (G), and electrical current (Isc) were analyzed *ex vivo* under physiological conditions with the Ussing chamber system. Stroke or sham mice were euthanized by cervical dislocation 3 or 24 hours after surgery and a fragment from the jejunum (~1 cm^2) was used for each chamber of the experiment, with minimum 4 replicates from the same animal. The tissue was moisturized with Krebs glucose solution (KCl 461 mM, NaH_2PO_4 x $2H_2O$ 114 mM, $CaCl_2$ x $2H_2O$ 250 mM, $MgSO_4$ x $7H_2O$ 116 mM, NaCl 115 mM, $NaHCO_3$ 22 mM, $C_6H_{12}O_6$ 11 Mm, pH 7.4) and the muscular layers were removed with the help of a scalpel blade. Next, we transferred the intestinal fragment to the Ussing chamber, with the basal layer exposed to Krebs solution and the mucosal layer exposed to Krebs mannitol solution (KCl 461 mM, NaH_2PO4 x $2H_2O$ 114 mM, $CaCl_2$ x $2H_2O$ 250 mM, $MgSO_4$ x $7H_2O$ 116 mM, NaCl 115 mM, $NaHCO_3$ 22 mM, $C_6H_{14}O_6$ 11 mM, pH 7.4) to maintain osmotic balance and avoid sodium mediated glucose transport. After 15 minutes of tissue incubation, we measured the electrical parameters with Acqknowledge software (Biopac, Goleta, CA, USA) at time point 0, and every 15 minutes, up to 60 minutes. To assess paracellular gut permeability 250 µl of a FITC dextran 4KDa solution in Krebs mannitol was added to the mucosal layer chamber (final concentration 1,000 µg/ml). To keep hydrostatic pressure, another 250 µl of Krebs glucose solution was added in the basolateral layer chamber. Aliquots from the basal layer chamber were taken at time point 0 and every 15 minutes up to 60 minutes. FITC Dextran fluorescence from the aliquots was analyzed on a 96 plate in a spectrophotometer with a 485 nm excitation laser and 528 nm emission laser (Infinite F200 Pro, TECAN). Concentrations of the samples were calculated by interpolation in a standard curve. To confirm tissue viability, we added carbachol (10^{-5} M), an agonist of cholinergic receptors that induces ion release in healthy tissue. Replicates that were unresponsive to carbachol at the end of the experiment were excluded from subsequent analysis.

Gene expression

We extracted RNA from the ileum and the liver using Trizol® Reagent (Life Technologies, Invitrogen, Carlsbad, CA, USA) followed by PureLink™ RNA Mini Kit (#12183018 A, Invitrogen). For targeted RNA profiling, RNA quantity and quality were analyzed using Qubit RNA Hs. The expression levels of 248 immune-related and 6 additional housekeeping genes were obtained using the Mouse Inflammation V2 panel (# NA-XT-CSO-MIN2-12, Nanostring Technologies, Diagnóstica Longwood, Zaragoza, Spain). A minimum of ~370 ng of total RNA was used to quantitate the expression levels of immune-related genes using the nCounter platform (Nanostring Technologies).

For RT-PCR, RNA quantity and quality were assessed using ND-1000 micro-spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Total RNA was reverse transcribed using a mixture of random primers (#4387406, High-Capacity cDNA Reverse Transcription kit, Applied Biosystems, Foster City, CA). From the RNA obtained, 1 µg of total RNA was reverse transcribed, and the final product was diluted six times in RNAse-free water. RT-PCR was carried out with Taqman™ system (#4440038, Applied Biosystems™) using iCycler iQ™ Multicolor Real-Time Detection System (Bio-Rad, Hercules, CA, USA). We quantified by normalizing cycle threshold (Ct) values with Hprt1 housekeeping gene Ct. Analysis was carried out with the 2−ΔΔCT method. The Taqman probes used were as follows: Hprt1, #Mm00446968_m1 ; Mbl2, #Mm00487623_m1; Tlr7, #Mm00446590_m1; Ccl5, #Mm01302427-m1; Cxcl10, #Mm00445235_m1; Ccl2, #Mm00441242_m1; Il1b, #Mm00434228_m1; Slc10a2, #Mm00488258_m1; Slc10a1, #Mm00441421_m1; Fabp6, #Mm00434315_m1; Nr1h4, #Mm00436425_m1; Fgf15, #Mm00433278_m1; Nr0b2, #Mm00442278_m1; Gpbar1, #Mm04212121_s1; Cyp7a1, #Mm00484150_m1; Cyp27a1, #Mm00470430_m1; Cyp7b1, #Mm00484157_m1; Cyp8b1, #Mm00501637_s1; Akr1d1, #Mm01165275_m1; Abcb1a, #Mm00440761_m1; Abcb1b, #Mm00440736_m1; Abcb4, #Mm00435630_m1; Abcb11, #Mm00445168_m1; Abcc2, #Mm00496899_m1; Abcg2, #Mm00496364_m1; Rela, #Mm00501346_m1; Nfkb1, #Mm00476361_m1.

Isolation of gut regions and tissue processing for flow cytometry

The small intestine was removed and tissue isolation separating intraepithelial lymphocytes (IELs) and lamina propria (LP) was performed. Peyer's patches (PP) and mLN were excised, counted, and homogenized with a Dounce tissue grinder (DWK Life Sciences) with Harvest medium (RPMI 1640, FBS 5%, Pen-Strep 20 U/mL-20 µg/mL, gentamicin 50 ng/mL). Tissue was filtered through a 100-µm filter and centrifuged at 4ºC, 7 minutes, 300 x *g*, and resuspended in an annotated volume of FACS solution (PBS, EDTA 2 mM, FBS 2%, NaN_3 0.05%) supplemented with Fc-block (1:200, BD Pharmingen). An aliquot (50 µl) was stained for flow cytometry.

