Aus der Klinik für Neurologie, Abteilung für Experimentelle Neurologie
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Impact of the gut microbiota on the outcome after experimental stroke

zur Erlangung des akademischen Grades
Medical Doctor - Doctor of Philosophy (MD/PhD)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

von

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aus Warszawa

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"Knowledge is like a sphere, the greater its volume,
the larger its contact with the unknown"

Blaise Pascal

Table of contents

Abstract (English)

Stroke is the second-leading cause of death worldwide and tremendous efforts are being made to fully characterize the pathophysiology of this disease and to identify new therapeutic targets. Importantly, stroke is associated with a high rate of complications contributing to the worsening of outcome.

In the recent years, microbiota, particularly the commensal bacterial population of the gut, gathered attention of neuroscientists. Gut microbiota not only affects the development of the immune system and plays an important role in host metabolic processes, but also contributes to the physiological function of the central nervous system. We hypothesized that gut microbiota may impact the outcome of cerebral ischemia and tested this hypothesis in an experimental stroke model using microbiota-depleted mice after broad-spectrum antibiotic pretreatment. The broad-spectrum antibiotics, implemented in our study, are often used in clinical practice for treating post-stroke infections.

We subjected microbiota-depleted C57BL/6J mice with and without continuous antibiotic treatment, microbiota-depleted mice recolonized with conventional microbiota and conventionally colonized animals to experimental stroke (middle cerebral artery occlusion, MCAo) and sham operation. We monitored general health parameters, assessed infarct size using magnetic resonance imaging and histology, investigated intestinal samples histologically and measured main immune parameters.

We observed significantly increased mortality in microbiota-depleted MCAo mice when antibiotic treatment was stopped before operation, as compared to sham-operated animals and conventionally colonized mice. All microbiota-depleted animals developed severe colitis. We showed that continuous antibiotic treatment or recolonization with conventional microbiota before surgery prevents the development of this phenotype. We did not find any differences in infarct volumes between either of the investigated groups. In line with previous studies, MCAo animals developed immunosuppression on day 5 after experimental stroke.

In summary, this is the first report showing that complex intestinal microbiota protects the host from severe lethal complications after experimental stroke. In this study, we took the advantage of an extreme model of microbiota depletion, nevertheless our results may have direct clinical implications, what needs to be tested in the clinical

setting. Antibiotics used in treatment of severe post-stroke pneumonia in combination with post-stroke immunosuppresion may disturb commensal microbiota, as well as host immunological barriers. This in turn may have similar effects, as in the mouse model, but also systemic, proinflammatory and in consequence negative effects on the ischemic brain.

Abstract (German)

Der Schlaganfall ist die zweithäufigste Todesursache weltweit und enorme Anstrengungen werden unternommen, um die Pathophysiologie dieser Erkrankung besser zu verstehen und um neue therapeutische Ansätze zu entwickeln. Schlaganfälle sind mit einer hohen Rate von Komplikationen verbunden, die zur Verschlechterung der Prognose beitragen.

Mikrobiota, besonders die kommensale Bakterienpopulation des Darms, erregte in den letzten Jahren vermehrt die Aufmerksamkeit von Neurowissenschaftlern. Die Darmmikrobiota beteiligt sich nicht nur an der Entwicklung des Immunsystems und spielt eine wichtige Rolle bei den Wirt-Stoffwechselprozessen, sondern trägt auch zu der physiologischen Funktion des zentralen Nervensystems bei. Wir stellten die Hypothese auf, dass Darmmikrobiota einen Einfluss auf die Prognose nach Schlaganfall hat und testeten diese Hypothese in einem experimentellen Modell der zerebralen Ischämie in Mikrobiota-depletierten Mäusen nach Breitspektrum-Antibiotika Vorbehandlung. Die hier verwendeten Antibiotika werden häufig in der klinischen Praxis zur Behandlung von Schlaganfall-assoziierten Infektionen verwendet.

Wir unterzogen Mikrobiota-depletierte C57BL/6J Mäuse mit und ohne kontinuierliche Antibiose, Mikrobiota-depletierte Mäuse nach Rekolonisation mit konventioneller Mikrobiota und konventionell kolonisierte Tiere einem experimentellen Schlaganfall (Okklusion der mittleren zerebralen Arterie, MCAo) oder einer sogenannten Sham-Operation. Wir beobachteten den generellen Gesundheitszustand der Tiere, untersuchten die Infarktgröße mittels Magnetresonanztomographie und Histologie, analysierten Darmproben mittels Histologie und bestimmten die Hauptimmunparameter. Wir stellten eine signifikante Erhöhung der Sterblichkeitsrate bei Mikrobiota-depletierten MCAo Mäusen ohne kontinuierliche Antibiose im Vergleich zu Sham-operierten Tieren und konventionell kolonisierten Mäusen fest. Alle Mikrobiota-depletierten Tiere entwickelten eine schwere Colitis. Wir konnten zeigen, dass dieser Phänotyp durch kontinuierliche Antibiose oder Rekolonisation mit konventioneller Mikrobiota vor der Operation verhindert werden kann. Wir fanden keine Unterschiede in den Infarktvolumina zwischen den untersuchten Gruppen. Die MCAo Tiere entwickelten eine Immunsuppression an Tag 5 nach dem experimentellen Schlaganfall, was im Einklang mit früheren Studien steht.

Zusammenfassend zeigt diese Arbeit erstmalig, dass die komplexe Mikrobiota des Darmes vor schweren, tödlichen intestinalen Komplikationen nach experimentellem Schlaganfall schützt. Obwohl für diese Studie zu den Folgen der Mikrobiota-Depletion eine sehr breite, über die normale klinische Praxis hinausgehende Antibiose eingesetzt wurde, haben diese Befunde möglicherweise auch klinische Implikationen, die in klinischen Studien untersucht werden sollten. So könnte die antibiotische Therapie der häufigen, schweren Schlaganfall-assoziierten Pneumonien in Kombination mit der Schlaganfall-induzierten Immundepression sowohl eine Veränderung der Darmmikrobiota als auch der immunologischen Barriere verursachen. Damit können ähnliche Effekte wie im Maus-Modell verbunden sein, aber auch systemische, proinflammatorische und in Konsequenz negative Effekte auf das ischämische Hirn.

Affidavit

I, Katarzyna Winek, certify under penalty of perjury by my own signature that I have submitted the thesis on the topic "Impact of the gut microbiota on the outcome after experimental stroke". I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The section on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) corresponds to the URM (s.o) and are answered by me. My contribution in the selected publication for this dissertation corresponds to those that are specified in the following joint declaration with the responsible person and supervisor.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date _____ Signature

Detailed Declaration of Contribution

Katarzyna Winek had the following share in the following publication:

Depletion of cultivatable microbiota by broad-spectrum antibiotic pretreatment worsens outcome after murine stroke
Winek K, Engel O, Koduah P, Heimesaat MM, Fischer A, Bereswill S, Dames C, Kershaw O, Gruber AD, Curato C, Oyama N, Meisel C, Meisel A, Dirnagl U.
Stroke. 2016

- idea and design of the study (together with Ulrich Dirnagl and Andreas Meisel)
- project management and coordination
- all *in vivo* procedures with support from co-authors: MCAo and sham surgery,

handling, clinical examination and extensive monitoring, recolonization, infarct volume measurement by MRI, behavioural tests [data not shown], pathological examination, samples collection and preservation

- design of FACS investigations (together with Claudia Dames and Caterina Curato)
- direct laboratory supervision of Priscilla Koduah, MSc, during her master thesis (contribution to experiment II)
- data interpretation with support from co-authors
- statistical analysis of acquired data
- preparation of figures
- writing the publication (with support from co-authors)
- coordination of the review-process and writing responses to the reviewers

(edited/complemented by Ulrich Dirnagl, Andreas Meisel, Markus Heimesaat, André Fischer, Stefan Bereswill)

Signature, date and stamp of the supervising University teacher

_____

Prof. Ulrich Dirnagl

Signature of the doctoral candidate

_____

Katarzyna Winek

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Journals 1 - 20 (of 192) [1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10] **Page 1 of 10**

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Journals 1 - 20 (of 192) [1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10] **Page 1 of 10**

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Depletion of Cultivatable Gut Microbiota by Broad-Spectrum Antibiotic Pretreatment Worsens Outcome After Murine Stroke

Katarzyna Winek, MD; Odilo Engel, DVM; Priscilla Koduah, MSc; Markus M. Heimesaat, MD; André Fischer, PhD; Stefan Bereswill, PhD; Claudia Dames, MSc; Olivia Kershaw, PhD; Achim D. Gruber, PhD; Caterina Curato, PhD; Naoki Oyama, MD; Christian Meisel, MD; Andreas Meisel, MD; Ulrich Dirnagl, MD

Background and Purpose—Antibiotics disturbing microbiota are often used in treatment of poststroke infections. A bidirectional brain–gut microbiota axis was recently suggested as a modulator of nervous system diseases. We hypothesized that gut microbiota may be an important player in the course of stroke.

