SUPPLEMENTAL MATERIAL

Depletion of cultivatable gut microbiota by broad-spectrum antibiotic pretreatment worsens outcome after murine stroke

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Detailed materials and methods:

Animals and housing

During the experiments, female C57Bl/6J mice (Forschungseinrichtung für Experimentelle Medizin, FEM, Charité Berlin, Germany) after microbiota depletion by quintuple antibiotic treatment[1] in the Institute of Microbiology and Hygiene, (Charité Berlin, Germany) and SPF littermates were housed in the animal facility of the Department of Experimental Neurology (Charité Berlin, Germany).

Recolonization of microbiota-depleted mice with intestinal microbiota from SPF littermates

Two days before recolonization the antibiotic cocktail was withdrawn and replaced by autoclaved tap water. Approximately 7 fresh fecal pellets were collected from individual SPF AB (-/-) littermates and homogenized in 10 ml sterile phosphate buffered saline (PBS). To reconstitute microbiota-depleted mice with a complex conventional microbiota, the recolonization group was challenged with 0.3 ml of the supernatant from the fecal suspension by oral gavage on two consecutive days[1]. To assure proper establishment of the complex microbiota, mice were recolonized four days before inclusion in stroke experiments.

Middle cerebral artery occlusion (MCAo)

Surgical interventions were performed under pathogen-reducing conditions. Mice were anaesthetized with a combination of 1.5-2% isoflurane in 70% nitrous oxide and 30 % oxygen. Throughout the operation body temperature was maintained at 36.5°C ± 0.5°C using a heating plate. After a midline ventral neck incision a silicon hardener-coated nylon filament (7019PK5Re, Doccol Corp. Redlands, California USA) was introduced into the internal carotid artery (over the common carotid artery) and inserted up to the origin of the left middle cerebral artery (MCA) occluding the origin of the vessel and causing ischemic lesion in the territory supplied by MCA. The filament was left on place for 60 minutes, followed by reperfusion. Sham operations were performed by inserting the filament to shortly occlude the MCA and withdrawn immediately.

Infarct volume assessment using magnetic resonance imaging (MRI)

Mice were anaesthetized using 1.5-2% isoflurane in a 1:2 oxygen/nitrous oxide mix. Measurement was conducted using a 98/38 mm RF Coil, with an inbuilt MR-compatible physiology temperature and a monitoring unit operating on Paravision software platform (Bruker, Karlsruhe, Germany). Physiological body temperature was maintained using a heated water jacket. Axial T2 weighted images covering the region between the olfactory bulb and the cerebellum were achieved with a Turbo RARE sequence (imaging parameters: 256×256 in plane resolution, 20 slices with a thickness of 500 µm, FOV 28 mm, TR 4200 ms, TE 36 ms, acquisition time 6 min). Infarct sizes were calculated using Analyze 5.0 software (Analyze Direct, Overland Park, KS, USA).

Microbiological investigation of fecal samples

Fecal samples were collected on day 0 before MCAo and during the experiments (as shown in Fig.1). Samples were stored overnight at +4°C if analysis took place on the next day or immediately frozen and stored at -20°C for further analysis.

