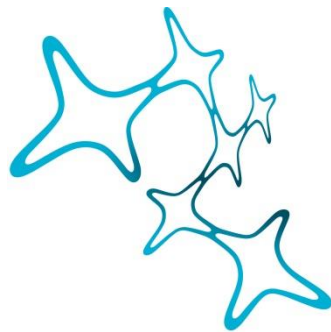

THE GUT MICROBIOME MODULATES POST STROKE OUTCOME

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ABSTRACT

15 million people suffer from stroke per year. Fundamentally, stroke is caused by a lack of oxygenated blood to brain tissue which results in tissue death. This entails a complex pathophysiology which encompasses 3 phases. Within minutes to hours, brain resident cells initiate excitotoxicity leading to irreversible neuronal death. From days to months, peripheral recruitment of immune cells to the brain drives neuroinflammation and exacerbates stroke outcome. Finally, within months to years, there is an increase in neuronal plasticity which enables reorganisation of cortical networks and restoration of broken circuits. Despite decades of research and intricate understanding of the physiological processes occurring after stroke, only one acute therapy is approved for use in clinics. An interesting therapeutic target for scientific researchers is modulation of the peripheral host immune system. Experimental research has shown that polarisation of the immune cell sub populations towards pro-/anti-inflammatory state can exacerbate or alleviate stroke outcome respectively. Polarised immune cell subsets migrate from peripheral secondary lymphoid organs to the brain lesion. While the intestinal immune compartment contains the majority of the immune cells in the body, it is the intestinal lumen that is the home to 1000 different readily adapting bacterial species. The gut microbiota has been shown to intimately interact with the immune system and alter the function of particular immune cell subsets. Recent experimental evidence has indicated a potential role for the interaction the gut microbiota and immune system in brain disease. We hypothesised that the gut microbiota could therefore play a role in the outcome of stroke. Within this thesis we explore the gut microbiota and its derived metabolites in experimental ischemic stroke models. This thesis incorporates four publications which have unravelled different aspects of how the gut microbiota affects stroke. The key experimental findings within this thesis can be summarised in five key concepts. 1) The gut microbiota and stroke have a bidirectional interaction, both having the ability to change the other. 2) The gut microbiota alters peripheral immune cells which after stroke, were shown to migrate to the brain and alter the inflammatory milieu. 3) The presence of the gut microbiota, or treatment with healthy gut microbiota transfer, improved stroke outcome. 4) Small changes in the gut microbiota can alter response to stroke immunotherapies. 5) Short-chain fatty acids, the dietary metabolites derived from the gut microbiota, improve functional post stroke recovery. Taken together, I hope this thesis reflects and demonstrates the interesting therapeutic potential of gut microbiota manipulation for treatment of stroke. The addition of microbiota-based treatments may not only be a stand-alone therapy to aid recovery after stroke, but additionally could be a practical add-on for existing procedural treatments.

LIST OF ABBREVIATIONS

ACTH – Adrenocorticotrophic hormone
AD – Alzheimer’s Disease
AhR – Aryl hydrocarbon receptor
 $\alpha\beta$ – Alpha beta glycoprotein
AMPA - α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ASD – Autism spectrum disorder
BDNF – Brain-derived neurotrophic factor
Ca²⁺ - Calcium
CARD9 - Caspase recruitment domain-containing protein 9
CD28SA - CD28 superagonistic antibody
CD - Cluster of differentiation
CFSE - Carboxyfluorescein succinimidyl ester
CIMT - Constraint-induced movement therapy
CR – Charles River Laboratories
CRF – Corticotropin-releasing factor
CT – Computed tomography
DAMPs - Danger associated molecular patterns
dMCAo – Distal middle cerebral artery occlusion
EAE – Experimental autoimmune encephalitis
EGF – Epidermal growth factor
Ex-GF – Germ-free recolonised
FDA – Food and drug administration
FFAR2 – Free fatty acid receptor 2
FFAR3 – Free fatty acid receptor 3
FGF – Fibroblast growth factor
FITC - Fluorescein isothiocyanate
fMCAo – Filamentous middle cerebral artery occlusion
fMRI – Functional magnetic resonance imaging
FMT – Fecal microbiota transplant
GALT – Gut-associated lymphoid tissue
 $\gamma\delta$ – Gamma delta glycoprotein
GF – Germ-free
GI – Gastrointestinal tract

Har – Harlan (now known as Envigo)
HDAC - Histone deacetylases
HIF-1 – Hypoxia-inducible factor 1-alpha
HPA – Hypothalamic-pituitary-adrenal axis
ICAM1 – Intercellular adhesion molecule 1
IDO1 - Indoleamine 2, 3-dioxygenase 1
IFN γ - Interferon gamma
IL - Interleukin
iTreg – “Induced” Treg
K⁺ - Potassium
LP – Lamina propria
M cells – Microfold cell
MALT – Mucosa-associated lymphoid tissues
MCT1 – Monocarboxylate transporter 1
MLN – Mesenteric lymph nodes
MMPs – Matrix metalloproteinases
MRI - Magnetic resonance imaging
mRS - Modified rankin scale
MS – Multiple sclerosis
Na⁺ - Sodium
NDMA - N-methyl-D-aspartate receptor
NF- κ B - Nuclear factor kappa-light-chain-enhancer of activated B cells
nTreg – “Natural” Treg
PD – Parkinson’s disease
PET - Positron-emission tomography
PP – Peyer’s patches
PT – Photothrombosis
RNA – Ribonucleic acid
rRNA – Ribosomal RNA
rtPA - Alteplase
SAA - Serum amyloid A
SCFA – Short-chain fatty acid
SFB – Segmented filamentous bacteria
SMCT1 – Sodium-coupled monocarboxylate transporter 1
SPF – Specific pathogen free

SSRIs – Serotonin reuptake inhibitors

STAT3 - Signal transducer and activator of transcription 3

Tac – Taconic Biosciences

Th1 – Type 1 T helper cells

Th17 – Type 17 T helper cells

Th2 – Type 2 T helper cells

TNF α – Tumor necrosis factor alpha

Tregs – Regulatory T cells

TrkB – Tropomyosin receptor kinase B

VCAM1 – Vascular cell adhesion molecule 1

INTRODUCTION

AN INTRODUCTION TO STROKE

Stroke is the leading cause of death and cause of disability worldwide (JohnsonNguyen et al., 2019). According to the world stroke organization, a remarkable 15 million people suffer from a stroke per year with 5.8 million of those patients who die. The majority of stroke patient survivors experience long term commodities such as dysphagia, paralysis and dementia. This leads to what is known as the socioeconomic burden of stroke; patients unable to return to work in addition to high prevalence of secondary diseases and costly hospitalization for rehabilitation. Together, this unequivocally demonstrates the absolute need for stroke research, to understand its pre-disposing factors, complex pathophysiology and post-stroke comorbidities. Essentially, stroke is caused by an occluded artery preventing oxygenated blood flow to brain tissue resulting in tissue death and loss of function. The aetiology of stroke itself is categorized into two main sub-types; haemorrhagic and ischemic. Haemorrhagic stroke accounts for 15% and amounts when a cerebral artery is ruptured causing bleeding into the brain tissue. Ischemic stroke is the most common form of stroke accounting for 85% of all cases. This occurs when a terminal cerebral artery is occluded, and results in necrotic core tissue and surrounding at risk yet salvageable penumbra tissue.

NEUROINFLAMMATION

The brain consumes extensive amounts of energy for proper functioning; however, following an ischemic injury is unable to maintain energy stores. Under an ischemic event the continuous supply of oxygen is removed and thus cells undergo rapid K^+ loss leading to membrane depolarisation (Katsura, Kristian et al., 1994). During this period, large amounts of Na^+ and Ca^{2+} enter cells encouraging glutamate release, and consequently initiates a wave of repetitive peri-infarct depolarisations (Dirnagl, Iadecola et al., 1999). The large amounts of glutamate release leads to over stimulation of post synaptic excitatory receptors (NMDA, AMPA and kainite) further allowing influx of Ca^{2+} . The elevated Ca^{2+} influx triggers a cascade of intracellular cytoplasmic events activating proteolytic enzymes and forcing free-radicals and cytochrome C from mitochondria. This cellular toxicity then induces cell death via apoptosis (delayed physiological and pathological conditions) or necrosis (acute, hypoxia conditions) depending on the stimulus (Chan, 2001) . Necrotic tissue can then secrete danger associated molecular patterns (DAMPs) such as ATP to further activate brain resident cells such as microglia. Simultaneously, these events occur within the acute phase after stroke lasting minutes to hours, and were coined excitotoxicity.

Within the sub-acute phase after stroke, neuroinflammation is the key driver of lesion progression. An elevated concentration of intracellular calcium stores is responsible for an increase in pro-inflammatory transcription factors such as NF- κ B (Oneill & Kaltschmidt, 1997), HIF-1 (Ruscher, Isaev et al., 1998) and STAT3 (Planas, Soriano et al., 1996). The up-regulation of these transcription factors induces a mass secretion of classic proinflammatory cytokines from brain resident cells such as IL-1 β , TNF α , and IL-6 (Lambertsen, Biber et al., 2012, Rothwell & Hopkins, 1995) and chemokines (Asensio & Campbell, 1999, Mirabelli-Badenier, Braunersreuther et al., 2011). In turn, endothelial cells respond to the proinflammatory milieu with a higher expression of adhesion molecules displayed on the surface. The adhesion molecules such as ICAM-1, VCAM-1, selectins and integrins work to enhance the recruitment of peripheral immune cells to the brain parenchyma (Frijns & Kappelle, 2002). Furthermore, the release of nitrous oxide from various cell sources (Chen, Mou et al., 2017) and matrix metalloproteinases (MMPs) work to dilate and degrade basement membrane and tight-junction proteins redesigning the extracellular matrix, and easing the migration of potential infiltrating immune cells into the brain parenchyma (Rosenberg, 2002).

IMMUNE CELL INFILTRATION INTO THE BRAIN

Immune cell recruitment from the periphery involves both chemokines and adhesion molecules. The innate and adaptive immune system are both engaged during this process (Iadecola & Anrather, 2011, Planas, 2018). For a temporal illustration of the immune cell infiltration into the brain, please refer to Figure 1. In the periphery, the innate immune system, is the first to respond and particularly neutrophils have been detected within one hour of stroke onset, peaking at 1-3 days post lesion (Gelderblom, Leypoldt et al., 2009). Whilst a higher number of neutrophils have been associated with worst stroke outcome, the therapeutic blockage of neutrophil infiltration has delivered controversial results (Jickling, Liu et al., 2015). The next cell types are macrophage and monocyte populations along with microglia cells rapidly increasing in cell number, peaking at around 4 days post ischemia. Initially it was thought that the presence of monocytes at the lesion following ischemia exacerbated stroke outcome. However, experimental evidence has suggested that upon monocyte depletion, ischemic stroke injury is worsened (Gliem, Mausberg et al., 2012).

Whilst the adaptive immune cells are present in the brain from as early as 24 hrs after ischemia, it is thought that they impose their effect within sub-acute phases. In support of this hypothesis, it was shown that T cells are increased in cell number within the brain up until 30 days post lesion in experimental mice models (Stubbe, Ebner et al., 2013). Furthermore, current research and discussion hints at the concept of T cells having a positive impact on neuroregeneration and not just having a detrimental role (Cramer, Benakis et al., 2019). T and B lymphocytes are derived from bone marrow

stem cells; however, T cells require further maturation in the thymus. In order for the lymphocytes to be fully functional, antigen must be presented in a secondary lymphoid organ. This enables cell division, differentiation and migration which in turn leads to clearance of specific proteins. The main secondary lymphoid organs are: lymph nodes, tonsils, spleen, Peyer's patches (PP) and mucosa associated lymphoid tissue (MALT). From here immune cells migrate to the brain after stroke and activate microglia and other brain resident cells. Importantly, the neuroinflammatory properties derived from lymphocytes has mainly been attributed to T cells and their cytokine release. This has been demonstrated in a number of elegant studies showing that T cells worsen stroke outcome, for example, lymphocyte deficient mice replenished with T cells had a worsened injury, whereas this affect was not seen with B cells (Hurn, Subramanian et al., 2007, Kleinschnitz, Schwab et al., 2010). Despite this, a recent growing body of evidence has suggested that B cells also contribute to the neuroinflammatory processes (Javidi & Magnus, 2019, Seifert, Vandenbark et al., 2018). T cells are the more widely investigated cell type in stroke, and can be classified into CD8⁺ cytotoxic cells and CD4⁺ helper cells. The latter, CD4⁺ T cells come in a variety of different subsets for example pro-inflammatory Th1, Th17, Th2 and anti-inflammatory regulatory T cells (Tregs). Classically it is was shown that upon treatment with anti-CD25, a key receptor found important for the suppressor function of Tregs, mice had significantly worse outcome at 7 days post lesion (Liesz, Suri-Payer et al., 2009), clarifying the neuroprotective properties of Tregs in stroke. Th1 cells mainly secrete proinflammatory cytokines including: IL-2, IL-12 IFN γ and TNF α . Th1 cells protect against bacterial infection, and in stroke have also been found in ischemic lesions where they contribute to secondary neuronal damage (Arumugam, Granger et al., 2005). Th2 cells secrete mainly anti-inflammatory cytokines such as IL-4, IL-5, IL-10 and IL-13, they aid in resolution of parasitic infection and also support antibody-mediated responses (Mosmann & Sad, 1996). Th17 cells secrete proinflammatory IL-21, IL-22 and IL-17. Th17 cells were shown to activate neutrophils and are also highly implicated in autoimmune disease. A subset of unconventional T cells (T cell receptor composed of $\gamma\delta$ glycoprotein instead of $\alpha\beta$) called $\gamma\delta$ cells, were found to be the main source of proinflammatory cytokine IL-17 in the brain, which exacerbated stroke outcome (Shichita, Sugiyama et al., 2009). Interestingly, it is now known that microglia are the main antigen presenting cells within the brain suggesting that potentially lymphocytes are re-stimulated in the brain parenchyma (Schettters, Gomez-Nicola et al., 2018).

After stroke, T cells migrate to the brain from secondary lymphoid organs. The spleen has been by far the most investigated secondary lymphoid organ after stroke. It was shown that following ischemia the spleen reduces in size and the number of circulating lymphocytes increase suggesting that they are derived from the spleen (Offner, Subramanian et al., 2006). However, the only experiment

performed which tracked cells from the spleen to the brain after stroke failed to show an increase in CFSE positive cells in the brain after injection of the dye in the spleen (Seifert, Hall et al., 2012). Another major secondary lymphoid tissue is the Gut-associated-lymphoid-tissue (GALT). A remarkable 70% of all immune cells from the body reside in the gut, insinuating that the GALT is a highly influential area for priming of the immune cells prior to brain infiltration post stroke. Interestingly, after stroke, T cells appear to have a higher migratory rate compared to B cells (Benakis, Brea et al., 2016b, Morton, Sefik et al., 2014). Furthermore, we found that 25% of all T cells found within the brain after stroke were originating from the GALT (Singh, Roth et al., 2016). More information about the importance of the immune cells residing in the gut will be listed in the next section.

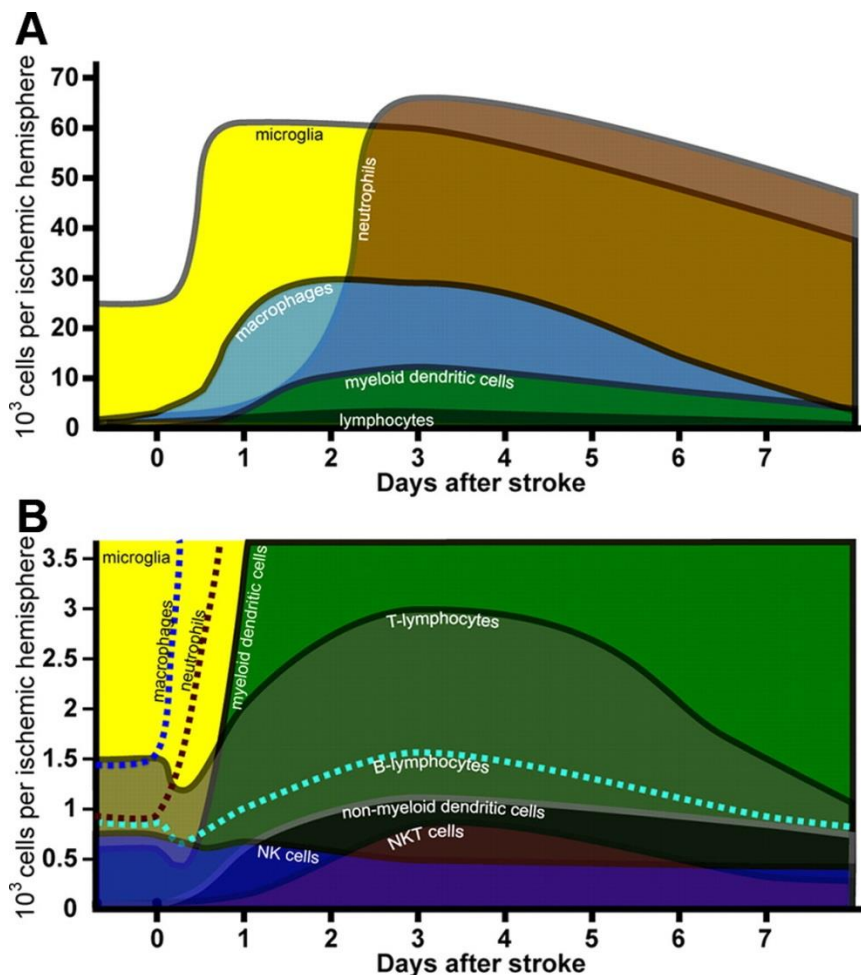


Figure 1. Time-dependent immune cell infiltration after stroke. Temporal dynamics of immune cell infiltration into the post stroke ischemic hemisphere following 60 mins transient occlusion of the middle cerebral artery. A is an overview of number of cells per hemisphere. B represents a higher magnification of the number of cells and different types found in the brain. Extracted from (Gelderblom et al., 2009).

Many mechanisms occurring during the recovery phase after stroke are akin to those employed during brain development. The physiological processes involved in motor and sensory recovery of the debilitated limb is often termed neuroregeneration. The extent of neurodegenerative processes is generally assessed in human patients and animal models using behavioural tests. The nurturing or boosting of these regenerative processes therefore leads to improved functional recovery after stroke. The brain is topographically organised and provides information about the anatomical structure, and function in the brain, i.e. there is an area of the brain associated with hand sensory and motor function. This concept has been clearly depicted in homunculus models of the motor and sensory cortex (Penfield & Boldrey, 1937). Furthermore, the control centers for i.e. limb movement are distributed over many neuronal networks some even existing in the other hemisphere (Brus-Ramer, Carmel et al., 2009, Gonzalez, Gharbawie et al., 2004). This is an important factor to consider after stroke, and shines light on the importance of the contralesional hemisphere during recovery. Resting state fMRI in stroke patients has indicated that the homotypical contralateral region receives less neuronal inhibition from the stroked ipsilateral hemisphere. This indicated that the contralateral region is therefore more active after stroke. During the recovery period, the ipsilateral hemisphere gradually gains more functional activity, restoring inhibition to the contralateral hemisphere, and therefore decreasing the contralateral activity. It has been shown that after stroke, eventually the contralateral hemisphere activity reduces back to “normal state” and is accompanied with motor improvements (Rehme & Grefkes, 2013). This was found to be comparable in experimental stroke performed in rodent models. With fMRI in combination with behavioural tests researchers saw an extensive activation of the contralesional hemisphere after stroke which over time decreased, and at this time the peri-infarct territory slowly re-gained activity (Dijkhuizen, Ren et al., 2001).

Within the peri-infarct region there is a rapid loss of neuronal dendritic spines in acute phases after stroke (Brown, Wong et al., 2008). The peri-infarct area undergoes a decidedly specific rewiring and remapping, and processes involving synaptic plasticity are highly upregulated (Jablonka, Witte et al., 2007). The gene regulation in stroke specific plasticity compared to experience dependant plasticity has been well defined (Carmichael, Archibeque et al., 2005). Furthermore, the growth promoting neurotrophic factors has been extensively studied and it is well considered that factors such as brain derived neurotrophic factor (BDNF) improves functional recovery after stroke (Ploughman, Windle et al., 2009). The functional reorganisation after stroke is mainly governed by two classes of plasticity: homeostatic and Hebbian (Murphy & Corbett, 2009). Homeostatic plasticity is defined as a negative feedback loop which aims to restore the attenuated synaptic activity back to baseline (Turrigiano &

Nelson, 2004). After a stroke the infarct region displays a decrease in synaptic activity which is compensated by the surrounding penumbra hyperexcitability. Interestingly, it is thought that the hyperexcitability phase within the peri-infarct region after stroke is a result from homeostatic plasticity overcompensating for the loss of synaptic activity. On the other hand, Hebbian plasticity favours already existing strong functioning synapses. It uses existing robust synapses to redistribute connections and enable the functioning of circuits (Buma, Kwakkel et al., 2013). Taken together it suggests that the main plasticity mechanisms occurring after stroke, first begins with homeostatic plasticity and then develops into Hebbian plasticity.

The survival of synapses and spines after stroke is partly orchestrated via microglia pruning (Zhang, Malik et al., 2014). The cross talk of microglia and neurons is key for successful reorganisation and functional integration of new connections. Microglia perform activation-or complement-dependent pruning of synapses in the post ischemic brain (Sandvig, Augestad et al., 2018). During neuroinflammation, the preference for microglia to phagocytose synapses is mainly driven by the neuronal activity itself, i.e. less active pre-synaptic inputs are more likely to be phagocytosed (Schafer, Lehrman et al., 2012, Wu, Dissing-Olesen et al., 2015). Additionally, the activation state of microglia largely influences synaptic pruning and reorganisation after stroke. Upon pro-inflammatory stimuli, microglia take on an activated phenotype, which, in stroke has been associated with lower rates of phagocytosis (Faustino, Wang et al., 2011). Evidence for this is provided in studies where microglia are pharmacologically entirely depleted and spine turnover rate was reduced (Reshef, Kudryavitskaya et al., 2017) or specifically in stroke where overall neuronal coverage was altered (Szalay, Martinecz et al., 2016).

The activation state of microglia can be assessed by their phenotypic markers and morphology. Classically within acute phases it is known that that activated or more amoeboid shaped microglia are pro-inflammatory, whilst the ramified microglia are resting-state and anti-inflammatory. Unfortunately, no study to date has done a time course to investigate the longer-term changes of microglia following stroke, however it is likely that the amoeboid shape in the stroke hemisphere is maintained much longer than 5d after stroke (Heindl, Gesierich et al., 2018). There are many stimuli which can influence microglia activation after stroke. Within the acute phase, the initial sterile inflammation forces damaged cells to release DAMPs such as lipids, nucleic acid and proteins which activate microglia (Gulke, Gelderblom et al., 2018). Later sub-acute or chronic phases, pre-primed infiltrating immune cells from the periphery have the potential to influence microglia activation state via cytokine release. For example release of IFN- γ from Th1 cells has been shown to cause microglia activation and worsen stroke outcome (Chabot, Charlet et al., 2001). Recently the role of T cells

affecting neurons in post stroke regeneration has come into discussion. The effects of T cells expressing glucocorticoid-induced TNF receptor was shown to deteriorate neural stem cell progenitors in the brain, whereas Tregs were shown to promote survival of the neural stem cell populations after stroke (Wang, Xie et al., 2015). Further investigation needs to be complete to elucidate the exciting role of brain invading immune cells and the direct effect on neurons in regeneration after stroke.

STROKE THERAPIES AND TREATMENTS

ACUTE PHASE

Typically, a stroke is recognised by weakness in face or arm, sudden confusion or speech difficulties, problems seeing in eyes, sudden trouble walking and dizziness. At the hospital a stroke diagnosis is given using a combination of physical examination, blood diagnostics, and a brain scan within 1 hour. Computed tomography (CT) scan helps diagnose an ischemic or haemorrhagic stroke. Magnetic resonance imaging (MRI) provides detailed information about brain structure identifying also smaller, not yet demarcated lesions and approximate information about the time of stroke onset. An echocardiography or carotid ultrasound is given later to confirm the aetiology of the stroke.

The only pharmacological intervention for ischemic stroke still lies with utilisation of one drug. Intravenous alteplase (rtPA) a thrombolytic agent, is administered to 7-12% of all stroke patients globally (Craig, Middleton et al., 2019). The rtPA actively converts plasminogen (precursor found in the liver) into plasmin. This then allows for the conversion of the fibrin clot into fibrin degradation products. The drug rtPA reduces post-stroke disability as seen with the Modified Rankin Score (mRS) score at 3-6 months (Hankey, 2017). Despite this, rtPA has the limitation of time, as the drug must be administered within 4.5 hours of ischemic stroke, and still poses threat for potential bleeding from the brain.

The second approved therapy typically for larger clots, where the rtPA is not feasible, involves surgical removal of the clot itself. Endovascular thrombectomy considerably reduces disability and has recently shown multiple positive results in randomised controlled trials (Campbell, Donnan et al., 2016). Despite these two promising therapies tackling the acute phase following stroke, large efforts in research have been focusing on discovering treatment for the neuroinflammatory phase after stroke (Lo, 2008). However, treatments shown to be beneficial in experimental models, often ineffective or even worsen stroke outcome, and therefore fail to be translated into clinics. In order to

prevent the chances of a second clot forming, patients are also given anti-platelets, anti-coagulants, anti-hypertensives and statins. To summarise, the target of the acute phase therapy is primarily the clot, the main goal is to salvage the threatened brain tissue and limit tissue damage (Lin, Finklestein et al., 2018).

CHRONIC PHASE

The chronic phase treatments tend to promote neural growth and regeneration in the existing tissue. This phase poses as an attractive therapeutic window, as a longer treatment time-frame is available. Additionally, the effectiveness of the treatment can be measured by a range of parameters. In humans depending on the location of the lesion the list of deficits to assess include: motor, sensory, cognitive, attention, language, visual, coordination, and gait disturbances, among others. Most interesting for translational research are the motor deficits, which are practical to measure and assess in rodent models. Rodent studies have shown long-lasting if not permanent behavioural motor deficits after stroke (Girard, Murray et al., 2014, Murphy & Corbett, 2009).

While spontaneous recovery does occur after stroke, it is highly variable and potentially could be enhanced, in order to encourage regeneration after stroke. To date there is no small molecule or biologic approved by the Food and Drug Administration (FDA). Conventional therapies such as physical, occupational and speech therapies are offered to recovering stroke patients. Other therapies include constraint-induced movement therapy (CIMT), this is the physical restraint of the less-active arm. CIMT was shown to significantly improve motor defects which persisted up until 1 year after stroke (Wolf, Winstein et al., 2006). Other experimental research therapies which have not yet been approved can be broken down into two categories; biological interventions and computer-based therapies. For the scope of this thesis I will focus on the biological interventions.

Currently the most promising drugs to enhance motor recovery post stroke are serotonergic and dopaminergic (Cramer, 2015, Lin et al., 2018, Viale, Catoira et al., 2018). A systematic review showed that trials using serotonin reuptake inhibitors (SSRIs) primarily to treat symptoms of post-stroke depression, also showed improvement in disability (Mead, Hsieh et al., 2013). However, phase III trials failed to show an improved functional outcome after stroke (Dennis, Forbes et al., 2019). Similarly, dopaminergic inhibitors displayed improved motor recovery in initial studies, however multi-centre trials revealed no difference in the primary end-point (the ability to walk independently) (Ford, Bhakta et al., 2015, Lin et al., 2018, Scheidtmann, Fries et al., 2001). Taken together, the unsuccessful trials in stroke patients highlight the complex modulation of excitatory/inhibitory synaptic and cortical network balance.

Growth factors work to promote angiogenesis and neural sprouting to facilitate stroke recovery. Whilst many have been shown to be neuroprotective in rodent stroke models such as fibroblast growth factor (FGF) (Kawamata, Dietrich et al., 1997) epidermal growth factor (EGF) (Jin, Sun et al., 2004) and Brain derived growth factor (BDNF) (Schabitz, Schwab et al., 1997), clinical trials fail to show improvement as shown with trafermin (basic FGF) (Bogouslavsky, Victor et al., 2002). Finally, new investigations focus on the physical administration of cells, in hope of replacing lost cells after stroke, or engineering them to secrete neuroprotective agents into the brain parenchyma. Transfer of cells has been performed with different cell lines and administration routes. At 24-48hrs post stroke, patients were administered allogeneic marrow-derived cells intravenously (Hess, Wechsler et al., 2017). At 6-60 months post stroke, improvements were also seen with stereotaxic implanted cells in the ipsilateral hemisphere from allogeneic immortalized human fetal neurons (Kalladka, Sinden et al., 2016) and allogeneic modified marrow-derived mesenchymal cells (Steinberg, Kondziolka et al., 2016). However, these treatments still remain to be further investigated and are not yet approved for the clinic.