Methods—We investigated the outcome of focal cerebral ischemia in C57BL/6J mice after an 8-week decontamination with quintuple broad-spectrum antibiotic cocktail. These microbiota-depleted animals were subjected to 60 minutes middle cerebral artery occlusion or sham operation. Infarct volume was measured using magnetic resonance imaging, and mice were monitored clinically throughout the whole experiment. At the end point, tissues were preserved for further analysis, comprising histology and immunologic investigations using flow cytometry.

Results—We found significantly decreased survival in the middle cerebral artery occlusion microbiota-depleted mice when the antibiotic cocktail was stopped 3 days before surgery (compared with middle cerebral artery occlusion specific pathogen-free and sham-operated microbiota-depleted mice). Moreover, all microbiota-depleted animals in which antibiotic treatment was terminated developed severe acute colitis. This phenotype was rescued by continuous antibiotic treatment or colonization with specific pathogen-free microbiota before surgery. Further, infarct volumes on day one did not differ between any of the experimental groups.

Conclusions—Conventional microbiota ensures intestinal protection in the mouse model of experimental stroke and prevents development of acute and severe colitis in microbiota-depleted mice not given antibiotic protection after cerebral ischemia. Our experiments raise the clinically important question as to whether microbial colonization or specific microbiota are crucial for stroke outcome. **(*Stroke*. 2016;47:1354-1363. DOI: 10.1161/STROKEAHA.115.011800.)**

Key Words: animal model ■ antibiotic ■ brain–gut microbiota axis ■ ischemic stroke ■ microbiota depletion

Stroke is the second leading cause of death worldwide and the most frequent cause of long-term disability in adults in developed countries.[1] Despite progress in understanding the pathophysiology of damage of this devastating disease, all efforts to establish pharmacological brain-protective strategies based on this knowledge have been futile. This has led stroke researchers to shift focus from neuroprotection to other modifiable determinants of outcome, in particular complications. Taken together, complications, such as infections, increased intracranial pressure, and sarcopenia explain at least 20% of the overall outcome of stroke patients.[2] Infections, particularly pneumonia, are the most common complication after stroke, contributing to increased mortality and worsening the neurological outcome. A substantial number of stroke patients are treated with antibiotics, often including combinations of broad-spectrum antimicrobial agents. Effects of this treatment on commensal microbiota have been neglected and the impact of microbiota on stroke outcome has not been investigated to date.

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Commensal microbiota, in particular that of the gut, has recently entered center stage in biomedicine due to the advances in DNA sequencing and bioinformatics. Gut microbiota not only defends the host from invading pathogens, but also stimulates angiogenesis,[3] regulates fat storage[4] and controls gut permeability.[5] This was demonstrated by sequencing whole microbiotic genomes (microbiomes), by using germ-free (ie, abiotic) animal models and selectively colonizing with specific microorganisms. Specific microbiota is required for proper organ development, including the immune system and brain, and in many other physiological functions. Consequently, germ-free mice develop altered structures of the brain, blood–brain barrier, and immune organs[6,7] and are resistant to diet-induced obesity because of changes in metabolic profiles.[4] Disturbances in the gut microbiota have been linked to several pathologies, including inflammatory bowel disease,[8] obesity,[9] type I diabetes mellitus,[10,11] as well as neurological conditions, such as multiple sclerosis, Guillain–Barré syndrome, nociceptive pain or stress, anxiety,[12] and neurodevelopmental disorders.[13]

Little to nothing is known, however, about the modulating impact of gut microbiota on acute central nervous system (CNS) injury in stroke or, in reverse, the impact of stroke on the composition or functional profile of microbiota. This is surprising, because there are important reasons to suspect a relevant interplay between the lesioned brain in stroke and the microbiome, particularly the gut microbiome. Stroke causes immunodepression, which renders the organism susceptible to bacterial infections.[14–16] Indeed, infections after stroke have a major impact on stroke outcome. Pneumonia, for example, is the most frequent cause of death in acute stroke.[1] Immunodepression after stroke leads to a breakdown of epithelial barriers,[17,18] making the gut an important potential source of invading bacteria. Stroke modulates the activity of the autonomic nervous system, which contributes to immunodepression, but also affects gut motility and permeability.[19,20] Further, because of their increased susceptibility to infection, many stroke patients are treated with potent combinations of antibiotics, with possibly drastic consequences for the commensal bacterial microbiota.

We speculate that gut microbiota may be an important direct or indirect modulating factor for stroke outcome by (1) causing infection, (2) modulating the immune or autonomic nervous system and thus other complications (eg, sarcopenia), or (3) exerting metabolic or humoral effects on the brain. As a first step to test this hypothesis, we choose to study the effect of extensively depleting commensal microbiota on stroke outcome in a well-characterized murine model of focal cerebral ischemia, in a clinically relevant setting, specifically by using combinations of potent antibiotics. These broad-spectrum antibiotic-treated animals were termed as microbiota-depleted, and they did not harbor any bacteria cultivatable on standard microbiological media.[21] We decided to choose this previously established model to avoid confounders present in germ-free animals, such as deficiencies in the immune system or altered brain physiology, biochemistry, and anatomy.[7,22,23] In our study, we found no effect of microbiota depletion on the brain lesion (infarct volume), but we did find a protective effect of gut microbiota on survival.

Materials and Methods

Detailed description of materials and methods can be found in the online-only Data Supplement.

Animals and Housing

Female C57BL/6J mice (Forschungseinrichtung für Experimentelle Medizin, FEM, Charité Berlin, Germany) after microbiota depletion by quintuple antibiotic treatment[21] were placed in autoclaved, individually ventilated cages lined with autoclaved chip bedding and kept on a 12-hour light/dark cycle with ad libitum access to food (autoclaved, standard chow, complete feed for rats and mice maintenance; Sniff, Soest, Germany) and autoclaved water. Mice were 11 to 28 weeks old at the time of the experiment. All experiments were conducted in accordance with the European directive on the protection of animals used for scientific purposes and approved by Landesamt für Gesundheit und Soziales, Berlin, Germany.

Generation of Microbiota-Depleted Mice

Eight-week old female C57BL/6J mice (Forschungseinrichtung für Experimentelle Medizin, FEM, Charité Berlin, Germany) harboring a conventional microbiota were transferred to autoclaved cages and treated with quintuple antibiotic cocktail consisting of ampicillin (1 g/L; Ratiopharm), vancomycin (500 mg/L; Cell Pharm), ciprofloxacin (200 mg/L; Bayer Vital), imipenem (250 mg/L; MSD), and metronidazole (1 g/L; Fresenius) in the drinking water available ad libitum according to the previously published protocol.[21] The microbiological status of mice was controlled every week as described previously.[21] Cultural and molecular methods revealed that the intestinal microbiota was virtually depleted 8 weeks after broad-spectrum antibiotic treatment.[21,24]

Experimental Stroke (Middle Cerebral Artery Occlusion)

Surgical procedures were conducted under microorganism-reducing conditions (sterile gown, gloves, instruments, surgical hand wash before operations, nonsterile helpers during surgery). Middle cerebral artery occlusion (MCAo; experimental focal cerebral ischemia) was performed according to the standard operating procedure from the Department of Experimental Neurology, Charité Berlin, Germany.[25]

Magnetic Resonance Imaging

Infarct volume was assessed using magnetic resonance imaging (Bruker 7T PharmaScan 70/16) on day one after MCAo.

Flow Cytometric Analysis of Spleens, Mesenteric Lymph Nodes, and Peyer's Patches

Cells were phenotyped on LSR Fortessa flow cytometer with FACSDiva software (BD Biosciences, Heidelberg, Germany) using antibodies specified in the online-only Data Supplement. Data were analyzed with FlowJo software (Tree Star Inc, Ashland, OR).