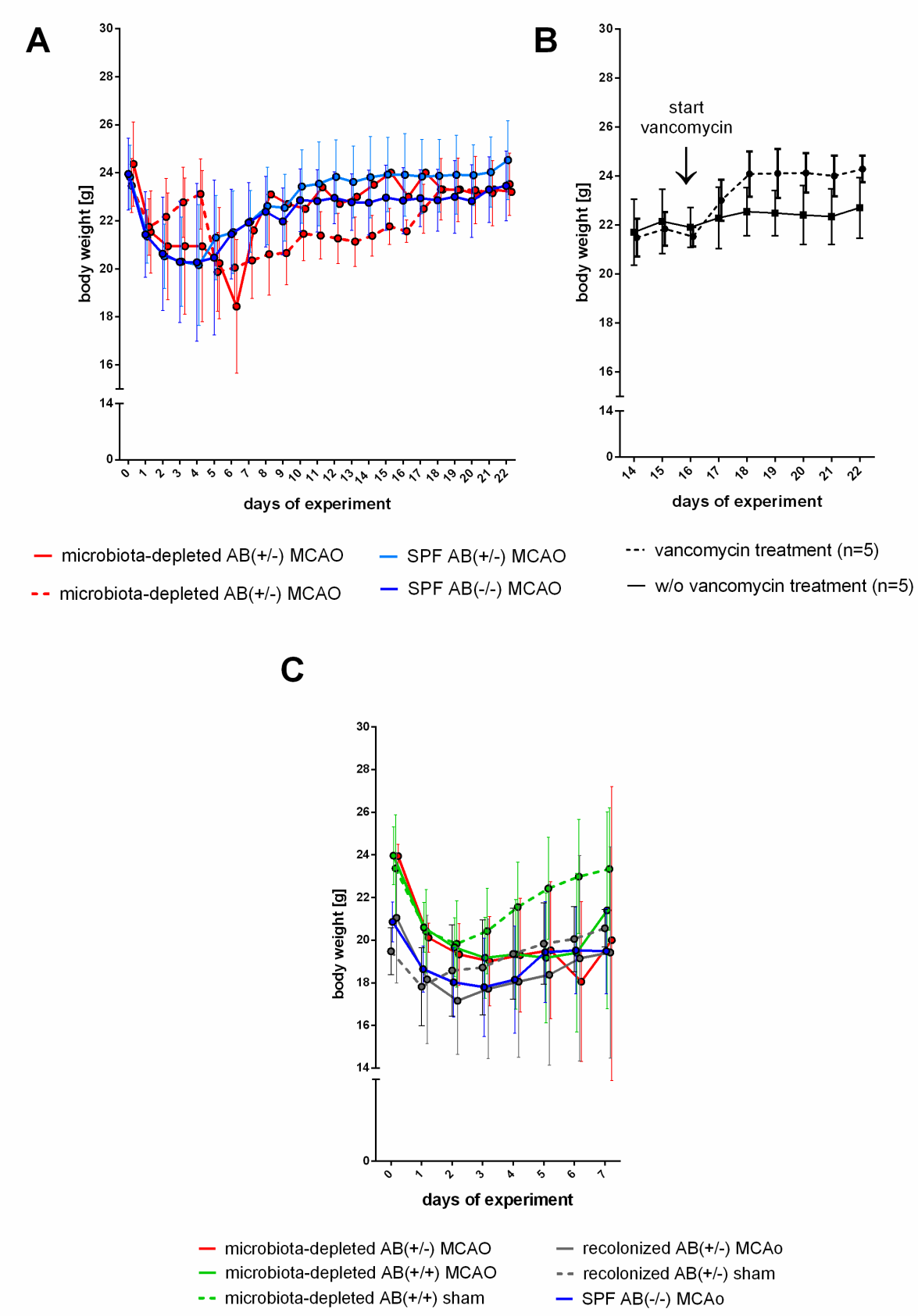
Fluorescent activated cell sorting (FACS) analysis of spleens, mesenteric lymph nodes (MLN) and Peyer's patches (PP)

Single cell suspensions from PP, spleens and MLN were prepared by forcing the tissues through a fine wire mesh. To obtain single cell suspensions from PP, the patches were initially digested 30 minutes at 37°C in RPMI containing 10% FCS (Biochrom, Berlin, Germany) and 3.5mg collagenase A (Roche, Basel, Switzerland). In spleen samples RBCs were lysed with erythrocyte lysis buffer (Qiagen, Hilden, Germany). Cells were washed, resuspended in RPMI 1640 medium containing penicillin, streptomycin, 2 mM glutamine, 10% FCS (Biochrom, Berlin, Germany) and $2x10^6$ cells were stimulated for four hours with 25ng/ml PMA and 1µg/ml ionomycin. Cytokine secretion was inhibited by addition of 5µg/ml Brefeldin A after the first hour. For cell phenotyping, the following fluorescently labeled anti–mouse monoclonal antibodies (BD Bioscience, BioLegend or eBioscience, Heidelberg, Germany) were used: CD19 (6D5), CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), TCRγδ (GL3), CD11b (M1/70), CD11c (N418), IFNγ (XMG1.2) and IL-17 (TC11-18H10.1). Dead cells were excluded by using LIVE/DEAD Fixable Aqua (Invitrogen, Waltham Massachusets, USA). The following lineage markers were combined for the analysis: T cells (CD11b-CD3+CD19), T helper cells (CD11b-CD19-CD3+CD4+), cytotoxic T cells (CD11b-CD19-CD3+CD8+), B cells (CD11b-CD3-C19+) and γδ T cells (CD11b-CD19-CD3+TCRγδ+).

