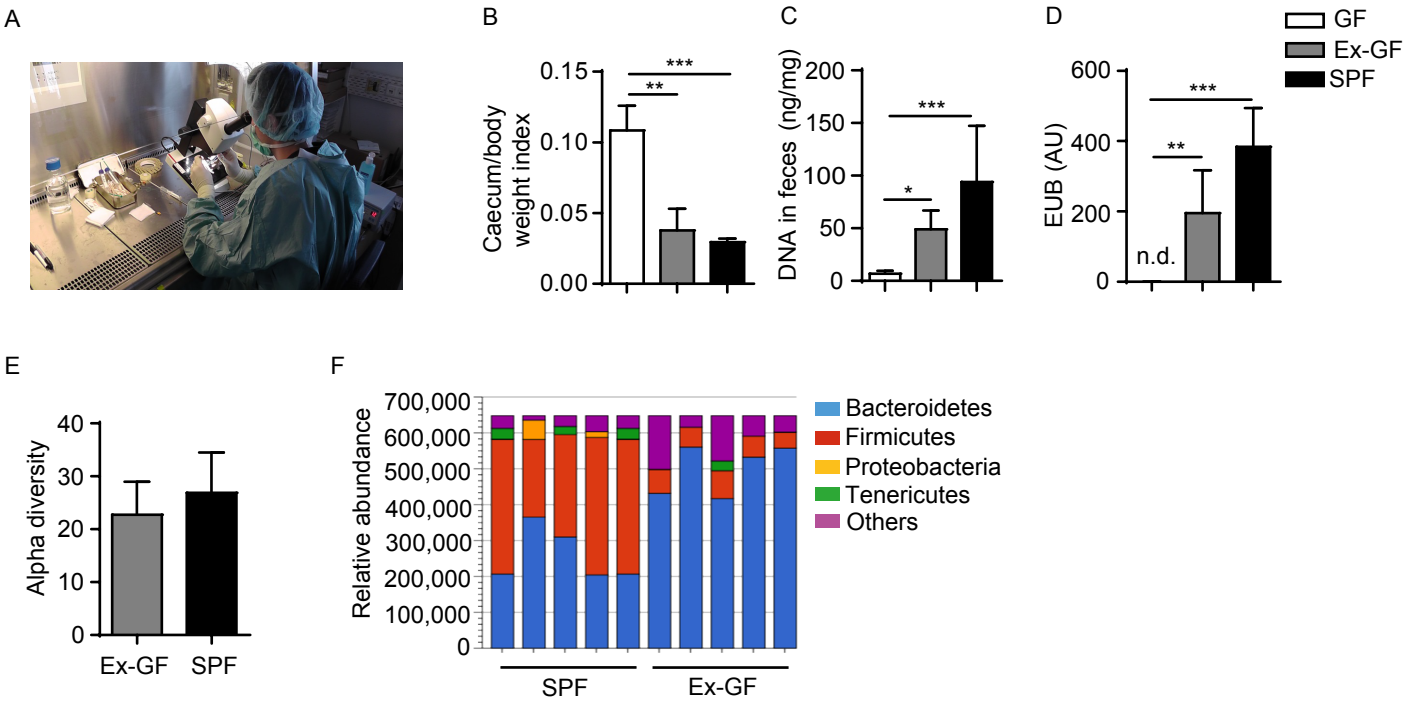
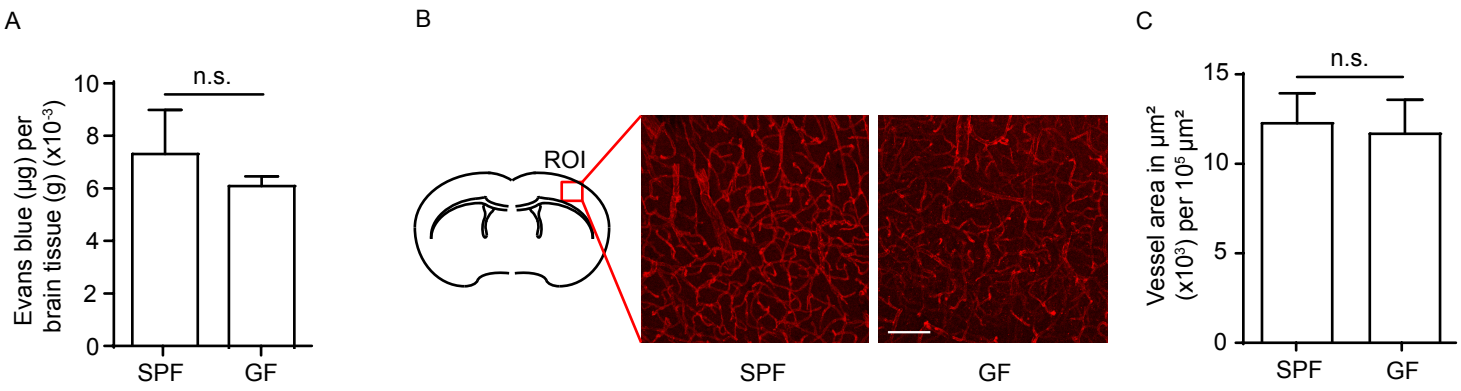
Supplementary Figure 1