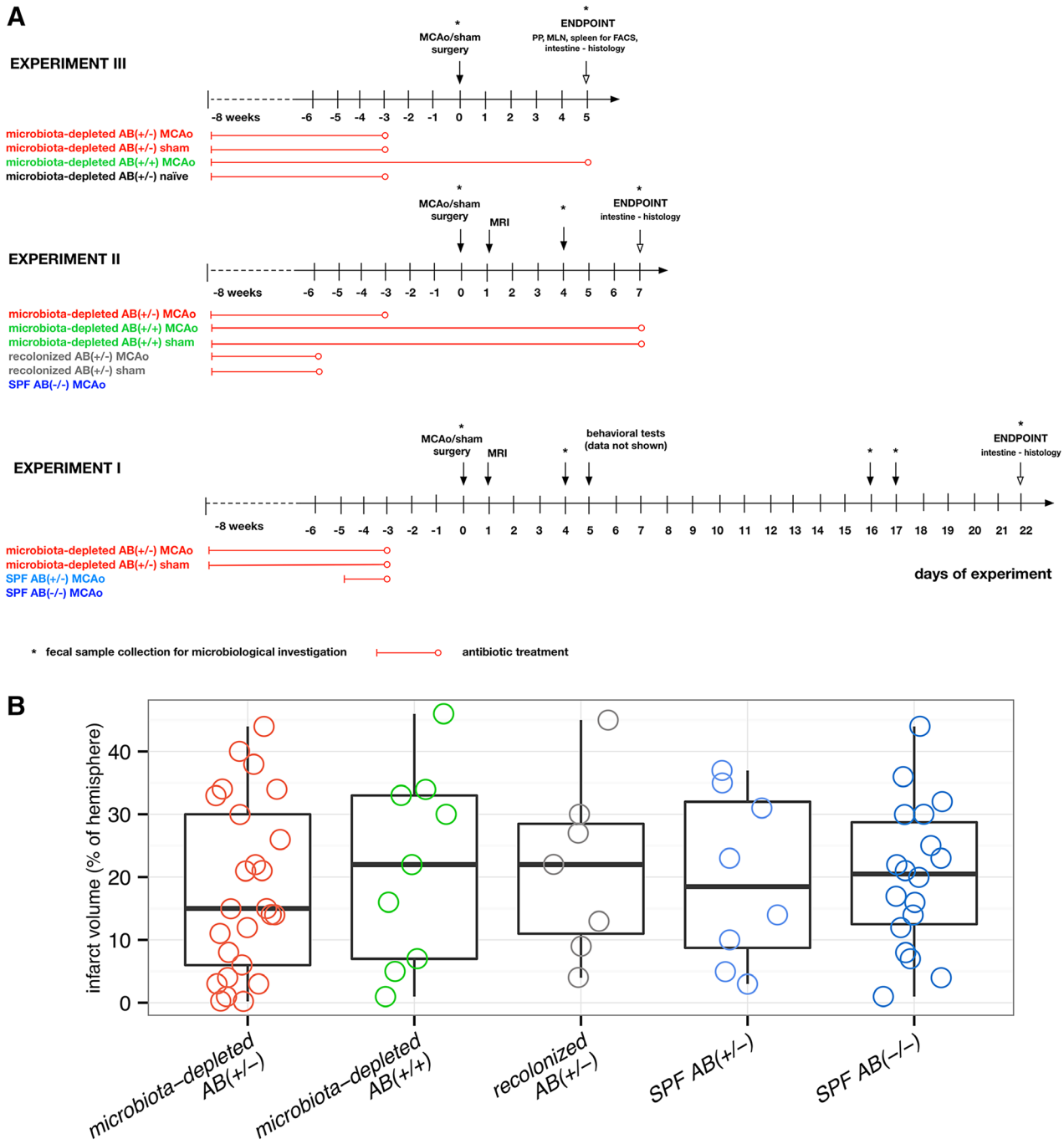

Figure 1. Experimental setup and infarct volume. **A**, Experimental setup (3 independent experiments). Experimental groups: microbiota-depleted AB(+/–) MCAo/sham, with antibiotic treatment stopped 72 h before surgery; microbiota-depleted AB(+/+) MCAo/sham, with antibiotic treatment during the entire experiment; recolonized AB(+/–) MCAo/sham, microbiota-depleted mice recolonized with SPF microbiota; antibiotic treatment was stopped 48 h before recolonization; microbiota-depleted AB(+/–) naïve, microbiota-depleted animals without any surgical intervention with antibiotic cocktail stopped 72 h before the experiment; SPF AB(–/–) MCAo, conventionally colonized (specific pathogen-free microbiota) mice without any antibiotic treatment, subjected to MCAo surgery; SPF AB(+/–) MCAo, SPF mice with antibiotic treatment for 48 h up to 72 h before surgery. **B**, Infarct volume assessed by MRI at day one after MCAo did not differ between investigated groups. Microbiota-depleted AB(+/–) n=25, microbiota-depleted AB(+/+) n=9, recolonized AB(+/–) (microbiota-depleted recolonized with SPF microbiota) n=7, SPF AB(+/–) (SPF with antibiotic treatment for 48 h) n=8, SPF AB(–/–) n=18. Box plot with whiskers minimum to maximum. No statistically significant differences were found when comparing all experimental groups (Kruskal Wallis test with Dunn's post hoc) or when comparing microbiota-depleted AB(+/–) with SPF AB(–/–) mice Mann–Whitney test). FACS indicates flow cytometric analysis; MCAo, middle cerebral artery occlusion; MLN, mesenteric lymph nodes; MRI, magnetic resonance imaging; PP, Peyer's patches; and SPF, specific pathogen-free.

Microbiological Investigation of Fecal Samples

Cultural analyses of aerobic, microaerophilic, and anaerobic bacterial species abundant in the fecal samples were performed as described previously.[21]

Hematoxylin and Eosin Staining of Intestinal Samples

Swiss rolls of intestinal segments were isolated during necropsy,[26] immersion-fixed in 4% paraformaldehyde, and embedded in

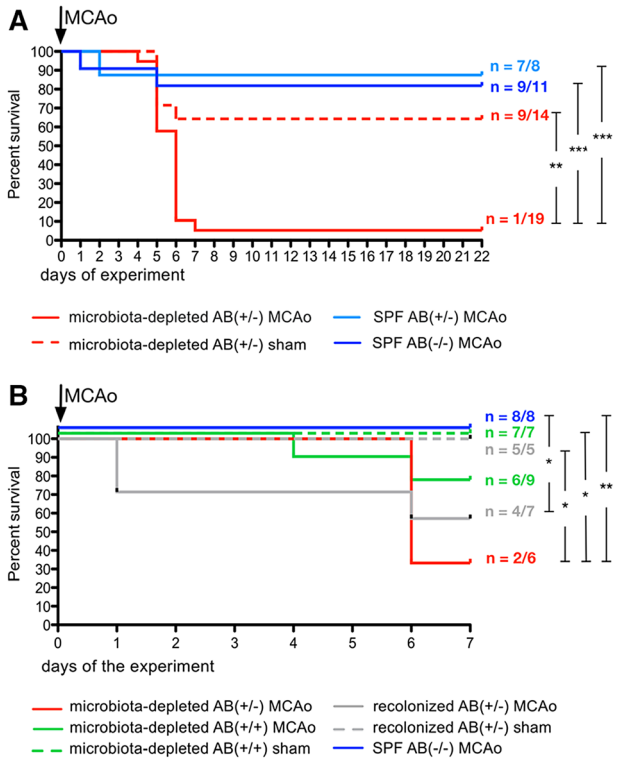

Figure 2. Survival analyses. **A**, Kaplan–Meier curve from experiment I. Extensive depletion of microbiota significantly decreases survival after cerebral ischemia. Microbiota-depleted mice after experimental stroke without antibiotic protection have significantly lower survival rate than do sham-operated animals and SPF AB(–/–) mice. Numbers indicate surviving animals/animals included in the experiment in the investigated group. Statistically significant differences were found between microbiota-depleted AB(+/–) MCAo vs microbiota-depleted AB(+/–) sham (P=0.002; Chi square =9.402); microbiota-depleted AB(+/–) MCAo vs SPF AB(–/–) MCAo (P<0.001; Chi square =16.526); and microbiota-depleted AB(+/–) MCAo vs SPF AB(+/–) group (P<0.001; Chi square =12.163) using log-rank (Mantel–Cox) test. **B**, Kaplan–Meier curve from experiment II. Survival rate of microbiota-depleted animals subjected to MCAo is improved by continuous antibiotic treatment or recolonization before experimental stroke with SPF microbiota. Numbers indicate surviving animals/animals included in the experiment in the investigated group. Statistically significant differences were found in comparing SPF AB(–/–) MCAo vs recolonized AB(+/–) MCAo (P=0.044; Chi square =4.048), microbiota-depleted AB(+/–) MCAo vs recolonized AB(+/–) sham (P=0.029; Chi square =4.762); microbiota-depleted AB(+/–) MCAo vs microbiota-depleted AB(+/+) sham (P=0.013; Chi square =6.222); microbiota-depleted AB(+/–) MCAo vs SPF AB(–/–) MCAo (P=0.008; Chi square =6.933) with log-rank (Mantel–Cox) test. MCAo indicates middle cerebral artery occlusion; and SPF, specific pathogen-free.

paraffin. 5-µm-thick sections were cut, dewaxed, stained with hematoxylin and eosin following standard protocols.

Methods to Prevent Bias and Exclusion Criteria

Cages with animals were randomly assigned to experimental groups. Operations, and daily health examination were performed unblinded because of the microorganism-reducing conditions required during surgery and handling. The exclusion criteria were (1) unsuccessful stroke, based on magnetic resonance imaging investigation in the experiments I and II or histological assessment in the experiment III, and (2) death on day of the surgery (Table I in the online-only Data Supplement).

Statistics

Statistics were performed using SPSS Statistics (IBM SPSS Statistics for Macintosh, Version 20.0.; IBM Corp, Armonk, NY). Sample size calculations (G*Power 3.1[27]) assumed effect size Cohen's d=1 for comparison primarily between conventionally colonized MCAo animals and microbiota-depleted MCAo mice using t test. For the calculation, we implemented α=0.05 and β=0.8 for all experiments and drop-out rate of 10% because of MCAo and 5% to the sham surgery. Total sample sizes were n=57 in experiment I, n=46 in experiment II, and n=27 in experiment III. The original data of this study is available online: http://dx.doi.org/10.6084/m9.figshare.1476224.