Statistics for supplementary figures

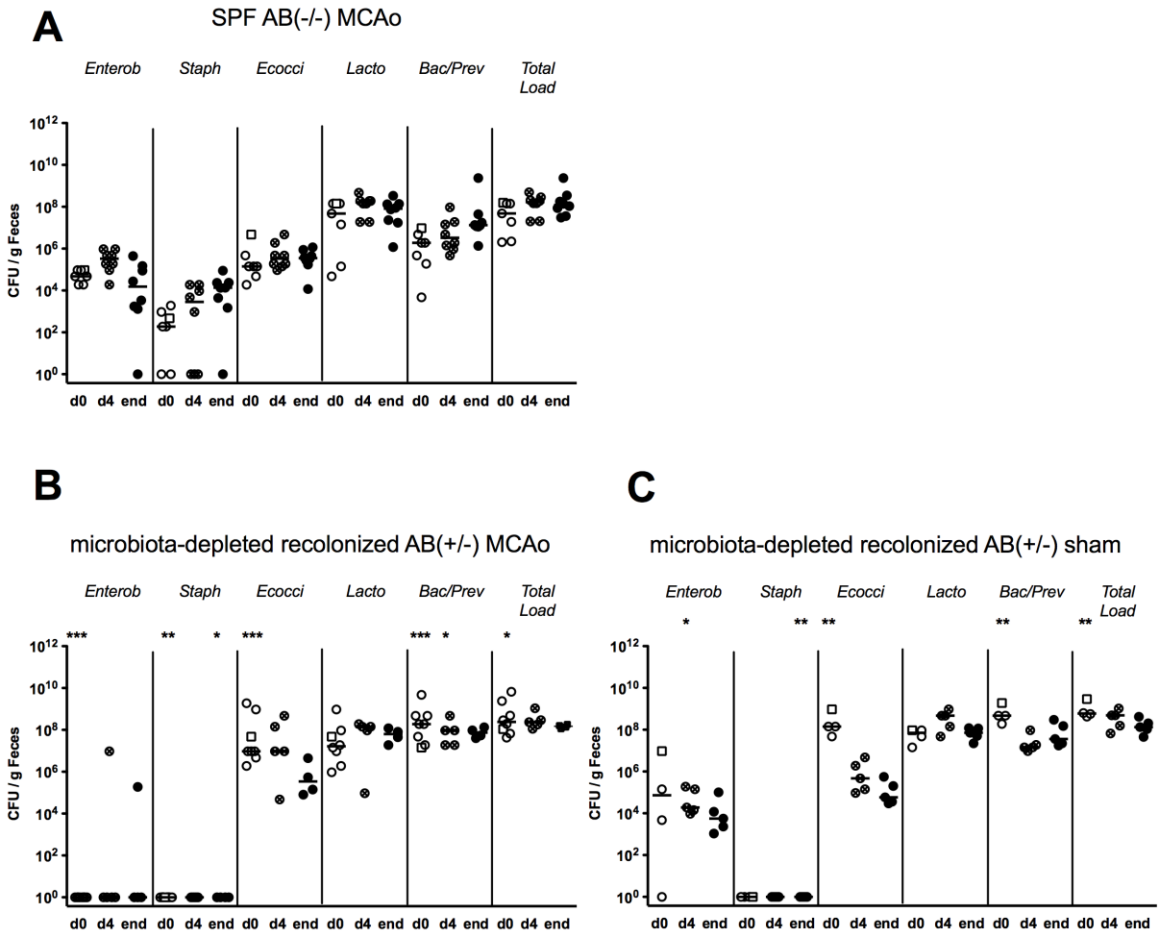
Statistics was performed using SPSS Statistics (IBM SPSS Statistics for Macintosh, Version 20.0. Armonk, NY: IBM Corp.). Bacterial counts were compared between recolonization groups and SPF group using Mann-Whitney U test. For immunology data: statistical analyses comparing the groups for each lymphocyte subpopulation (within one lymphatic organ) were conducted using Kruskal-Wallis Test with Dunn's post hoc.