Flow cytometry

We used CD16/CD32 BD FcBlock, Aqua live/dead cell fixable (# L34957, Molecular Probes, Eugene, OR, USA) for exclusion of dead cells, and fluorescent beads (# 7547053, Beckman Coulter, Brea, CA, USA) for absolute cell counting. Staining for leukocyte populations was carried out with antibodies against: CD45-BV780 (30-F11, BD Horizon, Franklin Lakes, NJ, USA), CD45R-V540 (RA3-6B2, BD Biosciences, Franklin Lakes, NJ, USA), CD3-Alexa Fluor 647 (17A2, BD Pharmingen, San Diego, CA, USA), CD8-BV605 (53-6.7, Biolegend, San Diego CA, USA), CD4-Alexa Fluor 700 (GK1.5, Biolegend), γδTCR-PE/Cy7 (GL3, eBioscience, San Diego, CA, USA), NK1.1-PerCP/cy5 (PK136, Tonbo Biosciences, San Diego, CA, USA), CD103-BV711 (M290, BD Biosciences), and CD69-Alexa Fluor 488 (H1.2F3, Biolegend). For the study of Tregs we used

the following antibodies: CD3-V450 (0032-U100, Tonbo Biosciences), CD8-BV605 (100744, Biolegend), CD45-BV780 (47-0451-82, eBioscience), CD4-FITC (MCA4635F, AbD Serotec, Oxford, UK), CD152-PE (106306, Biolegend), CD25-APC (102012, Biolegend), and FoxP3-PE/Cy7 (25-5773, eBioscience). For intracellular cytokine staining we used: CD45-BV780 (47-0451-82, eBioscience), CD3-V450 (75-0032-U100, Tonbo Biosciences), CD8-BV605 (100744, Biolegend), CD4-AF700 (100430, Biolegend), CD11b-APC/Cy7 (557657, BD Pharmingen), IFNγ-PerCP/Cy5.5 (45-7311-82, eBioscience), IL17A-APC (17-7177-81, eBioscience), and IL4-AF488 (53-7041-82, eBioscience). Data acquisition was performed in a FACS LSRII (BD Biosciences) with FACS Diva software (BD Biosciences). Flow-count fluorescent beads (Beckman Coulter) were added into each tube to determine absolute cell number per lymphoid tissue. Data were analyzed with FlowJo v10.6.0 (FlowJo-BD, Ashland, OR, USA).

ELISA Assays

Plasma was stored at -80ºC. Calprotectin concentration was measured with the Mouse ELISA Kit (S100A8/S100A9) (# ab263885, Abcam, Cambridge, UK). IgA concentration was measured from the intestinal content. The dissected gut was flushed with 12 mL cold PBS (4-10 ºC) containing protease inhibitors (#11836170001, cOmplete Protease Inhibitor Cocktail, Merck, Darmstadt, Germany). The fluid was centrifuged at 2,000 x*g* for 20 min at 4ºC. The supernatant was diluted 1:1,000 with PBS for the ELISA assay (Mouse IgA ELISA kit, #ab157717, Abcam).

Immunofluorescence

Ischemia or sham-operation was induced in CX3CR1$^{eGFP/+}$ mice. At 48h, mice were anesthetized with isoflurane and perfused through the heart with heparinized saline. The gut was obtained, flushed with HBSS buffer with 5% FBS, washed in the same buffer with 1mM DTT. The ileum was dissected out, fixed in 4% paraformaldehyde for 15 min, washed, cryopreserved in 30% sucrose and kept at -20ºC until 14 µm-thick sections were obtained in a cryostat. Cell nuclei were stained with DAPI. Images (3 sections, and 3 images per section) were taken in a confocal microscope (Dragonfly, Andor, Belfast, Northern Ireland, UK). We used the Fiji processing package of ImageJ to calculate the % of green fluorescent cells over total cell number (nuclei).

Blood cell counts

Blood was withdrawn from the inferior vena cava at the time of euthanasia and collected in K3 EDTA microtubes (Sarstedt 41.1395.005) for hematological examination. The hematological analysis was carried out using the DH36 veterinarian hematological analyzer (Shenzhen Dymind Biotechnology Co. LTD, Shenzhen, China).

Metabolomics

Liver and cecal content samples were collected from control, sham operated and ischemic mice after transcardial perfusion with cold 1% PBS. Liver samples were excised quickly, homogenized in ice-cold 0.5x PBS on ice and an aliquot of ~200 µl of liver was collected and immediately freeze-clamped using liquid nitrogen. Flushed cecal content was centrifuged at 4ºC for 10 min at 3,600 rpm, the precipitate was then weighed and stored at -80ºC. Bile acids were extracted from liver and cecal contents.[22] Briefly, liver tissue was lysed in cold MeOH:H_2O (1:1, v:v) in a 1:3 ratio (sample:solvent) with a TissueLyser LT homogenizer (QIAGEN, Hilden, Germany), using 3 mm stainless steel beads, at 40 Hz in 30-s intervals 5 times. Liver homogenates were, then, centrifuged at 4ºC for 10 min at 12,000 rpm and two aliquots of the supernatant were collected and dried out in a Speedvac (Savant SPD131DDA concentrator,

ThermoFisher Scientific, Waltham, MA, USA). Frozen pellets of flushed cecal content were instead homogenized with methanol (MeOH) in a 1:10 ratio (sample:solvent). Samples were stored at −20°C for 20 min for bile acid extraction, followed by sonication in an ice bath for 10 min, and centrifugation at 14,000 rpm for 15 min at 4°C. The supernatant was collected and dried out in a Speedvac. The resulting liver and cecal content samples were reconstituted in ACN:H_2O (20:80, v:v) before the injection. Untargeted-based metabolomics was performed using an Ultimate 3000 HPLC system coupled to an Orbitrap ELITE™ Hybrid Ion Trap-Orbitrap Mass Spectrometer (ThermoFisher Scientific). Samples were eluted using (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. Five µL of the samples were injected onto an Agilent mRP-Recovery C18 column (100 × 0.5 mm, 5 µm) thermostated at 55°C, and compounds were eluted at 100 µL/min. The gradient started from 6% to 96% of B in 20 min, keeping constant for 6 min and returned to starting conditions in 0.2 min, finally by keeping the re-equilibration at 96% of B for 8.8 min. The MS was operating in full scan mode from 70 to 1,700 m/z at 60,000 resolution and data were collected in profile mode in negative ESI. MS/MS spectra were collected at the end of the analysis in data-dependent mode by using a mass inclusion list targeting the BAs at 60,000 resolution. High collision dissociation (HCD) fragmentation was performed at 90, 120, and 180 normalized collision energy (NCE).

For data analysis, Compound Discoverer (CD, ThermoFisher Scientific) was used to align the data and the Metaboprofiler node in CD (freely available from OpenMS, http://www.openms.de/downloads/) to extract the signals and group them into features. For BAs profiling, individual species of BAs were listed by their characteristic retention time and the experimental mass. BAs were identified by the exact mass and by the elucidation of MS/MS spectra obtained using the LC-MS/MS analysis.