Results

Extensive Depletion of Gut Microbiota by Broad-Spectrum Antibiotic Pretreatment Does Not Affect Volume of the Ischemic Brain Lesion 1 Day After Experimental Stroke

In 3 series of experiments, we used microbiota-depleted mice generated by an 8-week broad-spectrum antibiotic regimen.[24] In the first experimental set, we aimed to assess long-term outcome of focal cerebral ischemia (MCAo) using this model (Figure 1A). Considering the immunomodulatory properties of antibiotics[28,29] and their possible neuroprotective or neurotoxic effects,[30] we stopped the antibiotic treatment in the AB(+/–) groups 72 hours before surgical intervention. Additionally, we introduced a specific pathogen-free (SPF) AB(+/–) control group treated with the quintuple antibiotic cocktail only for 48 hours up to 72 hours before operation and conventionally colonized mice without any antibiotic treatment SPF AB(–/–). To further characterize effects of the antibiotic regime and extensive depletion of commensal microbiota on the outcome of focal cerebral ischemia in the next experimental series, we additionally investigated the AB(+/+) groups, in which the treatment with antibiotic cocktail was continued up to the end of the experiments, and the groups recolonized with intestinal microbiota derived from SPF AB(–/–) littermates. We performed microbiological investigations of fecal samples in all experimental series and did not find any cultivatable microorganisms in samples from microbiota-depleted AB(+/–) and microbiota-depleted AB(+/+) mice at the time point of surgery.

Because bacterial metabolites, products, and antigens may have contributed directly or via interactions with the immune system to the development of the ischemic lesion, we assessed the infarct volume by magnetic resonance imaging on day one after focal cerebral ischemia in the first 2 series of experiments (Figure 1A). We did not find any statistically significant differences in stroke volume 24 hours after MCAo between any of the groups under investigation (Figure 1B).

Extensive Depletion of Gut Microbiota Decreases Survival After Experimental Stroke

In the first series of experiments, in which the antibiotic treatment was terminated 72 hours before operation, microbiota-depleted mice subjected to sham operation and MCAo surprisingly developed acute and severe diarrhea 5 to 6 days after surgery. Survival rate in the microbiota-depleted

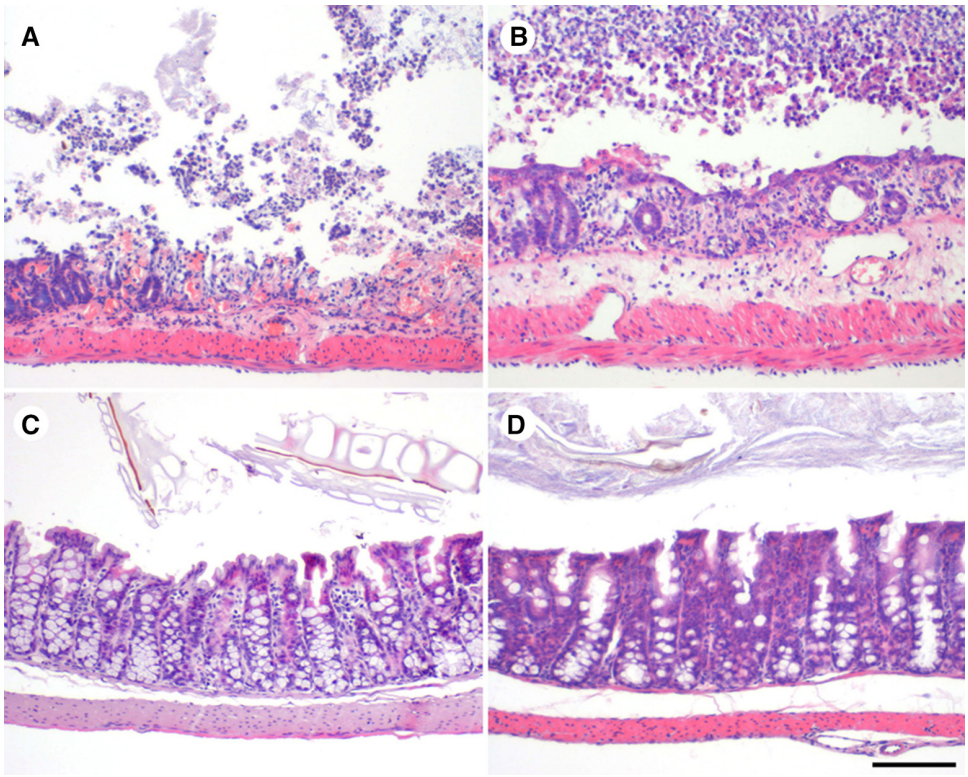

Figure 3. Histopathologic examination of intestinal samples. Depletion of microbiota before experimental stroke leads to the development of acute and severe colitis in the first week after cerebral ischemia. Histopathology of representative intestinal samples (hematoxylin and eosin staining; bar =100 μm) showed severe, acute erosive to ulcerative colitis present in microbiota-depleted AB(+/–) mice subjected to MCAo (**A**) and sham operation (**B**). In mice with continuous antibiotic treatment (**C**, microbiota-depleted AB(+/+) sham mouse) or recolonization with SPF microbiota (**D**, microbiota-depleted recolonized AB(+/–) MCAo mouse), colitis was prevented. MCAo indicates middle cerebral artery occlusion; and SPF, specific pathogen-free.

AB(+/–) MCAo group was significantly lower than that of SPF AB(–/–) mice. It was also lower than that in microbiota-depleted sham-operated animals (Figure 2A) that showed intestinal symptoms similar to those in the MCAo mice (weight loss, diarrhea, crouched position; Figure IA in the online-only Data Supplement). We were able to reproduce this finding in the second set of experiments, in which symptoms in the microbiota-depleted AB(+/–) MCAo group started 6 days after surgery (Figure 2B and Figure IC in the online-only Data Supplement). We strictly monitored all mice in the experiment, checking general well-being every 4 hours. Within ≈4 hours after onset of diarrhea, affected mice displayed symptoms resembling shock: crouched position, rough fur, lethargy, and difficulties in breathing.[31] These symptoms are indicative of an acute progression of systemic sequelae, and the affected animals were euthanized in compliance with the humane end points in our experiments.

Non-Neurological Symptoms in Microbiota-Depleted Mice Undergoing Surgery Are Linked to Severe Colitis

We next examined histopathologic changes in hematoxylin and eosin–stained intestinal samples derived from microbiota-depleted and SPF animals. Remarkably, we observed acute multifocal to segmental erosive–ulcerative and necrotizing colitis in those microbiota-depleted animals, which during the experiment had displayed apparent clinical colitis symptoms, such as weight loss and diarrhea.[32] Conversely, we did not find any histopathologic abnormalities in intestinal samples from SPF AB(–/–), SPF mice after short antibiotic treatment =SPF AB(+/–), or in samples from animals recolonized with conventional microbiota (Figure 3). Although during the experiments microbiota-depleted mice were kept in individually ventilated cages with autoclaved equipment and were handled under microorganism-reducing conditions (until day 5 in the experiment I because of behavioral testing planned from day 5, data not shown, and during the entire experiment II and III), we suspected possible microbial contamination and spontaneous recolonization of microbiota-depleted animals during experimental procedures. To empirically test this right from the first experiment, we divided surviving animals into 2 groups: with antibiotic treatment starting on day 16 (vancomycin 5 g/L in the drinking water ad libitum, n=5) and without antibiotic intervention (n=5). As early as one day after starting the treatment, we noted an increase in body weight in the treated group and incipient resolution of symptoms (Figure IB in the online-only Data Supplement). In some stool samples collected from microbiota-depleted mice on different days of the experiment (day 4–17), several microorganism such as *Clostridium species* (spp.), *Bacillus* spp., and Staphylococci were detected, whereas other samples were culture-negative (Table II in the online-only Data Supplement).