RELIABILITY AND REPRODUCIBILITY

Striving and achieving reproducibility among experimental research is a high priority within many fields. As mentioned previously, there is a clear struggle and roadblock between basic experimental research and successful translation into clinics. To tackle this, it is essential that reproducibility begins at the basic pre-clinical stage prior to use in the clinic. Despite this knowledge, still, only a small fraction of experimental basic stroke research is able to be replicated (Llovera & Liesz, 2016, McNutt, 2014). The main obstacles within replication of pre-clinical research include; insufficient power, missing cross-validation of data, study design, exclusion criteria, models used, experimental animals, researchers, statistics and analysis strategy, sharing of original data. To overcome these obstacles and “bridge the gap” between basic and clinical research, pre-clinical randomised control trials are being utilised (Llovera, Hofmann et al., 2015). While strict homogenisation of protocols enables reproducibility, there are some hidden confounding variables which are difficult to control but still may alter experimental results. Animal husbandry is thought to be a standardised procedure, however there are many confounding factors which cause the environments in which the animals live to be completely different. Chow, water, bedding, type of caging, frequency of bedding change, circadian rhythms, animal keeper, co-housing, drugs, commercial breeder purchased are some examples of the subtle differences which can alter the gut microbiota content (Figure 2).

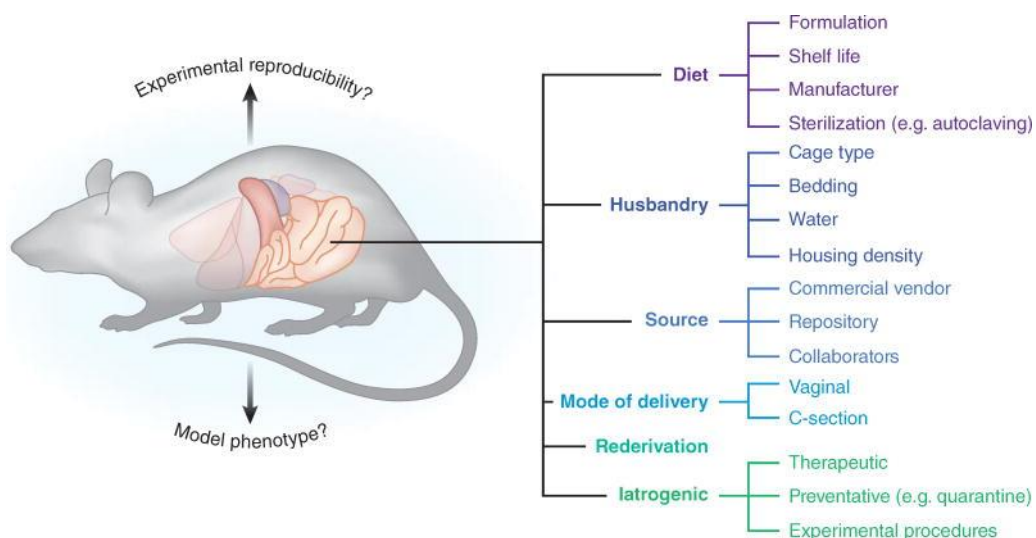


Figure 2. Animal husbandry alters the gut microbiota. Illustration highlighting the different variables in animal husbandry practice. These variables may affect the content of the gut microbiota and in turn alter experimental reproducibility or result in different results from the same genetic mouse model. Extracted from (Franklin & Ericsson, 2017).

It is important to acknowledge the potential existing difference between the gut microbiota of animals and can even reveal new mechanistic information about diseases. A study investigating bone marrow density serendipitously recognised that bone density was associated with presence of certain gut bacteria (McCabe, Irwin et al., 2013). Furthermore, other studies have shown than mice originating from The Jackson Laboratory or Charles River Laboratories (CR) are absent of *segmented filamentous bacteria* (SFB), a bacterium intimately interacting with the gut mucosa and priming Th17 cells (Ivanov, Frutos et al., 2008). Recently the difference between commercial breeder microbiome composition and the effects on immune system balance has been investigated in context of disease. The study showed by using mice from different commercial breeders that the *Enterobacteriaceae* bacterial family determine the susceptibility to *Salmonella* infection (Velazquez, Nguyen et al., 2019). Therefore, the gut microbiota could potentially affect disease models and act as a link to help understand the differences found in irreproducible experimental research. On the other hand, representation of the diverse gut microbiota as found in humans could potentially be modelled in a single study with use of mice from different research centres and mouse breeders.

AN INTRODUCTION TO THE GUT MICROBIOTA

Over the last 15 years, the field of gut microbiota research has blossomed. It is now considered as an important confounding factor in many diseases and is even recognised as a therapeutic target. While it is important that viruses, fungi, phages and yeast also reside in the gut, the majority of microbiome research is focused on bacteria. The gastrointestinal tract is colonised by 10^{14} bacterial cells, and

weighs on average 1 kg (Gill, Pop et al., 2006). A recently revised quantification of the gut microbiota has shown us that we are equal part human cells to bacterial cells (Sender, Fuchs et al., 2016), but perhaps the most remarkable information lies within the genetic material. There is 200 times more bacterial genetic material in our bodies compared to human genetic material. Despite this, the gut microbiota has an essential physiological function within our body such as: maintaining integrity of the gut epithelium (Natividad & Verdu, 2013), metabolism of indigestible food sources (den Besten, van Eunen et al., 2013) pathogenic control (Baumler & Sperandio, 2016) and host immunity regulation (Gensollen, Iyer et al., 2016).

The human genome project revealed that the human genome was in fact much smaller than our bacterial genome (Lander/Int Human Genome Sequencing et al., 2001). This encouraged the initiation of the human microbiota project in 2007. Now in 2014 a second integrative human microbiome project has been launched, which aims to investigate diseases specific and longitudinal microbiome changes (Huttenhower/Gevers et al., 2012, Turnbaugh, Ley et al., 2007). 16S ribosomal sequencing has become a powerful tool in order to assess the bacterial communities found within the gut microbiota. Essentially, 16S ribosomal RNA (rRNA) is highly conserved between bacterial species and contains 9 highly variable regions. This allows species to be easily identified. In terms of taxonomy, the phyla present in the adult gut are Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia. Firmicutes and Bacteroidetes compile 90% of the gut microbiome (Arumugam, Raes et al., 2011). For a further break down of species and their taxonomic classification refer to Figure 3.

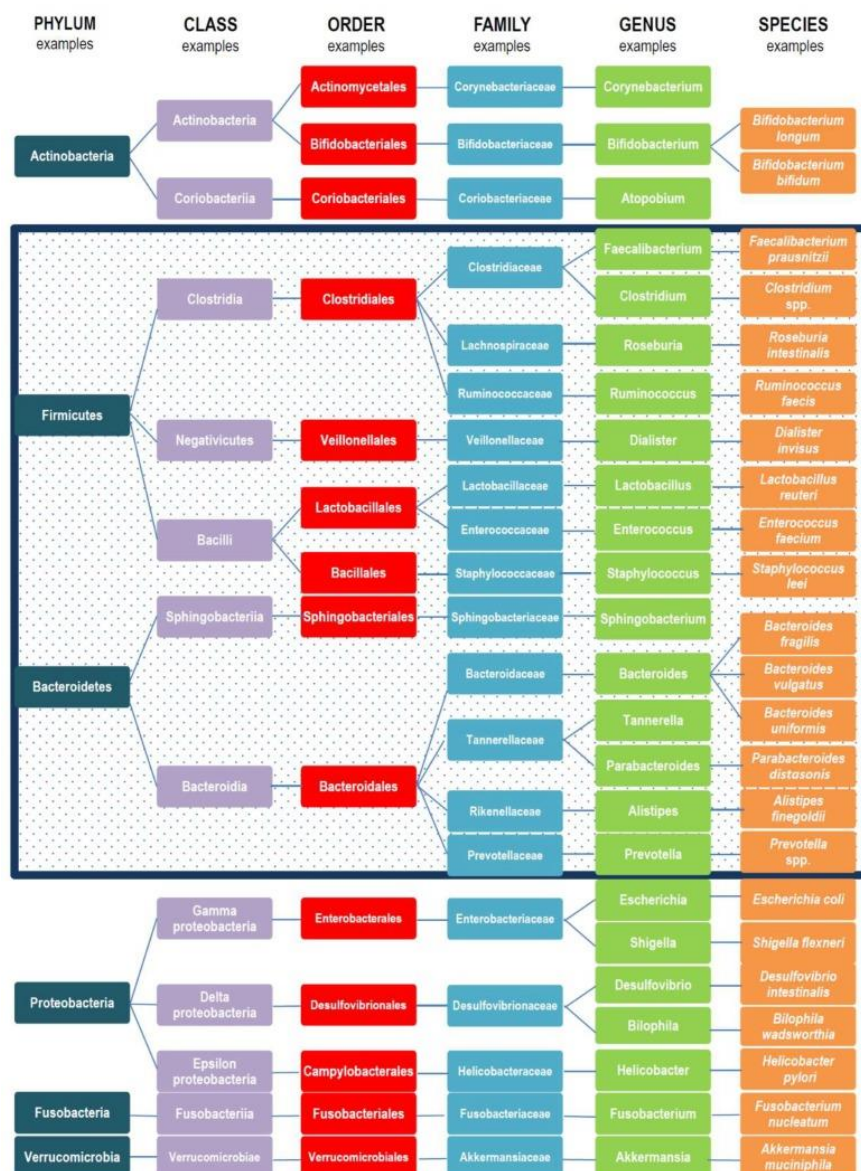


Figure 3. Taxonomic tree of bacteria from the gut microbiome. Taxonomic classification of different bacteria found in the gut microbiome. The box contains Firmicutes and Bacteroides phyla which represent 90% of the gut microbiota composition. Extracted from (Rinninella, Raoul et al., 2019).

There are many contributing factors which may potentially alter the gut microbiome composition of an individual, and in fact within an individual the gut microbiota alters over life, seasons and even within same day. The adult gut microbiota composition is challenged by diet, drugs, exercise, body mass and disease on a regular basis and adapts accordingly (Rinninella et al., 2019). Richness and a high diversity of bacterial species within the gut microbiota is considered “healthy” (Rinninella et al., 2019). Regular antibiotic use has been shown to completely decrease diversity of the gut microbiota. Some patients treated with antibiotics experienced complete eradication of bacterial species showing no complete restoration (Dethlefsen & Relman, 2011). The overuse of antibiotics and the

induced microbiome changes has now been linked to the differences in polarisation of the immune system and the development of an allergic disease (Droste, Wieringa et al., 2000).

One method to study the impact of the gut microbiota composition and its effects within the body is by faecal microbial transfer (FMT). FMT works to transfer the composition of a donor microbiota to another recipient. Recent research has shown the alterations observed in the gut microbiota of obese mice can be transferred into recipient mice and in turn induce obesity, thus highlighting the causality of the gut microbiota in disease pathophysiology (Wang, Huang et al., 2018). In experimental research germ-free (GF) mice or antibiotic-treated mice that have complete absence or depletion of their microbiota are used to investigate the influence of the gut microbiota on the host. GF mice are reared in complete sterile environments. Due to this fact they have immunological, developmental defects and alterations to their anatomical gut structures (Round & Mazmanian, 2009). On the other hand, mice treated with antibiotics have depleted gut bacterial population similar to GF mice, however have no developmental defects due to lack of bacterial exposure. One draw-back of using antibiotics as a tool to deplete gut microbiota is that antibiotics have off-target effects. These off-target effects include reduced protein aggregation, and countering neuroinflammation found in neurodegenerative diseases (Santa-Cecilia, Leite et al., 2019). This makes it challenging to investigate the gut microbiota importance in brain disease, and using antibiotics as a method to deplete the bacterial species. Furthermore, there is a risk that only specific bacterial populations are susceptible to treatment, therefore leaving bacterial populations which are antibiotic resistant. While both these approaches have pros and cons a combination of the two of these methods to validate one another is ideal to investigate the gut microbiota (Kennedy, King et al., 2018).

GUT MICROBIOTA AND THE IMMUNE SYSTEM

While the gut is the home of many readily adapting bacterial species, we know that the majority of immune cells are also located here. This allows the immune cell priming in the GALT to be highly influenced by the content of the gut microbiota. The GALT landscape consists of many highly specialised regions which allows interaction of the gut microbiota with the immune system (Figure 4). The lamina propria (LP) spans the entire gut, supports epithelium and contains the highest number of innate immune cells. The mesenteric lymph nodes (MLN) found in the gut (ileocolic), contain germinal centres, are found in the ileum and contain antigen presenting cells, which present antigen to local T and B cells. The intraepithelial cells are found throughout the epithelium of the gut and contain highly specialised lymphocytes which do not require priming. Finally, the PP are grape

like looking structures containing germinal centers, are located along the ileum and contain microfold cells (M cells). M cells have unique pocket-like structures which are found directly on the follicle-associated epithelium by the lumen of the gut. They allow dendritic cells located here to gain direct contact and sample the content of the gut lumen. This then allows antigen presentation within the germinal centers to local T and B cells. Within the MLN activated B cells undergo what is termed class switching. Class switching changes the type of immunoglobulin produced by the B cell from one isotype to another. In the MLN antigen presentation for example from dendritic cells switches the immunoglobulin isotype to IgA (Lycke & Bemark, 2017). B cells secrete IgA which transcytosed into the gut lumen, and prevents bacterial translocation across the tight gut barrier (Macpherson, Gatto et al., 2000, Macpherson & Uhr, 2004)

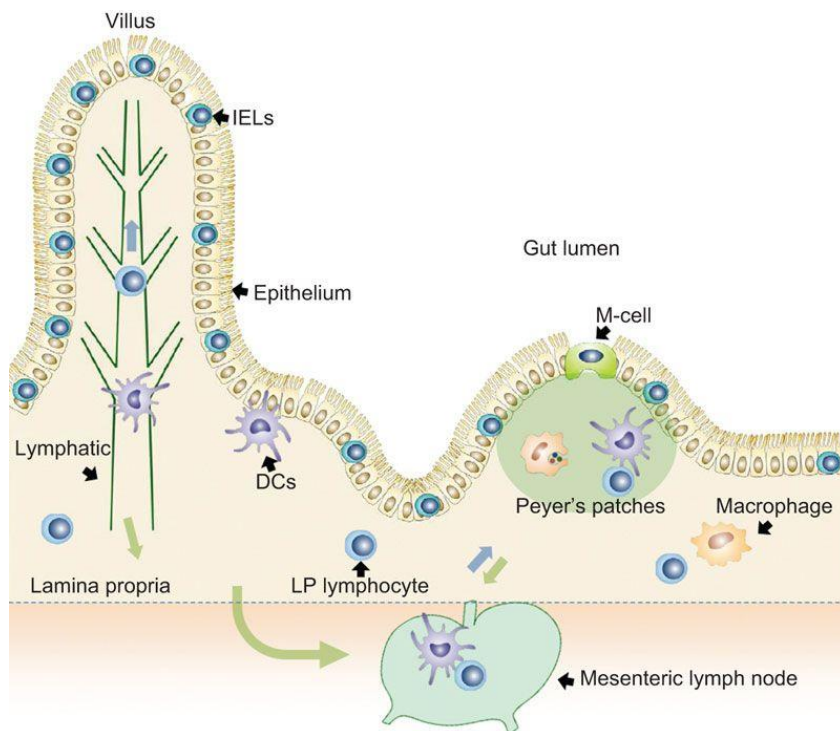


Figure 4 The Gut-associated-lymphoid (GALT) tissue landscape. A schematic highlighting the highly specialised areas in the GALT where immune cells reside and interact with the gut microbiota content. DC, dendritic cell; IEL, intraepithelial lymphocyte; LP, lamina propria; M-cell, microfold cell. Extracted from (Wu, Zhang et al., 2014).

To date there is a reasonable amount of research articles indicating the effect of specific bacterial species on the adaptive immune system. Most prominently, the effects of the commensal microbial species are shown to alter the homeostatic balance of Treg and Th17 cells whilst additionally there are few studies reporting effects on Th1 or Th2 cells (Zhao & Elson, 2018). Specifically, the gut microbiota appears to target cells located outside of the thymus i.e iTregs (Tregs induced outside the thymus from mature CD4⁺ cells) are altered but not nTregs (Tregs which are developed in the thymus) (Josefowicz, Niec et al., 2012). Mono-colonisation of GF mice with *Bacteroides fragilis* from

the *Bacteroidetes* phylum promoted differentiation of CD4⁺ cells toward a Treg phenotype, thereby increasing Foxp3 expression and the immunosuppressive cytokine IL-10. Authors reported that it was polysaccharide A, a molecule released *Bacteroides fragilis* which was responsible for the induction of Tregs (Round & Mazmanian, 2010). Additionally, the unique glycosphingolipids produced by *Bacteroides fragilis* has also been shown to lower host invariant natural killer T cells in a colitis model (An, Oh et al., 2014). Other studies showed that not just single bacterial species but groups of gram-positive bacteria were involved in the accumulation of Tregs within the gut. Specifically, oral gavage with 46 strains of bacteria from the *Clostridium* genus identified an environment rich in transforming growth factor beta and altered Treg function (Atarashi, Tanoue et al., 2011, Furusawa, Obata et al., 2013). On the contrary from the *Firmicutes* phylum SFB are gram-positive bacteria which intimately interact with the PP. GF mice colonised with SFB showed an increased abundance of host cell secreted serum amyloid A proteins in the terminal ileum. Furthermore, it was shown the SFB presence affected type 3 innate lymphoid cells prompting the releasing of IL-22, the release of SAA from epithelial cells. This was found to prime dendritic cells and was able to induce Th17 cells and IL-17 secretion (Atarashi, Tanoue et al., 2015, Farkas, Panea et al., 2015, Goto, Panea et al., 2014, Ivanov, Atarashi et al., 2009, Sano, Huang et al., 2015).

Dietary microbial metabolites and the immune system

Following the recent increase in focused research of gut microbiota and the immune system, it has become more evident that is not only the bacterial species themselves but also the microbial metabolites produced from our diet which can affect the immune system. The dietary metabolites derived from the gut microbiota are estimated at a total of 10% of metabolites found in human blood (Wikoff, Anfora et al., 2009). As mentioned previously, one of the main functions of the gut microbiota is to digest dietary complex carbohydrates for their own energy source. Short-chain fatty acids (SCFA) are bioactive bacterial metabolites derived from bacterial fermentation of dietary fibre which otherwise is not digested by host enzymes. SCFA are 1-6 carbons in length, with acetate (C2), propionate (C3) and butyrate (C4) being the most abundant in the intestine (Cummings, Pomare et al., 1987). *Bacteroidetes* and *Firmicutes* mainly produce SCFA and it is considered that acetate and propionate are mainly produced by *Bacteroidetes* whereas butyrate is mainly produced by *Firmicutes* phyla (Hoverstad & Midtvedt, 1986, Macfarlane & Macfarlane, 2003). The majority of butyrate is rapidly absorbed by the colonocytes, whereas propionate and acetate enter the systemic circulation. Propionate is mainly absorbed by the liver whereas acetate has been shown to reach very distant organs such as the brain (Frost, Sleeth et al., 2014, Morrison & Preston, 2016). In general, data suggests that SCFA impose their effect via activation of the FFAR2 and FFAR3 receptors, or by entry into cells via monocarboxylate transporters MCT1 and SMCT1. Furthermore, SCFA can passively

diffuse into the cell where they act as histone deacetylase inhibitors (Brown, Goldsworthy et al., 2003, Hinnebusch, Meng et al., 2002, Kamp & Hamilton, 2006, Miyauchi, Gopal et al., 2004, Waldecker, Kautenburger et al., 2008). SCFA are considered to be anti-inflammatory, and in-vitro it was shown that SCFA inhibit the release of proinflammatory cytokines (Chang, Hao et al., 2014). SCFA were also shown to act as chemoattractants for neutrophils (Le Poul, Loison et al., 2003). Other innate phagocytic cell types such as dendritic cells, microglia and monocytes have also been shown to have anti-inflammatory properties following SCFA treatment. Additionally, the adaptive immune system responds to SCFA. SCFA influences B cells and increases antibody production (Kim, Qie et al., 2016) and most importantly it is now well considered that SCFA expand the Treg population (Arpaia, Campbell et al., 2013, Smith, Howitt et al., 2013).

Another dietary metabolite tryptophan, like fibre, is also metabolised by bacterial species resulting in indole derivative production. Indole derivatives such as: indole, tryptamine, indoleethanol, indolepropionic acid, indolelactic acid, indoleacetic acid, skatole, indolealdehyde and indoleacrylic acid, have been shown to act as ligands for the aryl hydrocarbon receptor (AhR). The AhR is widely expressed on immune cells and therefore the indole derivatives act as immunomodulators (Stockinger, Di Meglio et al., 2014). Indoleamine 2, 3-dioxygenase 1 (IDO1) is an endogenous enzyme required for the metabolism of tryptophan to the AhR ligand, kynurenine. It was found that under high levels of tryptophan IDO1^{-/-} mice had an impact on the microbiome with an expansion of bacteria from the *Lactobacillus* genus. The *Lactobacillus* expansion led to an increase in bacterial derived indoles. The high levels of indoles in turn induced immune cells to secrete IL-22 (Zelante, Iannitti et al., 2013). Furthermore, in the irritable bowel syndrome mouse CARD9^{-/-} model, mice had an altered microbiota composition with a decrease in the *Lactobacillus* genus. The reduction of *Lactobacillus* once again led to an impaired metabolism of tryptophan to indole derivatives, this was linked to the inflammatory milieu in this disease model. However upon inoculation of these mice with *Lactobacilli* strain, intestinal inflammation was reduced (Lamas, Richard et al., 2016). Taken together this highlights the impact of microbial metabolites over endogenous metabolites which also play a role in activation of the immune system.

BRAIN DISEASE AND THE GUT MICROBIOTA

The earliest piece of evidence to suggest the gut microbiota plays a role in brain disease pathophysiology was found in 1993 within multiple sclerosis (MS); a demyelinating disorder of the central nervous system. The study reported that dirtier animal facilities, i.e. facilities with higher amounts of bacteria, had greater prevalence of the experimental mouse of MS, spontaneous experimental autoimmune encephalitis (EAE) (Goverman, Woods et al., 1993). In contrast, a study

performed using EAE mice reared under GF conditions found that the absence of the gut microbiota led to protection from disease onset (Berer, Mues et al., 2011). Furthermore, it was also shown that antibiotics and probiotics modulated EAE via proinflammatory cytokine reduction (Mangalam, Shahi et al., 2017, Ochoa-Reparaz, Mielcarz et al., 2009).

Parkinson disease (PD) is linked to decreased dopamine production from neurons within the substantia nigra pars compacta. This is accompanied with alpha-synuclein accumulation, deposited in surviving neurons, also known as Lewy bodies. Patients often have gastrointestinal complaints and gut motility problems for example constipation (Noyce, Bestwick et al., 2012) and recently the progression of PD has been associated with gut dysbiosis (Minato, Maeda et al., 2017). In experimental GF models of PD, mice experience less inflammation within the gut and additionally less alpha synuclein compared to SPF mice. Furthermore, in the same GF PD mouse models, FMT with human PD microbiota revealed the exacerbation of motor deficits (Sampson, Debelius et al., 2016).

The pathological hallmarks of Alzheimer disease (AD) include amyloid plaques and neurofibrillary tangles, this is accompanied with cognitive decline and dementia (Braak & Braak, 1991). There was a clear indication that the gut microbiota played a role in AD pathology when antibiotic treatment was administered, resulting in fewer plaques and less microglia activity (Cattaneo, Cattane et al., 2017). Additionally, it was shown that probiotic treatment of AD patients for 12 weeks improved cognitive function (Leblhuber, Egger et al., 2018). This indicated that the AD gut dysbiotic microbiome was beneficially altered with antibiotic treatment, but additionally, physically adding a cocktail of “healthy” bacteria, was beneficial. Some studies suggest the reduction of bacterial populations found in dysbiotic AD microbiota or in aged and dementia patients were specifically butyrate producers (Biagi, Nylund et al., 2010). Therefore, suggesting that FMT of SCFA-producing bacteria could be beneficial in alleviating AD symptoms.

Finally, stroke has only recently been linked to gut microbial dysbiosis in 2015. Interestingly it was reported that patients with Crohn’s disease, an ailment also associated with a disturbed gut microbiota, had an increased stroke prevalence (Keller, Wang et al., 2015). More specifically in stroke patients it was found that the gut microbiota was indeed disturbed (Yin, Liao et al., 2015). Based on the changes in the gut microbiome composition after stroke, a study has combined the common bacterial populations which alter after stroke in comparison to controls, and formulated a computerised gut microbiome dysbiosis index. They causally showed that patients with a high gut dysbiosis were linked to a worse outcome (Xia, You et al., 2019). Experimental mouse research on

microbiota in stroke has resulted in an array of conflicting studies. First, it was shown that antibiotic pre-treatment did not alter stroke outcome or immune cell populations (Winek, Engel et al., 2016). Shortly after, another study reared a mouse strain to contain antibiotic resistant gut microbiota. This enabled comparison of mice with antibiotic sensitive gut microbiota and allowed the investigation of the direct effects of antibiotics on the gut microbiota, ruling out any off-target systemic effects. In contrary to the first study it was reported that antibiotics decreased infarct volume, and was associated with higher number of Tregs plus lower number of $\gamma\delta$ T cells within the gut. Furthermore the study showed a lower migration of $\gamma\delta$ T cells to the meninges and lower levels of IL-17 after stroke (Benakis et al., 2016b). Whilst investigating the effects of gut microbiota with antibiotic treatment results in eradication certain populations of bacteria, on the other hand adding bacteria to the existing gut microbiota can also be used. FMT treatment from young mouse donors improved stroke outcome assessed by infarct volume (Spychala, Venna et al., 2018). Over the last decade, the key role of the gut microbiota in many brain diseases has been highlighted. New research is beginning to unravel and identify the key mechanism in how exactly gut microbiota potential can modulate the outcome of these diseases via bi-directional gut-brain interactions.

MECHANISM OF GUT MICROBIOTA BRAIN INTERACTION

Understanding the bi-directional interaction of the gut microbiota and brain is key to unravelling how the gut microbiota can potentially impose therapeutic effects within brain disease. These pathways are indirect and direct actions of the gut microbiota on the brain and have been classified into three main pathways; biochemically, immune and via neural pathways. Within this section, I will highlight the evidence which has been proposed to support these various pathways, as shown in Figure 5.

Biochemically. As mentioned previously, the gut microbiota produces a variety of bioactive and even neuroactive substances (Kim, Yun et al., 2018). Whilst bacteria produce these primarily for their own energy sources and survival, due to their resemblance in structure, they can additionally affect host cells. Interestingly, 90% of serotonin, and 50% of dopamine within our body is produced within our guts. This is largely dependent on our dietary input, however, is strongly regulated by the gut microbiota metabolism. Furthermore, it has been shown that bacteria within the gut microbiota are capable of producing serotonin and dopamine (Lyte, 2011). A study performed in GF mice identified a 60% reduction of serotonin (Yano, Yu et al., 2015). These neuroactive compounds released by the gut microbiota may have an indirect (acting on host cells) or direct (travelling via the blood stream) effect on the brain. A recent investigation in schizophrenic mice models showed modulation of glutamine and GABA pathways within the brain after FMT of schizophrenic patient microbiota (Zheng, Zeng et al., 2019). Despite this interesting finding, a substantial amount of work needs to be performed to

determine whether or not the gut microbial metabolites directly alter host neurotransmission (Strandwitz, 2018). Other metabolites produced by the gut microbiota such as SCFA and tryptophan, also have the ability to enter the blood stream via the distal ileum portal vein and have systemic effects elsewhere within the body. Small molecules such as SCFA cross the blood brain barrier and passively enter the brain (Frost et al., 2014). Not only does biochemical communication occur from the gut to the brain but also from the brain to the gut via the hypothalamus-pituitary-adrenal axis (HPA). Under stressful conditions, the HPA axis releases cortisol into the systemic circulation. Specifically, research has shown that the chronic stress and increased cortisol release altered immune cells to an inflammatory state, increased intestinal permeability and altered the composition of the gut microbiota (Bailey, Dowd et al., 2011).

Immune. As previously described, the gut microbiota holds an intimate interaction with the immune system. Depending on the bacterial content of our gut microbiota, the antigens, or bioactive mediators available to local immune cells differ, and thus the polarisation and priming of the immune cells changes accordingly. The cells within our immune system are able to travel via the systemic blood circulation from the gut and are recruited into the brain. As discussed in the earlier sections, dysbiotic gut microbiota obtained from animals with brain disease does alter the immune cells. While currently research has been focused on the priming of the immune cells from specific bacterial species namely SFB inducing proinflammatory Th17 cells, and *B. fragilis* inducing anti-inflammatory Treg cells (Ivanov et al., 2009, Round & Mazmanian, 2010). More effort needs to be focused on the effect of the entire disease microbiome signature and immune populations within the gut.