Supplementary Figure 1. Maintanance of germfree status and efficient germfree mouse colonization. (A) Image of the surgical setup used under sterile conditions for stroke induction and mouse handling. The body/caecum weight index (B), total DNA load in feces (C) and eubacterial abundance (D) were determined as markers of intestinal colonization at the end of the experiment. Results demonstrate successful germfree status in the GF group as well as effective colonization of Ex-GF mouse by co-housing. Quantitative analysis of alpha diversity (E) and (F) differential phyla abundance between SPF and Ex-GF littermates as measures of successful gut bacterial colonization (n=7 per group, mean ±SD). *=p<0.05, **=p<0.01, ***=p<0.001. n.d.: not detected

Supplementary Figure 2



Supplementary Figure 2. No differences in blood-brain-barrier integrity or vascular morphology between GF and SPF mice. **(A)** Mice were given an intraperitoneal injection of Evans blue dye, 2 h later perfused and fluorometrically analyzed for the concentration of Evans blue in the brain parenchyma. The data represent the amount of extravasated Evans blue per g of the brain tissue in SPF and GF mice (n= 4 per group). **(B)** For the analysis of vascular morphology, brain coronal sections of SPF and GF mice were stained for the endothelial marker CD31. Representative images illustrate the region of interest (ROI) in the cerebral cortex for SPF and GF mice as specified in the scheme. Magnification 10x, scale bar: 100 µm. **(C).** No differences in the total vessel area in the cortical ROIs were detectable (n= 3 per group). (*p<0.05, Student's t-test). All bar graphs: mean±SD, n.s. non-significant (p>0.05).

SUPPLEMENTARY METHODS

"The gut microbiome primes a cerebroprotective immune response after stroke"

Animal experiments. All animal experiments were performed under the institutional guidelines for the use of animals for research and were approved by the governmental ethics committee of Upper Bavaria (Regierungspraesidium Oberbayern, license number 2532-65-2014). Wild type C57BL/6J mice were obtained from Charles River Laboratories. Germfree (GF) C57BL/6J and GF Rag1$^{-/-}$ female mice were obtained from the Clean Mouse Facility, University of Bern, Switzerland. Animals were randomized to the treatment groups and all analyses were performed by investigators blinded to group allocation. Unblinding was done after the completion of statistical analysis. All mice were 10-12 weeks of age at time of stroke induction. All animal experiments were performed and reported according to the ARRIVE guidelines [1].

GF mice handling and intestinal bacterial colonization. GF mice were housed in sterile HAN-gnotocages and received sterile food pellets and water as conventional SPF and colonized (Ex-GF) mice. All surgical procedures, mouse handling and cage changes were performed in a laminar flow microbiological safety cabinet. GF mice were colonized through co-housing with conventional SPF mice for 3 weeks. Co-housing was maintained also after stroke induction in all experimental groups.

Permanent distal MCA occlusion model (cMCAo). Focal cerebral ischemia was induced as described previously by permanent occlusion of the MCA distal of the lenticulostriate arteries[2]. In brief, the mice were anesthetized with an i.p. injection of fentanyl (0.05 mg/kg), midazolam (5 mg/kg) and medetomidine (0.5 mg/kg). The skin incision was performed to expose the skull, a burr hole was drilled in the temporal bone, and the MCA was permanently occluded using high frequency electrocoagulation forceps. Immediately after surgery, anesthesia was antagonized by intraperitoneal injection of a combination of naloxon (1.2 mg/kg), flumazenil (0.5 mg/kg), and atipamezol (2.5 mg/ kg). After recovery, mice were returned to their cages with ad libitum access to water and food. Sham surgery was performed by the same surgical procedures without coagulation of the exposed MCA. During the surgical procedure, body temperature of mice was kept at 37°C. The overall mortality rate for the experimental model was less than 5%. Exclusion criteria were subarachnoid hemorrhage or death during surgery.