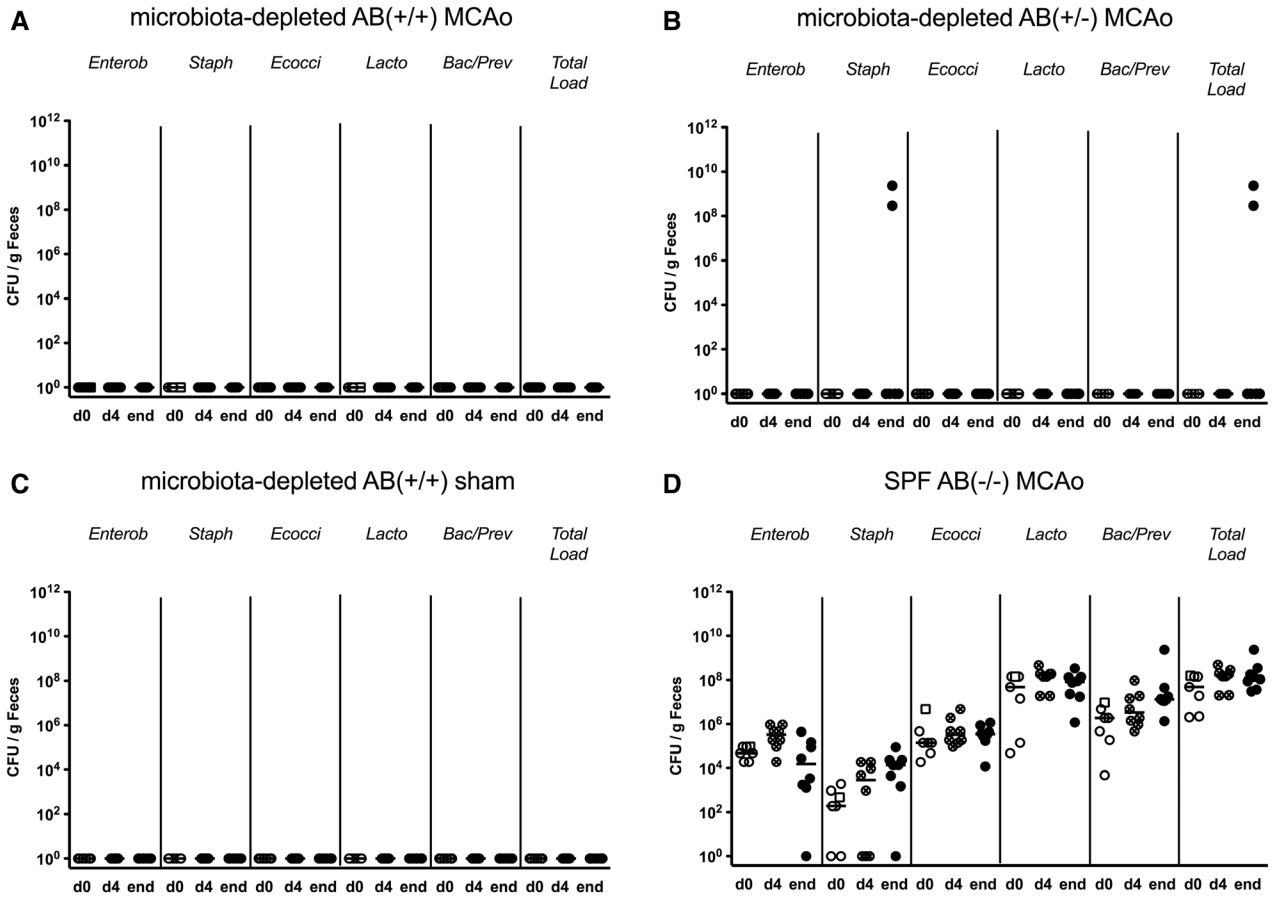

Figure 4. Microbiological analyses of fecal samples from experiment II. Analysis of fecal samples from (**A**) microbiota-depleted AB(+/+) MCAo animals, (**B**) microbiota depleted AB(+/-) MCAo, (**C**) microbiota-depleted AB(+/+) sham and (**D**) SPF AB(-/-) MCAo mice. Most microbiota-depleted animals remain culture-negative for 7 days of the experiment. Samples were collected on day of the surgery (d0), day 4 (d4), and at the end point (day 7 and day 6 for animals that have reached humane end points), respectively. Data presented as individual points and median. Samples from excluded animals (exclusion on day 1) are marked with squares. *Bac/Prev* indicates *Bacteroides/Prevotella* spp.; *Ecocci*, *Enterococci*; *Enterob*, *Enterobacteriaceae*; *Lacto*, *Lactobacilli*; MCAo, middle cerebral artery occlusion; SPF, specific pathogen-free; *Staph*, *coagulase-negative Staphylococci*; and Total load, total bacterial count are presented as the colony-forming units (CFU) per gram feces.

Continuous Antibiotic Treatment or Recolonization With Microbiota From SPF Littermates Improves the Outcome After Cerebral Ischemia

To elucidate potential pathogenic mechanisms of colitis development after stroke in microbiota-depleted AB(+/−) mice, we next investigated whether continuous preventative antibiotic treatment or restoration of commensal microbiota in microbiota-depleted mice could rescue the observed phenotype. We recolonized 2 groups of microbiota-depleted mice slated for MCAo or sham operation with intestinal microbiota from SPF AB(−/−) littermates. We observed that this intervention restored main bacterial groups of the gut microbiota. *Bacteroides/Prevotella* spp. and Enterococci appeared to recolonize the intestinal niche earlier than did other bacterial families and increased total bacterial load. Furthermore, recolonized animals had lower counts of the *Enterobacteriaceae* and Staphylococci, albeit the differences were <2 log and thus not considered to be biologically relevant (Figure II in the online-only Data Supplement). We did not observe bacterial growth in samples from microbiota-depleted AB(+/+) or in the microbiota-depleted AB(+/−) mice: only 2 samples were positive for Staphylococci at the end point (Figure 4A–4C). When comparing survival rates among microbiota-depleted mice, we observed that antibiotic treatment during the experiment, as well as recolonization with intestinal microbiota from SPF AB(−/−) littermates protected the mice from colitis and improved survival rate. Mortality rate in the recolonized MCAo group was still higher than in the SPF AB(−/−) MCAo group; nevertheless, recolonized animals did not develop symptoms of colitis (Figures 2B and 3 and Figure IC in the online-only Data Supplement). Furthermore, in our experiments, mortality on the first day after stroke appeared to be linked primarily to the severity of ischemic damage.

Microbiota-Depleted MCAo Mice Show Systemic Immunodepression on Day 5 After Cerebral Ischemia

In a third set of experiments, we investigated whether depletion of the intestinal microbiota might influence immune parameters after focal cerebral ischemia. We assessed main immune cell populations and percentages of interferon gamma (IFNγ) and interleukin 17 (IL-17) secreting cells after ex vivo stimulation with phorbol 12-myristate

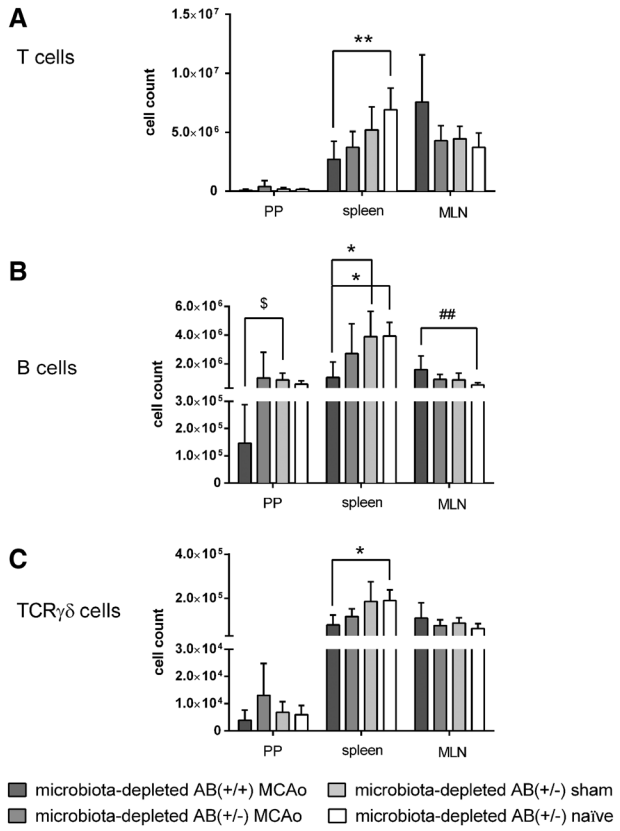

Figure 5. Flow cytometric analysis of immune cell populations from Peyer's Patches, spleen, and mesenteric lymph nodes (MLN) on day 5 after cerebral ischemia. Cell counts for (**A**) T cells, (**B**) B cells, and (**C**) T cell receptor γδ+ (TCRγδ+) cells. Microbiota-depleted mice show systemic immunodepression an day 5 after cerebral ischemia. Data are expressed as mean+standard deviation (SD). No statistically significant differences were found between group with continuous antibiotic treatment, and group with antibiotics stopped before surgery. Microbiota-depleted AB(+/+) MCAo n=8 (n=7 for Peyer's patches), microbiota-depleted AB(+/–) MCAo n=7, microbiotadepleted AB(+/–) sham n=6, and microbiota-depleted AB(+/–) naïve n=6. Statistical analyses comparing the groups within one lymphatic organ were conducted using Kruskal–Wallis Test with Dunn's post hoc. MCAo indicates middle cerebral artery occlusion; and TCR, T cell receptor. 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.

13-acetate (PMA) and ionomycin from spleens, mesenteric lymph nodes, and Peyer's patches. Assessment took place on day 5 after surgery in microbiota-depleted AB(+/+) MCAo, AB(+/–) MCAo, AB(+/–) sham, and AB(+/–) naïve animals. Overall, we observed that cell counts of T cells (including CD4+ and CD8+ T cells subsets), B cells, and T cell receptor γδ+ (TCRγδ+) cells in spleens from mice subjected to cerebral ischemia tended to be lower than cell counts in naïve and sham-operated groups. In the AB(+/+) MCAo group, numbers of B cells and CD8+ cytotoxic T cells, as well as percentages of IFNγ-producing CD8+, TCRγδ+, and CD4+ helper T cells were significantly lower compared with sham-operated animals (Figures 5 and 6 and Figure IIIA in the online-only Data Supplement). Moreover, in Peyer's patches, B cell numbers and percentage of IFNγ-producing TCRγδ+ cells were significantly decreased in the AB(+/+) MCAo mice compared with the sham-group (Figure 5 and Figure IIIB in the online-only Data Supplement). In contrast, in mesenteric lymph nodes, we observed significantly increased numbers of B cells in AB(+/+) MCAo animals (Figure 5) as compared with the naïve group and higher percentages of IL-17-producing CD4+ and TCRγδ+ cells than in sham-operated animals (Figure 6 and Figure IIIC in the online-only Data Supplement).