Supplementary figures

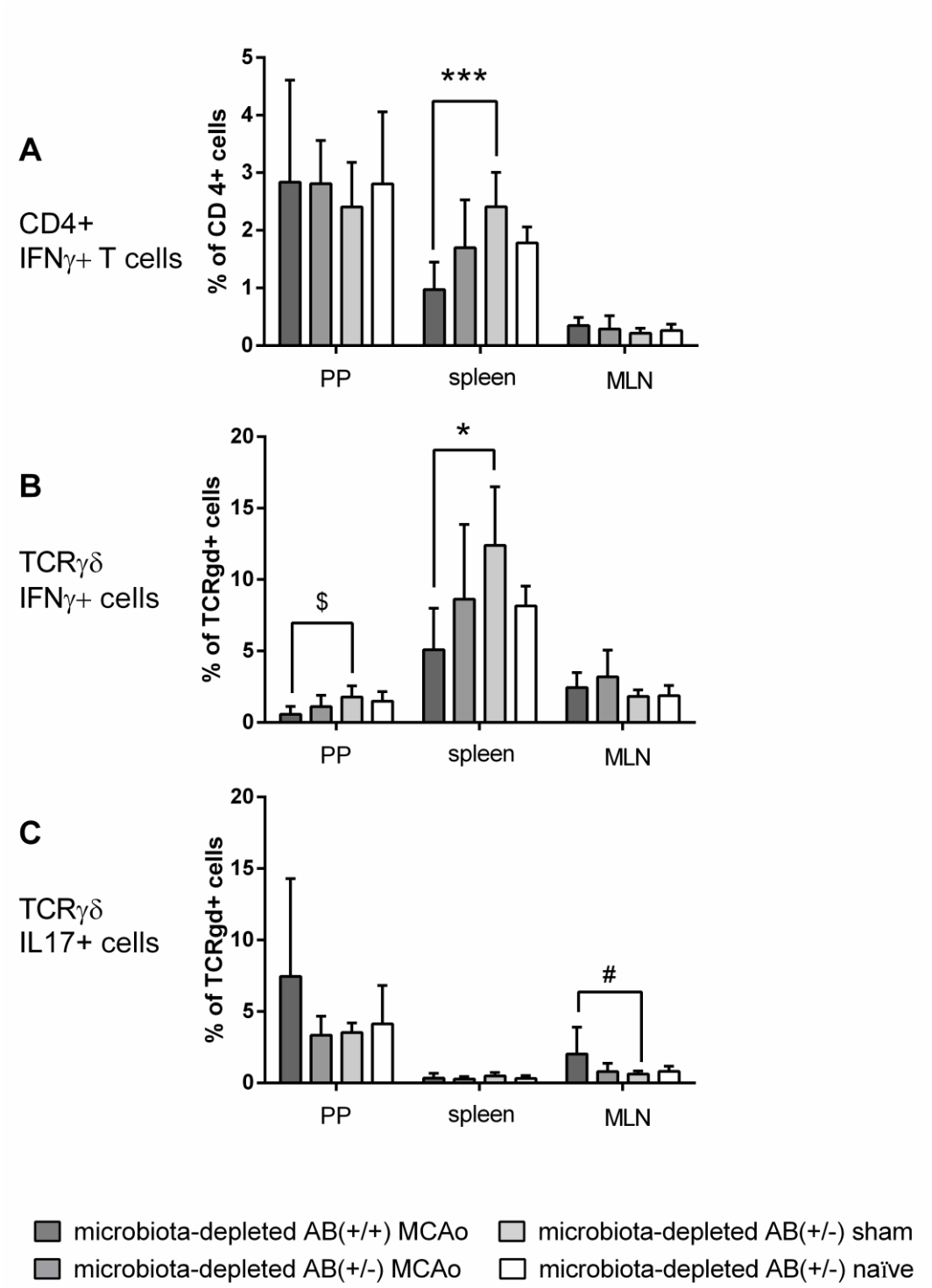


Supplementary Figure I (SFig.I) Weight of animals from experiment I and II

A) Body weight curve from the entire experiment I. We observed acute decrease in body weight of microbiota-depleted AB(+/-) MCAo and sham animals on day 5 and 6 parallel to the onset of diarrhea. **B)** Empiric vancomycin treatment improved the general state and symptoms in microbiota-depleted mice. Vancomycin (5g/l in drinking water ad libitum) was implemented in the first experiment. Mice with colitis symptoms were divided into two groups - with vancomycin treatment and without. Already one day after starting the administration of vancomycin we observed increase in the body weight in the treated group and mild resolution of the colitis symptoms. **C)** Body weight curve from experiment II**.** We observed acute decrease in body weight of microbiota-depleted AB (+/-) MCAo animals on day 6, linked with the onset of colitis symptoms.