Statistics

Statistical comparisons of parametric data were carried out using *t*-test, one-way ANOVA, or two-way ANOVA, and post-hoc analysis was carried out with the Sidak multiple comparison test or Tukey's multiple comparisons test, as appropriate. For data not conforming normality we used non-parametric comparisons of two groups with the Mann-Whitney test and for multiple groups we used the Kruskal-Wallis test. We used the Chi-Square or Fisher's exact test for analyses of proportions. BAs levels in plasma from control, ischemic and sham-operated mice were compared using one-way ANOVA and Tukey's HSD post-hoc analysis in order to exclude changes associated with the surgical intervention. BAs levels from sham-operated mice and ischemic mice were then compared using moderated t-test and the resulting p-values adjusted using the Benjamini-Hochberg FDR (q=0.05). Differences were considered statistically significant ($p<0.05$) in the comparison between the sham operated vs. the control mice. Statistical analyses were performed with GraphPad Prism version 8.3.0. Data from 16S profiling gut microbiota were total sum scaling and cumulative sum scaling normalization to account for the non-normal distribution of taxonomic count data for diversity and abundance analyses, respectively. The dissimilarity between microbial communities (beta-diversity) were assessed by Bray-Curtis distance method and visualized using PCoA (Principal Coordinate Analysis); statistical significance of sample groupings was calculated based on permutational Manova (Permanova). Further differential abundance analyses were performed through DeSeq2 test and taxonomic profiles at the family level were presented as stacked bar-plots. Statistical analyses were computed on the software R v3.2.5 and MicrobiomeAnalyst. Data were considered statistically significant at $p < 0.05$. The number of mice per group, and the p values are indicated in the figure legends.

Supplementary Figures

Figure S1

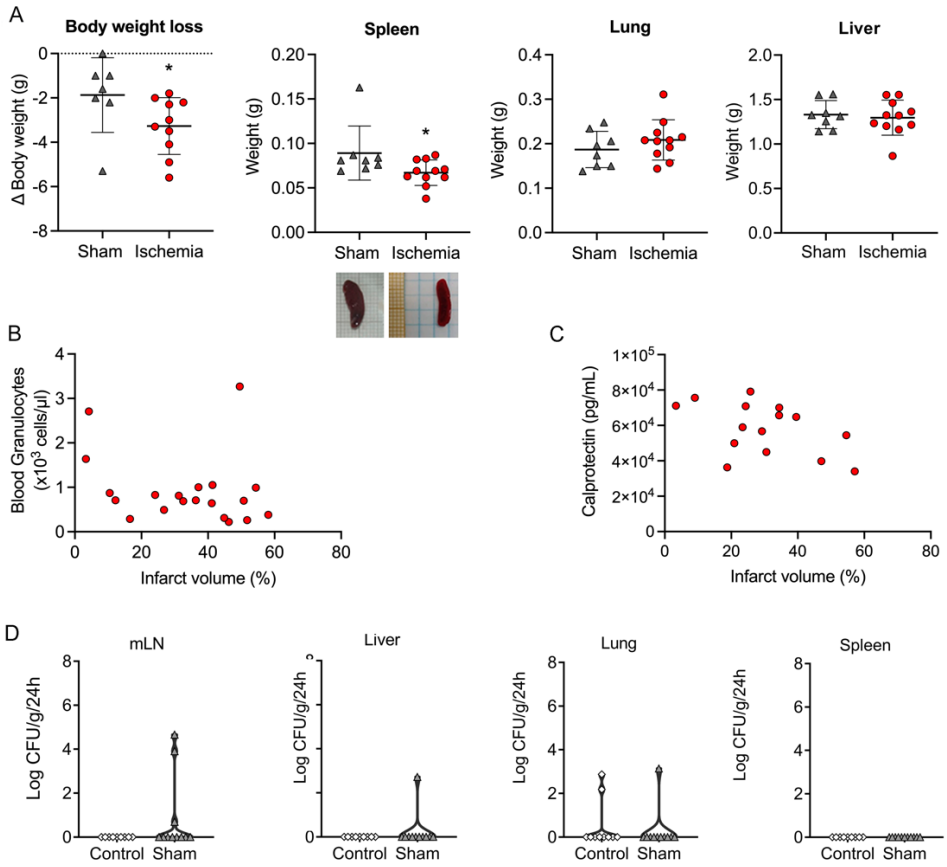


Fig. S1 General features of ischemic mice and organ bacteria growth. A) Mice showed body weight loss two days after ischemia (n=10) versus sham-operation (n=7) (*p=0.031). The size of the spleen seemed smaller after ischemia and the weight was reduced (*p=0.048), whereas the weight of the lung and liver was not affected (Mann-Whitney test) (n=10 ischemic, n=8 sham). B) Blood granulocyte counts did not correlate with infarct volume (r^2=0.057, p=0.310; n=20). C) Plasma calprotectin concentration did not correlate with infarct volume but showed a trend for inverse relationship (r^2=0.228, p=0.072; n=15). D) The number of CFUs growing from tissues of control (n=9) and sham-operated (n=11) mice were similar. These groups were pooled together for comparison with the ischemic group.