Infarct volumetry. Perfused brains were removed at 5 days after stroke induction and frozen on dry ice. Coronal cryosections (20 μm thick) were cut at 400 μm intervals. The sections were stained with cresyl violet consistent with standard protocols and scanned at 600 dpi. Infarct area was measured in each section using ImageJ software. The total infarct volume was calculated by integrating the measured areas and intervals between the sections. Percent infarct volumes normalized to SPF or SPF WT group was presented.

Cell preparation from lymphoid organs. Mice were deeply anesthetized with ketamine (120 mg/kg) and xylazine (16 mg/kg). The mice were then transcardially perfused with normal saline. The spleen, and Peyer's patches (PPs) were removed into cold phosphate buffer saline solution. The organs were homogenized and filtered through 40 μm cell strainers; in the spleen samples, the erythrocytes were lysed using isotonic ammonium chloride buffer. The total cell counts per organ were measured using an automated cell counter (Bio-Rad). The cells were then stained with different antibodies and washed before flow cytometric analysis.

Cell preparation from brain hemispheres. Brain homogenates were prepared for FACS as previously described[3]. Briefly, after perfusion with saline, the ipsilateral brain hemispheres were removed and collected in 200μl Dulbecco´s Modified Eagle Medium (DMEM) +10% fetal calf serum (FCS). The samples were digested in 2ml of digestion mix [DMEM + 10%FCS + 0.4% DNASEI (#11284932001, Roche) + 3% CollagenaseD (#11088866001, Roche)], 10 min at 37ºC, and then mechanically dissociated. Cerebral mononuclear cells were subsequently isolated using a 70% and 40% discontinuous Ficoll gradient.

Flow cytometry analysis. The following mouse antigen-specific antibodies were purchased from eBioscience: CD3 FITC (17A2), CD4 PerCP Cy5 (clone RM4 –5), CD45 eF450 (30-F11), FoxP3 PE (NRRF-30), RORγt APC (AFKJS-9). To quantify the various cell populations, cells were stained with specific antibodies in accordance with the manufacturer's protocols. For intracellular transcription factor staining, cell suspensions from the spleen, mLN and PPs were stained using the Foxp3/Transcription Factor Staining Kit (eBioscience). Stained cells were measured in a FACSVerse flow cytometer (BD Biosciences) and analyzed using FlowJo version 10 (TreeStar).

Iba1-staining of brain sections. The animals were anesthetized with a lethal dose of Ketamine (120 mg/kg) and Xylazine (4 mg/kg). The abdomen and thorax were opened and an incision was made into the right atrium. By applying a puncta to the left ventricle 20 ml of cold isotonic 0.9 % Saline were used for cardiac perfusion. For fixation, mice were additionally perfused

with 15 ml ice-cold 4 % Paraformaldehyde (PFA) in PBS. After PFA-perfusion, the brains were rapidly removed and post-fixed in 4 % PFA for 24 hours and dehydrated in 30 % sucrose for 48 hours. Using a vibratome (Leica) 100 µm coronal sections were prepared and collected in 0.1 M phosphate-buffered saline (PBS). For free-floating staining, the sections were blocked with goat serum blocking buffer in 48-well plates and stained with 1:200 anti-Iba1 (rabbit, Wako, #019-19741) and anti-rabbit coupled to Alexa-fluor 594 (goat anti-rabbit, Thermo Fisher Scientific, # A-11012). Nuclei were stained with 4′,6-diamidin-2-phenylindol (DAPI, Invitrogen, #D1306) 1:5000 in 0.01 M PBS. Sections were mounted on microscope slides (Menzel-Gläser Superfrost ® Plus, Thermo Fisher Scientific, #3502076) and covered with a coverslip (Menzel-Gläser 24-60 mm, #1, BB024060A1, Wagner and Munz) using aqueous mounting medium (Fluoromount^{TM}, Sigma-Aldrich, #F4680-25ML). Images were acquired using a Zeiss confocal microscope with a 40x magnification (objective: EC Plan-Neofluar 40x/1.30 Oil DIC M27) with a size of 1024 x 1024 pixels and a depth of 8 bits. Images were collected in Z-stacks with a slice-distance of 0.4 µm for analysis of microglial morphology. Cell count and microglial morphology was assessed at a ROI 900µm distant from the border of the ischemic core and the homotypic area of the contralateral hemisphere.