Neural. The vagus nerve (cranial nerve X) acts as a direct link from the gut to the brain. Interestingly 20% of its efferent fibres send signals from the brain to the gut, and 80% of the fibres send signals from the gut to the brain (Agostoni, Chinnock et al., 1957, Thayer & Sternberg, 2009). There are two types of nerves which perform as chemosensory (detecting metabolites and substances released from the gut microbiota) and also mechanosensory (which detect physical stimulation of nerve endings) both acting to communicate with the brain. There are several studies indicating this powerful connection. First within the gut, microbiota absence has been shown to alter the excitability within the ENS which was reversible upon FMT or colonisation with bacteria (Keita & Soderholm, 2010, Neufeld, Mao et al., 2013). Additionally, a study investigating anxiety and depression saw a beneficial effect of administration of *L. rhamnosus*. The improvement in symptoms from the bacteria were however not seen when the vagus nerve was surgically severed (Bercik, Park et al., 2011, Bravo, Forsythe et al., 2011, Siopi, Saha et al., 2019). Additionally, bacterial-derived SCFA

were able to stimulate vagal afferents (Lal, Kirkup et al., 2001). The physical interaction between bacteria and the vagus mechanosensory afferents still needs to be further elucidated.

Taken together gut microbiota communicates with the brain along multiple different pathways, which enable potential alteration of brain disease. While on the one-hand very fascinating, this provides challenge to researchers to pin-point the exact pathway in which the gut microbiota may alter brain disease.

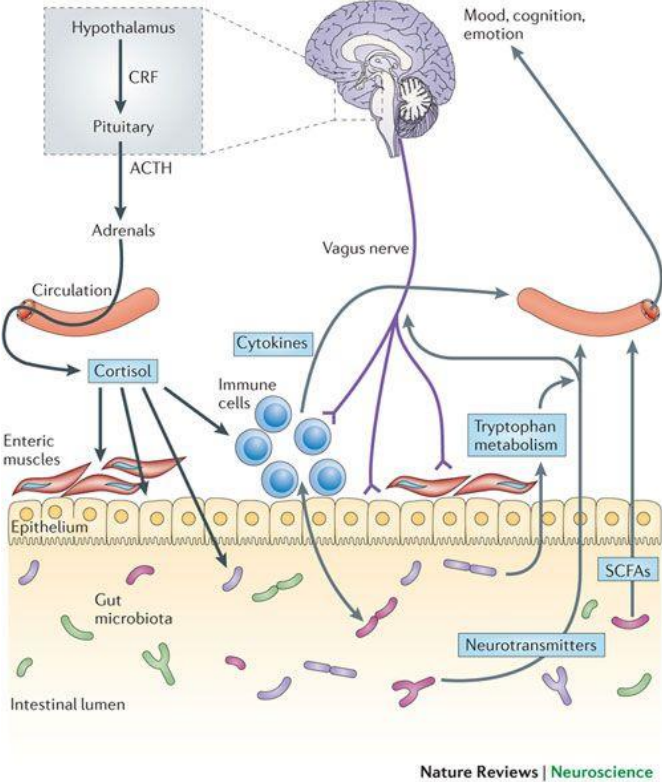


Figure 5. The bidirectional communication between the gut microbiota and the brain. This illustration highlights the complex interactions between gut microbiota and the brain. It shows the three pathways which are considered the main routes of communication. Biochemical, shown in black arrows, immune shown in grey arrows and neural shown in purple arrows. SCFA; Short-chain fatty acids, ACTH, adrenocorticotrophic hormone; CRF, corticotropin-releasing factor. Extracted from (Cryan & Dinan, 2012)

AIMS

The major goals of this thesis were to 1) investigate the extent of gut microbiota alterations after stroke and the bi-directional interaction between the gut and the brain. 2) Reflect how differences in the gut microbiota composition may contribute to discrepancies in stroke research. 3) Elucidate the role of the gut microbiota in the recovery phase after stroke.

To address the first aim, we used 16S RNA sequencing and revealed differences in the gut microbiota composition between sham and different experimental stroke surgeries. We then utilised GF mice treated with FMT in order to investigate the altered stroke microbiome vs. sham microbiome on the intestinal immune system. Immune cell labelling was used to track cells from the gut to the brain and to show the significance of gut microbiome immune cell polarisation on stroke outcome. Finally using GF mice, we characterised whether gut microbiota presence affected stroke outcome.

For the second aim, we obtained mice from 3 commercial breeders and characterised the content of their gut microbiome and intestinal immune compartment. The hypothesis was that the potentially different microbiome composition from different commercial breeders would partly explain stroke studies which were unable to be reproduced in different laboratories. From our previous study, we learnt that dMCAo stroke model did not alter the microbiome composition. We used the dMCAo in order to investigate the prevailing gut microbiome composition from the respective breeders, rather than stroke induced changes. Additionally, we used CD28 superagonist, as a therapeutic to reduce stroke infarct volume. In experimental research, this drug had previously inconclusive results, and within this study we addressed the role of the gut microbiota in this phenomenon.

For the third aim we set out to investigate the microbiota in the recovery phase after stroke and assessed the multimodal features which characterise regeneration. As a result, from the first study we saw that the composition of the gut microbiome was different; therefore we hypothesised that the key bioactive metabolites produced by the gut microbiota would also differ. Here we administered SCFA within the drinking water as a 4-week pre-supplementation paradigm. We utilised a photothrombotic (PT) stroke model to target motor areas of the cortex and performed specific motor deficit assessment. The automated assessment of affected limb motor deficits allowed us to observe the effects of SCFA on functional recovery after stroke. Furthermore, we employed *in vivo* widefield calcium imaging to investigate network connectivity and Golgi-Cox staining to investigate synaptic alterations. Finally, we investigated brain resident (microglia) and peripheral (lymphocytes)

immune cells and their potential involvement in altered synaptic changes following SCFA supplementation.

MICROBIOTA DYSBIOSIS CONTROLS THE NEUROINFLAMMATORY RESPONSE AFTER STROKE

SUMMARY

In this first study we characterised to what extent the gut microbiota composition changes after stroke and how this affects acute stroke outcome. Using faecal 16S RNA sequencing, we initially identified a decrease in species diversity. We mainly observed an expansion of the *Bacteroides* phylum in mice after fMCAo compared to sham surgery. We then hypothesised that the stroke itself induces gastrointestinal (GI) paralysis. Using a FITC dextran oral gavage bolus, we were able to track the fluorescence in various segments of the gut and identify a reduction of motility after stroke. The reduced gut motility seen after fMCAo was also associated with an increased protein leakage of the gut and furthermore, an increase in stress hormones such as catecholamine. Surgical ileus was performed to disrupt gastrointestinal motility and confirmed that gut motility alterations induced gut microbial dysbiosis. Interestingly there was no change in gut motility within the dMCAo model, and no significant alterations in gut microbiota composition. For a next step, we utilised the faecal gut microbial dysbiosis induced by fMCAo and not dMCAo stroke models to investigate the role of the gut microbiota in stroke. This question was addressed using FMT donors from mice which underwent fMCAo surgery. The fMCAo microbiome samples were then transferred into GF mice, which then underwent dMCAo surgery. From our previous experiment, we knew that dMCAo would not alter the microbiome content, and thus allow us to investigate the direct effects of the fMCAo stroke-dysbiotic microbiome on dMCAo stroke outcome. The fMCAo faecal receiver experienced larger strokes, with greater behavioural deficits and a more proinflammatory immune system. Using CFSE dye we labelled all cells in the PP, and then identified these cells within the brain after stroke. Here we discovered that 25% of all T cells in the brain after stroke were originating from the gut. This showed that the dysbiotic microbiota composition polarised a proinflammatory immune response, which later contributed to the inflammatory milieu and worsened stroke outcome. Furthermore, we showed that FMT treatment with a healthy microbiome was beneficial and reduced the severity of stroke, which was associated with a higher number of Tregs. This beneficial effect of healthy microbiome FMT was not evident in Rag^{-/-} mice, a transgenic model absent in T and B lymphocytes. This confirmed that the immune modulation via the gut microbiome was affecting stroke outcome.

REFERENCE

Author contributions: A.L. designed research; V.S., S.R., G.L., R.S., and A.L. performed research; V.S., S.R., D.G., B.S., and A.L. analysed data; B.S., M.D., and A.L. wrote the paper. V.S. and S.R. contributed equally to this work.

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Microbiota Dysbiosis Controls the Neuroinflammatory Response after Stroke

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Acute brain ischemia induces a local neuroinflammatory reaction and alters peripheral immune homeostasis at the same time. Recent evidence has suggested a key role of the gut microbiota in autoimmune diseases by modulating immune homeostasis. Therefore, we investigated the mechanistic link among acute brain ischemia, microbiota alterations, and the immune response after brain injury. Using two distinct models of acute middle cerebral artery occlusion, we show by next-generation sequencing that large stroke lesions cause gut microbiota dysbiosis, which in turn affects stroke outcome via immune-mediated mechanisms. Reduced species diversity and bacterial overgrowth of bacteroidetes were identified as hallmarks of poststroke dysbiosis, which was associated with intestinal barrier dysfunction and reduced intestinal motility as determined by *in vivo* intestinal bolus tracking. Recolonizing germ-free mice with dysbiotic poststroke microbiota exacerbates lesion volume and functional deficits after experimental stroke compared with the recolonization with a normal control microbiota. In addition, recolonization of mice with a dysbiotic microbiome induces a proinflammatory T-cell polarization in the intestinal immune compartment and in the ischemic brain. Using *in vivo* cell-tracking studies, we demonstrate the migration of intestinal lymphocytes to the ischemic brain. Therapeutic transplantation of fecal microbiota normalizes brain lesion-induced dysbiosis and improves stroke outcome. These results support a novel mechanism in which the gut microbiome is a target of stroke-induced systemic alterations and an effector with substantial impact on stroke outcome.

Key words: inflammation; microbiota; stroke; T cells

Significance Statement

We have identified a bidirectional communication along the brain–gut microbiota–immune axis and show that the gut microbiota is a central regulator of immune homeostasis. Acute brain lesions induced dysbiosis of the microbiome and, in turn, changes in the gut microbiota affected neuroinflammatory and functional outcome after brain injury. The microbiota impact on immunity and stroke outcome was transmissible by microbiota transplantation. Our findings support an emerging concept in which the gut microbiota is a key regulator in priming the neuroinflammatory response to brain injury. These findings highlight the key role of microbiota as a potential therapeutic target to protect brain function after injury.

Introduction

The inflammatory reaction to sterile tissue injury is a critical pathophysiological component in organ-specific injuries, includ-

ing ischemic stroke (Iadecola and Anrather, 2011; Chamorro et al., 2012), myocardial infarction (Hofmann and Frantz, 2015), and other conditions (Huang et al., 2007). Previous reports have shown that T cells play a defining role in secondary neuroinflammation after brain ischemia (Shichita et al., 2009; Liesz et al., 2013c; Schwartz and Raposo, 2014). Experimental approaches using pharmacological or genetic lymphocyte depletion paradigms (Yilmaz et al., 2006; Kleinschnitz et al., 2010) or pharma-

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ecological approaches to block cerebral lymphocyte invasion (Liesz et al., 2011; Neumann et al., 2015) have established a primary role for lymphocytes, particularly T cells, as crucial mediators of an inflammatory collateral damage to the injured brain after stroke. Previous work from our group and others has proposed that T_{helper} cell subpopulations might have differential effects on stroke outcome: proinflammatory Th1, Th17, and $\gamma\delta$ T cells have been associated with increased inflammatory damage and worse outcome, whereas regulatory T cells (T_{regs}) have been shown to suppress an overshooting neuroinflammatory reaction to brain injury (Liesz et al., 2009a; Shichita et al., 2009; Gelderblom et al., 2012; Liesz et al., 2013c). In contrast to primary autoimmune diseases of the CNS, acute brain ischemia induces a rapid local and peripheral immune activation (Iadecola and Anrather, 2011; Shichita et al., 2012; Liesz et al., 2015). T cells are recruited to the injured brain already within the first days after stroke (Gelderblom et al., 2009; Zhou et al., 2013) and peripheral T-cell activation can also be observed (Offner et al., 2006; Yilmaz et al., 2006; Liesz et al., 2009b; Vogelgesang et al., 2010). Nevertheless, the mechanisms of peripheral T-cell activation, specifically T_{helper} cell polarization, after acute brain injury are still elusive.

Several lines of evidence suggest that the gut microbiota is a key regulator of T-cell homeostasis and is intricately involved in the maturation of the immune system and maintaining the mutual coexistence of host and microbe (Ivanov et al., 2009; Hooper et al., 2012; Arpaia et al., 2013). Recently, the functional relation between the gut microbiota and brain function, termed the “gut–brain axis,” has become an emerging field in neuroscience and neuroimmunology (Collins et al., 2012; Cryan and Dinan, 2012). The gut microbiota has been attributed a decisive role in autoimmune diseases of the CNS (Berer et al., 2011; Lee et al., 2011). A recent report by I (Benakis et al., 2016) has demonstrated that antibiotic-treatment-induced dysbiosis of intestinal microbiota influences poststroke neuroinflammation and outcome in an experimental stroke model. However, the impact of stroke on the microbiota composition and the contribution of stroke-specific microbiota alterations on neuroinflammation were previously unknown. Therefore, the aim of this work was to investigate microbiota alterations after brain ischemia and their role in the poststroke neuroinflammatory reaction.

In this study, we observe dysbiosis of the gut microbiota via stress-mediated intestinal paralysis after stroke. In turn, microbiota dysbiosis is causally linked to changes in T-cell homeostasis, induction of a proinflammatory response, and deterioration of stroke outcome. Fecal microbiota transplantation to normalize poststroke dysbiosis is associated with an improved stroke outcome. Altogether, our data point to a novel and highly complex interplay between the brain and the gut microbiota after acute brain injury in which the microbiota is a target of stress-mediated pathways resulting dysbiosis, as well as an effector of immune homeostasis with profound impact on stroke outcome.

Materials and Methods

Animal experiments. All animal experiments were performed consistent with the guidelines for the use of experimental animals and were approved by the governmental committee of Upper Bavaria (Regierungspraesidium Oberbayern #2532-65-2014). Wild-type C57BL/6J mice and *Rag1*^{-/-} male mice were obtained from Charles River Laboratories. Germ-free (GF) C57BL/6J and GF *Rag1*^{-/-} female mice were obtained from the Clean Mouse Facility, University of Bern, Switzerland. A priori sample size calculation was based on either variance and effect size from previous studies or on preliminary pilot experiments performed during this study. Data were excluded from all mice that died after surgery. Animals were randomized to treatment groups and all analyses were performed by investigators blinded to group allocation. Unblinding was

performed after completion of statistical analyses. All animal experiments were performed and reported consistent with the ARRIVE guidelines (Kilkenny et al., 2010).

GF mouse handling and intestinal recolonization. GF mice were housed in sterile HAN-gnotocages and received the same sterile water and pelleted food as conventional or recolonized mice. All surgical procedures, cage changes, and behavioral tests of GF mice were performed in a disinfected laminar flow microbiological safety cabinet. GF mice were recolonized with gut microbiota obtained from sham or post-filament middle cerebral artery (MCA) occlusion model (fMCAo) donor mice by gastric gavage of freshly prepared cecum microbiota. In brief, the donor mice were killed, cleaned with 70% ethanol, and the peritoneal cavity was opened to expose the cecum. The contents of the cecum were collected in sterile tubes, diluted in sterile water to 200 mg/ml, and centrifuged at 2000 rpm for 10 min to remove the large particles. The supernatants were collected into fresh sterile tubes and each recipient mouse received a single 200 μ l bolus of the freshly prepared microbiota suspension.

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 (Llovera et al., 2014). In brief, the mice were anesthetized with an intraperitoneal injection of fentanyl (0.05 mg/kg), midazolam (5 mg/kg), and medetomidine (0.5 mg/kg). The skull was exposed by skin incision, 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. Throughout the surgical procedure, body temperature was maintained at 37°C using a feedback-controlled heating pad. The overall mortality rate in this experimental group was <5%. Exclusion criteria were subarachnoid hemorrhage or death during surgery.

Transient fMCAo. Mice were anesthetized with isoflurane delivered in a mixture of 30% O₂ and 70% N₂O. An incision was made between the ear and the eye to expose the temporal bone. A laser Doppler probe was affixed to the skull above the MCA territory and the mice were placed in the supine position. An incision was made in the midline neck region and the common carotid artery and left external carotid artery were isolated and ligated; a 2 mm silicon-coated filament (catalog #701912PKRe; Doccol) was inserted into the internal carotid artery and MCA occlusion was confirmed by a corresponding decrease in blood flow (i.e., a decrease in the laser Doppler flow signal). After 60 min of occlusion, the animals were reanesthetized and the filament was removed. For the survival period, the mice were kept in their home cage with facilitated access to water and food. Sham-operated mice received the same surgical procedure except the filament was inserted and immediately removed. Body temperature was maintained throughout surgery using a feedback-controlled heating pad. The overall mortality rate in this group (excluding the sham-operated animals) was ~10%. Exclusion criteria were as follows: insufficient MCA occlusion (a reduction in blood flow to >20% of the baseline value), mice that died during the surgery, and mice without induction of brain ischemia as quantified postmortem by histological analysis.

Cylinder test. GF mice that were inoculated with a fecal suspension obtained from control and fMCAo-induced mice were tested for forepaw use and asymmetry by measuring behavioral asymmetry and general activity on the first and third days after cMCAo surgery. The test was performed in a sterile laminar flow safety cabinet. Mice were placed in a transparent acrylic glass cylinder (diameter: 8 cm; height: 25 cm) in front of two mirrors and videotaped (Llovera et al., 2014). To assess independent forelimb use, contact with one forelimb during full rear and landing with only one forelimb after full rear were scored by performing a frame-by-frame analysis of the recorded videos. All rearing movements during the trial were counted and used as a measure of the animal's overall activity.

Fecal microbiota transfer (FMT). Freshly defecated feces were collected from restrained mice and placed in sterile tubes. Donor mice (Charles River) were housed under SPF conditions and the same ones were used for all recipient mice in a given group. After collection, the fecal pellets

were immediately mixed in sterile water (to 200 mg/ml) and the suspension was centrifuged at 2000 rpm for 10 min. The supernatant was collected and 200 μ l was administered to each mouse by gastric gavage. FMT treatment was administered daily for the survival period starting on the same day of stroke induction. For FMT experiments, the recipient mice were housed in cages with sterile bedding and *ad libitum* access to sterile water. For the fMCAo model, the mice did not have access to food to eliminate any confounding effects of differing food consumption between the fMCAo, sham-operated, and treatment groups. Therefore, fMCAo and FMT recipient mice received three daily oral doses of water-suspended ultra-fine ground chow containing the same composition and caloric intake as the pelleted food (equivalent to \sim 2.5 mg of chow).

Infarct volumetry. Perfused brains were removed at the indicated time points after stroke induction and frozen on powdered dry ice. Coronal cryosections (20 μ m thick) were cut at 400 μ m 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. For the fMCAo model, an edema correction for infarct volume was performed using the following formula: (ischemic area) = (direct lesion volume) – [(ipsilateral hemisphere) – (contralateral hemisphere)]. The total infarct volume was calculated by integrating the measured areas and intervals between the sections.

Preparation of cells from lymphoid organs. Mice were deeply anesthetized with ketamine (120 mg/kg) and xylazine (16 mg/kg). Blood was obtained by intracardiac puncture and collected into EDTA test tubes; the plasma was isolated by centrifugation at 3000 rpm for 10 min and stored at -80°C until further use. The mice were then transcardially perfused with normal saline and the spleen and Peyer's patches (PPs) were removed into cold Hank's balanced salt solution. The organs were homogenized and filtered through 30–40 μ m 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). For the isolation of mucosal leukocytes, the distal ileum was dissected carefully to remove the muscle layer. The mucosal tissue was then minced into small pieces and incubated in RPMI medium containing 10 μ g/ml DNase I and 0.5 mg/ml collagenase D (Roche) for 10 min at 37°C . The cell suspensions were then mechanically homogenized, filtered, and washed before flow cytometric analysis.

Isolation of brain-invading leukocytes. Mice were deeply anesthetized with ketamine (120 mg/kg) and xylazine (16 mg/kg) and transcardially perfused with normal saline. Brains were carefully removed and ipsilateral hemispheres were used for preparation of cells as described previously (Liesz et al., 2011). Briefly, brains were cut into small pieces and digested in RPMI medium containing 10 μ g of DNase I and 0.5 mg/ml collagenase D (Roche) for 10 min at 37°C . The cell suspension was mechanically homogenized and passed through a 40 μ m cell strainer (Falcon). The cell suspension was resuspended with 40% Percoll solution (GE Healthcare) and overlaid on a 70% Percoll solution. After centrifugation at 2100 rpm for 25 min at room temperature, cells at the interphase were collected and washed in isotonic buffer.

Flow cytometry analysis. The following mouse antigen-specific antibodies were purchased from eBioscience/BioLegend: CD3 FITC (17A2), CD4 PerCP Cy5 (clone RM4–5), CD45 eF450 (30-F11), CD11b PerCP Cy5.5 (M1/70), IFN- γ FITC (XMG1.2), and IL-17 APC (17B7). To quantify the various cell populations, cells were stained with specific antibodies in accordance with the manufacturer's protocols. For intracellular cytokine staining, cell suspensions from the spleen and PPs were resuspended in RPMI medium containing 10% heat-inactivated fetal calf serum, 1% penicillin/streptomycin, and 10 μ M 2-mercaptoethanol. The cells were plated at a density of 3×10^5 cells/well on anti-CD3 antibody (clone 145-2C11; eBioscience)-coated 96-well plates, costimulated with soluble CD28 antibody (clone 37.51; eBioscience), and incubated for 40 h at 37°C and 5% CO_2 . After incubation, GolgiPlug (BD Biosciences) was added to the cells for 4 h, after which the cells were harvested for intracellular staining using the Foxp3/Transcription Factor Staining Kit (eBioscience). Stained cells were measured in a FACScyte flow cytometer (BD Biosciences) and analyzed using FlowJo version 10 (TreeStar).

FISH and histochemical staining. The distal ileum was ligated on both sides with surgical sutures, removed, fixed in 4% paraformaldehyde, and

6- μ m-thick transverse sections were prepared. The sections were mounted on glass slides and FISH was performed in an equilibrated humidity chamber at 46°C in hybridization buffer containing 0.9 M NaCl, 20 mM Tris-HCl, pH 7.2, 30% formamide, and 0.01% SDS for 3 h. Probes EUB³³⁸: Cy5-GCT GCC TCC CGT AGG AGT-Cy5; EUB³³⁸-III: Cy5-GCT GCC ACC CGT AGG TGT-Cy5) were used at a final concentration of 5 ng/ μ l. Sections were hybridized in 20 μ l of hybridization buffer containing the EUB³³⁸ or EUB³³⁸-III probe. The sections were then placed in prewarmed (48°C) wash buffer containing 100 mM NaCl, 20 mM Tris-HCl, pH 7.2, and 5 mM EDTA in autoclaved Milli-Q water (Millipore) for 10 min. SYTOX green (1:5000) was used to counterstain the nuclei. Images (1024 \times 1024 pixels, 400 Hz scan rate) were acquired as z-stacks using a 63 \times glycerol objective on a confocal microscope (TCS SP5 X; Leica).

Immunohistology. For analysis of labeled T cells from PPs, 12 μ m coronal brain sections at bregma height were stained with anti-mouse CD3 polyclonal antibody (1:50; Abcam) and goat anti-rabbit Alexa Fluor 488 (1:200; Jackson Laboratories) antibodies. After nuclear staining with DAPI, sections were analyzed for the presence of CM-DiI⁺ CD3⁺ DAPI⁺ cells using confocal microscopy (TCS SP5 X; Leica).

Tracing migration of leukocytes from PPs to the brain. Forty-eight hours after cMCAo or sham surgery, cells within PPs were labeled by microinjection of fluorescent-cell-staining dyes CFSE (25 μ M in 2 μ l of PBS per PP) or CM-DiI (5 μ M in 2 μ l of PBS per PP) (Life Technologies). Mice were killed 24 h after cell labeling and brains prepared either for flow cytometric analysis (CFSE labeling) or immunohistological analysis (CM-DiI labeling) as described above.

Postoperative ileus model. Mice were anesthetized with isoflurane delivered in a mixture of 30% CO_2 and 70% N_2O . Before surgery, an injection of carprofen (5 mg/kg) was administered to the mice. After this, mice were fixed with surgical tape in the supine position on a feedback-regulated heating pad. The abdominal cavity was then opened 2 cm in length along the linea alba. With two moist sterile cotton applicators, the small intestine content was carefully touched from the pylorus to the cecum as described previously (Vilz et al., 2012). After surgery, the mice were sutured, monitored in a recovery chamber, and then returned to their home cages. Fecal samples were collected before and 3 d after the surgery for microbial DNA isolation and metagenomic analysis. Directly afterward, an oral bolus of FITC-dextran was administered for the gut motility test.

Gastrointestinal motility analysis. Mice received a 100 μ l oral bolus of 50 mg/ml FITC-dextran (70,000 kDa; Sigma-Aldrich) in 0.9% PBS. One hour after administration, the mice were killed and the entire intestinal tract from the stomach to the colon was removed and imaged using a chemiluminescence detection system (Fusion FX7). To quantify gastrointestinal transit (i.e., motility), the complete gastrointestinal tract was divided into segments, each segment was flushed with distilled water, and the fluorescence of the purified recovered flushing solution was measured using a fluorescence plate reader (Promega). The values obtained were normalized to blank controls and expressed as the percentage of fluorescence per intestinal segment.

ELISA for albumin quantification. Mice were killed 3 d after fsham or fMCAo surgery and feces and plasma samples were collected for measurement of albumin concentrations. Sampled feces (50 mg/ml) and plasma (1:50000) were diluted in assay buffer (50 mM Tris, 0.1 M NaCl, 0.05% Tween 20, pH 7.4) and ELISA was performed with corresponding albumin standard dilutions. Albumin was quantified by ELISA and the ratio of feces to plasma albumin are presented (Bethyl Laboratories).

Bacterial cultures. Mice were deeply anesthetized and perfused transcardially with normal saline. The cecum's contents were then transferred to sterile tubes under strict aseptic conditions. A fixed amount of cecum content from each mouse was mixed in PBS to 50 mg/ml and dilutions were prepared. Two dilutions (1:40,000 and 1:400,000) from each sample were plated on blood agar plates (Oxoid) and incubated at 37°C for 48–72 h. To generate an anaerobic environment, the plates were placed in anaerobic jars containing AnaeroGen paper sachets (Oxoid). After the incubation period, the plates were examined for bacterial growth and the number of colonies was determined.

Microbial community analysis. DNA from mouse feces was isolated using the QIAamp Fast DNA Stool Mini Kit (Qiagen). The total amount

of DNA in each sample was measured using a Qubit fluorometer (Life Technologies). 16S rRNA amplicons were generated using primers corresponding to the hypervariable regions V1–V3 (primer 27F: AGA GTTGTGATCCTGGCTCAG; primer 534R: ATTACCGCGGCTGCTGG), 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 cutoff of $1e^{-5}$. A BLAT similarity search for the longest cluster representative was performed against the Ribosomal Database Project (RDP) database. Rarefaction curves, α diversity, and phylogenetic analysis were performed and phylogenetic trees were drawn using the MG-RAST platform. Read counts were normalized logarithmically for subsequent analysis. Principal component analysis plots were drawn using normalized values and Bray–Curtis distance. Differences between group means for genera were analyzed using ANOVA or Student's *t* test. *p*-values were adjusted for comparison of multiple comparisons using Bonferroni correction. The DNA sequences have been deposited in MG-RAST (<http://metagenomics.anl.gov/>) under accession number 9778. The linear discriminant analysis (LDA) effect size (LEfSe) method was used to identify microbial taxa showing a statistically different relative abundance between the microbiota after sham and fMCAo surgery (Segata et al., 2011). Experiments were performed with an α error set to 0.05 and the LDA threshold set to 2.0.

Quantitative RT-PCR. Brain tissue from the ipsilateral and contralateral hemispheres was lysed in 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). The quantitative expression of various cytokines was measured using predesigned RT² qPCR Primer Assays and 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 (i.e., cyclophilin). All assays were performed in duplicate.

Statistical analysis. Data were analyzed using GraphPad Prism version 6.0. All summary data are expressed as the mean \pm SD. All datasets were tested for normality using the Shapiro–Wilk normality test. The groups containing normally distributed data were tested using a two-way Student's *t* test (for two groups) or ANOVA (for more than two groups). The remaining data were analyzed using the Mann–Whitney *U* test. Similar variance was assured for all groups, which were compared statistically. Differences with a *p*-value <0.05 were considered to be statistically significant. *p*-values were adjusted for comparison of multiple comparisons using Bonferroni correction. More detailed methods for analyzing the amplicon sequencing results and the metabolomics data are provided in the respective Materials and Methods sections.