Figure S2

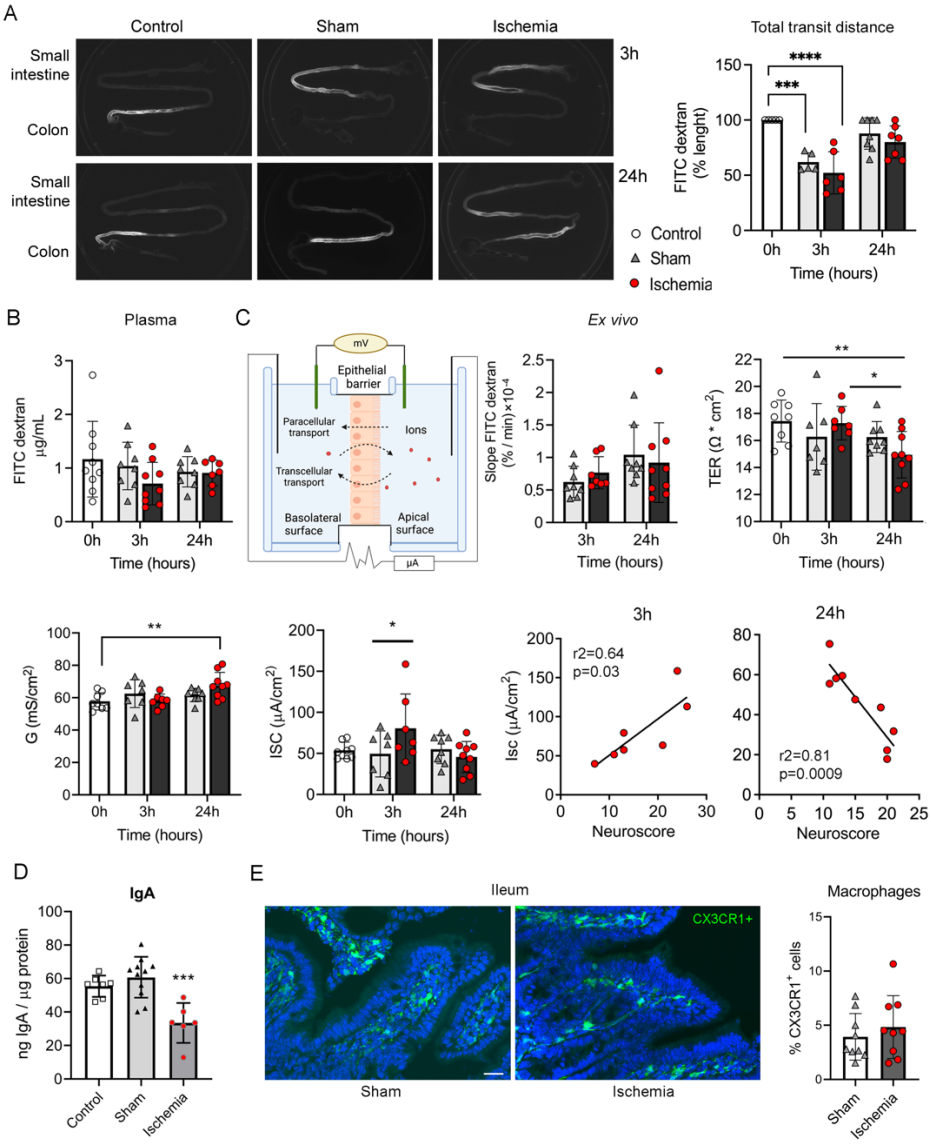


Fig. 2 Ischemia induces alterations in gut epithelial properties. A) Mice received 4-kDa fluorescein isothiocyanate dextran (FITC-Dextran) (500 mg/Kg) orally at either 3h or 24h after ischemia or sham-operation, and control naïve mice. One hour later, the mice were euthanized to study the intestinal transit and the presence of FITC-dextran in the plasma. The length of intestine containing FITC was similar in ischemic mice (n=6) and sham-operated mice (n=5) at 3h, but both groups showed less transit compared to control naïve mice (n=5). At 24h, both ischemic mice (n=7) and sham-operated mice (n=8) showed values similar to control (Kruskal-Wallis test, and Dunn's test, ** p=0.005; ***p=0.0006). B) The plasma concentration of FITC dextran shows no differences between groups. C) Schematic representation of the *ex vivo* study of small intestine epithelial permeability to ions performed in the Ussing chamber. Schema generated with BioRender.com. The gut was obtained at 3h (n=7) and 24h (n=9) following ischemia, and 3h (n=7) and 24h (n=8) sham-operation, and controls (n=8). Leakage of 4 kDa-FITC-dextran from the apical (mucosal) to the basolateral (serosal) side, as assessed by the slope of the increase in FITC-dextran accumulation in the side of the basolateral surface with time, did not differ between sham-operated and ischemic mice (Two-way ANOVA by group and time point). Transepithelial electrophysiological parameters in jejunum following ischemia were measured conducting 2-4 replicates per each animal. The transepithelial resistance (TER) decreased slightly from 3h to 24h post-ischemia (*p=0.0169) and at 24h post-ischemia is was lower than in controls (**p=0.0080) (One-way ANOVA and Sidak's multiple comparisons test). Transepithelial tissue conductance G 24h after ischemia increased vs.

controls (**p=0.009) and tended to increase vs. sham mice (p=0.07); t-test (E). The short-circuit current (Isc) increased 3h post-ischemia versus sham operation (One-way ANOVA and Sidak's multiple comparisons test, *p=0.0433). ISC values 3h post-ischemia positively correlated with the values of the neurological score (Neuroscore) (higher value corresponds to worse neurological deficit) (Pearson r^2= 0.64, p=0.03, n=7), whereas the ISC values 24h post-ischemia were negatively correlated with the neuroscore (r^2=0.81, p=0.0009, n=9). D) IgA content of the intestinal wash 2 days post-ischemia in control (n=7), sham-operated (n=11) and ischemic (n=6) mice. IgA concentration -expressed as ng/μg of intestinal wash protein- decreased in the ischemic mice (p=0.0015 vs. sham-operated mice, and p=0.0457 vs. controls) (Kruskal-Wallis test). E) Microscopic images of the ileum representative of sham-operated and ischemic CX3CR1-EGFP transgenic mice 2 days after surgery (n=9 per group) show $CX3CR1^+$ macrophages. Counting the $CX3CR1^+$ cells (green) and cell nuclei (DAPI, blue) showed that the % of macrophages was not significantly different between groups. Values are expressed as the mean ± SD.