Analysis of microglial morphology. Confocal Z-stack images were processed and microglial morphology features were extracted using custom written scripts in MATLAB (R216b, The MathWorks, Natick, Massachusetts, USA), with dependencies on the Image Processing Toolbox as well as Statistics and Machine Learning Toolbox. Statistical analysis and data visualizations were performed in RStudio [4] using R version 3.2.2 [5] and the packages ROCR [6], plyr [7], beeswarm [8] and corrplot [9]. A Kruskal-Wallis test with post-hoc Bonferroni-correction was applied for multilevel comparisons between groups. The detailed protocol and properties of the MATLAB script have been previously described[10].

Metagenomic sequencing. Total DNA was isolated from mice fecal contents by using stool DNA isolation kit (Qiagen). Same amount of DNA was amplified by using following primers and settings:16S rRNA amplicons were generated using primers corresponding to the hypervariable regions V1-V3 (primer 27F: AGA GTTTGATCCTGGCTCAG; primer 534R: ATTACCGCGGCTGCT GG), and the PCR products were purified. Libraries were prepared using the standard tagmentation procedure (Nextera XT; Illumina). All samples were sequenced on an Illumina MiSeq platform using a 300 bp paired-end approach. Amplicon sequencing datasets were analyzed using the Metagenomics (MG)-RAST pipeline (version 3.3.6) (Meyer et al., 2008). Low-quality reads (comprising 0.01% of all reads) were trimmed using the

SolexaQA program; only high-quality reads were included in the subsequent analysis. 16S rRNA genes were identified by performing a BLAT (Kent, 2002) search, and amplicons were clustered at 97% identity with an e-value cut off of 1e-5. A BLAT similarity search for the longest cluster representative was performed against the Ribosomal Database Project (RDP) database. Diversity, and phylogenetic analysis were performed using the MG-RAST platform. Read counts were normalized logarithmically for subsequent analysis. The DNA sequences have been deposited in MG-RAST (http://metagenomics.anl.gov/) under accession number 9778.

Bacterial DNA isolation and PCR. DNA was isolated from mouse feces using the QIAamp Fast DNA Stool Mini Kit (Qiagen). The total amount of DNA in each sample was measured using a Qubit (Life Technologies). Equal amounts of DNA from all samples were used for the PCR reactions and were amplified using SYBR Green ROX qPCR Mastermix (Qiagen) in a LightCycler 480 II (Roche). For the quantitative analysis of 16S rRNA sequences, PCR was performed using the following primers. EUB (eubacteria): Uni forward 340: ACT CCT ACG GGA GGC AGC AGT; Uni reverse 54: ATT ACC GCG GCT GCT GGC. Each run was performed in duplicate for each DNA sample. A linear dilution-amplification curve was obtained from diluted pooled samples, and the relative expression of each gene was calculated using the standard curve method.

Quantitative RT-PCR. Brain tissue from the ipsilateral and contralateral hemispheres was lysed in the Qiazol Lysis Reagent (Qiagen) and total RNA was extracted using the MaXtract High Density kit (Qiagen). RNA was purified using the RNeasy Mini Kit in accordance with the manufacturer's instructions (Qiagen). Equal amounts of RNA from each sample were used to synthesize cDNA with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Pre-designed RT2 qPCR Primer Assays (Ambion) were used for the following genes: *Foxp3* (PPM05497F), *Il*-10 (PPM03017C), *Il-17A* (PPM03023A), *Il-1β* (PPM03109F) and *Tnf-α* (PPM03113G). The quantitative expression was measured using SYBR Green ROX qPCR Mastermix (Qiagen) in a LightCycler 480 II (Roche). A linear dilution-amplification curve was obtained from diluted pooled samples. Using this curve, the expression of each gene was measured relative to the expression of the housekeeping gene encoding peptidylprolyl isomerase A. All assays were performed in duplicates.

Statistical analysis. Sample size was estimated according to effect size of microbiota manipulation on stroke based on our previous reports[11, 12] and on the variability of the used

stroke model as previously published[2]. Data were analyzed using GraphPad Prism version 6.0. All summary data are expressed as the mean SD. All data sets were tested for normality using the Shapiro–Wilk normality test. Groups containing normally distributed data were analyzed with either t test (two groups) or ANOVA (>two groups). The groups contain non-normally distributed data were tested using Mann Whitney U test (two groups) or using Kruskal-Wallis test (for more than two groups). Similar variance was assured for all groups, which were compared statistically. A p value < 0.05 was considered to be statistically significant.

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