Discussion

We aimed at investigating the effects of microbiota depletion with broad-spectrum antibiotics on the outcome of experimental stroke. We hypothesized that uncompromised gut microbiota acts as an important modulator of stroke outcome. We applied a well-characterized murine model of experimental stroke in animals after depletion of microbiota with broad-spectrum antibiotics frequently used in intensive care medicine. Our main findings were that the absence of cultivatable microbiota at the time of induction of focal cerebral ischemia (1) does not affect infarct sizes 1 day later and (2) induces excessive mortality manifesting between days 5 and 7 (3) and that this mortality was prevented by continuous antibiotic treatment or by the recolonization of microbiota-depleted mice with a complex conventional intestinal microbiota from SPF littermates. This is the first demonstration that gut microbiota can affect outcome after acute CNS injury.

Stroke may interfere with normal gut microbiota–host interaction on many levels. After a brain lesion, the CNS engages in intense signaling with the immune system, resulting in a decrease of immune cell numbers and their functionality. This is termed stroke-induced immunodepression.[14–16] Altered systemic immunity after CNS lesion may lead to the breakdown of mucosal barriers and to the translocation of bacteria and their products (such as bacterial cell wall constituents or toxins) to the host blood stream or lymphatic organs, further impacting the immune system and providing costimulation in deleterious immune–brain cell interaction. Furthermore, a direct impact of brain lesions on bacterial microbiota is expected through the effects of the sympathetic nervous system, as well as the vagus nerve: acute brain lesions affect the outflow of the autonomic nervous system. This may reduce gut motility and increase its permeability via the enteric nervous system and ultimately lead to bacterial translocation, infection, and sepsis. Antibiotic therapy in patients with brain lesions further disturbs the composition or even eradicates commensal bacterial communities and produces bacterial fragments, which may act as toxins and costimulants. Moreover, changes in nutrition, which might affect the gut microbiome diversity,[33] are often a consequence of acute stroke, with dysphagia and unconsciousness mandating parenteral or tube feeding.

To date, only few studies have addressed the effects of brain lesions on the gut and its microbiota. In an experimental model of stroke, stress prior MCAo induced bacterial translocation and contributed to negative outcome.[17] Schulte-Herbrüggen et al demonstrated in a mouse model of stroke similar to the one used in our study that 24 hours after focal cerebral ischemia, T- and B cell counts are reduced in the Peyer's patches.[34] Swidsinski et al found signs of ulcerative

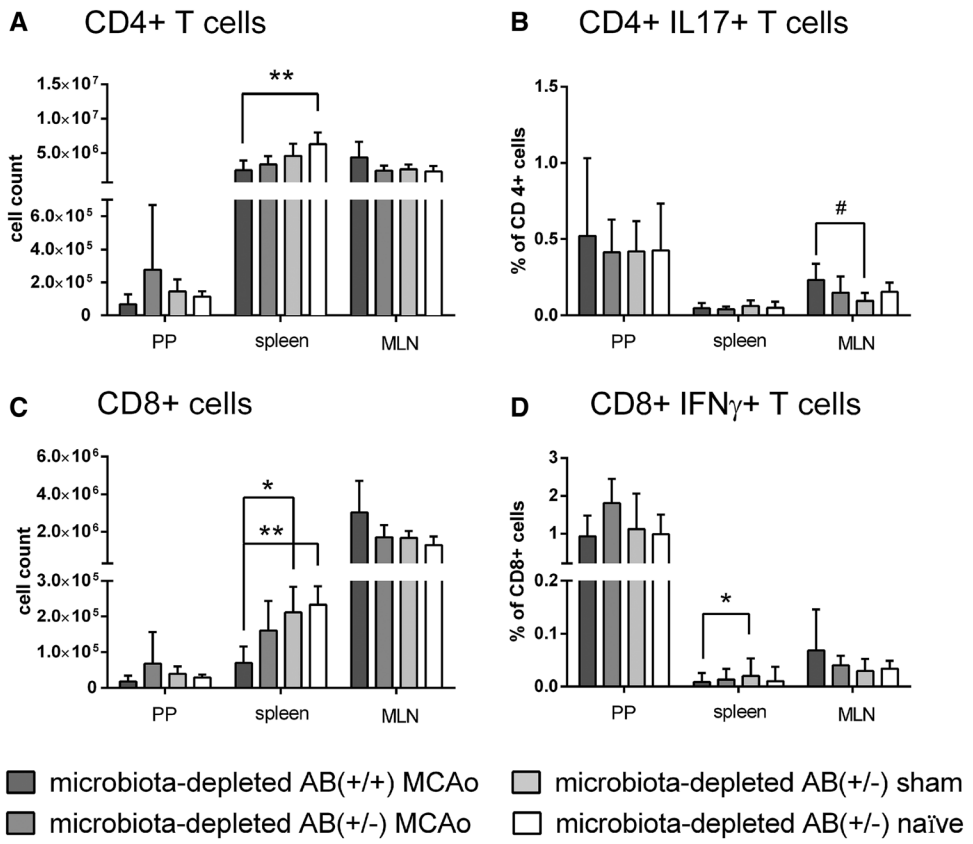

Figure 6. Flow cytometric analysis of immune cell populations from Peyer's Patches, spleen, and mesenteric lymph nodes (MLN) on day 5 after cerebral ischemia. Microbiota-depleted mice show systemic immunodepression an day 5 after cerebral ischemia. Cell counts for (**A**) CD4+ cells and (**C**) CD8+ cells. (**B**) Percentage of CD4+ cells producing interleukin 17 and (**D**) percentage of CD8+ cells producing interferon gamma. Data are expressed as mean+standard deviation (SD). No statistically significant differences were found between group with continuous antibiotic treatment, and group with antibiotics stopped before surgery. Microbiota-depleted AB(+/+) MCAo n=8 (n=7 for Peyer's patches), microbiota-depleted AB(+/-) MCAo n=7, microbiota-depleted AB(+/-) sham n=6, and microbiota-depleted AB(+/-) naïve n=6. Statistical analyses comparing the groups within one lymphatic organ were conducted using Kruskal–Wallis Test with Dunn's post hoc. MCAo indicates middle cerebral artery occlusion. 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.

colitis and dramatic changes in gut microbiota composition in stool samples from patients with acute stroke,[35] Hayakawa et al described a sudden decrease in commensal organisms and an increase in potentially harmful bacteria after severe insults, as is seen in cerebral vascular disease.[36] Karlson et al reported that patients with symptomatic atherosclerosis have altered gut metagenome with enrichment in the Collinsella genus and reduced levels of β-carotene in serum.[37]

A prediction based on these previous reports would suggest a gut–brain interaction after stroke with possible negative effects on the acute outcome post ischemia. Our findings did not provide evidence for such deleterious effects, in particular on infarct volume, although minor to moderate effects could not be ruled out given the inherent high variability of modeling stroke in rodents and limitations in sample size. In addition, delayed (beyond day one) effects on infarct volume or recovery could not be ruled out in view of the high morbidity and mortality of the microbiota-depleted animals after MCAo.

In contrast to a putative deleterious role of a depleted gut microbiota after stroke, we found that a complex conventional intestinal microbiota (or continuous antibiotic treatment) protects the host, whereas a compromised physiological resistance to colonization after completed antibiotic treatment may lead to severe illness and even death.[38] It is well known that stroke induces a long-lasting immunosuppression with lymphopenia and altered cellular immune function, impairing antibacterial defense in a conventionally colonized host. Prass et al demonstrated previously that MCAo animals have reduced counts of T, NK, and B cell subsets in spleen and reduced cytokine excretion (eg, IFNγ and TNFα) as measured by ex vivo tests as early as 12 hours after experimental stroke.[39] After MCAo in wild-type animals not treated with antibiotics, T and B cell counts were significantly reduced in Peyer's patches compared with sham-operated animals, while no differences were found for natural killer cells and macrophages. Moreover, no significant changes in intraepithelial and lamina propria lymphocytes subsets were observed after cerebral ischemia compared with controls.[34] In our experiments, MCAo animals showed reduced counts of T and B cells and a reduced percentage of IFNγ secreting lymphocytes on day 5 after stroke as compared with naïve and sham mice. This was observed mainly in spleens and to lesser extent in Peyer's patches. Because we found no significant differences between microbiota-depleted AB(+/−) and AB(+/+) MCAo

animals in immune cell numbers or function in secondary lymphoid organs, antibiotic treatment is unlikely to have mitigated depressed immunity in the gut. Moreover, survival rates of microbiota-depleted AB(+/−) sham-operated animals were higher compared with microbiota-depleted AB(+/−) MCAo group, suggesting that stroke-induced immunodepression–mediated systemic immune response syndrome might contribute to poor stroke outcome in the microbiota-depleted state.