Figure S3

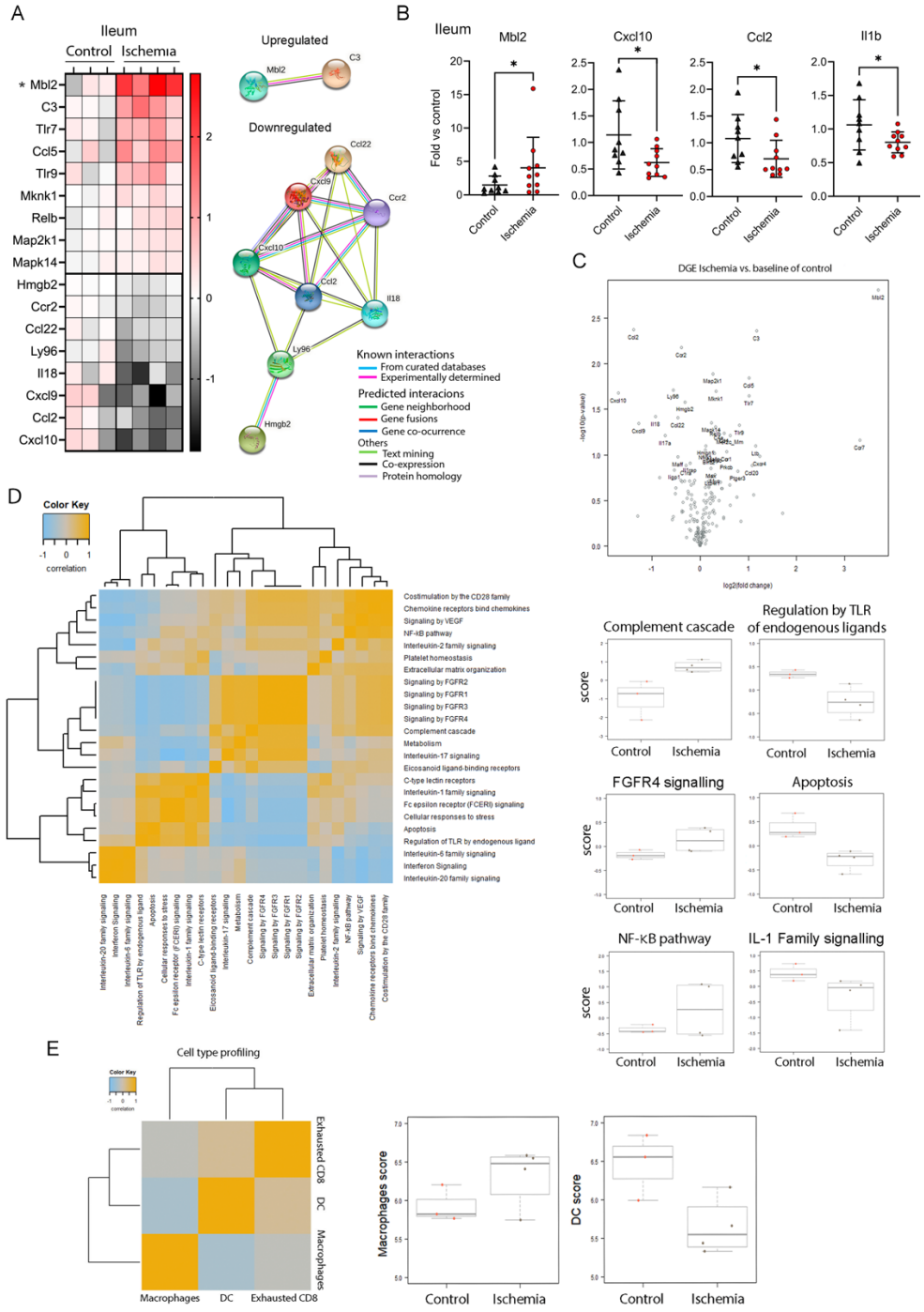


Fig. S3 RNA analysis of inflammatory gene expression in the ileum and liver. A) mRNA expression of a panel of inflammatory genes (Nanostring) in the ileum of mice 2 days post-ischemia (n=4) or controls (n=3). Data analyses of the average log-scale gene expression (nCounter Advanced Analysis 2.0) identified ischemia-induced 17 differentially expressed genes (DEG) ($p<0.05$), 9 genes were upregulated and 8 genes were downregulated after ischemia. However, adjusted p-values for multiple comparisons did not reach significance. String (https://string-db.org/) interactions found between these genes are represented for illustrative purposes. B) We validated some of the upregulated or downregulated genes in the ileum by RT-PCR in a larger sample of n=9 controls and n=10 ischemic mice: Mbl2 *p=0.047; Cxcl10 *p=0.017; Ccl2 *p=0.014; and Il1b *p=0.037 (one-tailed Mann-Whitney test). C) Volcano plot of Nanostring data (A) showing the genes most differently expressed. D) Pathway analysis showing

relationships between different pathways. Gold means highly correlated pathways and blue means opposite pathways. We selected some of the pathways to compare the scores assigned to the samples of each group, showing some of the upregulated and downregulated pathways after ischemia. E) Heatmap of correlation matrix of cell type measurements shows the correlation between different cell types. The heatmap indicates that the abundance of macrophages is inversely related to the abundance of dendritic cells (DCs). The plots on the right showing the raw cell type measurements versus respective covariate provide an estimate of abundance (cell type score calculated in log2 scale) for each condition. The results indicate a tendency to increase the function of macrophages over DCs after ischemia.