Supplementary Figure II (SFig.II) Microbiological analyses of microbiota-depleted recolonized AB (+/-) groups and SPF AB (-/-) MCAo animals
Main microbial groups were restored by recolonization with conventional SPF microbiota. Total load=total bacterial count and *Enterob=Enterobacteriaceae, Staph=coagulase-negative Staphylococci, Ecocci=Enterococci, Lacto=Lactobacilli, Bac/Prev=Bacteroides/Prevotella* spp. are presented as the colony forming units (CFU) per gram feces. Samples from animals excluded from the study (exclusion on day 1) are marked with squares. Groups recolonized with conventional microbiota were separately compared with SPF AB(-/-) group using Mann-Whitney U test. Significance levels are marked *p≤0.05; **p≤0.01; ***p≤0.001



Supplementary Figure III (SFig.III) FACS analysis of immune cell populations from Peyer's Patches, spleen and mesenteric lymph nodes (MLN) on day 5 after cerebral ischemia
Microbiota-depleted MCAo AB(+/+) n=8 (n=7 for Peyer's patches), microbiota-depleted MCAo AB(+/-) n=7, microbiota-depleted sham AB(+/-) n=6, microbiota-depleted naïve AB(+/-) n=6. Statistical analyses for each lymphocyte subpopulation comparing the groups within one lymphatic organ were conducted using Kruskal-Wallis Test with Dunn's post hoc. Statistically significant differences were found between microbiota-depleted AB(+/+) MCAo and microbiota-depleted AB(+/-) sham group in the percentage of cytokine producing cells: **A)** CD4+ cells (IFNγ+ in spleen), **B and C)** TCR γδ cells (IFNγ in spleen and PP and IL17+ in

MLN). Significance levels are marked as follows: Peyer's patches (PP) $p≤0.05; $$p≤0.01; $$$p≤0.001; spleen *p≤0.05; **p≤0.01; ***p≤0.001; mesenteric lymph nodes (MLN) #p≤0.05; ##p≤0.01; ###p≤0.001.

Study	Animal ID	Experimental group	Exclusion on day 1 due to:
Experiment I	32	microbiota-depleted AB(+/-) MCAo	no stroke in MRI
	41	SPF AB(+/-) MCAo	no stroke in MRI
	51	SPF AB(-/-)MCAo	MRI-image analysis not conclusive
	53	SPF AB(-/-) MCAo	no stroke in MRI
	56	SPF AB(-/-) MCAo	no stroke in MRI
Experiment II	5	microbiota-depleted AB(+/+) MCAo	death on the day of surgery
	31	microbiota-depleted recolonized AB(+/-) MCAo	no stroke in MRI
	36	SPF AB(-/-) MCAo	death on the day of surgery
	45	microbiota-depleted recolonized AB(+/-) sham	death on the day of surgery

Supplementary Table I (STab.I) Animals excluded from the study
All animals excluded from the analyses with respective group and fulfilled exclusion criteria are reported in the table above.