Although the immunodepression observed after MCAo may promote bacterial infections, it does not provide a direct mechanistic explanation for the development of severe colitis in the absence of continuous antibiotic treatment. In a murine model of chronic psychosocial stress, which is known to lead to spontaneous colitis, Reber et al recently observed that animals rapidly developed mucosal immunosuppression and epithelial barrier defects associated with increased bacterial load in intestinal tissue shortly after stress onset. Later development of colitis was associated with hyperreactivity of mucosal immune cells in the elevated presence of bacterial antigens, both of which can be prevented by prolonged antibiotic treatment before and during chronic stress.[40] Whether similar mechanisms are operational in our model of MCAo-associated colitis needs to be further elucidated.

Our study in a murine model of stroke in which we simulated the rather unphysiological scenario of virtually depleted microbiota, nevertheless raises some intriguing clinical questions because intestinal microbiota may contribute to stroke outcome in antibiotic-treated patients. Our findings indicate that the presence of an uncompromised complex intestinal microbiota may be critical for the outcome after cerebral ischemia. In the clinical scenario, considering pooled post-stroke bacterial infection rate of 30% in the first week after stroke onset,[1] it is probable that many stroke patients are treated with antibiotics. Representative data of large stroke populations are missing. Nevertheless, in the recently published large PASS trial investigating the effect of preventive antibiotics in acute stroke on 3-month outcome, 20% of stroke patients in the control group were treated with antibiotics.[41] Because ischemic brain lesion in many of these patients produced an immunodepressed state, they may be prone to recolonization by facultative or obligatory bacterial pathogens. This would further increase their risk of infection (in particular pneumonia).

We speculate that understanding gut microbiota–brain cross talk will contribute to a better understanding of the pathophysiology of acute CNS disorders and related complications and may lead to the improvement of current clinical practice or entirely new treatment strategies.

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Disclosures

None.

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Depletion of Cultivatable Gut Microbiota by Broad-Spectrum Antibiotic Pretreatment Worsens Outcome After Murine Stroke

Katarzyna Winek, Odilo Engel, Priscilla Koduah, Markus M. Heimesaat, André Fischer, Stefan Bereswill, Claudia Dames, Olivia Kershaw, Achim D. Gruber, Caterina Curato, Naoki Oyama, Christian Meisel, Andreas Meisel and Ulrich Dirnagl

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SUPPLEMENTAL MATERIAL

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

Katarzyna Winek MD, Odilo Engel DVM, Priscilla Koduah MSc, Markus M. Heimesaat MD, André Fischer PhD, Stefan Bereswill PhD, Claudia Dames MSc, Olivia Kershaw PhD, Achim D. Gruber PhD, Caterina Curato PhD, Naoki Oyama MD, Christian Meisel MD, Andreas Meisel MD, Ulrich Dirnagl MD

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.

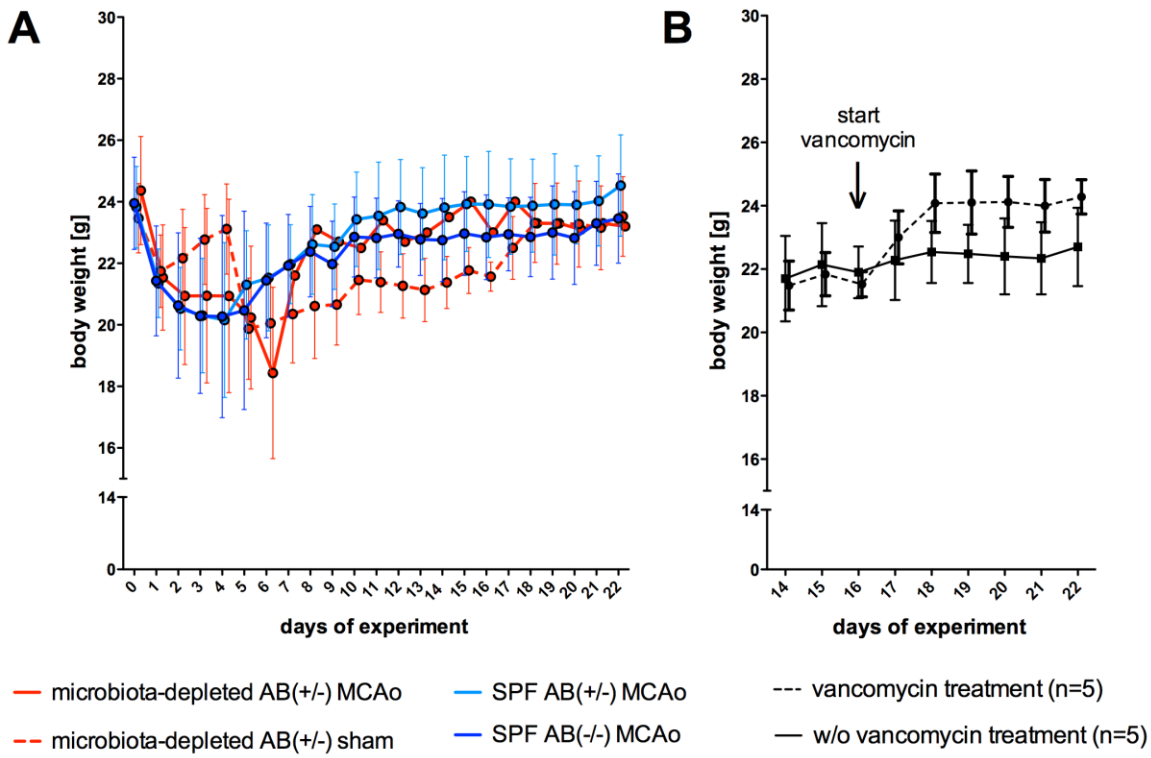
Flow cytometric analysis of spleens, mesenteric lymph nodes (MLN) and Peyer's patches (PP)

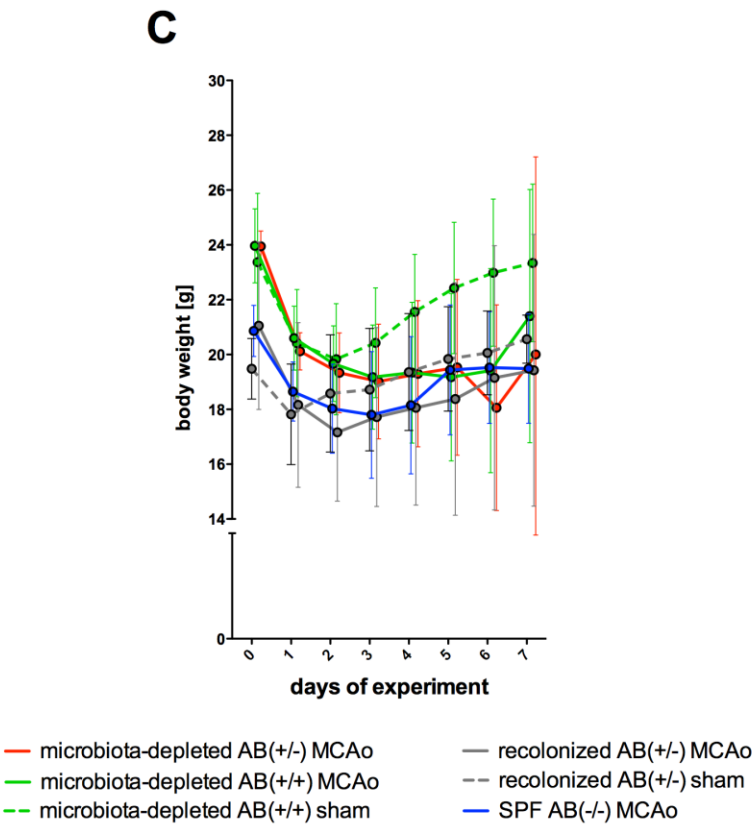
Single cell suspensions from PP, spleens and MLN were prepared by forcing the tissues through a fine wire mesh. To obtain single cell suspensions from PP, the patches were initially digested 30 minutes at 37°C in RPMI containing 10% FCS (Biochrom, Berlin, Germany) and 3.5mg collagenase A (Roche, Basel, 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 were 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.