Figure S4

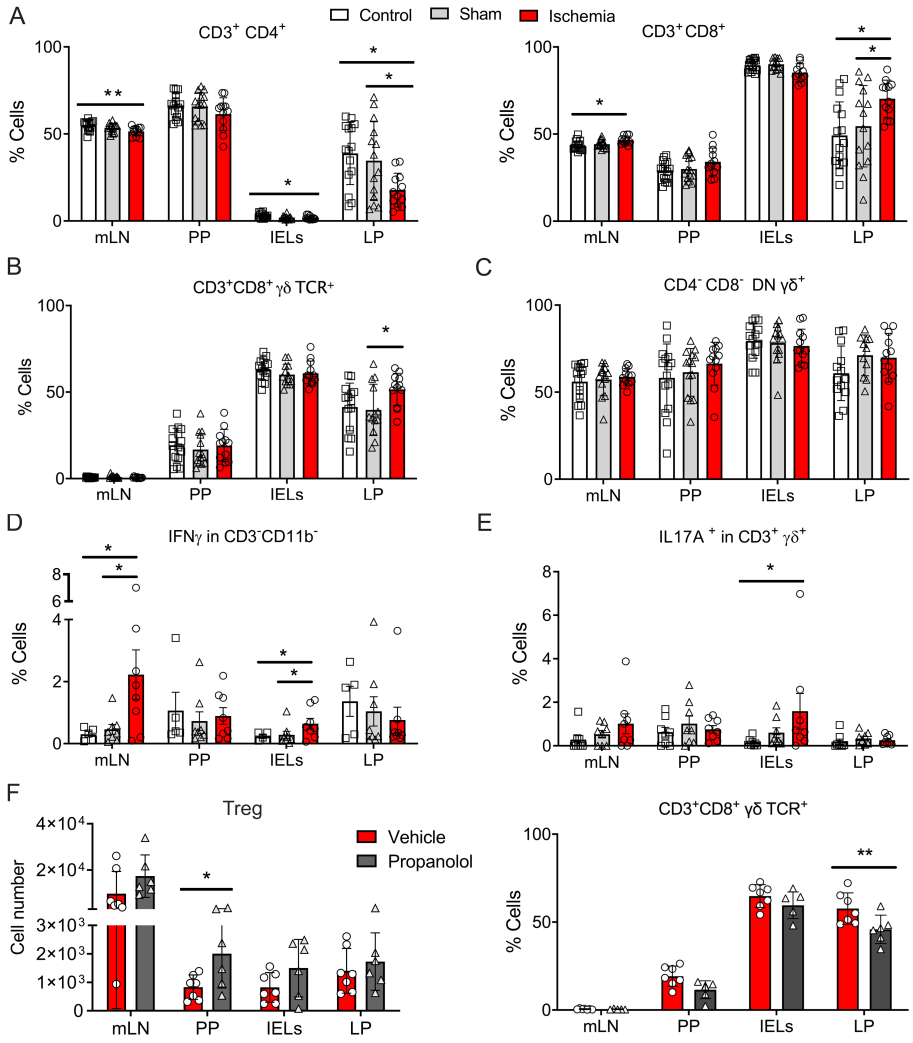


Fig. S4. Ischemia-induced alterations in blood lymphocyte populations. We studied by flow cytometry the mesenteric lymph nodes (mLN), and gut lymphoid tissue by mechanical separation of the Peyer's Patches (PP), intraepithelial lymphocytes (IELs), and Lamina Propria (LP). The populations of gut lymphocytes was studied 2 days after ischemia (n=12) or sham-operation (n=14) and in naïve control mice (n=15) (A-E). A) Ischemia reduced the % of $CD3^+CD4^+$ T lymphocytes in the LP (**p=0.008 vs. controls and *p=0.021 vs. sham) and mLN (**p=0.005 vs. controls) (one-way ANOVA and Holm-Šídák's test), and the IELs (*p=0.015 vs. controls; Kruskal-Wallis test and Dunn's test). In turn, ischemia increased the % of $CD3^+CD8^+$ T cells in the LP (*p=0.010 vs. control and sham; one-way ANOVA and Holm-Šídák's test), and mLN (**p=0.005 vs. control, Kruskal-Wallis test and Dunn's test). B) Ischemia increased the percentage of $CD3^+CD8^+\gamma\delta TCR^+$ cells in the LP (*p=0.038 vs. control and sham). C) In all regions, most $CD3^+CD4^-CD8^-$ double negative (DN) lymphocytes are $\gamma\delta$ TCR^+. D,E) Cytokine production was assessed by flow cytometry using intracellular staining in ischemic (n=8), sham (n=8) and control (n=5) mice. D) Ischemia increased the % of $IFN\gamma^+$ $CD3^-CD11b^-$ lymphocytes in the mLN (*p=0.035 vs. sham and controls) and IELs (*p=0.035 vs. control and sham); one-way ANOVA and Holm-Šídák's tests. Ischemia also increased the % of $IL17A^+$ $CD3^+$ $\gamma\delta$ TCR^+ lymphocytes in the IELs (*p=0.021 vs. controls, Kruskal-Wallis test and Dunn's test). F) Mice received propranolol (30 mg/Kg, n=6) or the vehicle (saline, n=7) via i.p. starting immediately prior to ischemia and then at 4, 8, 12, 24, 28, 32 and 36 hours after reperfusion. Propranolol attenuates ischemia-induced reduction of Treg in the PP (*p=0.034, *t*-test), and globally reduced the ischemia-induced increase in the percentage of $CD3^+CD8^+$ $\gamma\delta$ TCR^+ cells (group effect ***p=0.0008; Two-way Anova), particularly in the LP (**p=0.005 post-hoc Holm-Šídák's test). Points correspond to individual values for each mouse. Bars represent the mean±SD.

Figure S5

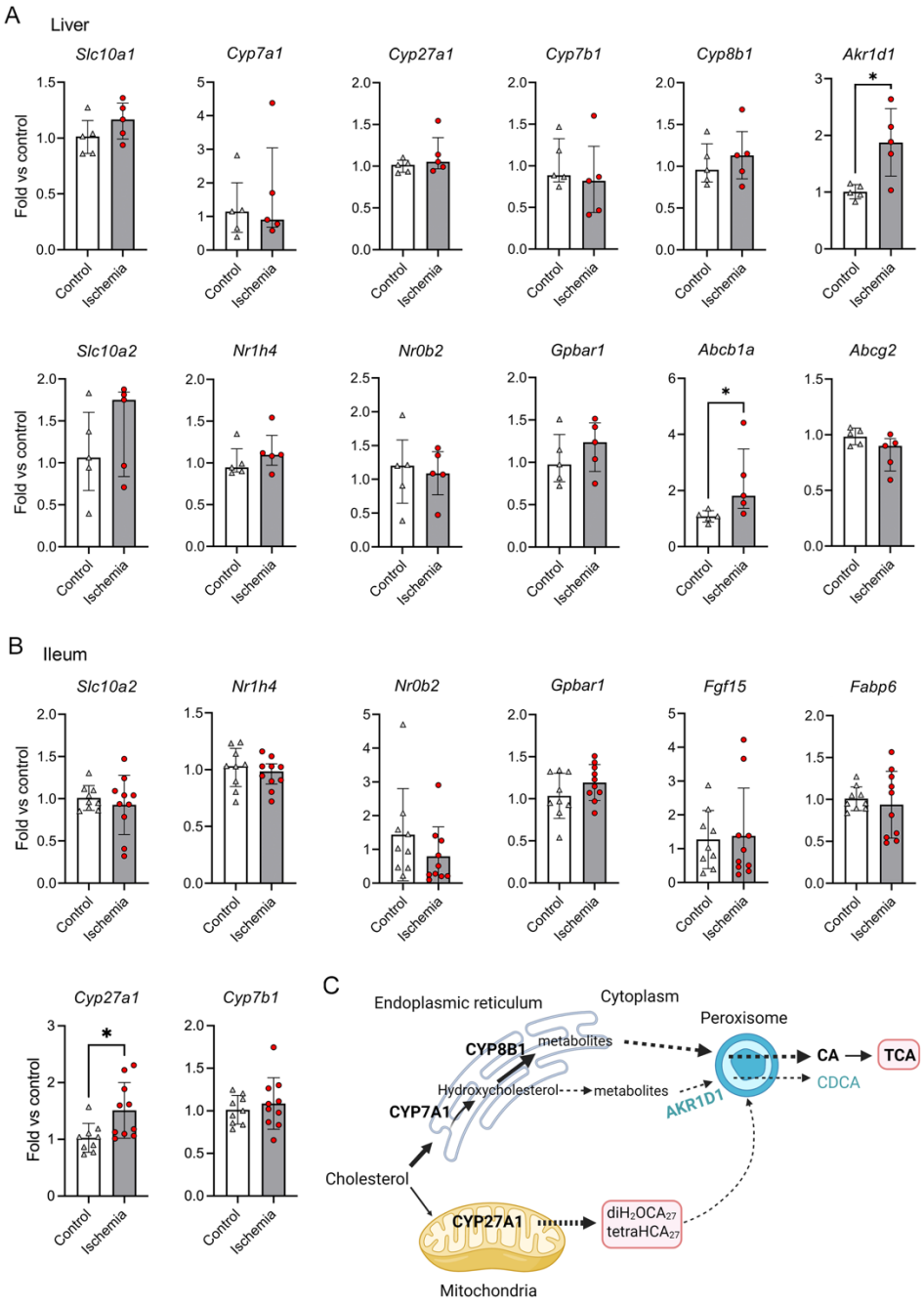


Fig. S5. Gut and liver mRNA expression of molecules involved in Bile Acid (BA) synthesis. mRNA expression in: (A) liver 2 days after ischemia (n=5) vs. controls (n=5) (*p=0.0317, Mann-Whitney test), and (B) ileum 2 days after ischemia (n=10) vs. controls (n=8) (*p=0.0125, Mann-Whitney test). Values are expressed as fold versus mean control. Points show values of individual mice and bars represent the median and interquartile range. Genes involved in BA synthesis: *Cyp7a1, Cyp27a1, Cyp7b1, Cyp8b1, Akr1da;* transport: *Slc10a1, Slc10a2, Fabp6*; efflux: *Abcb1a, Abcg2*; receptors and/or negative feedback regulation*: Gpbar1, Nr1h4*, *Nr0b2, Fgf15.* C) Simplified schematic representation of liver BA biosynthetic pathways from cholesterol through metabolites generated in the endoplasmic reticulum or the mitochondria (forming acidic BAs: diH_2OCA_{27} and $tetraHCA_{27}$) that are further metabolized in peroxisomes for generation of primary BAs cholic acid (CA) and chenodeoxycholic acid (CDCA), and the conjugated primary taurocholic acid (TCA). TCA is the main liver BA metabolite, and levels increase after ischemia (see Fig. 3).