Results

Stroke induces intestinal microbiota dysbiosis

To investigate the impact of acute stroke on the gut microbiota, we used fMCAo, an experimental stroke model resulting in large hemispheric lesions (mean \pm SD of all control experiments in this study: 72 ± 18 mm³; Fig. 1*a*). Next-generation sequencing of gut microbiota composition in mice after fMCAo and controls (naive and sham treatment) revealed substantial changes 3 d after severe stroke (Fig. 1*b*). We observed significant reduction in species diversity as a key feature of after stroke microbiota dysbiosis (Fig. 1*c*). Hallmarks of poststroke dysbiosis included changes in specific bacterial genera within the most abundant phyla: Firmi-

cutes, Bacteroidetes, and Actinobacteria (Fig. 1*d*). We next performed an indicator taxa analysis using LEfSe algorithm analysis for the detection of specific features that were statistically different between sham- and fMCAo-operated mice. Using this analysis strategy, we identified several significantly changed species after stroke induction consistent with the above-shown phylogenetic tree analysis; however, we also identified corresponding changes in higher-order taxa of the respectively altered species (Fig. 1*e*). These results suggested a consistent and specific impact of brain injury on microbiota populations within the most abundant bacterial phyla.

Extensive brain injury impairs gastrointestinal function

Next, we investigated which mechanisms link acute brain injury and microbiota dysbiosis. It was reported previously that patients with severe brain injuries experience reduced gastrointestinal motility (Olsen et al., 2013; Engler et al., 2014). Consistent with these clinical observations, we detected severe gastrointestinal paralysis after large infarcts induced by the fMCAo stroke model using a gastric fluorescent bolus tracking technique (Fig. 2*a,b*). Reduced gastrointestinal motility was associated with overgrowth of intestinal bacteria after severe experimental stroke compared with sham surgery (Fig. 2*c*). Due to this functional gastrointestinal impairment, we further investigated the impact of stroke on intestinal barrier function. We did not observe bacterial translocation or transmucosal bacterial invasion after stroke by *in situ* hybridization with a general bacteria (EUB 388) probe (Fig. 2*d*). However, we measured a significantly increased protein leakage by feces/plasma albumin ratio after fMCAo compared with sham-treated controls (Fig. 2*e*). These results suggested a functionally, but not morphologically, impaired intestinal barrier after fMCAo in association with poststroke intestinal paralysis. Previous reports from our group and others have identified dysregulation of the autonomous nervous system and a pronounced stress response after stroke as one of the key pathways involved in mediating systemic effects on remote organ function after acute brain injury (Meisel et al., 2005; Chamorro et al., 2007; Chamorro et al., 2012; Liesz et al., 2013b; Mracsko et al., 2014). We detected increased blood catecholamine levels after extensive stroke in the fMCAo model to be associated with poststroke intestinal paralysis (Fig. 2*f*). Consistent with our results, a recent report by Houlden et al. (2016) demonstrated poststroke intestinal changes as a consequence of the catecholaminergic stress response. It was shown previously that the severity of the brain injury is correlated with the extent of secondary systemic immunomodulation (Meisel et al., 2005; Liesz et al., 2009b). Consistent with these previous immunological findings, induction of only small cortical lesions in a second stroke model (cMCAo, mean infarct volume in this model: 8 ± 2 mm³) caused no significant change in the microbiota composition and species diversity (Fig. 2*g,h*). Small brain lesions in the cMCAo model also did not induce gastrointestinal paralysis (Fig. 2*i*). These results indicate that infarct severity might be the key factor determining intestinal dysfunction, although we cannot ultimately exclude the possibility that other factors differing between the investigated stroke model also contributed to this effect.

To investigate the causality of gut motility changes after stroke and observed microbiota dysbiosis, we further determined effects of inducing gastrointestinal paralysis by physical intestinal manipulation, a model for postsurgical ileus (Vilz et al., 2012). Postsurgical ileus mimicked the disturbed gastrointestinal motility pattern of stroke animals (Fig. 3*a*) and recapitulated general features of microbiota dysbiosis such as reduced α diversity (Fig.

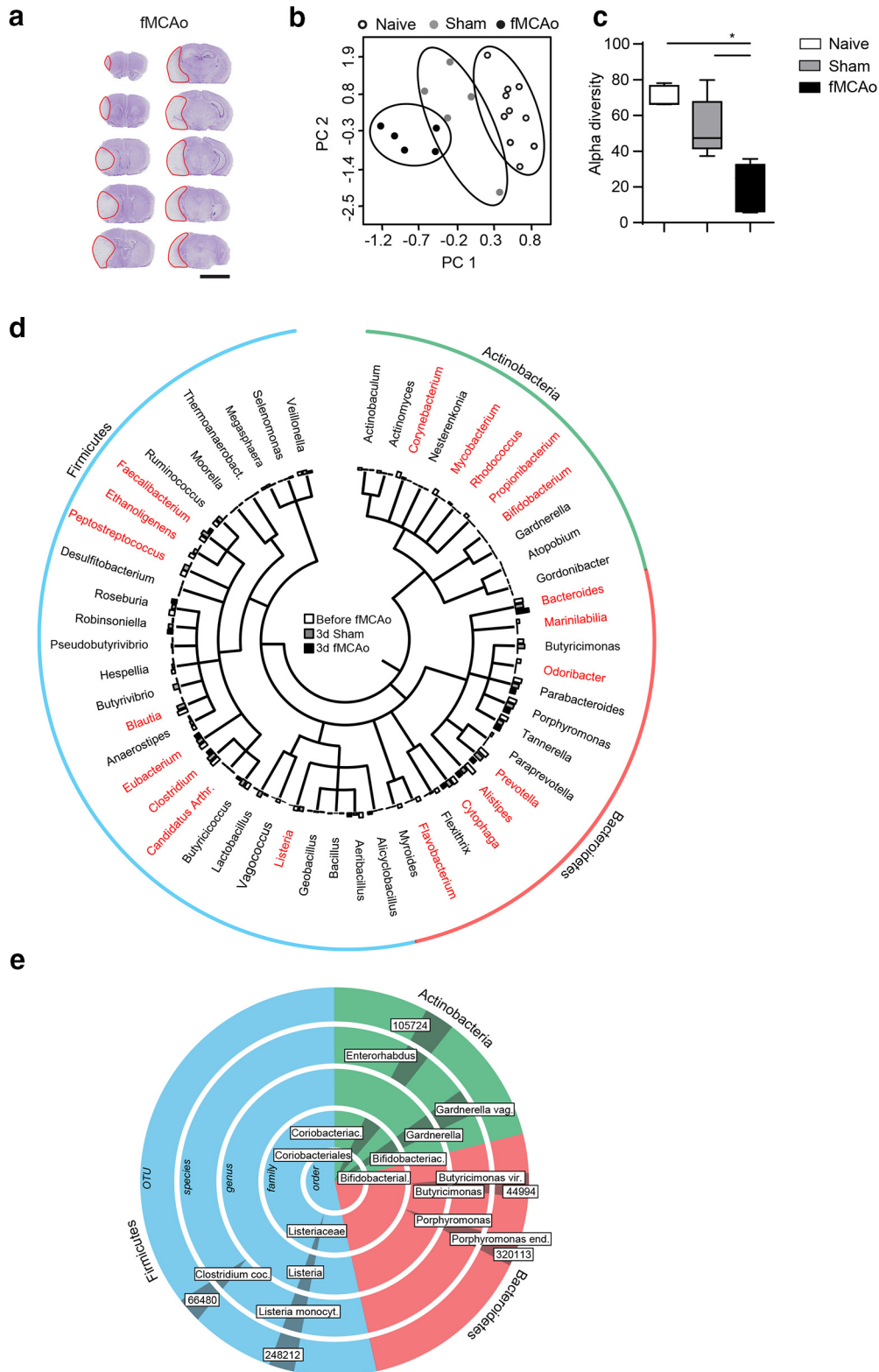


Figure 1. Severe stroke induces microbiota dysbiosis. **a**, Representative images of cresyl-violet-stained coronal brain sections 3 d after fMCAo. In each section, the infarct area is outlined in red (5 mm). **b**, Principal component (PC) analysis of the intestinal microbiota by taxonomic abundance patterns in naive mice (before sham or fMCAo surgery) and after sham and fMCAo surgery. **c**, Quantitative analysis of α diversity confirms significantly reduced species diversity of the gut microbiota after brain injury in the fMCAo model (Mann–Whitney U test). **d**, Phylogenetic tree illustrating the distribution of the identified bacterial genera within the most abundant phyla (Actinobacteria, Bacteroidetes, and Firmicutes) comparing post-fMCAo mice with sham-operated and before-fMCAo mice. Genera that were significantly altered in the fMCAo group compared with the sham and before-fMCAo groups are indicated in red (ANOVA, $n = 5$ mice per group). **e**, LefSe algorithm analysis was performed for indicator taxa analysis identifying features that are statistically different between sham- and fMCAo-operated mice. Labels in boxes define significantly regulated operational taxonomic units (OTU, numbers) and higher order taxa, respectively.

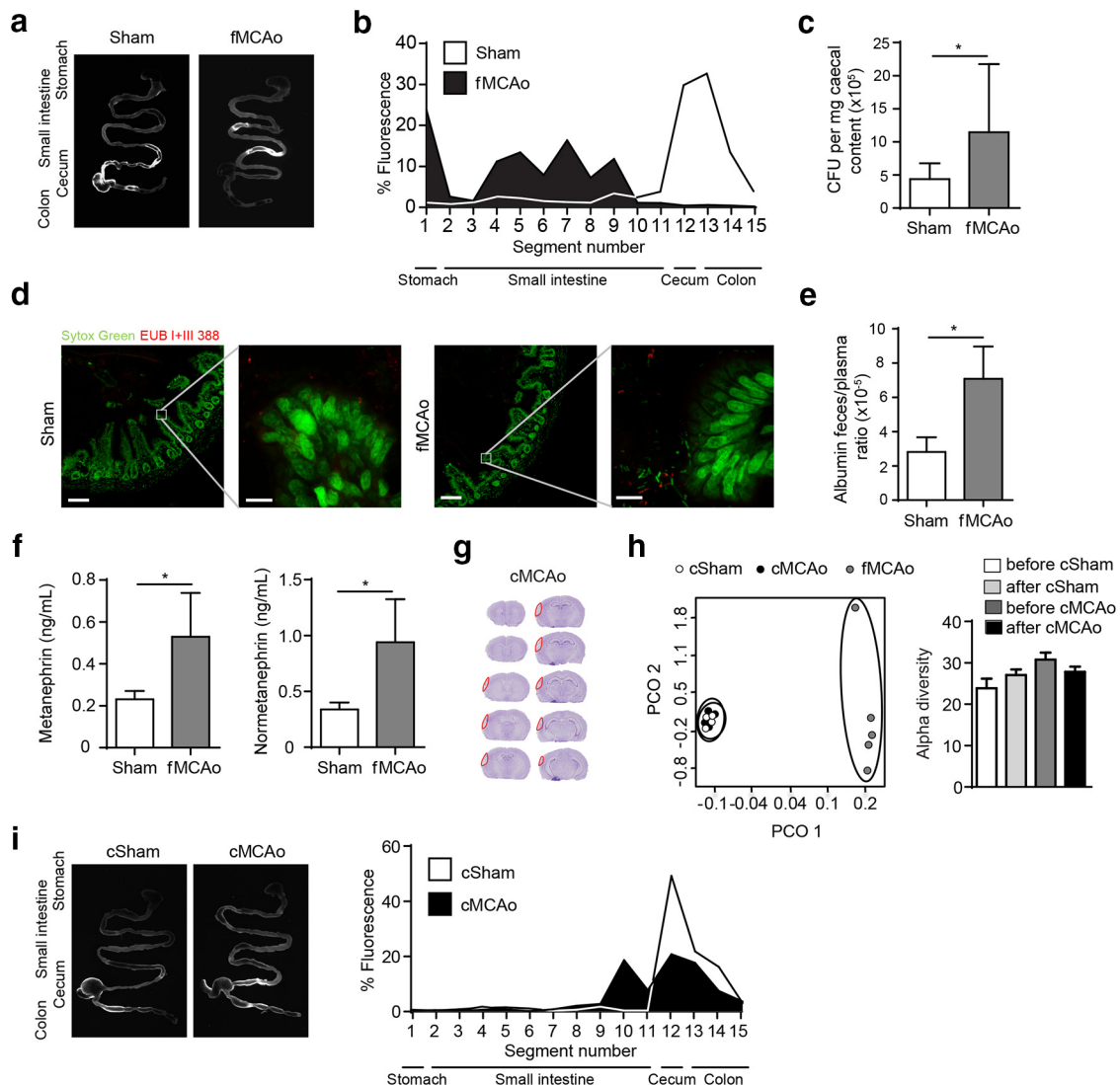


Figure 2. Lesion severity determines gastrointestinal dysfunction. *a*, Intestinal motility was measured 24 h after fMCAo or sham surgery. Representative fluorescence images of the complete gastrointestinal tract 60 min after gastric instillation of FITC-dextran showing retention of the fluorescent bolus in the upper gastrointestinal tract as a marker of severely impaired motility after fMCAo compared with sham mice. Arrowheads indicate cecum. *b*, Quantification of fluorescence intensity in intestinal segments. Note the retention of fluorescence signal in the upper gastrointestinal tract after fMCAo ($n = 8$ per group, 3 individual experiments). *c*, Number of colony forming units (CFUs) per milligram of murine cecal content cultured under anaerobic conditions after sham or fMCAo surgery ($n = 8–9$ mice per group, 2 independent experiments). *d*, No bacterial invasion into lamina propria was found in transverse sections of mouse ileum, which were hybridized using a general bacteria-specific EUB³³⁸ probe with Sytox green counterstaining. Left panels: Magnification, 20 \times ; scale bars, 100 μ m; right panels: magnification, 63 \times , scale bars, 10 μ m. *e*, Albumin concentrations were determined by ELISA and are represented as the ratio of the concentrations in feces and plasma from sham- and fMCAo-operated mice ($n = 5$ mice per group, 2 individual experiments). *f*, Plasma catecholamine levels, represented as catecholamine metabolite concentrations of metanephrine and normetanephrine, were significantly increased 24 h after fMCAo compared with sham surgery ($n = 10$ per group, 3 individual experiments). *g*, Representative cresyl-violet-stained coronal brain sections 3 d after stroke induction in the cMCAo model. The small cortical lesions are outlined in red. *h*, Principal component (PC) analysis (left) for fecal taxonomic abundance after csham and cMCAo compared with fMCAo, and α diversity for the indicated groups (right) illustrate that small cortical lesions do not affect microbiota composition. *i*, Representative fluorescence images (left) and quantitative analysis (right, $n = 6$) of gastrointestinal motility after csham and cMCAo surgery corresponding to data shown in *a* and *b*. Bar graph: * $p < 0.05$, mean \pm SD.

3*b,c*). Despite this, in-depth phylogenetic analysis also revealed substantial differences in specifically regulated taxa between stroke and mechanically induced dysbiosis (Fig. 3*d*). Collectively, these results suggest stress-mediated paralytic ileus as a potential cause of poststroke microbiota dysbiosis, although alternative CNS-specific pathways could be involved in poststroke dysbiosis.

Dysbiosis is causally linked to deteriorated stroke outcome

We next investigated whether poststroke dysbiosis has a functional impact on stroke outcome. To investigate this mechanism, we used a microbiota-transfer model into GF mice. We transplanted microbiota obtained from sham- and fMCAo-operated

mice into GF recipient mice; 3 d later, we induced cortical lesions by the cMCAo model, which does not alter microbiota composition *per se*, as shown above (Fig. 4*a*). Metagenomic analysis of recipients' feces samples at the time point of cMCAo induction (i.e., 3 d after transplantation) confirmed establishment of distinct microbiota in the recipient mice, which resembled the specific pattern of the microbiota donors (Fig. 4*b*). Remarkably, mice that had received microbiota from brain-injured animals developed significantly larger infarct volumes after cortical lesions in the cMCAo stroke model compared with littermates that were recolonized with sham-surgery microbiota (Fig. 4*c*). In addition, functional impairment in recipients of the dysbiotic

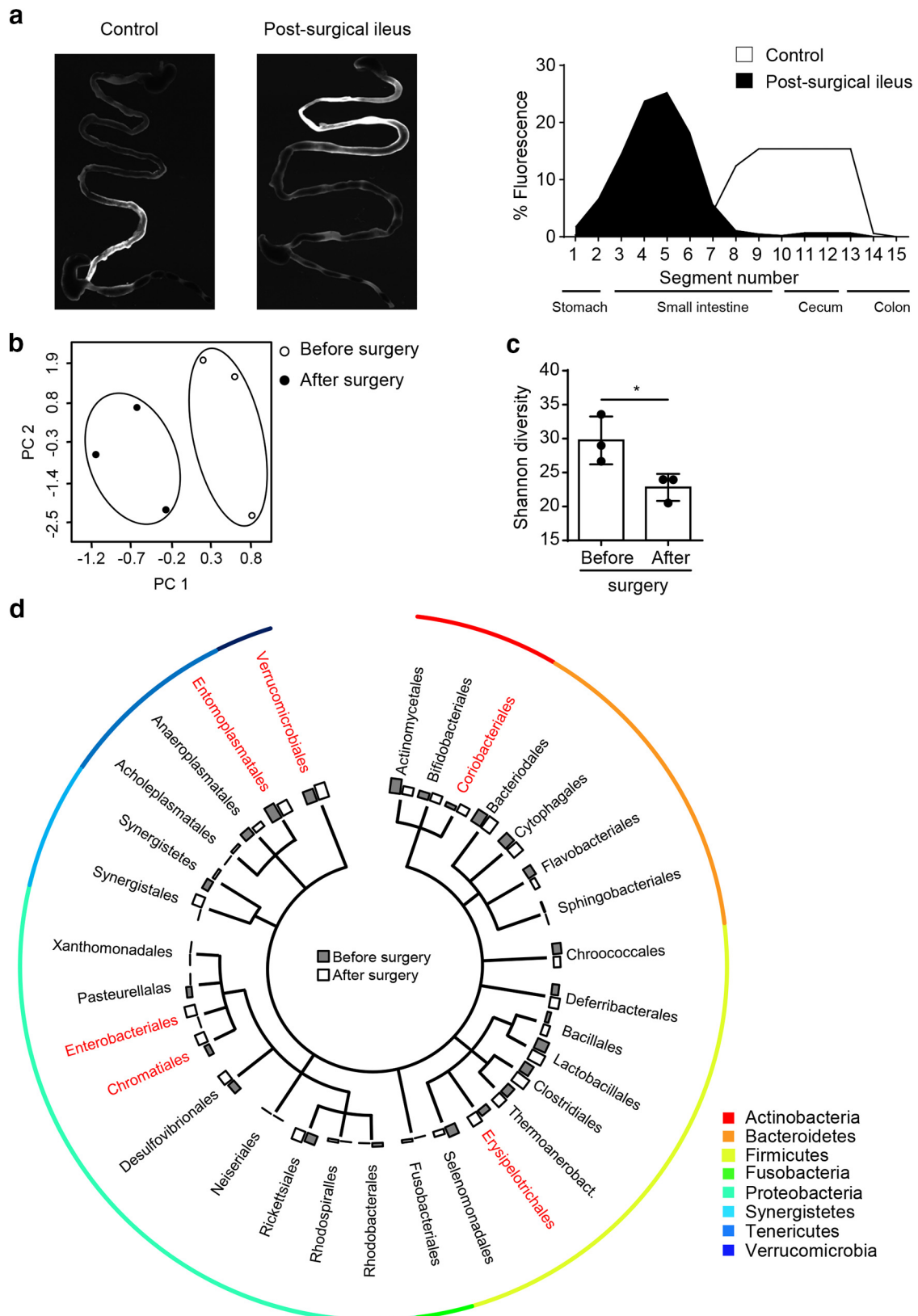


Figure 3. Postsurgical ileus induces intestinal motility dysfunction and dysbiosis of the gut microbiota. **a**, Intestinal motility was measured 3 d after surgical ileus induction or sham surgery. Shown are representative images of the gastrointestinal tract 60 min after an oral dose of FITC-dextran showing retention of fluorescence signal in the upper gastrointestinal tract (left). Corresponding quantification of fluorescence intensity per intestinal segment ($n = 3$ per group, right panel). **b**, Principal component (PC) analysis of the microbiota composition in mice before and 3 d after mechanical manipulation of the ileus reveals microbiota alteration. **c**, Analysis of Shannon diversity index show a significantly reduced species diversity induced by the postsurgical ileus model. **d**, Shown is a phylogenetic tree illustrating the distribution of the identified bacterial orders comparing microbiota before and 3 d after surgery. Orders that significantly differed are highlighted in red ($n = 3$ mice per group, t test [unpaired]). All bar graphs: $*p < 0.05$, mean \pm SD.

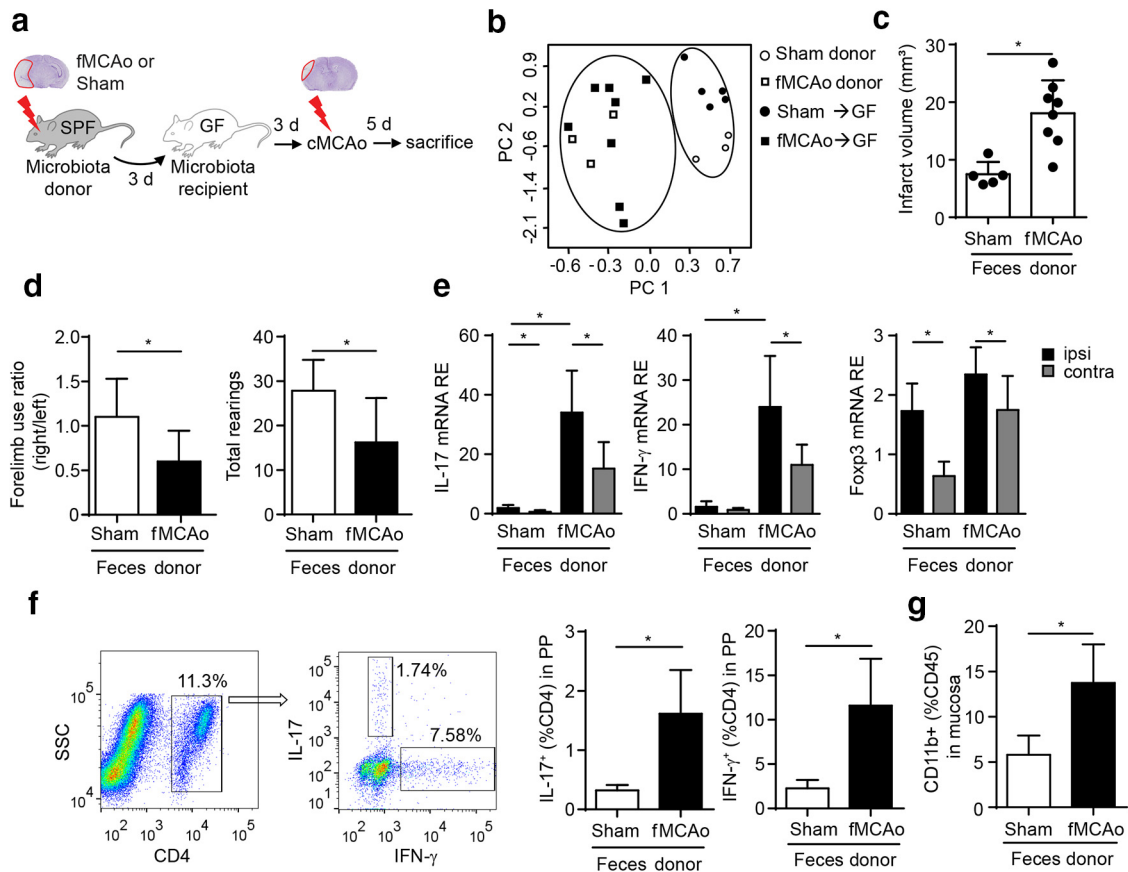


Figure 4. Brain ischemia-induced dysbiosis alters the poststroke immune reaction and exacerbates stroke outcome. **a**, Experimental design for recolonizing GF mice with gut microbiota from sham- or fMCAo-operated SPF donor mice. Three days after recolonization, mice underwent cMCAo induction and were killed another 5 d later for subsequent analysis. **b**, Principal component analysis of the microbiota composition in donor mice after sham and fMCAo surgery and microbiota composition in GF recipient mice 3 d after transplantation with donor microbiota. Analysis revealed a distinct pattern of the two donor populations before and after transplantation to recipient GF mice. **c**, Comparison of brain infarct volumes (cMCAo) in the recolonized recipient mice 5 d after cMCAo induction. **d**, Recolonizing mice with the gut microbiota obtained from post-fMCAo donors significantly increased brain lesion volumes and significantly reduced behavioral performance assessed by measuring forelimb use asymmetry (left) and total rearing activity (right) in the cylinder test ($n = 5–8$ per group, 2 individual experiments). **e**, Relative gene expression levels of IL-17, IFN- γ , and Foxp3 in the ipsilateral (ipsi) and contralateral (contra) hemispheres of GF recipient mice ($n = 5–8$ per group, 2 individual experiments). Recolonization with microbiota from fMCAo donor mice massively increased Th17 (IL-17) and Th1 (IFN- γ) expression compared with recipients of sham-microbiota. **f**, Representative dot plots with gating strategy for the flow cytometry analysis of Th17 (IL-17) and Th1 (IFN- γ) T cells in PPs (left). Percentages of IL-17 $^{+}$ and IFN- γ $^{+}$ T cells were significantly increased in PPs of mice recolonized with the fMCAo-microbiota (right panel). **g**, Percentage of CD11b $^{+}$ monocytes/macrophages in the mucosal layer of the small intestine (terminal ileum) was significantly increased in mice receiving the microbiota from fMCAo donors compared with sham microbiota. All bar graphs: $*p < 0.05$, mean \pm SD.

microbiota was exacerbated, as determined by forelimb use asymmetry and overall activity (rearing efforts) in the established cylinder test for this stroke model (Fig. 4*d*). We next analyzed the expression of the cytokines IL-17 and IFN- γ and of the transcription factor Foxp3 as markers of T_{helper} cell polarization in brains 5 d after cMCAo. Recipients of post-fMCAo microbiota exhibited massively increased expression of the proinflammatory IFN- γ and IL-17 cytokines, which are markers of Th1 and Th17 T cell polarization, respectively, and have previously been associated with worse outcome in experimental stroke models (Yilmaz et al., 2006; Liesz et al., 2009a; Shichita et al., 2009; Gelderblom et al., 2012). In contrast, Foxp3 expression, as a marker for neuroprotective T_{reg} cells, did not differ significantly between sham and fMCAo microbiota recipients (Fig. 4*e*). In addition, we performed flow cytometry analysis for intracellular cytokine expression of T cells in PPs to investigate the effect of the distinct microbiota on intestinal T-cell polarization. Here, we detected a >4-fold increased expression of proinflammatory Th17 (IL-17 $^{+}$) and Th1 (IFN- γ $^{+}$) cells in PPs of fMCAo microbiota compared with sham microbiota recipients, consistent with results obtained from brain tissue (Fig. 4*f*). These results indicate that

the dysbiotic microbiota after large (fMCAo) brain infarcts induces a predominantly proinflammatory Th1 and Th17 response, which is associated with increased infarct volume. This finding is consistent with previous studies showing that T-cell polarization can be induced by selected members of Bacteroidetes and Firmicutes (Round and Mazmanian, 2010; Magrone and Jirillo, 2013). In addition to lymphocyte polarization/activation, microbial products can regulate innate immunity and contribute to intestinal monocyte/macrophage maturation and activation (Bain and Mowat, 2014). Here, we found increased number of mucosal CD11b $^{+}$ monocytes in mice that were recipients of post-fMCAo microbiota (Fig. 4*g*). Together, these results indicate an activation of intestinal innate and adaptive immune cells in response to poststroke dysbiotic microbiota.