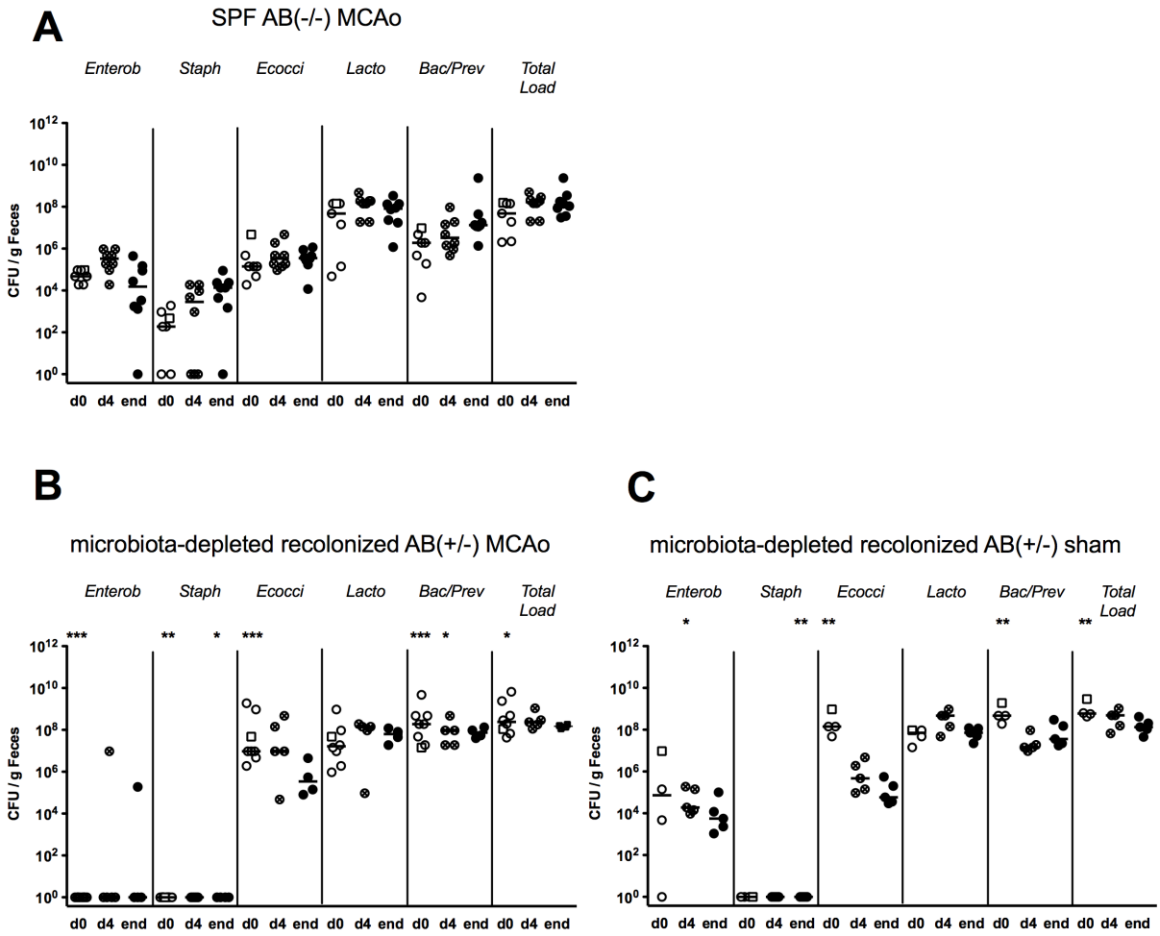
Supplementary figures



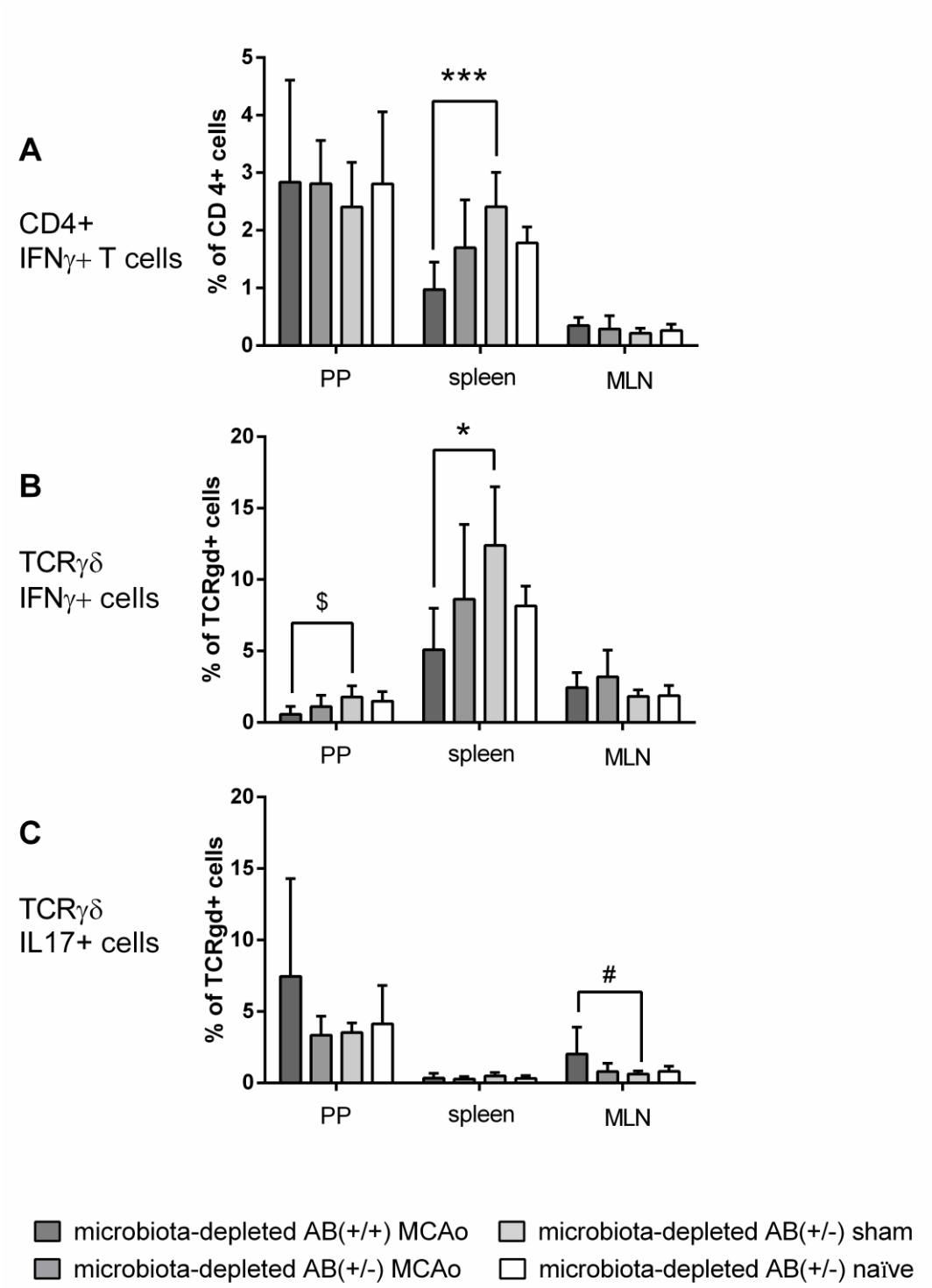


Supplementary Figure I (SFig.I) Weight of animals from experiment I and II

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) Flow cytometric 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.

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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

Curriculum vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Complete list of publications

Original contributions:

Depletion of cultivatable microbiota by broad-spectrum antibiotic pretreatment worsens outcome after murine stroke.
Winek K, Engel O, Koduah P, Heimesaat MM, Fischer A, Bereswill S, Dames C, Kershaw O, Gruber AD, Curato C, Oyama N, Meisel C, Meisel A, Dirnagl U.
Stroke. 2016 May;47(5):1354-63. **IF: 5.761**

Cholinergic pathway suppresses pulmonary innate immunity facilitating pneumonia after stroke.
Engel O, Akyüz L, da Costa Goncalves AC, **Winek K**, Dames C, Thielke M, Herold S, Böttcher C, Priller J, Volk HD, Dirnagl U, Meisel C, Meisel A.
Stroke. 2015 Nov;46(11):3232-40 **IF: 5.761**

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J Neurosci. 2015 May 20;35(20):7777-94 **IF: 6.344**

Review/Opinion articles:

Gut microbiota as therapeutic target in CNS diseases – implications for stroke
Winek K, Dirnagl U, Meisel A.
Neurotherapeutics. 2016 Oct;13(4):762-774. **IF: 5.054**

Gut microbiota impact on stroke outcome: Fad or fact?
Winek K, Meisel A, Dirnagl U.
J Cereb Blood Flow Metab. 2016 May;36(5):891-8. **IF: 5.407**

Impact factor according to Journal Summary List (ISI Web of Knowledge^{SM}) 2014

Manuscripts in preparation:

Exploratory investigation of intestinal function and bacterial translocation after focal cerebral ischemia in the mouse
Oyama N*, **Winek K***, Koduah P, Zhang T, Beckers Y, Dames C, Werich M, Kershaw O, Meisel A, Dirnagl U. *these authors contributed equally

Delayed autoreactivity and chronic inflammation are mediated by recruitment and activation of CD4 T cells and B cells in ischemic stroke.
Zhang T, **Winek K**, Dames C, Andrzejak E, Engel O, Dirnagl U, Meisel C, Meisel A.

Experimental stroke is accompanied by reduction in number of ciliated tracheal epithelial cells and diminished particle transport speed.
Dames C, **Winek K**, Perniß A, Krasteva-Christ G, Meisel C, Meisel A, Kummer W.

Die Bedeutung des intestinalen Mikrobioms beim ischämischen Schlaganfall
Winek K, Dirnagl U, Meisel A. (under revision in Aktuelle Neurologie)

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