Figure S6

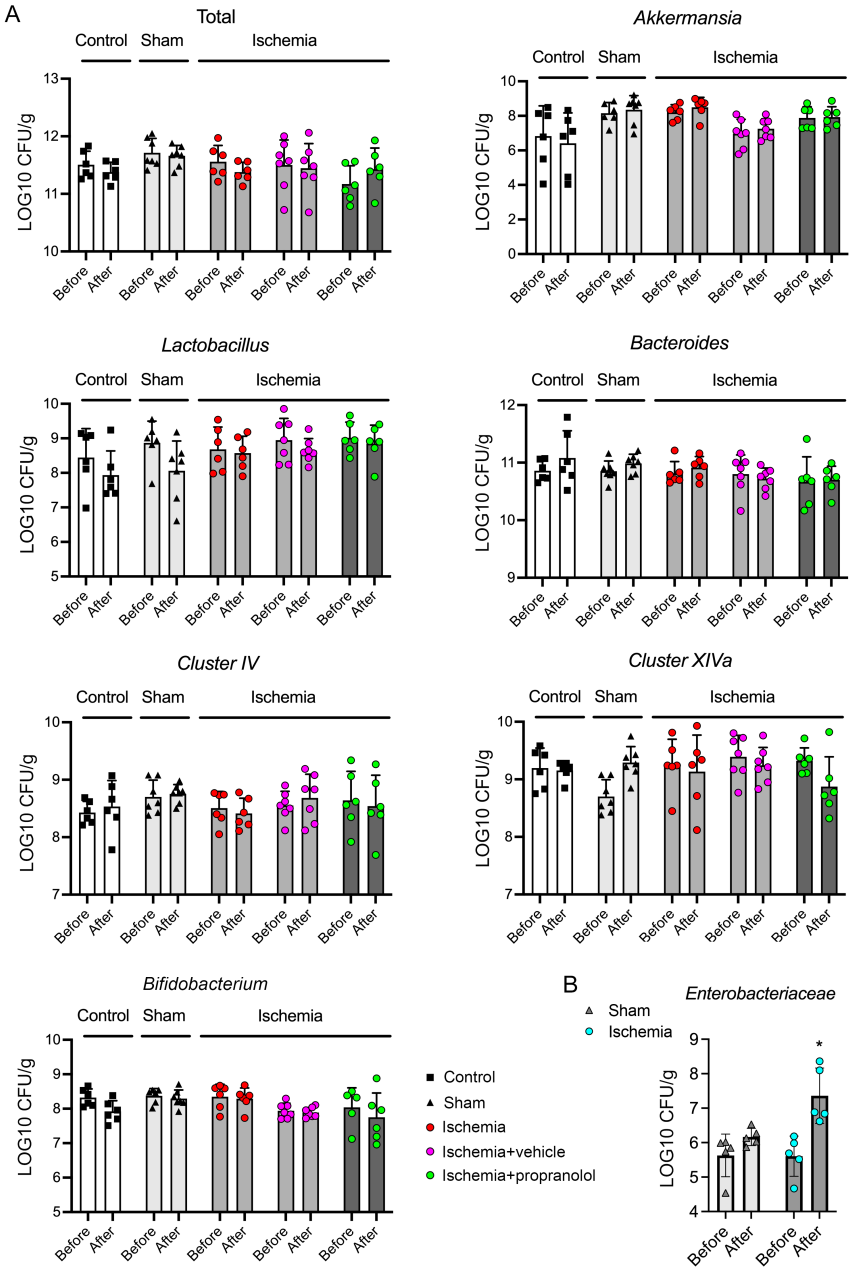


Fig. S6. Study of certain common bacteria in the feces by RT-PCR. A) Feces were collected before and two-days after ischemia or sham-operation and twice in naïve controls within the same time frame. All mice were obtained from Janvier (France). The groups control (n=6), sham (n=7) and ischemia (red dots) (n=6) were carried out in parallel, whereas the ischemic groups vehicle (n=7) and propranolol (n=6) were conducted in an independent experiment. The analysis of all feces was carried out in parallel. There were no differences between groups in total bacteria, *Lactobacillus, Bacteroides, Akkermansia, Bifidobacterium, Clostridium* Cluster IV and Cluster XIVa. We only detected increases in *Enterobacteriaceae* post-ischemia (see Fig. 5B and Fig. 6). B) In an independent experiment, we used mice from a supplier (Harlan, The Netherlands) different to our regular mouse supplier (Janvier, France) to ensure that the observed ischemia-induced *Enterobacteriaceae* expansion in the feces was not supplier-dependent. Ischemia (n=5), but not sham-operation (n=5), increased *Enterobacteriaceae* in the feces as seen before in mice obtained from another supplier (* two-way ANOVA, p=0.0123). Bars show the mean±SD.