T cells migrate from the intestine to the poststroke brain

Interestingly, the peripheral immune responses coincided precisely with the massively increased cerebral gene expression for proinflammatory cytokines, suggesting that peripherally activated and/or polarized T cells may migrate to the injured brain after cMCAo in GF recipient mice. To investigate potential T-cell

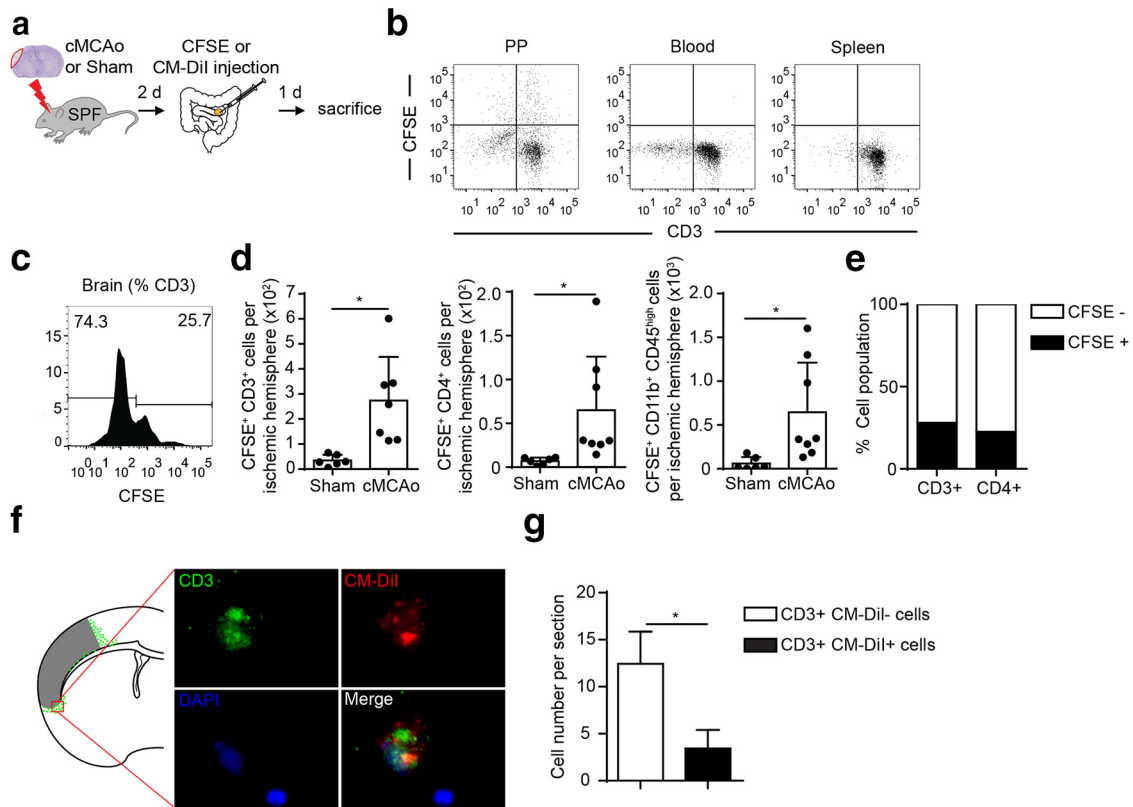


Figure 5. Lymphocytes migrate from PPs to the brain after stroke. *a*, Experimental design for tracing the migration of PP-derived lymphocytes in mice after cMCAo or sham surgery. CFSE or CM-Dil was injected in PP 2 d after the respective surgery and, 24 h later, brain and lymphoid organs were dissected and analyzed for dye-positive T cells. *b*, Validation of site-specific T-cell labeling with CFSE in PP 3 h after microinjection; T-cell labeling was not detectable in blood and spleen. *c*, Representative histogram for CFSE⁺ T cells (gated for CD45⁺ CD3⁺ expression) from flow cytometry analysis of brain homogenates of the ipsilateral hemisphere 24 h after CFSE microinjection in all detectable PPs. *d*, Quantification of flow cytometry analysis shows increased numbers of CFSE⁺ total T cells (CD3⁺) and T_{helper} cells (CD4⁺) and monocytes (CD11b⁺) in ischemic hemispheres 3 d after cMCAo compared with sham control. *e*, Percentage of CFSE-labeled CD3⁺ and CD4⁺ T cells identified in ischemic hemispheres 3 d after cMCAo and 24 h after PP labeling. *f*, Brain-invasive CM-Dil⁺ T cells derived from PPs were identified in the peri-infarct region and are illustrated as a cumulative map from five mice on one topographical coronal brain section at the bregma level. *g*, Quantification of CM-Dil- and CM-Dil⁺ T cells (CD3⁺) per one histological brain section used to generate the cumulative map shown in *f*.

migration from PPs to the brain, we used a fluorescent labeling technique by microinjection of either CFSE or CM-Dil in all detectable PPs of the mice intestines after cMCAo or sham surgery (Fig. 5*a*). We confirmed that this microinjection technique labeled cells only locally in PPs, but no systemic cell labeling by potentially diffusing dye occurred in blood or spleen (Fig. 5*b*). Consistent with a previous report (Benakis et al., 2016), T cells and monocytes fluorescently labeled after microinjection in PPs were detected in the ischemic hemisphere 3 d after cMCAo consistent with the previously demonstrated kinetics of post-stroke leukocyte invasion (Gelderblom et al., 2009; Liesz et al., 2011) (Fig. 5*c,d*). CFSE-positive cells derived from labeled PPs accounted for ~25% of total T cells and the T_{helper} cell subpopulation 3 d after cMCAo (Fig. 5*e*). We confirmed these findings in an independent experiment using CM-Dil as a lipophilic labeling dye and subsequent histological analysis (Fig. 5*f*). Here, superimposing localization of T cells from five mice on one coronal section map, we detected brain-invasive T cells surrounding the ischemic core consistent with previous reports using the same stroke model (Liesz et al., 2011; Zhou et al., 2013); moreover, the percentage of PP-derived T cells was consistent with results obtained from the above-shown flow cytometric analysis (Fig. 5*f,g*). These results unequivocally demonstrate the invasion of considerable numbers of T cells from the intestine to the peri-infarct tissue at risk in the postischemic brain with a remarkably fast kinetics.

Fecal microbiota transplantation is neuroprotective after stroke

Based on our results, we hypothesized that poststroke dysbiosis is a key novel target for modulating the systemic immune response after stroke. Therefore, we tested the use of FMT as a therapeutic approach to restore a healthy microbiome in animals after stroke. Indeed, FMT treatment starting at the day of stroke induction and performed once daily during the survival period significantly reduced the lesion after large stroke using the fMCAo stroke model for severe brain infarctions (Fig. 6*a*). This improvement in stroke outcome was associated with increased numbers of Foxp3⁺ T_{reg} cells in peripheral immune organs and in the ischemic brain after stroke (Fig. 6*b*). To further elucidate the contribution of lymphocytes to this effect, we examined FMT in a model of lymphocyte-deficient *Rag1*^{-/-} mice. FMT had no effect on lesion size in *Rag1*^{-/-} mice, supporting the notion that microbiome-mediated effects on brain injury are mediated by lymphocytes (Fig. 6*c*). Metagenomic analysis of microbial communities showed a normalization of stroke-induced dysbiosis and partially restored microbiota diversity by the FMT treatment in mice with extensive infarcts (Fig. 6*d,e*). More in-depth analysis revealed that FMT treatment increased abundance of several of the very same taxa, which were reduced by stroke-induction compared with sham treatment (Fig. 6*f*). These findings suggest that FMT therapy normalizes stroke-induced changes to micro-

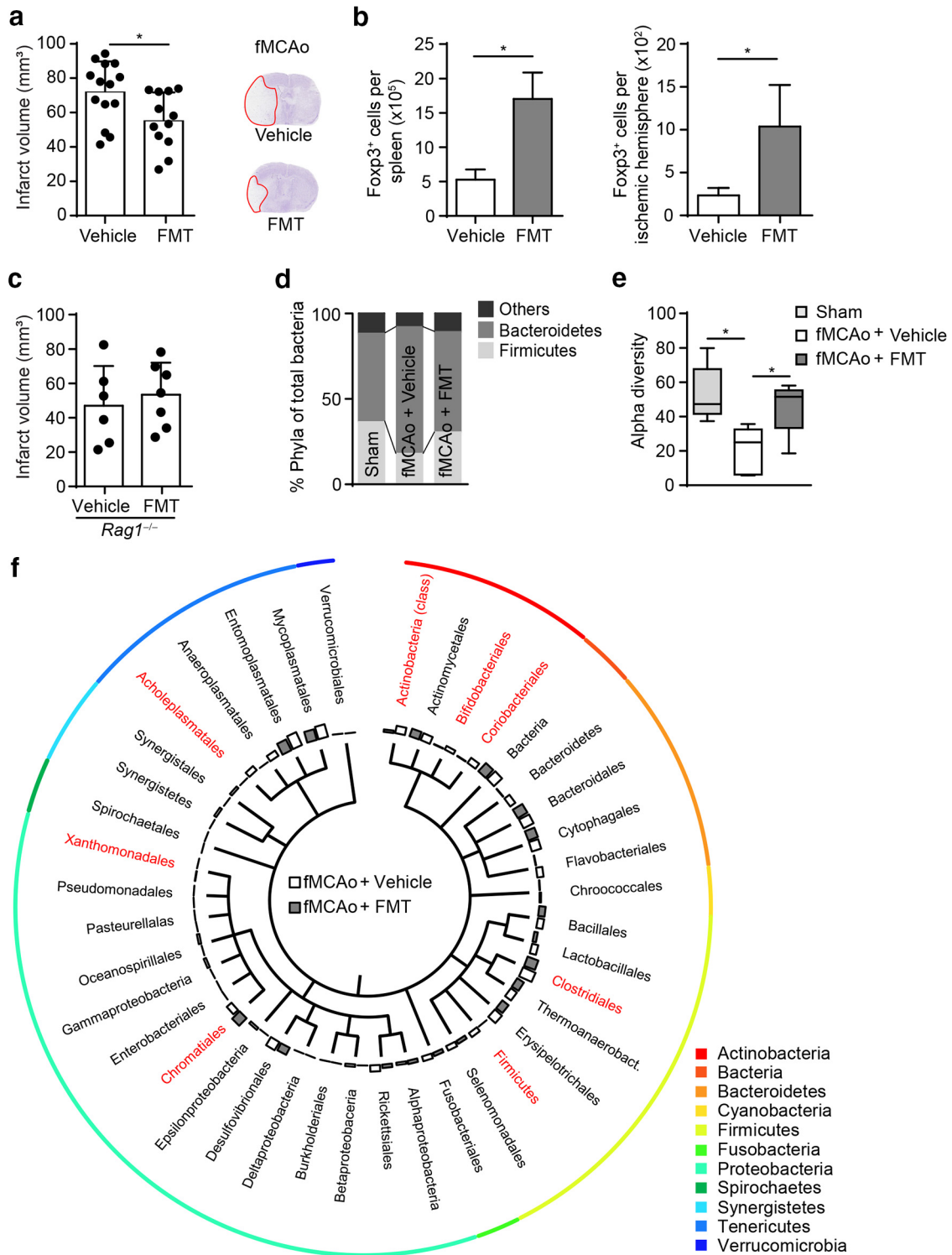


Figure 6. Fecal microbiota transplantation improves stroke outcome. **a**, Brain infarct volume 3 d after fMCAo was compared between mice receiving vehicle or FMT; FMT treatment significantly reduced brain lesion volume (three independent experiments). **b**, Flow cytometric analysis shows increased Foxp3⁺ T_{reg} cell counts in spleens and ischemic hemispheres after FMT treatment compared with controls 3 d after fMCAo (*n* = 5 per group). **c**, Brain infarct volume 3 d after fMCAo in lymphocyte-deficient *Rag1*^{-/-} mice did not differ between vehicle and FMT treatment, suggesting a lymphocyte-mediated effect (two independent experiments). **d**, FMT treatment normalized microbiota composition after fMCAo-induced dysbiosis as demonstrated by the taxonomic abundance of eubacterial phyla. Note the normalization in the abundance ratio between Firmicutes, Bacteroidetes, and the less abundant phyla (others) in the FMT-treated group. **e**, Analysis of the corresponding α diversity by the Shannon diversity index showing that FMT treatment partially reversed the reduced species diversity induced by fMCAo (*n* = 5 mice per group). **f**, Phylogenetic tree illustrating the distribution of the identified bacterial orders comparing vehicle- and FMT-treated mice 3 d after fMCAo. Orders that significantly differed between groups are highlighted in red (*n* = 5 mice per group, *t* test [unpaired]). All bar graphs: **p* < 0.05, mean \pm SD.

bial communities efficiently and might thereby exert a neuroprotective function after stroke.

Discussion

Our results support the concept of a bidirectional communication along the brain–gut–microbiome–immune axis. Recent reports have suggested that the microbiota plays an evident role in developmental and autoimmune brain disorders (Berer et al., 2011; Cryan and Dinan, 2012) and that antibiotic treatment-induced dysbiosis affects stroke outcome (Benakis et al., 2016). Here, we report that stroke itself markedly affects the intestinal microbial composition and that these changes in turn can determine stroke outcome.

In our study, we have demonstrated the substantial effects of brain injury on microbiota composition; these effects included reduction in microbiota species diversity and intestinal bacterial overgrowth with a preferential expansion of the Bacteroidetes phylum. In addition, we identified more specific stroke-induced changes on the bacterial genus and even the species level. These findings on poststroke dysbiosis are consistent with recent reports describing changes in intestinal microbiota composition in stroke patients (Karlsson et al., 2012; Swidsinski et al., 2012; Yin et al., 2015). Several of these features of microbiota alterations are of direct pathophysiological relevance; specifically, high microbiota diversity has been suggested as a key feature of a healthy microbiome (Claesson et al., 2012; Human Microbiome Project, 2012). Our analyses also revealed that the surgical procedure itself (i.e., sham surgery) also induced apparent changes in microbiota composition, thus highlighting the sensitivity of microbiota composition to stress responses (Carabotti et al., 2015). However, brain injury compared with sham surgery imposed an additional and substantial impact on microbiota composition and we were able to detect specific species and their corresponding higher-order taxa most significantly associated with acute brain injury compared with sham surgery using indicator taxa analysis.

Our results suggested that microbiota dysbiosis after stroke is associated with reduced gastrointestinal motility and intestinal paralysis in a postsurgical ileus model recapitulated several key features of poststroke dysbiosis. These findings have broad clinical implications; specifically, the intestinal dysfunction revealed by our animal model was recently reported in patients after acute brain injury (Bansal et al., 2009; Olsen et al., 2013). However, a decisive causality among poststroke stress response, impaired motility, and dysbiosis could not be clarified in this study. In addition, we cannot exclude other direct mediators released from the necrotic brain tissue affecting microbiota composition via currently unknown mechanisms. One potential alternative mechanism not investigated in this study is the release of proinflammatory alarmins such as ATP, HMGB1, and S100 proteins from the injured brain with potential direct effects on intestinal immunity and microbiota composition (Liesz et al., 2015; Singh et al., 2016).

Our results demonstrate that microbiota dysbiosis is an important factor in determining poststroke inflammation and thereby stroke outcome in an experimental stroke model. Numerous reports over the last decade have highlighted the importance of the secondary inflammatory response to brain injury as a key pathophysiological mechanism (Iadecola and Anrather, 2011; Chamorro et al., 2012). Moreover, immunotherapeutic strategies emerge to be tested progressively in human stroke patients and first studies report a beneficial effect of inhibiting cerebral lymphocyte invasion in stroke patients (Fu et al., 2014; Zhu et al., 2015). However, the role of the gut microbiota in stroke

patients was elusive until the very recent publication of an elegant report by Benakis et al. (2016), who observed alteration of lymphocyte populations in the intestinal immune compartment after induction of dysbiosis by antibiotic treatment. Dysbiosis-induced changes in the peripheral immune system had a striking impact on stroke outcome with changes in infarct volume by 60% between treatment groups. In contrast to our study, antibiotic-induced intestinal dysbiosis had a neuroprotective effect with reduced infarct volumes and improved functional outcome after amoxicillin treatment. Nevertheless, the underlying mechanisms of microbiota–brain communication identified in the study by Benakis et al. (2016) and our report are largely identical, such as alteration in T-cell homeostasis, changes in the $T_{reg}/Th17$ ratio, and migration of intestinal lymphocytes to the ischemic brain. The at-first-sight contradictory impact of dysbiosis on final stroke outcome might depend on the differential immune polarization by specific bacterial species. In fact, whereas antibiotic treatment induced T_{reg} expansion and reduction of Th17 cells (Benakis et al., 2016), poststroke dysbiosis investigated in our study itself favors an opposing pattern with predominant expansion of proinflammatory T-cell subpopulations. Future studies will be required to analyze the contribution of specific microbial species in this highly complex interplay to identify neuroprotective or harmful bacteria in stroke.

Results from our microbiota transplantation experiments to GF animals using microbiota from donors undergoing stroke induction or sham surgery have clearly demonstrated a causal link between poststroke microbiota dysbiosis and changes in peripheral immunity and finally a worse outcome after stroke. A potential limitation of this experiment was the only short recolonization time of 3 d from transplantation to stroke induction or 8 d from transplantation to analysis, because previous reports have suggested that recolonization of GF mice with a complete fecal microbial induces pronounced immune activation with a peak at ~4 d after the start of colonization (El Aidi et al., 2012). This short time period for recolonization was nevertheless chosen deliberately to avoid shifts in microbiota composition during longer recolonization periods. Therefore, whereas the results from this specific recolonization experiment might overestimate immunological differences, they nevertheless provide a first proof-of-concept for the causality between dysbiosis and poststroke immune alterations.

In our study, we have observed a substantial induction of proinflammatory Th1 and Th17 T_{helper} cell polarization by transfer of a dysbiotic microbiome. A consistent cytokine expression pattern with markedly increased IFN- γ and IL-17 levels were observed in brains of mice receiving the dysbiotic microbiota. Moreover, we could determine in a cell-tracking experiment from intestinal PPs to the ischemic brain that at least one-fourth of all brain-invading T cells within the acute phase after stroke are derived from the intestinal immune compartment, which is consistent with a previous report (Benakis et al., 2016). This observation of T-cell invasion and activation is consistent with numerous reports on the surprisingly fast kinetics of poststroke adaptive immunity (Schroeter et al., 1994; Jander et al., 1995; Gelderblom et al., 2009; Liesz et al., 2011; Chamorro et al., 2012). The central neuroinflammatory reaction starts as early as hours after stroke, with T cells invading the brain in substantial numbers between 3 and 5 d after brain injury (Gelderblom et al., 2009; Chamorro et al., 2012). Furthermore, we have detected previously substantial clonal expansion of T cells using spectratype analysis within the first week after acute brain injury (Liesz et al., 2013a). Therefore, initiation of an adaptive immune response

and particularly activation of T cells occurs within few days after stroke, consistent with results presented in this study. Specifically, it was shown previously that T_{helper} cell polarization occurs very early after brain injury and the $T_{\text{reg}}/Th17$ balance contributes to outcome within the first 3 d after stroke (Liesz et al., 2009a; Shichita et al., 2009; Kleinschnitz et al., 2010).

The assumption of intestinal priming of T_{helper} cells by the microbiota and rapid translocation to the ischemic brain after stroke was further corroborated by the results obtained in experiments in which we treated mice after extensive brain ischemia (fMCAo model) by FMT from healthy donors. FMT treatment reduced lesion size and was associated with expansion of regulatory (neuroprotective) T cells in the peripheral immune system and in the ischemic brain. These findings are consistent with numerous previous reports demonstrating a key role for differential T_{helper} cell priming in stroke pathophysiology, in which proinflammatory Th1, Th17, and $\gamma\delta$ T cells deteriorate post-stroke inflammation, whereas T_{reg} cells limit the inflammatory collateral damage (Shichita et al., 2009; Iadecola and Anrather, 2011; Chamorro et al., 2012).

Interestingly, the protective effect of FMT treatment was absent in lymphocyte-deficient $Rag1^{-/-}$ mice, supporting a key role of lymphocytes in mediating the microbiota's effect on brain function. However, the specific mechanisms underlying this complex phenomenon are still unclear and will need further investigations. Specifically, the interaction of microbiota-dependently activated T cells with other immunocompetent cells of the poststroke brain (microglia, astrocytes, endothelium, invading leukocyte subpopulations) requires further analyses. A previous report by Erny et al. demonstrated a key role of the gut microbiota in microglial function and proposed microbiota-derived short chain fatty acids as the underlying mediator of microbiota–microglia communication (Erny et al., 2015). Therefore, it remains to be elucidated whether the important role of microbiota-dependent T-cell polarization after stroke shown by us and others (Benakis et al., 2016) affects neuronal protection/toxicity directly or if it affects stroke outcome indirectly via microglial function, as was shown previously to occur by brain-invading lymphocytes (Appel, 2009; Lucin and Wyss-Coray, 2009; Liesz et al., 2011). In addition, we have also observed activation of intestinal monocytes/macrophages by a dysbiotic poststroke microbiome. Moreover, intestinal monocytes were detected to invade the brain in the acute phase after stroke. Therefore, in addition to T-cell polarization, brain-invading monocytes could also potentially play a role in microbiota-mediated effects on stroke outcome.

Our results suggest a novel concept in which dysbiosis of the gut microbiota is a consequence of acute brain injury and a key effector in poststroke immune alterations with considerable impact on stroke outcome. Our findings suggest that restoring the health and balance of the intestinal microbiome could add to the treatment of stroke patients.

References

Appel SH (2009) CD4+ T cells mediate cytotoxicity in neurodegenerative diseases. *J Clin Invest* 119:13–15. [CrossRef Medline](#)

Arpaia N, Campbell C, Fan X, Dikiy S, van der Veen J, deRoos P, Liu H, Cross JR, Pfeffer K, Coffey PJ, Rudenski AY (2013) Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature* 504:451–455. [CrossRef Medline](#)

Bain CC, Mowat AM (2014) Macrophages in intestinal homeostasis and inflammation. *Immunol Rev* 260:102–117. [CrossRef Medline](#)

Bansal V, Costantini T, Kroll L, Peterson C, Loomis W, Eliceiri B, Baird A, Wolf P, Coimbra R (2009) Traumatic brain injury and intestinal dysfunction: uncovering the neuro-enteric axis. *J Neurotrauma* 26:1353–1359. [CrossRef Medline](#)

Benakis C, Brea D, Caballero S, Faraco G, Moore J, Murphy M, Sita G, Rac-

chumi G, Ling L, Pamer EG, Iadecola C, Anrather J (2016) Commensal microbiota affects ischemic stroke outcome by regulating intestinal $\gamma\delta$ T cells. *Nat Med*.

Berer K, Mues M, Koutouros M, Rasbi ZA, Boziki M, John C, Wekerle H, Krishnamoorthy G (2011) Commensal microbiota and myelin autoantigen cooperate to trigger autoimmune demyelination. *Nature* 479:538–541. [CrossRef Medline](#)

Carabotti M, Scirocco A, Maselli MA, Severi C (2015) The gut-brain axis: interactions between enteric microbiota, central and enteric nervous systems. *Ann Gastroenterol* 28:203–209. [Medline](#)

Chamorro A, Amaro S, Vargas M, Obach V, Cervera A, Gómez-Choco M, Torres F, Planas AM (2007) Catecholamines, infection, and death in acute ischemic stroke. *J Neurol Sci* 252:29–35. [CrossRef Medline](#)

Chamorro Á, Meisel A, Planas AM, Urra X, van de Beek D, Veltkamp R (2012) The immunology of acute stroke. *Nat Rev Neurol* 8:401–410. [CrossRef Medline](#)

Claesson MJ, Jeffery IB, Conde S, Power SE, O'Connor EM, Cusack S, Harris HM, Coakley M, Lakshminarayanan B, O'Sullivan O, Fitzgerald GF, Deane J, O'Connor M, Harnedy N, O'Connor K, O'Mahony D, van Sinderen D, Wallace M, Brennan L, Stanton C, et al. (2012) Gut microbiota composition correlates with diet and health in the elderly. *Nature* 488:178–184. [CrossRef Medline](#)

Collins SM, Surette M, Bercik P (2012) The interplay between the intestinal microbiota and the brain. *Nat Rev Microbiol* 10:735–742. [CrossRef Medline](#)

Cryan JF, Dinan TG (2012) Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nat Rev Neurosci* 13:701–712. [CrossRef Medline](#)

El Aidy S, van Baaren P, Derrien M, Lindenbergh-Kortleve DJ, Hooiveld G, Levenez F, Doré J, Dekker J, Samsom JN, Nieuwenhuis EE, Kleerebezem M (2012) Temporal and spatial interplay of microbiota and intestinal mucosa drive establishment of immune homeostasis in conventionalized mice. *Mucosal Immunol* 5:567–579. [CrossRef Medline](#)

Engler TM, Dourado CC, Amâncio TG, Farage L, de Mello PA, Padula MP (2014) Stroke: bowel dysfunction in patients admitted for rehabilitation. *Open Nurs J* 8:43–47. [CrossRef Medline](#)

Erny D, Hrabé de Angelis AL, Jaitin D, Wieghofer P, Staszewski O, David E, Keren-Shaul H, Mhlahkoiv T, Jakobshagen K, Buch T, Schwierzeck V, Utermohlen O, Chun E, Garrett WS, McCoy KD, Diefenbach A, Staeheli P, Stecher B, Amit I, Prinz M (2015) Host microbiota constantly control maturation and function of microglia in the CNS. *Nat Neurosci* 18:965–977. [CrossRef Medline](#)

Fu Y, Zhang N, Ren L, Yan Y, Sun N, Li YJ, Han W, Xue R, Liu Q, Hao J, Yu C, Shi FD (2014) Impact of an immune modulator fingolimod on acute ischemic stroke. *Proc Natl Acad Sci U S A* 111:18315–18320. [CrossRef Medline](#)

Gelderblom M, Leyboldt F, Steinbach K, Behrens D, Choe CU, Siler DA, Arumugam TV, Orthey E, Gerloff C, Tolosa E, Magnus T (2009) Temporal and spatial dynamics of cerebral immune cell accumulation in stroke. *Stroke* 40:1849–1857. [CrossRef Medline](#)

Gelderblom M, Weymar A, Bernreuther C, Velden J, Arunachalam P, Steinbach K, Orthey E, Arumugam TV, Leyboldt F, Simova O, Thom V, Friese MA, Prinz I, Hölscher C, Glatzel M, Korn T, Gerloff C, Tolosa E, Magnus T (2012) Neutralization of the IL-17 axis diminishes neutrophil invasion and protects from ischemic stroke. *Blood* 120:3793–3802. [CrossRef Medline](#)

Hofmann U, Frantz S (2015) Role of lymphocytes in myocardial injury, healing, and remodeling after myocardial infarction. *Circ Res* 116:354–367. [CrossRef Medline](#)

Hooper LV, Littman DR, Macpherson AJ (2012) Interactions between the microbiota and the immune system. *Science* 336:1268–1273. [CrossRef Medline](#)

Houlden A, Goldrick M, Brough D, Vizi ES, Lenart N, Martinecz B, Roberts IS, Denes A (2016) Brain injury induces specific changes in the caecal microbiota of mice via altered autonomic activity and mucoprotein production. *Brain Behav Immun*. In press.