Animal ID	Group	day	Vancomycin start day 16	Species (without CFU quantification)
G4	microbiota-depleted AB(+/-) MCAo	d4		culture-negative
G8	microbiota-depleted AB(+/-) MCAo	d4		*Paenibacillus* sp.
G10	microbiota-depleted AB(+/-) MCAo	d4		*Paenibacillus* sp.
G13	microbiota-depleted AB(+/-) sham	d4		*Paenibacillus* sp.*; Bacillus* sp.
G14	microbiota-depleted AB(+/-) sham	d4		*Paenibacillus* sp.*; Bacillus* sp.
G15	microbiota-depleted AB(+/-) sham	d4		*Paenibacillus* sp.*; CNS*
G17	microbiota-depleted AB(+/-) sham	d4		*Paenibacillus* sp.*; Bacillus* sp.
G18	microbiota-depleted AB(+/-) sham	d4		*Bacillus sp.*
G19	microbiota-depleted AB(+/-) sham	d4		*Paenibacillus* sp*; 2 Bacillus* spp.
G20	microbiota-depleted AB(+/-) sham	d4		*Paenibacillus* sp.
G21	microbiota-depleted AB(+/-) sham	d4		*Bacillus* sp.; *Staph. aureus*
G23	microbiota-depleted AB(+/-) sham	d4		*Bacillus* sp.; *Staph. aureus*
G24	microbiota-depleted AB(+/-) sham	d4		*Bacillus* sp.; *Staph. aureus*
G25	microbiota-depleted AB(+/-) MCAo	d4		*Paenibacillus* sp.
G26	microbiota-depleted AB(+/-) MCAo	d4		culture-negative
G27	microbiota-depleted AB(+/-) MCAo	d4		*3 Bacillus* spp.
G28	microbiota-depleted AB(+/-) MCAo	d4		culture-negative
G30	microbiota-depleted AB(+/-) MCAo	d4		culture-negative
G31	microbiota-depleted AB(+/-) MCAo	d4		culture-negative
G34	microbiota-depleted AB(+/-) MCAo	d4		*3 Bacillus* spp.
G30 (cecum)	microbiota-depleted AB(+/-) MCAo	d5		*Paenibacillus* sp.*; Clostridium sordellii; Clostridium perfringens*
G31 (cecum)	microbiota-depleted AB(+/-) MCAo	d5		*3 Bacillus* spp.
G34 (cecum)	microbiota-depleted AB(+/-) MCAo	d5		*3 Bacillus* spp.
G14	microbiota-depleted AB(+/-) sham	d7		*Clostridium sordellii; Clostridium perfringens*
G17	microbiota-depleted AB(+/-) sham	d7		*3 Bacillus* spp.
G18	microbiota-depleted AB(+/-) sham	d7		*3 Bacillus* spp.
G20	microbiota-depleted AB(+/-) sham	d7		*3 Bacillus* spp.
G22	microbiota-depleted AB(+/-) sham	d7		*Clostridium sordellii; Clostridium perfringens*
G24	microbiota-depleted AB(+/-) sham	d7		*Clostridium sordellii; Clostridium perfringens; Staph. aureus; CNS*
G33	microbiota-depleted AB(+/-) MCAo	d7		*3 Bacillus* spp.
G14	microbiota-depleted AB(+/-) sham	d16		*Clostridium sordellii; Clostridium perfringens; Bacillus* sp.
G16	microbiota-depleted AB(+/-) sham	d16		*2 Bacillus* spp.
G17	microbiota-depleted AB(+/-) sham	d16		*2 Bacillus* spp.
G18	microbiota-depleted AB(+/-) sham	d16		*3 Bacillus* spp.
G20	microbiota-depleted AB(+/-) sham	d16		*Bacillus* sp.
G21	microbiota-depleted AB(+/-) sham	d16		*Clostridium sordellii; Clostridium perfringens; Bacillus* sp.
G24	microbiota-depleted AB(+/-) sham	d16		*Bacillus* sp.
G33	microbiota-depleted AB(+/-) MCAo	d16		*Clostridium sordellii; Clostridium perfringens*
G14	microbiota-depleted AB(+/-) sham	d17	-	*Clostridium sordellii; Clostridium perfringens*
G16	microbiota-depleted AB(+/-) sham	d17	+	culture-negative
G17	microbiota-depleted AB(+/-) sham	d17	+	*2 Bacillus* spp.

G18	microbiota-depleted AB(+/-) sham	d17	+	*Bacillus* sp.
G19	microbiota-depleted AB(+/-) sham	d17	+	*Bacillus* sp.
G20	microbiota-depleted AB(+/-) sham	d17	+	*Bacillus* sp.
G22	microbiota-depleted AB(+/-) sham	d17	-	*Clostridium sordellii*
G24	microbiota-depleted AB(+/-) sham	d17	-	*2 Bacillus* spp.
G33	microbiota-depleted AB(+/-) MCAo	d17	-	*Clostridium sordellii; Clostridium perfringens*

CFU = colony forming units, CNS = coagulase-negative staphylococci

Supplementary Table II (STab.II) Microbiological analysis of the stool samples from the first experimental series
In the first experimental series several recolonizing strains were found in stool samples from microbiota-depleted mice. Stool samples were collected on different time-points of the experiment (day) and stored overnight at 4°C when analysis took place next day or frozen by -20°C for further analysis. Identification of bacterial species was performed based on the assessment of growth on selective microbiological media.

References

1. Heimesaat MM, Bereswill S, Fischer A, Fuchs D, Struck D, Niebergall J, et al. Gram-negative bacteria aggravate murine small intestinal th1-type immunopathology following oral infection with toxoplasma gondii. *J Immunol*. 2006;177:8785-8795