Figure S7

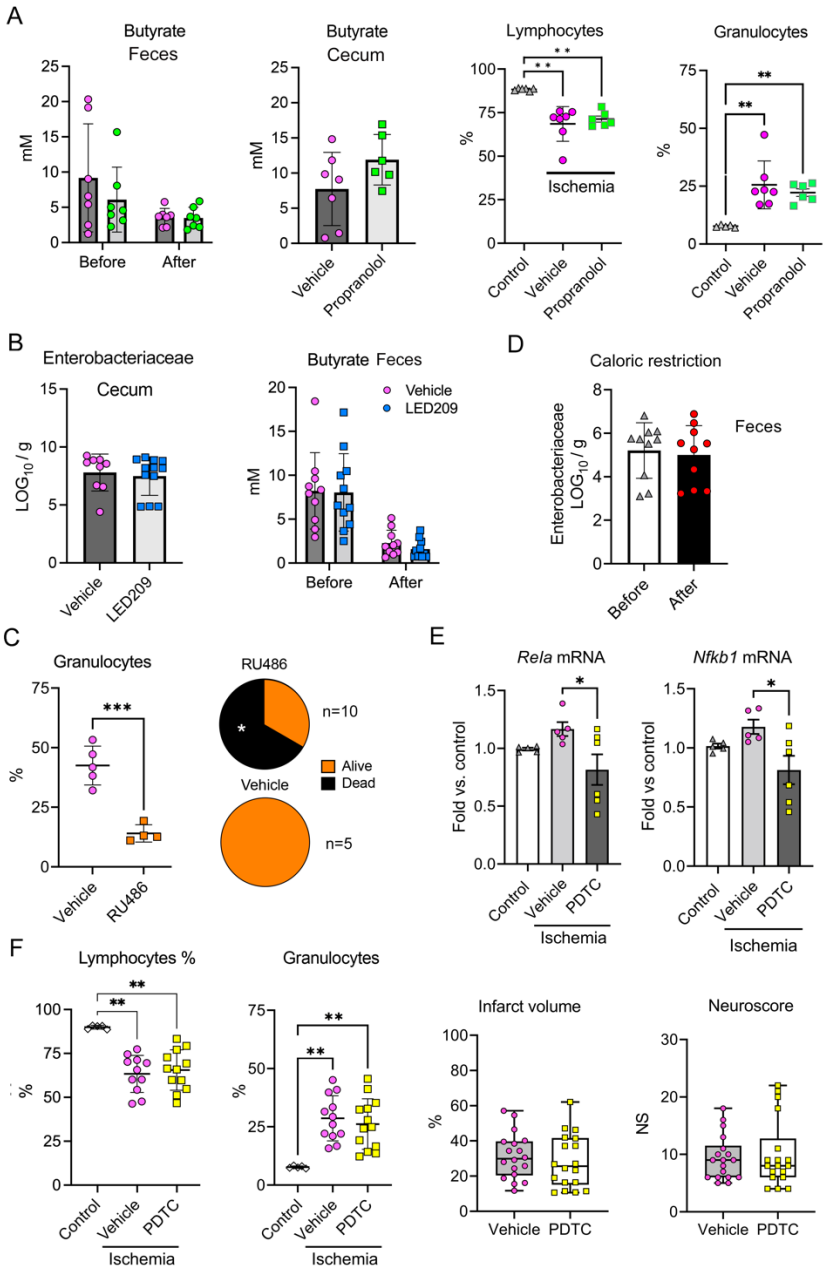
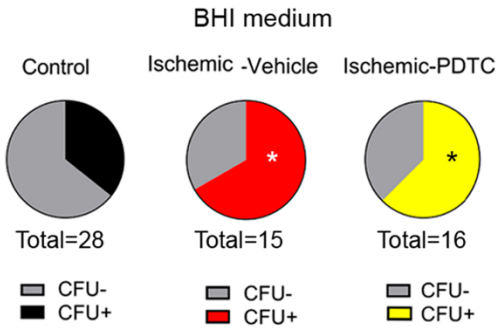


Fig. S7. Effect of treatments on ischemia-induced changes. A) Mice were treated with propranolol (30 mg/Kg) (n=7) or the vehicle (saline) (n=7) via i.p. immediately before induction of ischemia, 0h, and at 4h, 8h, 12h, 24h, 28h, 32h, and 36h post-ischemia. Butyrate before and after ischemia in feces and cecum of mice treated with propranolol did not differ from those receiving the vehicle. Propranolol did not prevent the ischemia-induced reduction of blood lymphocytes (**p=0.0056 ischemia-vehicle (n=7) vs. control (n=6); **p=0.0058 ischemia-propranolol vs. control, (n=6 per group) and increase in neutrophils (**p=0.0035 ischemia-vehicle vs. control; **p=0.0095 ischemia-propranolol vs. control) Kruskal-Wallis test and Dunn's test. B) LED209 (16 mg/Kg) (n=12) or the vehicle (n=10) were given orally 3h prior to induction of ischemia and at 24h post-ischemia. LED209 did not prevent the reduction of butyrate in feces induced by ischemia. C) RU486 (n=4) attenuated blood neutrophilia induced by stroke in the vehicle group (n=5). Treatment with RU486, but not the vehicle, caused some mortality (p=0.0606, Fisher's exact test). D) We subjected naïve mice to food restriction for two days by reducing food availability until 25% of their normal food intake. The content of Enterobacteriaceae in feces of mice under caloric restriction for two days did not change vs. mice under *ad libitum* feeding (n=10 per

group). E) Compared to the vehicle (n=5), PDTC (n=6) reduced the expression of the NF-κB p65 (*Rela*) and p50 (*Nfkb1*) subunits in the liver two days after brain ischemia (*p=0.0352 and *p=0.0189, respectively; One-way ANOVA and Holm-Šídák's multiple comparisons test). Values are expressed as fold vs. control (n=5). F) Ischemia induced blood lymphopenia in the vehicle group (n=12, **p=0.0026) and the PDTC group (n=13, **p=0.0054) compared with a group of non-ischemic naïve controls (n=5), and there were no differences between the PDTC and vehicle groups (p=0.7707); likewise, PDTC did not prevent the neutrophilia induced by ischemia (**p=0.0014 vehicle and **p=0.0045 PDTC vs. control, respectively) Kruskal-Wallis test and Dunn's multiple comparison test. Values are expressed as the mean±SD. PDTC did not modify infarct volume or the neurological score vs. the vehicle (n=18 per group). Data are shown as box and whiskers indicating minimum to maximum values. Points indicate the individual mice.

Figure S8



M	Control	M	Control	M	Ischemia vehicle	M	Ischemia PDTC
1	*Staphylococcus sciuri*	15	no CFUs	1	Not identified	1	Not identified
2	no CFUs	16	no CFUs	2	*Klebsiella pneumoniae*	2	no CFUs
3	no CFUs	17	*Escherichia coli*	3	*Escherichia coli*	3	*Escherichia coli*
4	no CFUs	18	no CFUs	4	*Enterococcus fecalis*	4	Not identified
5	Not identified	19	no CFUs	5	*Escherichia coli*	5	no CFUs
6	no CFUs	20	no CFUs	6	no CFUs	6	*Enterobacter cloacae* complex
7	no CFUs	21	Not identified	7	*Enterobacter cloacae* complex	7	*Enterobacter cloacae* complex
8	*Staphylococcus sciuri*	22	no CFUs	8	*Enterobacter cloacae* complex	8	*Escherichia coli*
9	no CFUs	23	no CFUs	9	*Staphylococcus sciuri*	9	*Enterococcus fecalis + E. coli*
10	*Enterobacter cloacae* complex	24	no CFUs	10	*Staphylococcus sciuri*	10	no CFUs
11	no CFUs	25	no CFUs	11	no CFUs	11	no CFUs
12	*E. Fecalis + S. aureus*	26	*Staphylococcus sciuri*	12	no CFUs	12	no CFUs
13	*Staphylococcus sciuri*	27	*Lactobacillus murinus*	13	*Enterobacter cloacae* complex	13	no CFUs
14	no CFUs	28	no CFUs	14	*Staphylococcus aureus*	14	*Staphylococcus aureus*
				15	no CFUs	15	Not identified
						16	*Staphylococcus sciuri*

Fig. S8. *PDTC does not prevent bacterial colonization of the lung after stroke*. The lungs of 31 ischemic mice and 28 controls were seeded on BHI agar plates. Ischemic mice were previously treated with PDTC (n=16) or received the corresponding vehicle (n=15). Graphs represent the proportion of mice showing CFUs in their lungs. Compared to controls, the incidence of mice with CFUs was significantly higher in ischemic mice either the vehicle group (*p=0.026) or the PDTC group (*p=0.043) (One-sided Chi-Square).