Huang Y, Rabb H, Womer KL (2007) Ischemia-reperfusion and immediate T cell responses. *Cell Immunol* 248:4–11. [CrossRef Medline](#)

Human Microbiome Project Consortium (2012) Structure, function and diversity of the healthy human microbiome. *Nature* 486:207–214. [CrossRef Medline](#)

Iadecola C, Anrather J (2011) The immunology of stroke: from mechanisms to translation. *Nat Med* 17:796–808. [CrossRef Medline](#)

Ivanov II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, Wei D,

- Goldfarb KC, Santee CA, Lynch SV, Tanoue T, Imaoka A, Itoh K, Takeda K, Umesaki Y, Honda K, Littman DR (2009) Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* 139:485–498. [CrossRef Medline](#)
- Jander S, Kraemer M, Schroeter M, Witte OW, Stoll G (1995) Lymphocytic infiltration and expression of intercellular adhesion molecule-1 in photochemically induced ischemia of the rat cortex. *J Cereb Blood Flow Metab* 15:42–51. [CrossRef Medline](#)
- Karlsson FH, Fåk F, Nookaew I, Tremaroli V, Fagerberg B, Petranovic D, Bäckhed F, Nielsen J (2012) Symptomatic atherosclerosis is associated with an altered gut metagenome. *Nat Commun* 3:1245. [CrossRef Medline](#)
- Kent WJ (2002) BLAT—the BLAST-like alignment tool. *Genome Res* 12:656–664. [Medline](#)
- Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG (2010) Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol* 8:e1000412. [CrossRef Medline](#)
- Kleinschnitz C, Schwab N, Kraft P, Hagedorn I, Dreykluft A, Schwarz T, Austinat M, Nieswandt B, Wiendl H, Stoll G (2010) Early detrimental T-cell effects in experimental cerebral ischemia are neither related to adaptive immunity nor thrombus formation. *Blood* 115:3835–3842. [CrossRef Medline](#)
- Lee YK, Menezes JS, Umesaki Y, Mazmanian SK (2011) Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci U S A* 108:4615–4622. [CrossRef Medline](#)
- Liesz A, Suri-Payer E, Veltkamp C, Doerr H, Sommer C, Rivest S, Giese T, Veltkamp R (2009a) Regulatory T cells are key cerebroprotective immunomodulators in acute experimental stroke. *Nat Med* 15:192–199. [CrossRef Medline](#)
- Liesz A, Hagmann S, Zschoche C, Adamek J, Zhou W, Sun L, Hug A, Zorn M, Dalpke A, Nawroth P, Veltkamp R (2009b) The spectrum of systemic immune alterations after murine focal ischemia: immunodepression versus immunomodulation. *Stroke* 40:2849–2858. [CrossRef Medline](#)
- Liesz A, Zhou W, Mracsko É, Karcher S, Bauer H, Schwarting S, Sun L, Bruder D, Stegemann S, Cerwenka A, Sommer C, Dalpke AH, Veltkamp R (2011) Inhibition of lymphocyte trafficking shields the brain against deleterious neuroinflammation after stroke. *Brain* 134:704–720. [CrossRef Medline](#)
- Liesz A, Karcher S, Veltkamp R (2013a) Spectratype analysis of clonal T cell expansion in murine experimental stroke. *J Neuroimmunol* 257:46–52. [CrossRef Medline](#)
- Liesz A, Rüter H, Purrucker J, Zorn M, Dalpke A, Möhlenbruch M, Englert S, Nawroth PP, Veltkamp R (2013b) Stress mediators and immune dysfunction in patients with acute cerebrovascular diseases. *PLoS One* 8:e74839. [CrossRef Medline](#)
- Liesz A, Zhou W, Na SY, Hämmerling GJ, Garbi N, Karcher S, Mracsko E, Backs J, Rivest S, Veltkamp R (2013c) Boosting regulatory T cells limits neuroinflammation in permanent cortical stroke. *J Neurosci* 33:17350–17362. [CrossRef Medline](#)
- Liesz A, Dalpke A, Mracsko E, Antoine DJ, Roth S, Zhou W, Yang H, Na SY, Akhisaroglu M, Fleming T, Eigenbrod T, Nawroth PP, Tracey KJ, Veltkamp R (2015) DAMP signaling is a key pathway inducing immune modulation after brain injury. *J Neurosci* 35:583–598. [CrossRef Medline](#)
- Llovera G, Roth S, Plesnila N, Veltkamp R, Liesz A (2014) Modeling stroke in mice: permanent coagulation of the distal middle cerebral artery. *J Vis Exp* e51729.
- Lucin KM, Wyss-Coray T (2009) Immune activation in brain aging and neurodegeneration: too much or too little? *Neuron* 64:110–122. [CrossRef Medline](#)
- Magrone T, Jirillo E (2013) The interaction between gut microbiota and age-related changes in immune function and inflammation. *Immun Ageing* 10:31. [CrossRef Medline](#)
- Meisel C, Schwab JM, Prass K, Meisel A, Dirnagl U (2005) Central nervous system injury-induced immune deficiency syndrome. *Nat Rev Neurosci* 6:775–786. [Medline](#)
- Meyer F, Paarmann D, D'Souza M, Olson R, Glass EM, Kubal M, Paczian T, Rodriguez A, Stevens R, Wilke A, Wilkening J, Edwards RA (2008) The metagenomics RAST server: a public resource for the automatic phylogenetic and functional analysis of metagenomes. *BMC Bioinformatics* 9:386. [CrossRef Medline](#)
- Mracsko E, Liesz A, Karcher S, Zorn M, Bari F, Veltkamp R (2014) Differential effects of sympathetic nervous system and hypothalamic-pituitary-adrenal axis on systemic immune cells after severe experimental stroke. *Brain Behav Immun* 41:200–209. [CrossRef Medline](#)
- Neumann J, Riek-Burchardt M, Herz J, Doeppner TR, König R, Hütten H, Etemire E, Männ L, Klingberg A, Fischer T, Görtler MW, Heinze HJ, Reichardt P, Schraven B, Herrmann DM, Reymann KG, Gunzer M (2015) Very-late-antigen-4 (VLA-4)-mediated brain invasion by neutrophils leads to interactions with microglia, increased ischemic injury and impaired behavior in experimental stroke. *Acta Neuropath* 129:259–277. [CrossRef Medline](#)
- Offner H, Subramanian S, Parker SM, Wang C, Afentoulis ME, Lewis A, Vandenbark AA, Hurn PD (2006) Splenic atrophy in experimental stroke is accompanied by increased regulatory T cells and circulating macrophages. *J Immunol* 176:6523–6531. [CrossRef Medline](#)
- Olsen AB, Hetz RA, Xue H, Aroom KR, Bhattarai D, Johnson E, Bedi S, Cox CS Jr, Uray K (2013) Effects of traumatic brain injury on intestinal contractility. *Neurogastroenterol Motil* 25:593–e463. [CrossRef Medline](#)
- Round JL, Mazmanian SK (2010) Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. *Proc Natl Acad Sci U S A* 107:12204–12209. [CrossRef Medline](#)
- Schroeter M, Jander S, Witte OW, Stoll G (1994) Local immune responses in the rat cerebral cortex after middle cerebral artery occlusion. *J Neuroimmunol* 55:195–203. [CrossRef Medline](#)
- Schwartz M, Raposo C (2014) Protective Autoimmunity: A Unifying Model for the Immune Network Involved in CNS Repair. *Neuroscientist* 20:343–358. [CrossRef Medline](#)
- Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, Huttenhower C (2011) Metagenomic biomarker discovery and explanation. *Genome Biol* 12:R60. [CrossRef Medline](#)
- Shichita T, Sugiyama Y, Ooboshi H, Sugimori H, Nakagawa R, Takada I, Iwaki T, Okada Y, Iida M, Cua DJ, Iwakura Y, Yoshimura A (2009) Pivotal role of cerebral interleukin-17-producing gammadeltaT cells in the delayed phase of ischemic brain injury. *Nat Med* 15:946–950. [CrossRef Medline](#)
- Shichita T, Hasegawa E, Kimura A, Morita R, Sakaguchi R, Takada I, Sekiya T, Ooboshi H, Kitazono T, Yanagawa T, Ishii T, Takahashi H, Mori S, Nishibori M, Kuroda K, Akira S, Miyake K, Yoshimura A (2012) Peroxiredoxin family proteins are key initiators of post-ischemic inflammation in the brain. *Nat Med* 18:911–917. [CrossRef Medline](#)
- Singh V, Roth S, Veltkamp R, Liesz A (2016) HMGb1 as a key mediator of immune mechanisms in ischemic stroke. *Antioxid Redox Signal* 24:635–651. [CrossRef Medline](#)
- Swidsinski A, Loening-Baucke V, Krüger M, Kirsch S (2012) Central nervous system and the colonic bioreactor: analysis of colonic microbiota in patients with stroke unravels unknown mechanisms of the host defense after brain injury. *Intestinal Research* 10:332–342. [CrossRef](#)
- Vilz TO, Overhaus M, Stoffels B, Websky M, Kalf J, Wehner S (2012) Functional assessment of intestinal motility and gut wall inflammation in rodents: analyses in a standardized model of intestinal manipulation. *J Vis Exp* 67: pii: 4086. [CrossRef Medline](#)
- Vogelgesang A, May VE, Grunwald U, Bakkeboe M, Langner S, Wallaschofski H, Kessler C, Bröker BM, Dressel A (2010) Functional status of peripheral blood T-cells in ischemic stroke patients. *PLoS One* 5:e8718–e8718. [CrossRef Medline](#)
- Yilmaz G, Arumugam TV, Stokes KY, Granger DN (2006) Role of T lymphocytes and interferon-gamma in ischemic stroke. *Circulation* 113:2105–2112. [CrossRef Medline](#)
- Yin J, Liao SX, He Y, Wang S, Xia GH, Liu FT, Zhu JJ, You C, Chen Q, Zhou L, Pan SY, Zhou HW (2015) Dysbiosis of gut microbiota with reduced trimethylamine-N-oxide level in patients with large-artery atherosclerotic stroke or transient ischemic attack. *J Am Heart Assoc* 4: pii: e002699. [CrossRef Medline](#)
- Zhou W, Liesz A, Bauer H, Sommer C, Lahrmann B, Valous N, Grabe N, Veltkamp R (2013) Posts ischemic brain infiltration of leukocyte subpopulations differs among murine permanent and transient focal cerebral ischemia models. *Brain Pathol* 23:34–44. [CrossRef Medline](#)
- Zhu Z, Fu Y, Tian D, Sun N, Han W, Chang G, Dong Y, Xu X, Liu Q, Huang D, Shi FD (2015) Combination of the immune modulator fingolimod with alteplase in acute ischemic stroke: a pilot trial. *Circulation* 132:1104–1112. [CrossRef Medline](#)

GUT MICROBIOME PRIMES A CEREBROPROTECTIVE IMMUNE RESPONSE AFTER STROKE

SUMMARY

Within the next publication we continued with the notion that the gut microbiota-induced immune cell alterations would alter stroke outcome. However, within this study we wanted to characterise the importance of the presence of the microbiome as a mediator of immunity. We compared three groups GF, Ex-GF (GF recolonised) and conventional specific pathogen free (SPF) mice allowing us to rule out potential confounding developmental immune deficits of GF mice as previously reported (Falk, Hooper et al., 1998). Due to the coprophagic behaviour of mice, GF mice were naturally colonised three weeks prior to experimentation by housing with SPF mice. We performed dMCAo surgery and saw that GF mice had larger stroke lesions compared to Ex-GF and SPF mice. This was associated with a higher microglial cell count and increased levels of proinflammatory cytokines; however, we did not observe an alteration in microglia morphology. This suggested that the absence of the microbiota was not neuroprotective and also worsened stroke outcome. In the peripheral immune compartment's, within spleen and the gut (PP) we saw a general expansion in the number of immune cells of Ex-GF, compared to GF mice. Additionally, this was not found to be in certain subsets of T cells as both proinflammatory Th17, and anti-inflammatory Tregs were both increased. The microbiota presence did not affect stroke outcome in GF Rag^{-/-} mice compared SPF Rag^{-/-} mice as infarct size did not change. This study once again highlighted the influential ability of the gut microbiome on T and B cells in the context of stroke outcome.

REFERENCE

Author contributions: VS, RS, SH, GL, SR, CB performed experiments; VS, RS, CB and AL analysed data, VS and AL wrote the manuscript; AL conceived the study and supervised the project.

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The gut microbiome primes a cerebroprotective immune response after stroke

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Abstract

Microbiome alterations have been shown to affect stroke outcome. However, to what extent the presence of a gut microbiome per se is affecting post-stroke neuroinflammation has not been tested. By comparing germfree mice with recolonized (Ex-GF) and conventional SPF mice, we were able to demonstrate that bacterial colonization reduces stroke volumes. Bacterial colonization increased cerebral expression of cytokines as well as microglia/macrophage cell counts in contrast to improved stroke outcome. Interestingly, the microbiome-mediated brain protection was absent in lymphocyte-deficient mice. These findings support the concept of lymphocyte-driven protective neuroinflammation after stroke under control of the microbiome.

Keywords

Stroke, microbiota, germfree, T cells, microglia, neuroinflammation

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Introduction

Stroke triggers a neuroinflammatory reaction which encompasses activation of brain resident microglia and invasion of leukocytes.¹ T cells are potent contributors to post-stroke neuroinflammation. While pro-inflammatory T_{helper} cell subpopulations (e.g. Th1, Th17) promote neuroinflammation, other populations with anti-inflammatory properties (e.g. T_{reg} cells) can be cerebroprotective. Current findings have highlighted the role of commensal gut microbiota in the regulation of T cell responses to brain ischemia.^{2,3} Recently, we have shown that stroke changes the bacterial composition in the gut, which was causally linked to a pro-inflammatory T cell polarization and worse stroke outcome.² Similarly, a study by Benakis et al.³ demonstrated the impact of antibiotic treatment-induced dysbiosis on stroke outcome. Although these first studies demonstrated a previously unrecognized key role of changes in microbiota composition to affect stroke outcome, we are still lacking the proof-of-concept that bacterial colonization per se affects stroke outcome and post-stroke neuroinflammation. In order to fill this

gap, we investigated stroke outcome and neuroinflammation in GF animals and colonized littermates (Ex-GF) and specifically analyzed the role of T cells as potential mediators along the gut-brain axis in stroke.

Material and methods

Detailed material and methods can be found in the online Supplementary material. All original raw data of this study as well as detailed protocols are available from the corresponding author upon reasonable request.

All animal experiments were performed under the institutional guidelines for the use of animals for

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research and were approved by the governmental ethics committee of Upper Bavaria (Regierungspraesidium Oberbayern). GF mice were housed in sterile HAN-gnotocages and received sterile food pellets and water. All surgical procedures including stroke induction, mouse handling and cage changes were performed under sterile conditions in a sterile microbiological laminar flow (Supplementary Figure 1(a)). Germfree status of GF mice was confirmed after surgery and survival period by 16s PCR of fecal samples. Littermates of GF mice were colonized through co-housing with conventional specific pathogen-free (SPF) C57BL/6J mice for three weeks and co-housing was maintained also after stroke surgery. 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 animal experiments were performed and reported according to the ARRIVE guidelines.¹

Results

Bacterial colonization is cerebroprotective after stroke

Stroke was induced in GF, Ex-GF and SPF mice in order to test the impact of bacterial colonization on stroke outcome (Figure 1(a)). All surgical procedures were performed in a sterile microbiological safety cabinet (Supplementary Figure 1(a)). Reduced caecum-body weight index increased total fecal DNA and eubacteria amount verified bacterial colonization in Ex-GF mice as well as maintaining the germfree status after surgery in GF mice (Supplementary Figure 1(b) to (d)). Sequencing of bacterial 16s rRNA showed comparable alpha diversity and abundance of bacterial phyla between Ex-GF and SPF mice as a key markers of successful colonization in the Ex-GF group (Supplementary Figure 1(e) and (f)). Quantification of infarct volume five days after stroke induction revealed an improved stroke outcome in Ex-GF and SPF mice compared to GF littermates (Figure 1(b)). This substantial effect of colonization on lesion volume has been consistently observed in three independent experiments performed by three different surgeons. After confirmation of comparable outcome and colonization in SPF and Ex-GF animals, we used Ex-GF generated by randomized colonization of GF littermates as the favourable “colonized” control group.

Gut microbiota increase the neuroinflammatory response after stroke

In order to test the impact of bacterial colonization on the local neuroinflammatory milieu, we investigated cell

counts and morphology of microglia/macrophages as well as key transcriptional markers of innate immune responses after stroke. In contrast to reduced lesion volumes, we found an increased number of microglia/macrophages in the ischemic hemisphere of Ex-GF mice compared to the GF group (Figure 1(c) and (d)). Automated analysis of microglial morphology⁴ revealed massive activation of microglia in the peri-infarct area characterized by enhanced sphericity index and reduced ramifications compared to the contralateral side (Figure 1(e)). While bacterial recolonization did not further affect already activated peri-lesional microglia/macrophages, a significant effect was observed on cell sphericity and ramifications in the contralateral hemispheres in Ex-GF mice (Figure 1(e)). Accordingly, we also observed a significant increase in the transcriptional regulation of pro-inflammatory cytokine expression associated with microglial activation such as *Il-1 β* and *Tnf- α* (Figure 1(f)).

Cerebroprotection is mediated by microbiome-induced T cell priming

Previous studies using models of altered microbiota composition have proposed modification in lymphocyte priming and particularly in T cell polarization as key mediators of the microbiome effect on stroke outcome.^{2,3,5} Therefore, we first sought to determine the impact of the gut microbiome on T cell priming in secondary lymphatic organs by flow cytometry. While the overall leukocyte count was unaltered between GF and Ex-GF animals, we detected increased T and B cells counts in spleens of Ex-GF compared to GF animals (Figure 2(a)). More specifically, we observed an increase in overall T_{helper} cell (CD4⁺) counts as well as polarized regulatory T cells (Foxp3⁺) and Th17 cells (RoR γ t⁺) in the intestinal immune compartments of the Peyer's patches (Figure 2(b) and (c)) and even more pronounced in the spleens of Ex-GF compared to GF mice at five days after stroke (Figure 2(d)). Interestingly, we observed a similar pattern in the ischemic brain with increased mRNA levels for specific T_{helper} cell subpopulations including *Foxp3* and *Il-10* (T_{reg}) and *Il-17* (Th17) in Ex-GF animals compared to GF littermates (Figure 2(e)). Additionally, we observed by flow cytometry also an increase in total T cell counts in the ischemic hemispheres of Ex-GF compared to GF animals (Figure 2(f)). These results implicate that a physiological gut microbiome is required for generating an adequate lymphocyte-driven immune reaction in response to brain injury and execute tissue protection. Because of this pronounced effect of bacterial colonization on post-stroke T cell responses, we examined the role of

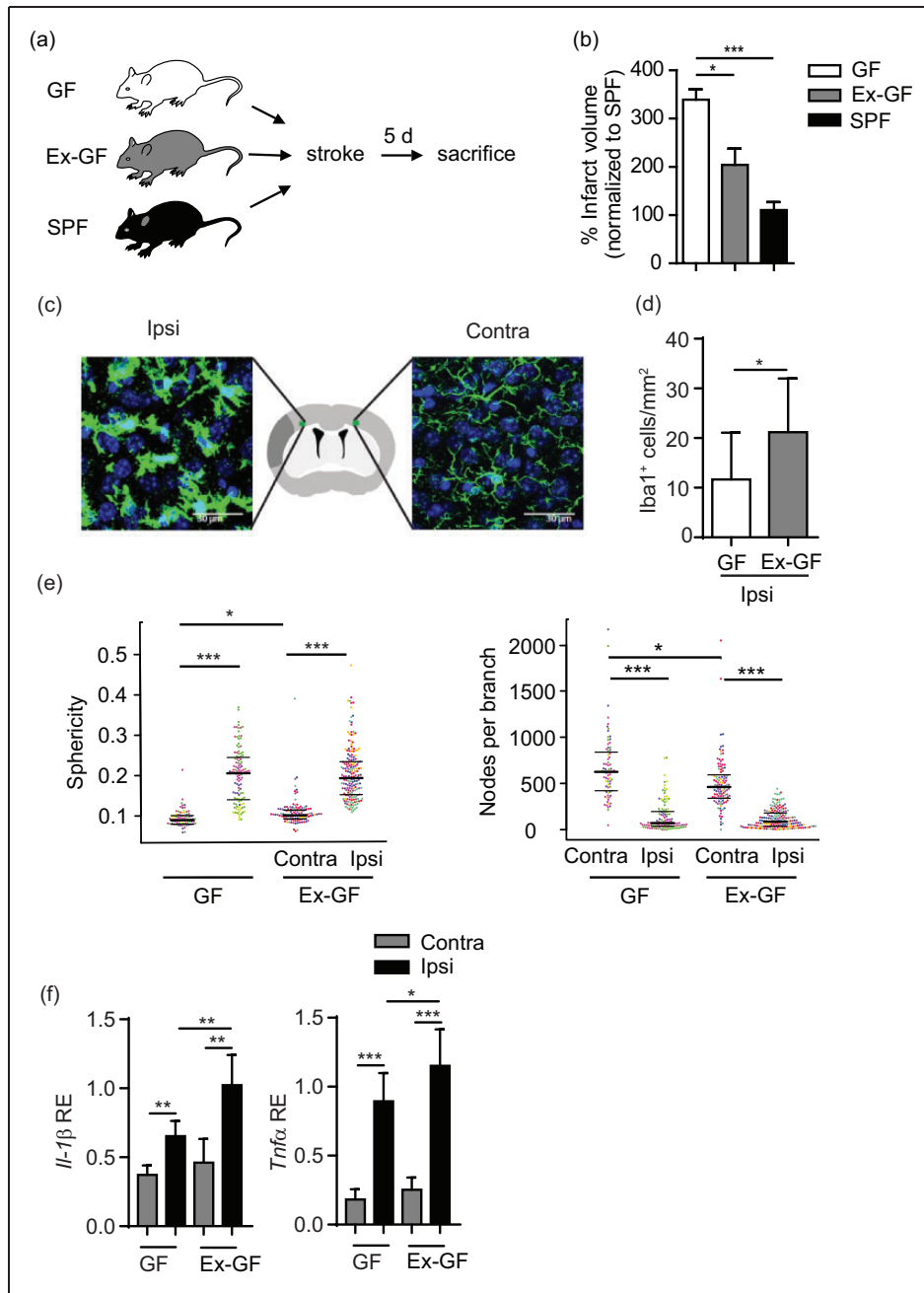


Figure 1. Bacterial colonization is cerebroprotective after stroke. (a) Schematic illustration of experimental paradigm. (b) Quantification of infarct volume in germfree (GF), colonized (Ex-GF) and SPF mice ($n = 12$ per group). Data are shown as mean \pm SD normalized to the mean of the SPF group as 100%. (c) Representative images of Iba-1 stained microglia/macrophages with schematic illustration of imaging locations in the peri-infarct area 900 μm distant from the border of the infarct core in the deep cortical layer 4/5 and homotypical contralateral area. (d) Analysis of microglia cell counts ($n = 5$ per group) in the ipsilateral cortex. (e) Analysis of microglial morphology showing increased sphericity (left) and reduced ramification (right) in ipsilateral hemispheres as markers of microglial activation. (f) Gene expression analysis by RT-PCR for the indicated genes of mainly microglial/macrophage origin in ipsilateral and contralateral hemispheres after stroke ($n = 10\text{--}12$ per group, mean \pm SD). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

lymphocytes in mediating the cerebroprotective effects of the microbiome in lymphocyte-deficient *Rag1*^{-/-} mice. Lesions were smaller in *Rag1*^{-/-} mice compared to wild-type C57BL/6J mice. However, we found no

difference in lesion volumes between GF *Rag1*^{-/-} mice and conventional *Rag1*^{-/-} mice, supporting the finding that lymphocytes play an important role in the microbiome-mediated effects on stroke

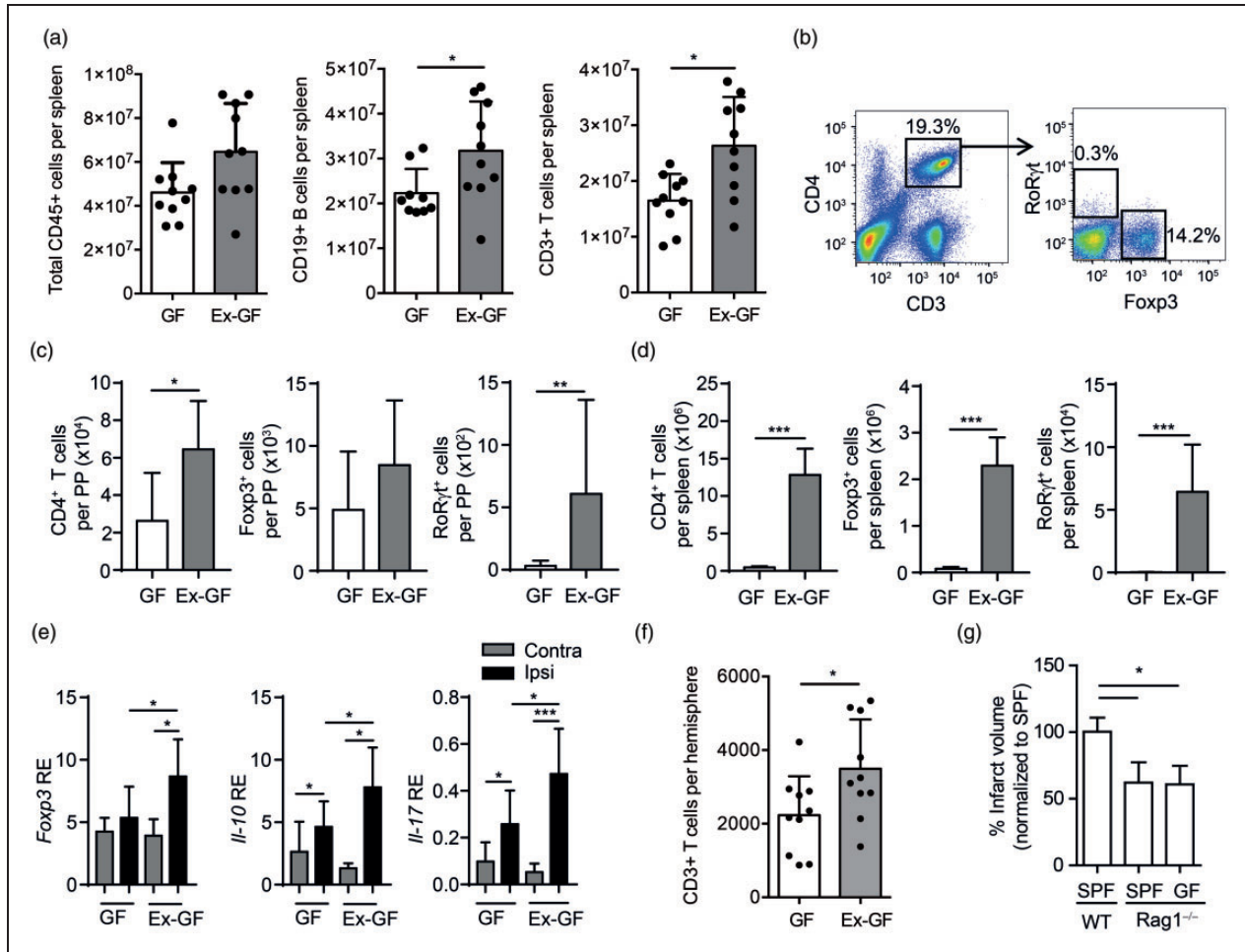


Figure 2. T cell priming by the gut microbiome is cerebroprotective. (a) Flow cytometric analysis of absolute cell counts per spleen in GF and Ex-GF animals for total leukocytes (CD45+), B cells (CD19+) and T cells (CD3+). While total leukocytes counts were unaffected, bacterial colonization significantly increase T and B cells counts in Ex-GF mice. (b) Representative gating strategy for the flow cytometric analysis of T_{helper} cell counts and subsets of Foxp3⁺ (T_{reg}) and RoRyt⁺ (T_{H17}) cells in (c) the Peyer's patches and in (d) the spleen. (e) (c) RT-PCR for expression T_{helper} cell markers *Foxp3*, *Il-10* and *Il-17* in ipsilateral and contralateral brain hemispheres five days after stroke ($n = 10-12$ per group). (f) Flow cytometric analysis of ipsilateral brain hemispheres five days after stroke in GF and Ex-GF revealing significantly increased T cell counts in the ischemic hemispheres of recolonized Ex-GF animals. (g) Brain infarct volumes in SPF WT, SPF Rag1^{-/-} and GF Rag1^{-/-} mice ($n = 7-9$ per group, mean \pm SD normalized to SPF group as 100%). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

(Figure 2(g)). Additionally, we did not detect a significant impact of gut bacterial colonization on blood-brain barrier integrity or cortical capillary density (Supplementary Figure 2).

Discussion

This study provides a proof-of-concept that the gut microbiome per se is cerebroprotective in experimental stroke. These are novel and relevant insights for the gut-brain axis field in stroke as well as other models of acute brain injury because previous studies have so far exclusively investigated the impact of alterations in

bacterial composition on disease outcome but did not test the relevance of the microbiome as a bona fide modulator of secondary neuroinflammation.

These experiments were so far lacking due to the technical difficulties of GF mouse handling and surgical stroke induction. We have overcome these technical limitations by on one side ensuring germfree status of animals despite surgical manipulation and on the other side establishing natural recolonization as comparable to conventional SPF mice on stroke outcome, which allows the study of littermates (GF and Ex-GF) for improved comparability. Ex-GF mice were chosen as a superior control group for colonized

animals compared to SPF mice which have substantial disadvantages including potential differences due to genetic drift, epigenetic modifications and housing conditions.

We have previously reported that large stroke lesions induce changes in the gut microbiome, leading to a pro-inflammatory over-activation of peripheral immune responses and worse stroke outcome.² In contrast, we show here that also the complete lack of a gut flora leads to enlarged brain lesions compared to colonized littermates or conventionally housed SPF mice. This notion contradicts the widely accepted concept that the secondary inflammatory response to tissue injury is per se “detrimental” and—in the case of acute brain injury—“neurotoxic.”¹ However, recent reports revised this paradigm of secondary neuroinflammation as “too much of a bad thing” by considering secondary neuroinflammation to be a physiologically relevant protective mechanism in which both excessive immune activation and immunosuppression can be harmful.⁶

By investigating germfree, lymphocyte-deficient *Rag1*^{-/-} mice, we provide evidence supporting a key role for lymphocytes along the gut-brain axis. Our results demonstrate that lymphocytes are required to generate a neuroinflammatory milieu that is associated with a reduction of secondary lesion expansion. We observed that a lack of bacterial colonization was associated significantly with an increased expression of pro-inflammatory cytokines in the ischemic hemisphere. On the other side, germfree animals displayed reduced overall microglia/macrophage cell counts and a reduction in pro-inflammatory cytokine expression. These findings could indicate a dysfunctional, non-physiological microglial response to the ischemic injury which is associated with secondary neurological deterioration. Moreover, lymphocyte-dependent neuroprotection under the control of the gut microbiome is likely to be mediated also via non-immunological mechanisms that contribute to lesion growth and recovery such as growth factor secretion by lymphocytes, modulation of neurogenesis or impaired vascular function^{7,8} although our results exclude direct major effects on vascular function. Additionally, while the role of B cells is less investigated in stroke-immunology as well as the gut-brain research field compared to T cells, the expansion of splenic B cell counts and the lack of mature B cells in *Rag1*^{-/-} mice warrant additional experiments investigating the contribution of B cells to the observed phenomenon.

Taken together, future experiments which were beyond the scope of this proof-of-concept study are urgently needed for the in-depth analysis of the potential pathways of lymphocyte–brain interaction that lead

to neuroprotection under control of the microbiome. Our findings open up several new questions such as the mode of microbiome interaction with intestinal lymphocytes, the involved bacterial mediators and/or antigens. Finally, the druggability of microbiome-lymphocyte interaction to prime a beneficial, pro-regenerative immune response is of great future interest. More detailed understanding of the gut-immune-brain axis in stroke could open up a new field of microbiome-targeted therapies for stroke patients.

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DNA sequences have been deposited in MG-RAST (<http://metagenomics.anl.gov/>) under accession number #9778. We thank Kathleen McCoy (University of Bern, Switzerland) for providing GF mice.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Authors' contributions

VS, RS, SH, GL, SR, CB performed experiments; VS, RS, CB and AL analyzed data, VS and AL wrote the manuscript; AL conceived the study and supervised the project.

Supplementary material

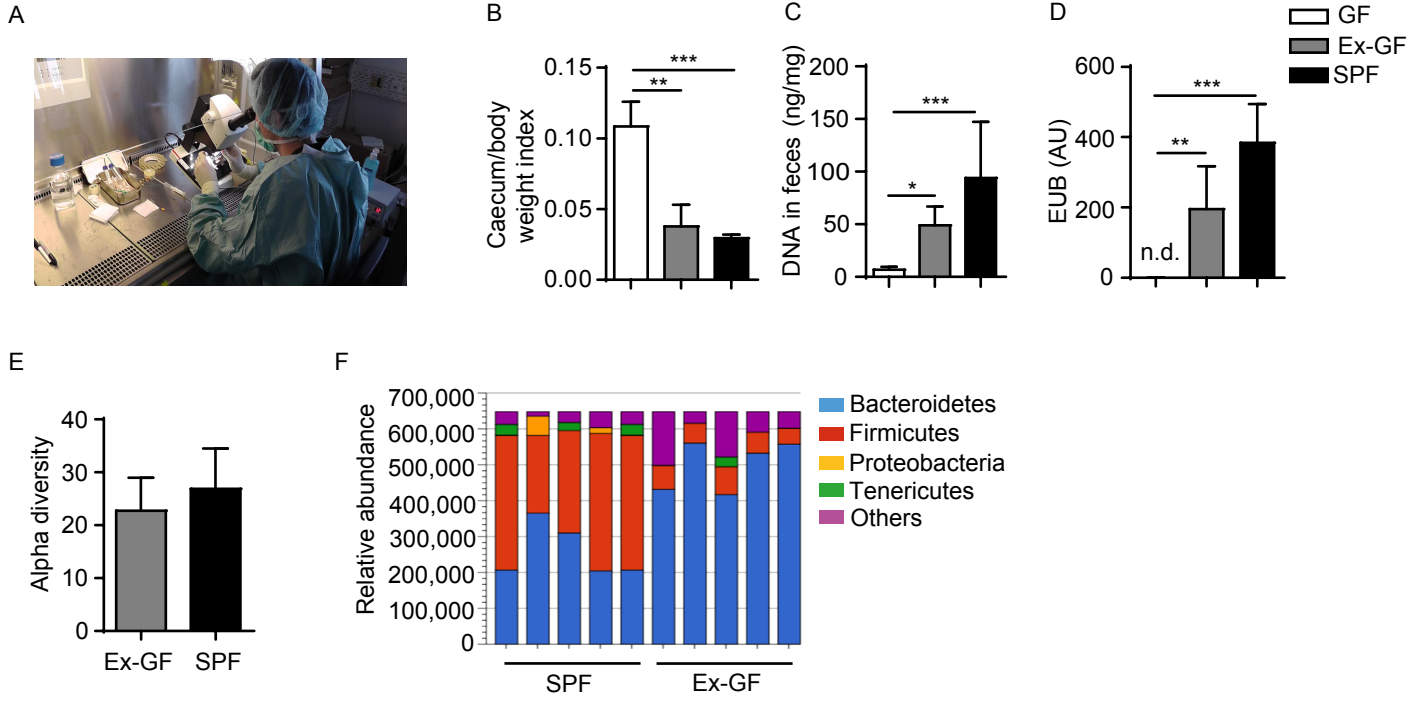
Supplementary material for this paper can be found at the journal website: <http://journals.sagepub.com/home/jcb>

References

1. Iadecola C and Anrather J. The immunology of stroke: from mechanisms to translation. *Nat Med* 2011; 17: 796–808.
2. Singh V, Roth S, Llovera G, et al. Microbiota dysbiosis controls the neuroinflammatory response after stroke. *J Neurosci*. 2016; 36: 7428–7440.
3. Benakis C, Brea D, Caballero S, et al. Commensal microbiota affects ischemic stroke outcome by regulating intestinal gammadelta T cells. *Nat Med* 2016; 22: 516–523.
4. Heindl S, Gesierich B, Benakis C, et al. Automated morphological analysis of microglia after stroke. *Front Cell Neurosci*. Epub ahead of print 19 April 2018. DOI: 10.3389/fncel.2018.00106.
5. Sadler R, Singh V, Benakis C, et al. Microbiota differences between commercial breeders impacts the post-stroke immune response. *Brain Behav Immun* 2017; 66: 23–30.

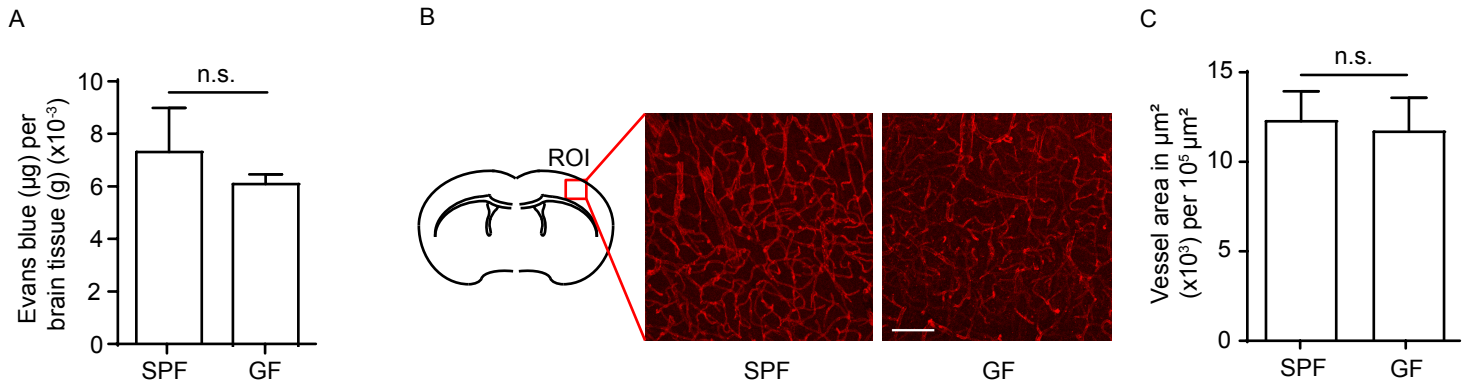
6. Schwartz M and Kipnis J. Protective autoimmunity and neuroprotection in inflammatory and noninflammatory neurodegenerative diseases. *J Neurol Sci* 2005; 233: 163–166.
7. Ziv Y, Ron N, Butovsky O, et al. Immune cells contribute to the maintenance of neurogenesis and spatial learning abilities in adulthood. *Nat Neurosci* 2006; 9: 268–275.
8. De Meyer SF, Denorme F, Langhauser F, et al. Thromboinflammation in Stroke Brain Damage. *Stroke* 2016; 47: 1165–1172.

Supplementary Figure 1



Supplementary Figure 1. Maintenance 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 \pm 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 \pm 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 guidelines¹.

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 arteries². 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 described³. Briefly, after perfusion with saline, the ipsilateral brain hemispheres were removed and collected in 200 μl 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 37°C, 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), ROR γt 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 FACSVerser 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 μ m 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[™], 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 μ m for analysis of microglial morphology. Cell count and microglial morphology was assessed at a ROI 900 μ m 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 described¹⁰.

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-1 β* (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 published². 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.

References

1. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: The arrive guidelines for reporting animal research. *PLoS Biol.* 2010;8:e1000412
2. Llovera G, Roth S, Plesnila N, Veltkamp R, Liesz A. Modeling stroke in mice: Permanent coagulation of the distal middle cerebral artery. *J Vis Exp.* 2014:e51729
3. Llovera G, Benakis C, Enzmann G, Cai R, Arzberger T, Ghasemigharagoz A, et al. The choroid plexus is a key cerebral invasion route for t cells after stroke. *Acta Neuropathol.* 2017;134:851-868
4. Team R. *Rstudio: Integrated development for r.* Boston, MA: RStudio, Inc.; 2016.
5. R Core Team. *A language and environment for statistical computing.* Vienna, Austria: R Foundation for Statistical Computing; 2016.
6. Sing T, Sander O, Beerenwinkel N, Lengauer T. ROCR: Visualizing classifier performance in r. *Bioinformatics.* 2005;21:3940-3941
7. Wickham H. The split-apply-combine strategy for data analysis. *Journal of Statistical Software.* 2011;40:1-29
8. Eklund A. Beeswarm: The bee swarm plot, an alternative to stripchart. R package version 0.2.3. 2016
9. Wei TS, Viliam. Corrplot: Visualization of a correlation matrix. R package version 0.77. 2016
10. Heindl S, Gesierich B, Benakis C, Llovera G, Duering M, Liesz A. Automated morphological analysis of microglia after stroke. *Front. Cell. Neurosci.* 2018
11. Sadler R, Singh V, Benakis C, Garzetti D, Brea D, Stecher B, et al. Microbiota differences between commercial breeders impacts the post-stroke immune response. *Brain Behav Immun.* 2017;66:23-30
12. Singh V, Roth S, Llovera G, Sadler R, Garzetti D, Stecher B, et al. Microbiota dysbiosis controls the neuroinflammatory response after stroke. *J Neurosci.* 2016;36:7428-7440

MICROBIOTA DIFFERENCES BETWEEN COMMERCIAL BREEDERS IMPACTS THE POST-STROKE IMMUNE RESPONSE

SUMMARY

Reproducibility of pre-clinical experiments and inter-laboratory differences are all components which contribute to the so-called reproducibility crisis. However, understanding the hurdle between laboratory and clinical care may aid to better understand failed trials. For the second aim, we designed a study to model the impact of the gut microbiota as a possible source for inconsistencies. We obtained C57BL/6 mice from 3 different commercial breeders across Europe CR, Taconic Biosciences (Tac) and Harlan (Har) (now known as Envigo)). 16S RNA sequencing analysis revealed substantial changes in the diversity and in the composition of the microbiota. Furthermore, we saw that Th17-inducing bacterial species named SFB, were lower or absent in the faeces of CR mice compared to Tac and Har mice. By flow cytometry we found that SFB presence correlated to higher levels of IL-17 cells, and lower levels of Tregs, as found in Tac and Har mice. On the contrary, CR mice had high percentages of Tregs and lower IL-17 cells. In another experiment we addressed whether SFB presence was causative of changes in gut immune cell polarisation. By cohousing SFB negative mice with SFB positive mice, we created SFB recolonised mice. This indeed was enough to increase IL-17+ cells and reduce Treg cell populations. We therefore decided to use this paradigm to test CD28SA an antibody previously shown to expand Tregs; however within basic stroke research in rodent models had contradictive results (Na, Mracsko et al., 2015, Schuhmann, Kraft et al., 2015). Upon treatment with CD28SA we saw that mice deficient of SFB did not experience a peripheral expansion of the Treg population compared to controls. This was on the contrary to SFB positive mice which did experience Treg expansion. Due to the changes within the immune system under CD28SA, SFB negative mice that underwent dMCAo surgery had significantly larger infarct volumes compared to mice who did not receive the treatment. Taken together this further highlighted the intimate interaction of the gut microbiota and immune system, but also showed that immunogenic therapies which experience a contraction in results may turn to the microbiota as a possible confounding factor.

REFERENCE

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Microbiota differences between commercial breeders impacts the post-stroke immune response



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ABSTRACT

Experimental reproducibility between laboratories is a major translational obstacle worldwide, particularly in studies investigating immunomodulatory therapies in relation to brain disease. In recent years increasing attention has been drawn towards the gut microbiota as a key factor in immune cell polarization. Moreover, manipulation of the gut microbiota has been found effective in a diverse range of brain disorders. Within this study we aimed to test the impact of microbiota differences between mice from different sources on the post-stroke neuroinflammatory response. With this rationale, we have investigated the correlation between microbiota differences and the immune response in mice from three commercial breeders with the same genetic background (C57BL/6). While overall bacterial load was comparable, we detected substantial differences in species diversity and microbiota composition on lower taxonomic levels. Specifically, we investigated segmented filamentous bacteria (SFB)—which have been shown to promote T cell polarization—and found that they were absent in mice from one breeder but abundant in others. Our experiments revealed a breeder specific correlation between SFB presence and the ratio of Treg to Th17 cells. Moreover, recolonization of SFB-negative mice with SFB resulted in a T cell shift which mimicked the ratios found in SFB-positive mice. We then investigated the response to a known experimental immunotherapeutic approach, CD28 superagonist (CD28SA), which has been previously shown to expand the Treg population. CD28SA treatment had differing effects between mice from different breeders and was found to be ineffective at inducing Treg expansion in SFB-free mice. These changes directly corresponded to stroke outcome as mice lacking SFB had significantly larger brain infarcts. This study demonstrates the major impact of microbiota differences on T cell polarization in mice during ischemic stroke conditions, and following immunomodulatory therapies.

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1. Introduction

Stroke is a devastating disease and the second most common cause of death (Donnan et al., 2008). Over the last decade, it is neuroinflammation occurring at hours to days post lesion, which has

been established as a key therapeutic target used to inhibit secondary infarct progression and improve stroke outcome (Dirnagl et al., 1999; Iadecola and Anrather, 2011). A critical event during post-stroke neuroinflammation is the invasion of circulating leukocytes into the ischemic brain (Gelderblom et al., 2009). In particular, it is during 3–5 days after stroke where the infiltration of T cells into the brain parenchyma is known to be key in exacerbating secondary neuronal apoptosis and worsening stroke outcome (Kleinschnitz et al., 2010). Interestingly, while pro-inflammatory T cell subpopulations (i.e. Th1 and Th17) are neurotoxic and contribute to the inflammatory collateral damage after stroke (Gelderblom et al., 2009; Shichita et al., 2009), the immunosuppressive subpopulation of regulatory T cells (Treg) have been shown to be neuroprotective after experimental brain ischemia

Abbreviations: Treg, T regulatory cells; GALT, gut-associated-lymphoid tissue; PP, Peyer's patches; CR, Charles River Laboratories; Tac, Taconic Biosciences; Har, Harlan Laboratories; Jax, The Jackson Laboratory; CD28SA, CD28 super-agonist; SPF, specific-pathogen-free; SFB, segmented filamentous bacteria; dMCAo, distal middle cerebral artery occlusion.

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(Liesz and Kleinschnitz, 2016; Liesz et al., 2009). The mechanisms of T cell polarization before entry into the brain has been a key question in the field of stroke-immunology; for example either the polarization towards a neuroprotective Treg or harmful IL-17/Th17 cell phenotype. The gut-associated-lymphoid tissue (GALT) is where the majority of immune cells reside in the body (Brandtzaeg et al., 1989). Furthermore, the gut itself harbors a readily adapting composition of bacteria known as the gut microbiota which acts as the main source of antigens and immune-active mediators within the gut (Lathrop et al., 2011). It has been reproducibly shown that segmented filamentous bacteria (SFB) are able to specifically increase IL-17-producing Th17 cells (Ivanov et al., 2009; Round and Mazmanian, 2009). It has become evident that the gut microbiota plays a key role in driving autoimmune mechanisms and the inflammatory response in a number of diverse brain disorders. For example, in one study using experimental autoimmune encephalomyelitis (EAE), the absence of the microbiota in germ-free mice was enough to alleviate the onset of symptoms (Berer et al., 2011) while the induction of Tregs by *B. fragilis* antigen was able to alleviate EAE symptoms (Ochoa-Reparaz et al., 2010). Finally, we have recently demonstrated that alteration of the gut microbiota can have a major impact on secondary neuroinflammation and outcome after experimental brain ischemia (Benakis et al., 2016; Singh et al., 2016).

Reproducibility of preclinical experiments has become a pressing challenge in translational research (Dirnagl et al., 2013; Laukens et al., 2016; McNutt, 2014). Small inter-laboratory differences can largely impact and alter the results of experiments hindering reproducibility. In retrospect, the microbiota could potentially influence and partially explain the discrepancies seen between similar studies with opposing results. Looking more into the influence of microbiota-driven immune cell changes may aid in understanding the hurdle between laboratory and clinical care (Duda et al., 2014; Ioannidis et al., 2014).

In this study we have identified highly functional and relevant differences in the microbiota composition of mice of the same genetic background (C57BL/6) but from different commercial breeders. We detected corresponding alterations in the intestinal immune compartment—particularly changes in T cell homeostasis—and abundance of specific bacterial species between the groups. We then tested the impact of these microbiota differences between commercial breeders on the immunological outcome of an exemplary immunotherapeutic approach using CD28 superagonistic antibodies (CD28SA) in a murine stroke model.

2. Results

2.1. C57BL/6 mice show clear breeder-dependent gut microbiota differences

Despite being housed in the same environment and fed the same diet 3 weeks prior to analysis, we observed significant differences in the microbiota composition of mice from the three independent breeder sources; Charles River Laboratories (CR), Taconic Biosciences (Tac) and Harlan Laboratories (Har). We did not detect significant differences between the tested groups regarding total bacterial density, indicating similar overall bacterial colonization of the intestine (Fig. 1A). In contrast, we observed significant differences in bacterial diversity between mice of different breeder source (Fig. 1B). Substantial differences between the microbiota communities of the three breeders became also evident in the principal component analysis (Fig. 1C). Furthermore, we noticed distinct differences in the relative abundance of specific bacterial families between mice from different breeders, while the overall taxonomic composition was consistent in mice from the same

breeder (Fig. 1D). Interestingly, we identified significant differences in the abundance of segmented filamentous bacteria (SFB), a bacterial species that has previously been shown to be associated with T_{Helper} cell polarization (Goto et al., 2014; Ivanov et al., 2009; Round and Mazmanian, 2009). As detected by semi-quantitative PCR, SFB were absent in all mice obtained from CR, but present in fecal samples from mice obtained from Tac and were most abundant in mice from Har (Fig. 1E). Finally, we performed a LEfSe analysis and identified several operational taxonomic units (OTUs) which were significantly more represented in one breeder compared to others, for example *Prevotella* in CR, *Parabacteroides* in Tac or *Rikenellaceae* in Har (Suppl. Fig. 1). This suggests that different commercial breeders supply mice with a significantly different microbiota composition.

2.2. Specific microbiota differences are associated with T cell polarization

After detecting specific differences in the gut microbiota of mice from different breeder sources, we investigated the associated polarization of immune cells within the Peyer's patches (PP) of the intestinal lymphoid tissue. Total and differential leukocyte counts in the PP and spleen did not differ between breeder groups (Suppl. Fig. 2). In contrast, we observed substantial differences in the abundance of T_{Helper} cell subpopulations between breeders, thus indicating that the breeder-specific microbiota composition may influence and impact immune polarization. We particularly analyzed the polarization of Treg (Foxp3+) and Th17 (IL-17+) cells (Fig. 1F). We observed high percentages of pro-inflammatory Th17 cells in mice received from Tac—in contrast to mice received from CR (SFB-deficient), which demonstrated high Treg cell counts but low pro-inflammatory Th17 cell counts (Fig. 1G). This was demonstrated clearly in the ratio of Foxp3 to IL-17 cells showing a much lower ratio when SFB's were present in the breeder mice. Interestingly, this effect was specifically confined to the intestinal immune compartment (PP) as Treg and Th17 populations did not differ in spleens between groups, however, general Treg properties such as cell frequency, Foxp3 and CD28 expression did not differ *per se* between spleen and PP (Suppl. Fig. 3B). These results indicated a potential association between SFB abundance in the intestinal microbiota and induction of pro-inflammatory T cell subpopulations.

2.3. Co-housing restores SFB content and shifts the T cell ratio in the periphery

In order to investigate whether differences in T cell polarization were indeed a cause of SFB presence within the intestinal microbiota, we co-housed SFB negative (Jax) and SFB positive mice for 2 weeks (Fig 2A). Due to the coprophagic behavior, SFB-negative mice were able to naturally recolonize themselves with SFB from the cohoused SFB-positive donor mice (Fig 2B). We then performed flow cytometric analysis to examine T cell subpopulations following SFB-colonization. SFB recolonized mice demonstrated a clear shift in their T cell polarization pattern resembling largely the effects observed in SFB-positive mice, particularly a consistent reduction in the Foxp3/IL-17 ratio (Fig. 2C). anti-inflammatory Treg cells were decreased and pro-inflammatory IL-17+ and IFN γ + cells were increased in SFB-recolonized animals, although no effect on ROR γ t expression was observed. In order to validate these crucial findings, we repeated this experiment using animals from CR as SFB negative mice, confirming a similar treatment pattern with reduction of the Foxp3/IL-17 ratio after recolonization in PP and spleen as in the primary experiment (Suppl. Fig. 4). These results demonstrate a causal relationship between intestinal SFB-colonization and specific T_{Helper} cell polarization in the intestinal immune compartment.

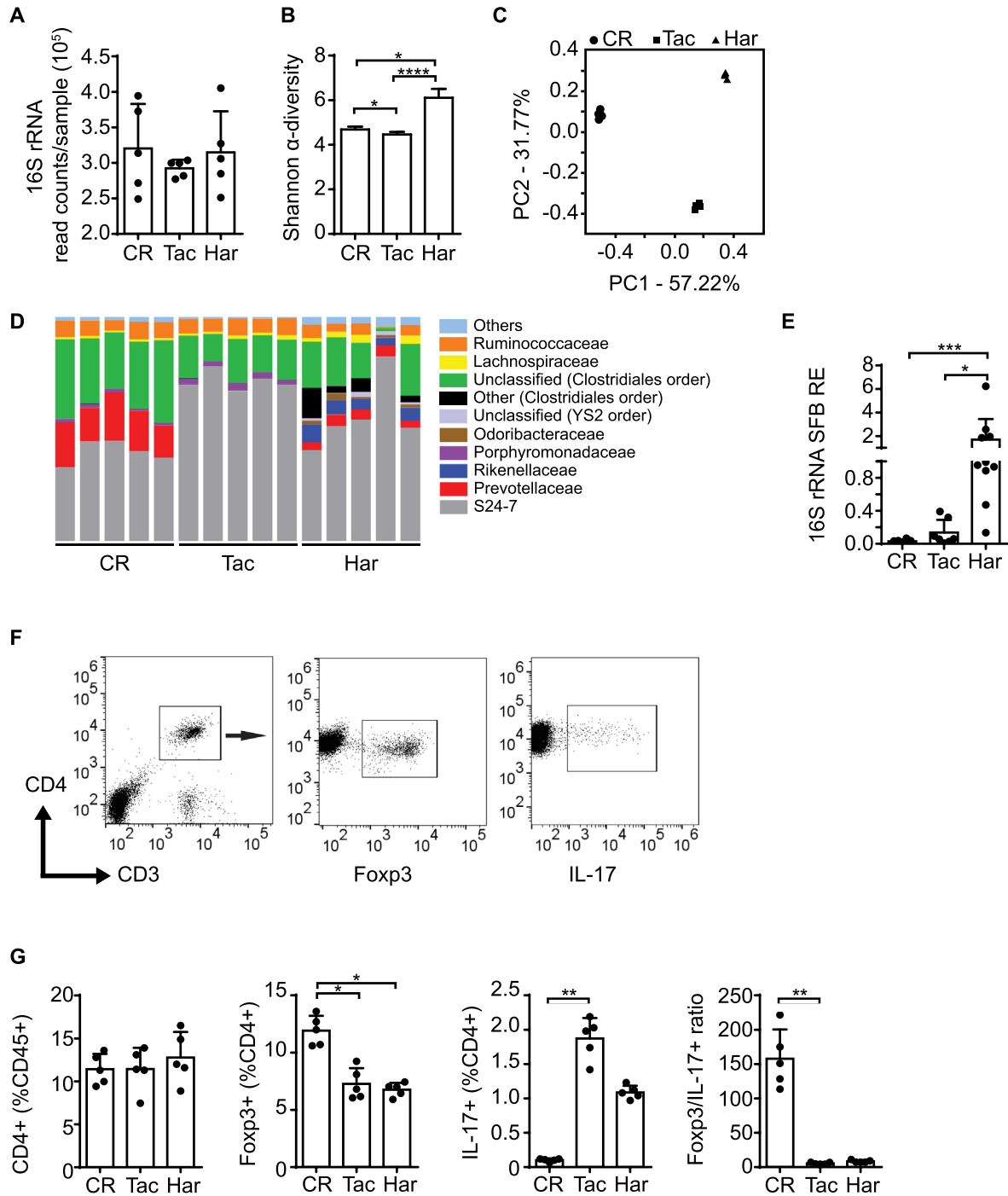


Fig. 1. Microbiota differences in C57BL/6 mice from different commercial breeders. (A) Total read counts per sample from the 16 S ribosomal gene sequencing. Kruskal-Wallis test with Dunn's multiple comparisons test ($n = 5$) (1 independent experiment). (B) Shannon α -diversity index for within-sample diversity of grouped data ($n = 5$ per group, mean + SE). (C) Two-dimensional Bray-Curtis β -diversity PCoA plot of the microbial fecal communities (1 independent experiment). (D) Taxonomic summary representing the distributions of bacteria found at family level. All families contributing <2% were grouped into the "Others" category (1 independent experiment). (E) Semi-quantitative qPCR of SFB to EUB 16S rRNA (CR $n = 8$, Tac $n = 7$, Har $n = 10$; 2 independent experiments). (F) Representative dot plots and gating strategy for flow cytometric analysis of Foxp3 and IL-17 populations from CD4+ T_{Helper} cells. (G) Analysis of the indicated T_{Helper} cell subpopulation based on the transcription factor and intracellular cytokine expression per breeder group. From left CD4+, Foxp3+, IL-17+ and Foxp3/IL-17 ratio ($n = 5$ per group, 1 independent experiment). Kruskal-Wallis test with Dunn's multiple comparisons test. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. CR = Charles River Laboratories, Tac = Taconic Biosciences, Har = Harlan Laboratories.

2.4. Microbiota-associated T cell polarization affects immunotherapeutic efficacy

Next, we investigated whether the microbiota-dependent T cell polarization between commercial breeders would affect stroke outcome of an immunotherapeutic treatment. Previously we have found that extensive brain injuries can alter the microbiota compo-

sition by affecting intestinal motility function (Singh et al., 2016). As a result of this, we chose the distal Middle Cerebral Artery occlusion (dMCAo) model for these experiments—inducing only moderate-sized cortical lesions—in order to exclude stroke-induced microbiota alterations. Based on previous studies with unexplained contradicting results, we selected the CD28 superagonistic antibody (CD28SA) as the exemplary immunotherapeutic

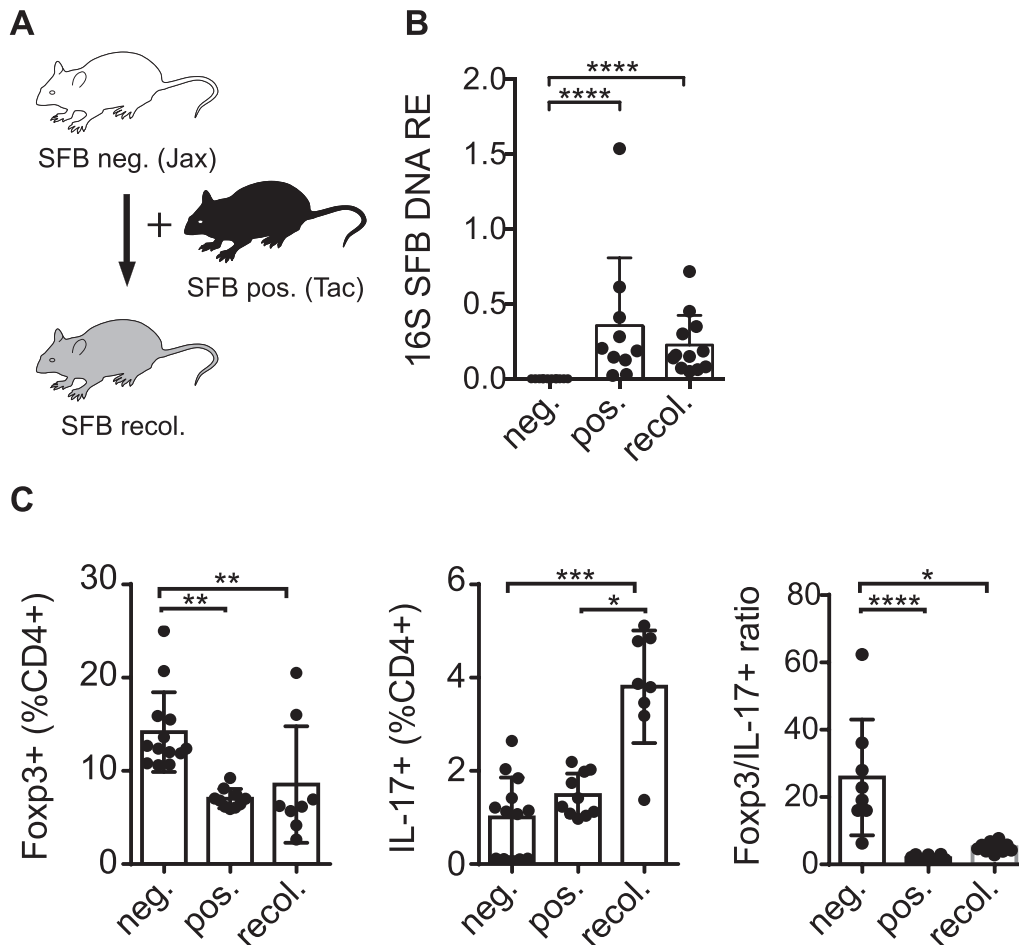


Fig. 2. SFB colonization is causally linked to T_{Helper} cell polarization. For natural recolonization of animals with a SFB-positive gut microbiome, SFB-deficient animals were co-housed with SFB-laden animals (see Methods section for technical details). (A) Schematic illustration of the co-housing experiment and the three investigated mouse groups: SFB-negative, SFB-positive and SFB-recolonized. (B) Semi-quantitative qPCR of SFB 16S rRNA relative expression, indicating efficient SFB recolonization by co-housing ($n = 12$ for SFB-negative and SFB-recolonized, $n = 10$ for SFB-positive). (C) Flow cytometric analysis of T_{Helper} cell polarization in Peyer's patches according to data shown in Fig. 1. SFB recolonization significantly alters T_{Helper} cell homeostasis and Fxp3:IL-17 ratio. ($n = 13$ for SFB-negative, $n = 8$ for SFB-recolonized, $n = 10$ for SFB-positive). Kruskal-Wallis test with Dunn's multiple comparisons test. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. Jax = The Jackson Laboratory, Tac = Taconic Biosciences, Har = Harlan Laboratories.

treatment (Na et al., 2015; Schuhmann et al., 2015). Control groups received no treatment after confirming no difference in T cell polarization between untreated and IgG isotype control treated animals (Suppl. Fig. 5A,B). Interestingly, we found that immunomodulation using CD28SA administered once at 3 h after stroke induction was able to consistently expand all Treg and Th17 cells in the spleen (Suppl. Fig. 5C), but resulted in a differential breeder-dependent T cell polarization in the PP (Fig. 3A). Specifically, CD28SA effectively induced Treg cells in (SFB positive) mice from Tac and Har but failed to induce this neuroprotective T cell population in CR mice, resulting in a significantly shifted balance between pro-inflammatory Th17 and immunosuppressive Treg cells. A similar, yet only partially significant, Treg expansion pattern was also observed in brains by performing Fxp3 RT-PCR of brain hemispheres after stroke. CD28SA induced a significant increase in Tac mice as well as a non-significant expansion in Har mice which was not observed in the CR group (Fig. 3B). The microbiota-associated shift in T cell homeostasis after CD28SA treatment was then correlated with the efficacy of this treatment on stroke outcome. We detected exacerbation of the infarct volume in CR mice—with a shift towards pro-inflammatory Th17 cell expansion—compared to unaltered infarct volumes in Tac and Har mice (Fig. 3C,D). These results propose that the microbiota composition, particularly the presence of SFB, strongly influences

the ability of CD28SA treatment to expand specific T_{Helper} cell sub-populations and therefore affect disease outcome.

3. Discussion

Our study has addressed the ongoing question of to what extent do microbiota differences between conventional laboratory mice obtained from different breeding sources impact the physiological immune homeostasis and the neuroinflammatory response after brain injury. We observed that specific changes in the microbiota composition were associated with immunological alterations. In particular, the presence of SFB was causally linked to T cell polarization and these changes prevailed after experimental stroke and influenced the efficacy of an immunomodulatory treatment. Our results indicate that even minimal differences in the microbiota composition may have a significant impact on the neuroinflammatory response and the outcome of immunomodulatory therapies. These findings are of high relevance for the design of future studies and interpretation of previous reports in immunity and neuroinflammation.

In accordance with previous reports, our study confirmed differences in the microbiota composition at lower taxonomic levels between mice from different commercial breeders (Ericsson

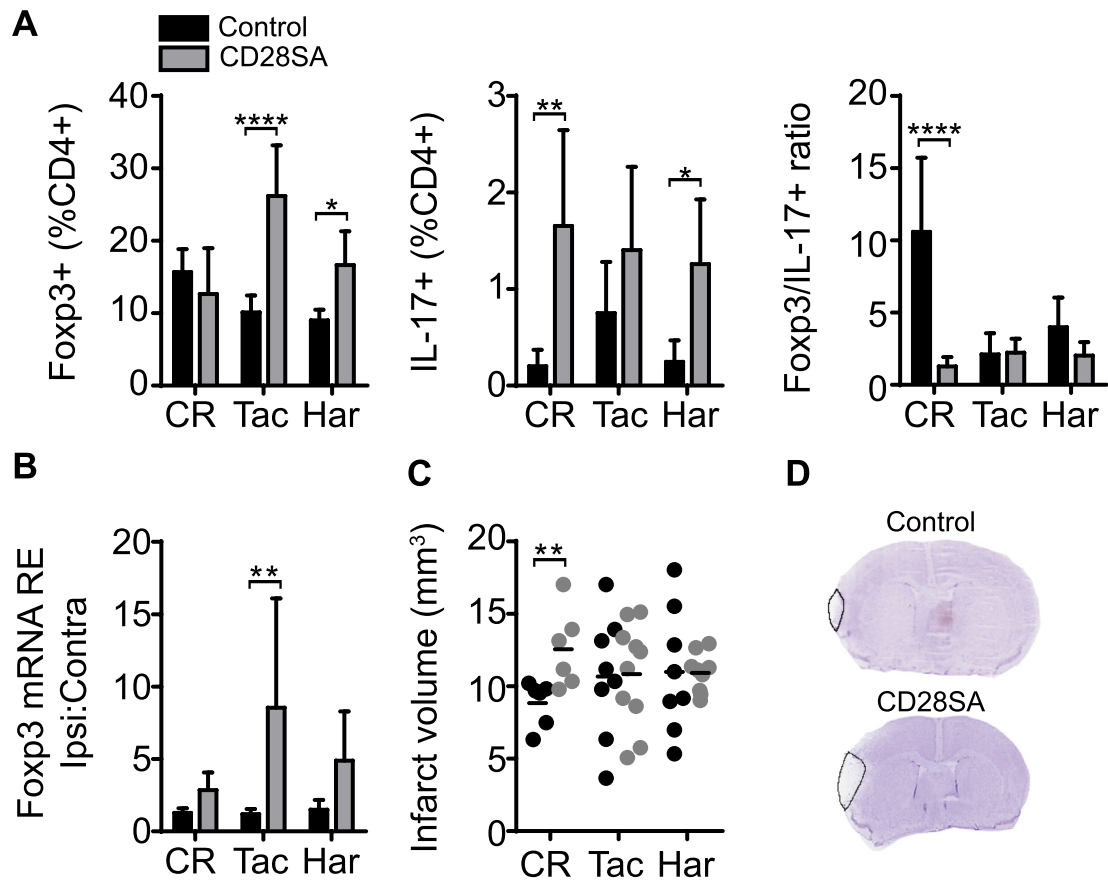


Fig. 3. Effect of CD28SA treatment differs between mice from different breeders. Mice from different breeders were administered either with CD28SA antibody or given no treatment after stroke induction. (A) Percentage of T_{Helper} cell subpopulations (Foxp3, IL-17 and ratio) in Peyer's patches (PP) according to the analysis strategy shown in Fig. 2 (for control: $n = 5$ per group (1 independent experiment), for CD28SA: CR = 6, Tac = 8, Har = 8 (2 independent experiments). 2way-ANOVA with Sidak's multiple comparisons test. (B) Semi-quantitative PCR analysis of Foxp3 expression in the ipsilateral and contralateral brain hemisphere after stroke induction per indicated treatment group (for control: CR = 7, Tac = 6, Har = 8; for CD28SA: CR = 6, Tac = 6, Har = 5; 2 independent experiments). (C) Infarct volumetry of coronal sections 5d post dMCAo (for control: CR = 6, Tac = 8, Har = 8; for CD28SA: CR = 6, Tac = 10, Har = 9; 2 independent experiments). Mann-Whitney U test, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. (D) Representative cresyl-violet staining for Control and CD28SA treated CR mice. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

et al., 2015; Ivanov et al., 2009). Interestingly, mice obtained from CR were devoid of SFB in their gut microbiota, this concurs with former studies, which report the same observation for animals from Jax. C57BL/6 mice bred at CR were historically obtained from Jax, supporting the concept that certain bacterial strains may be inherited (Faith et al., 2015). In the past, SFB have been identified as one of the most differentially abundant species in the microbiota of Jax and Tac mice (Ivanov et al., 2009). Furthermore, SFB are known to play a prominent role in the induction of Th17 polarization of T cells (Atarashi et al., 2015; Goto et al., 2014; Ivanov et al., 2009). This suggests that the differences in the abundance of single bacterial species within the gut microbiota can strongly influence immune homeostasis and the immune response during disease.

In fact, we have observed a striking difference in the microbiota-dependent polarization of T cells between the intestinal immune compartment (PP) and the spleen where the impact of microbiota differences between groups was less prominent. This finding further supports the concept of a site-specific priming of T cell homeostasis in the intestine by the microbiota but also gives rise to further questions on how this localized effect could potentially impact distant neuroinflammation. In this line, we have previously demonstrated using in vivo T cell-labelling approaches that approximately 25% of cerebral T cells after stroke are directly

recruited from Peyer's patches (Benakis et al., 2016; Singh et al., 2016). Taken together, it seems well conceivable that a substantial number of T cells in the brain have migrated from the intestinal immune compartment where they have received a microbiota-dependent "functional imprint" and thereby are able to modulate the post-ischemic neuroinflammatory response.

Although the mice were originating from the same genetic background, a potential limitation of comparing mice from different commercial breeders is genetic drift due to inbreeding at each breeding site over multiple generations. Taking our results into consideration this may have an impact on the immune system. Previous studies have addressed this exact question and reported that of a potential 1449 single-nucleotide-polymorphisms (SNPs), the Tac (C57BL/6NTac) and Har (C57BL/6NHsd) strains were identical, but differ from the original CR (C57BL/6J) at only 12 SNPs (Mekada et al., 2009; Zurita et al., 2011). These SNPs were found to be located in the NAD(P) transhydrogenase, a mitochondrial gene which is not associated with immune homeostasis or T cell polarization. Therefore, to finally investigate the T cell polarization changes induced by microbiota differences observed between breeders, we performed a microbiota recolonization experiment by co-housing of SFB-negative and SFB-positive mice resulting in efficient SFB recolonization of SFB-negative mice. Results from this experiment unequivocally demonstrated the causal link between

microbiota changes, specifically SFB (and potentially other associated and currently unknown bacterial species) and the ability to induce or change T cell polarization.

While the majority of T_{helper} cells in mice are naïve cells, approximately 85% based on previous reports, the smaller population of polarized cells determine disease course of T cell-driven pathologies (Hashiguchi et al., 2011). The microbiota-associated differences in T cell polarization did not have a significant impact on infarct volume under naïve conditions. Nevertheless, the influence of microbiota-dependent immune priming became evident when testing the efficacy of CD28SA treatment as a proof-of-concept immunomodulatory approach. We identified selective expansion of a T cell subpopulation upon treatment with the costimulatory CD28SA which was determined by the microbiota-dependent pre-polarization of the T cell population between mice of different breeder source. Mice lacking SFB in the gut microbiota were unable to efficiently expand the (neuroprotective) Treg population upon CD28 stimulation resulting in an increase of the pro-/anti-inflammatory T cell subpopulation balance. Finally, the differing immunomodulatory response to CD28SA resulted in significantly differing stroke outcomes between breeders, a finding which should be validated in a larger, fully-powered confirmatory study (Llovera et al., 2015). This specific example of CD28SA treatment in experimental murine stroke can serve as a proof-of-concept study, demonstrating the impact of microbiota alterations on the immunotherapeutic outcome. These findings are of significant relevance for the design of future preclinical studies particularly aiming at T cell homeostasis, activation or expansion in a variety of inflammatory disease models.

CD28SA treatment was chosen for this study with the rationale of providing potential explanations for contradictory findings using this drug in experimental stroke studies (Gauberti and Vivien, 2015; Na et al., 2015; Schuhmann et al., 2015; Veltkamp et al., 2015). Interestingly, the two original studies reporting exacerbation of infarct volume in one and reduction in the other manuscript used the very same stroke model, antibody and similar readout measures—however, mice were obtained from two different commercial breeders (Har and CR) (Na et al., 2015; Schuhmann et al., 2015). Based on the results from our study, it seems conceivable that differences in the microbiome of the animals may have been a relevant confounder explaining the contradictory outcome between these studies. Notably, in this study we did not observe a reduced infarct volume with CD28SA treatment in any breeder mouse. However, due to the lack of information on the microbiota composition in animals used in the previous studies investigating CD28SA in experimental stroke—particularly as mice housed in conventional animal facilities do not necessarily represent the microbiota composition of the original breeder—it cannot be concluded whether microbiota-dependent immune priming is the exact reason of failed reproducibility.

In light of the so-called “reproducibility crisis” which draws increasing attention in diverse research fields (McNutt, 2014) the findings of the present study introduce the gut microbiota as a relevant factor in the comparability of experimental studies. Strain, sex and age of laboratory animals have become factors commonly reflected in study design and reporting of experimental studies based on well-accepted guidelines (Kilkenny et al., 2010). Due to the intimate interaction of the microbiota and immune system, not only do we agree with previous publications which stress the importance of the microbiota composition in terms of reproducibility (Bleich and Hansen, 2012; Laukens et al., 2016), in addition we clearly show that the neuroinflammatory response is also affected by differences in microbiota composition between three commercial breeders. For this, future studies must further characterize the key immunogenic bacterial species of the microbiome in order to enable a targeted analysis and reporting of these confounders.

4. Materials and methods

4.1. Animals

All experimental protocols were approved by the responsible governmental committees (Regierung von Oberbayern, Munich, Germany). C57BL/6 inbred female mice were purchased as the C57BL/6J substrain from CR (Sandhofer Weg 7, 97633 Sulzfeld, Germany), C57BL/6NTac from Tac, (Bomholtvej 10, DK-8680 Ry, Denmark) and C57BL/6NHsd from Har (Kreuzelweg 53, 5961 NM Horst, Netherlands). In order to minimize genetic drift, inbred mice of the indicated strains were backcrossed at Jax, CR and Tac every fifth generation to original parent lines (Taft et al., 2006). On the day of arrival mice were aged 4–6 weeks. For a further 3 weeks mice were left to acclimatize to the housing environment (autoclaved sawdust, cellulose, gnawing wood piece, water and food (ssniff R/M-H) *ad libitum*). Mice were housed in autoclaved gnotobiotic cages under controlled temperatures (22 ± 2 °C), with 12 h light-dark cycle periods.

4.2. Co-housing experiments

Specific-pathogen-free (SPF) C57BL/6 mice (males; 6 weeks of age) were purchased from Jax and allowed to acclimatize in the animal facility for 1 week before being randomly assigned to experimental groups. C57BL/6 mice from Jax were transferred into an autoclaved mini-isolator cage and co-housed for 2 weeks with a C57BL/6 mouse from Tac tested to be positive for SFB. Mice received autoclaved drinking water and sterilized standard chow *ad libitum*. For the validation co-housing experiments, mice were received and left to acclimatize for 3 weeks. Three mice from CR were co-housed with two Tac mice for 2 weeks for natural recolonization. After, dMCAo was induced and mice were sacrificed 5 days post lesion.

4.3. Experimental stroke model

All surgical procedures were performed as previously reported (Llovera et al., 2014). In brief, mice were anaesthetized with isoflurane, delivered in a mixture of 30% O₂ and 70% N₂O. Throughout the surgical procedure body temperature was maintained at 37 °C with a mouse warming pad in addition Dexamethasone eye ointment was applied to both eyes. Skin was incised between the eye and the ear revealing the temporal muscle. The temporal muscle was removed from the skull using electrocoagulation forceps. Upon location of the middle cerebral artery (MCA) under the skull, a small hole was drilled to expose the MCA. The MCA was then permanently occluded using bipolar electrocoagulation forceps. After visual confirmation of the occluded artery the mouse was sutured. Mice were recovered in a nursing box at 32 °C for 15 min and then returned to home cages. Control mice received no CD28SA treatment. The brain was carefully removed from the skull 5 days post lesion, and placed immediately on dry ice. Microscopy slides were mounted with 20 μM thick coronal cryosections cut at 400 μM intervals. Sections were then stained with cresyl violet solution (C5042-10G, Sigma Life Science). For the infarct volume, all slides were scanned using 600dpi. Measurements were performed using the ImageJ software (NIH). Using the scale of 23.62 pixels/mm, the area of white or non-stained infarct tissue was measured and integrated into the total brain area.

4.4. CD28SA treatment

CD28SA (clone: D665) was administered intraperitoneally 3 h after electro-coagulation of the MCA. A single dose of 150 μg per

mouse diluted in sterile PBS was chosen based on previous experiments (Gogishvili et al., 2009). For control experiments reported in Suppl. Fig. 5, isotype control IgG1 (clone: MOPC-21) was administered intraperitoneally in a single dose of 150 µg per mouse under naïve conditions.

4.5. Flow cytometric analysis

Five days post lesion, mice were anaesthetized with intraperitoneal injection of ketamine and xylazine. Following saline perfusion, approximately 6 PP per mouse and the entire spleen were dissected and placed in ice-cold RPMI medium (Invitrogen), and immediately used for flow cytometric analysis. All cells were isolated under sterile conditions. Spleens were mashed through 40 µm cell strainers and treated with red blood cell lysis buffer. PP tissue was mashed through a 30 µm pre-separation filter (Miltenyi Biotec). For isolation of brain leukocytes, brains were taken from the skull, olfactory bulb and the cerebellum was removed and the hemispheres separated. Each hemisphere was then transferred into a 7 ml dounce homogenizer containing 3 ml of RPMI 1640 with phenol red and disrupted with a pestle. The sample was then mixed with Percoll to obtain a 30% solution. This was then slowly overlaid 70% Percoll using a 10 ml syringe with a 20G needle. The Percoll gradients were centrifuged with no brake at 500g for 30 min (18 °C). The layer of cells were then carefully removed and then used for flow cytometry. For intracellular cytokines, triplicates of 1×10^6 cells for spleen and duplicates for PP were plated with pre-coating of anti-mouse CD3e antibodies (clone BM10-37). Cells were then co-stimulated with CD28 antibodies (clone 37.51) and cultured for 40 h at 37 °C and 5% CO₂ in complete RPMI medium (10% heat-inactivated fetal calf serum, 1% penicillin/streptomycin, and 10 µM 2-mercaptoethanol). Following incubation, GolgiPlug (BD Biosciences) was added for a further 4 h. Cell surface markers were stained using the following antibodies: Anti-CD4 PerCP-Cy5.5 (clone RM4-5), Anti-CD45 eFluor450 (clone 30-F11), Anti-CD3 FITC (clone 17A2), Anti-CD8 PE (53-6.7), Anti-CD11b PE/Cy7 (clone M1/70) Anti-CD11c APC/Cy7 (clone, Anti-Ly6C APC (clone HK1.4), Anti-Ly6G PE/Cy7 (clone RB6-8C5), and Anti-CD28 PE/Cy7 (clone 37.51). Cells were then fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (ebioscience). The following antibodies were used for intracellular stainings (all eBioscience): Anti-IL-17A APC (clone eBio17B7) and Anti-Foxp3 PE (clone NRRF-30). Respective isotype antibodies were used as controls. Stained cells were analyzed on a BD FACSVerser flow cytometer (BD Biosciences) and analysis performed by FlowJo software (version 10.0).

4.6. Sequencing data analysis and taxonomic profiling

Feces samples were always collected at 9.00 am, before and 5 days post-dMCAo and placed immediately on dry ice. DNA isolation was performed using the QIAamp Fast DNA Stool Mini Kit (Qiagen). The total amount of DNA in each sample was measured using Qubit (Life Technologies). 16S rRNA amplicons were generated using primers corresponding to the hypervariable regions V1-V3. All samples were sequenced on an Illumina MiSeq platform. 18 million paired-end reads were generated at 300-bp length. Prior to taxonomic analysis, paired-end reads were pre-processed with Trimmomatic (Bolger et al., 2014) to retain only high-quality sequences, and joined using scripts from the BBMap/BBTools package (Bushnell B., sourceforge.net/projects/bbmap/). Joined reads were quality filtered during demultiplexing with QIIME (Caporaso et al., 2010), and chimeric sequences were removed with USEARCH v. 6.1. OTU picking, taxonomic classification and diversity analysis were then performed with QIIME v. 1.9. Open-reference OTU clustering and taxonomy assignment of sequences

were performed with UCLUST (Edgar, 2010) against the Silva database release 119 (Quast et al., 2013) at the 97% similarity level. OTUs with a number of sequences <0.01% of the total number of sequences were discarded as a second level of quality-filtering. Alpha diversity and beta diversity metrics were finally calculated, after rarefying over the minimum number of sequences per sample. The linear discriminant analysis (LDA) effect size (LEfSe) algorithm was used to detect microbial features having a statistically different relative abundance between the microbiota of the three different breeders. Experiments were performed with an alpha error set to 0.05 and the LDA threshold set to 4.0.

4.7. Quantitative RT-PCR

For feces analysis: Segmented filamentous bacteria (SFB) (forward: GACGCTGAGGCATGAGAGCAT and reverse: GACGGCAGC-GATTGTATTCA) and Eubacteria (EUB) (forward: ACT CCTACGGGAGGCAGCAGT and reverse: ATTACCGCGGCTGCGC) were used. The expression of the genes was quantified using a LightCycler 480 II (Roche). For the cohousing experiments: Total bacterial density of 16S rDNA sequences were amplified from fecal DNA using 0.2 µM of the universal bacterial r16S gene primers 16S-V2-101F (5'-AGYGGCGIACGGGTGAGTAA-3') and 16S-V2-361R (5'-CYIACGTCTGCCTCCCGTAG-3') in conjunction with Maxima SYBR Green/ROX qPCR Master mix 2× (Thermo Scientific). Amplifications were performed on a Chromo 4 Detector (Bio-Rad, Hercules, CA) using a two-step cycling protocol as previously described (Benakis et al., 2016). The standard curve was prepared using a plasmid containing an E. coli 16S V2 rDNA fragment. Using the same genomic DNA from each sample, real-time PCRs were completed using SFB primers as described above (Barman et al., 2008). For brain qPCR the following primers were used from Qiagen; Foxp3 (PPM05497F-200) and PPIA (PPM03717B). Gene expression was normalized to housekeeping gene PPIA using the standard curve technique.

4.8. Statistical analysis

All statistical analyses were performed using GraphPad Prism software (GraphPad, version 6.0). Sample size was chosen based on comparable experiments from previous experiments (Singh et al., 2016). A p value of <0.05 was regarded as statistically significant.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bbi.2017.03.011>.

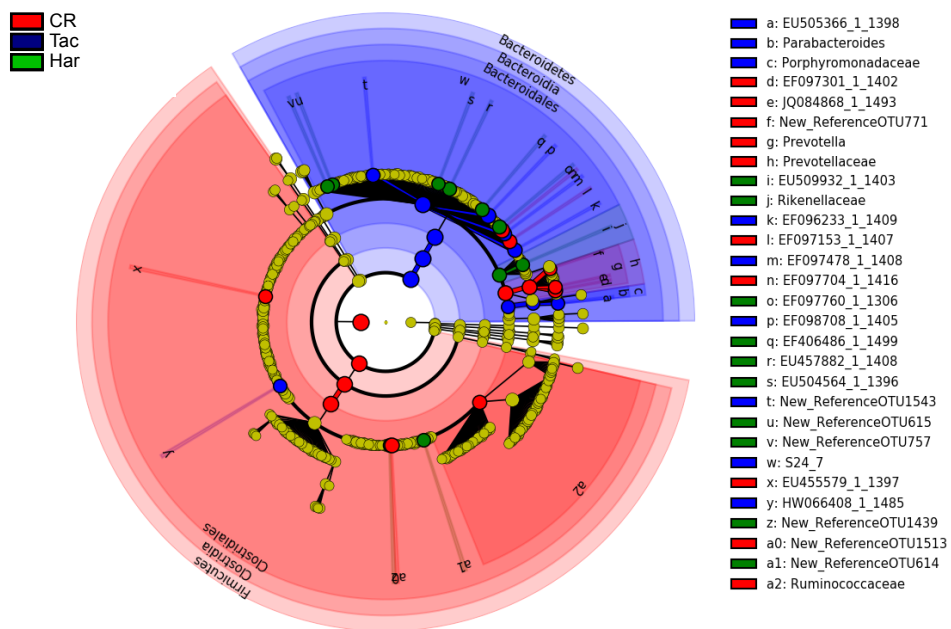
References

- Atarashi, K., Tanoue, T., Ando, M., Kamada, N., Nagano, Y., Narushima, S., et al., 2015. Th17 cell induction by adhesion of microbes to intestinal epithelial cells. *Cell* 163 (2), 367–380.
- Barman, M., Unold, D., Shifley, K., Amir, E., Hung, K., Bos, N., et al., 2008. Enteric salmonellosis disrupts the microbial ecology of the murine gastrointestinal tract. *Infect. Immun.* 76 (3), 907–915.

- Benakis, C., Brea, D., Caballero, S., Faraco, G., Moore, J., Murphy, M., et al., 2016. Commensal microbiota affects ischemic stroke outcome by regulating intestinal gamma delta T cells. *Nat. Med.* 22 (5), 516–523.
- Berer, K., Mues, M., Koutrolos, M., Al Rasbi, Z., Boziki, M., Johnner, C., et al., 2011. Commensal microbiota and myelin autoantigen cooperate to trigger autoimmune demyelination. *Nature* 479 (7374), U266–538.
- Bleich, A., Hansen, A.K., 2012. Time to include the gut microbiota in the hygienic standardisation of laboratory rodents. *Comp. Immunol. Microbiol. Infect. Dis.* 35 (2), 81–92.
- Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30 (15), 2114–2120.
- Brandtzaeg, P., Halstensen, T.S., Kett, K., Krajci, P., Kvale, D., Rognum, T.O., et al., 1989. Immunobiology and immunopathology of human gut mucosa – humoral immunity and intraepithelial lymphocytes. *Gastroenterology* 97 (6), 1562–1584.
- Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., et al., 2010. QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7 (5), 335–336.
- Dirnagl, U., Hakim, A., Macleod, M., Fisher, M., Howells, D., Alan, S.M., et al., 2013. A concerted appeal for international cooperation in preclinical stroke research. *Stroke* 44 (6), 1754–1760.
- Dirnagl, U., Iadecola, C., Moskowitz, M.A., 1999. Pathobiology of ischaemic stroke: an integrated view. *Trends Neurosci.* 22 (9), 391–397.
- Donnan, G.A., Fisher, M., Macleod, M., Davis, S.M., 2008. Stroke. *Lancet* 371 (9624), 1612–1623.
- Duda, G.N., Grainger, D.W., Frisk, M.L., Bruckner-Tuderman, L., Carr, A., Dirnagl, U., et al., 2014. Changing the mindset in life sciences toward translation: a consensus. *Sci. Transl. Med.* 6 (264).
- Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26 (19), 2460–2461.
- Ericsson, A.C., Davis, J.W., Spollen, W., Bivens, N., Givan, S., Hagan, C.E., et al., 2015. Effects of vendor and genetic background on the composition of the fecal microbiota of inbred mice. *PLoS ONE* 10 (2).
- Faith, J.J., Colomel, J.-F., Gordon, J.I., 2015. Identifying strains that contribute to complex diseases through the study of microbial inheritance. *Proc. Natl. Acad. Sci. U.S.A.* 112 (3), 633–640.
- Gauberti, M., Vivien, D., 2015. Letter by Gauberti and Vivien regarding article, “amplification of regulatory T cells using a CD28 superagonist reduces brain damage after ischemic stroke in mice”. *Stroke* 46 (2), e50–1.
- Gelderblom, M., Leyppoldt, F., Steinbach, K., Behrens, D., Choe, C.-U., Siler, D.A., et al., 2009. Temporal and spatial dynamics of cerebral immune cell accumulation in stroke. *Stroke* 40 (5), 1849–1857.
- Gogishvili, T., Langenhorst, D., Luhder, F., Elias, F., Elflein, K., Dennehy, K.M., et al., 2009. Rapid regulatory T-cell response prevents cytokine storm in CD28 superagonist treated mice. *PLoS ONE* 4 (2), 9.
- Goto, Y., Panea, C., Nakato, G., Cebula, A., Lee, C., Diez, M.G., et al., 2014. Segmented filamentous bacteria antigens presented by intestinal dendritic cells drive mucosal Th17 cell differentiation. *Immunity* 40 (4), 594–607.
- Hashiguchi, M., Hachimura, S., Ametani, A., Sato, T., Kojima, H., Kumagai, Y., et al., 2011. Naive CD4+ T cells of Peyer’s patches produce more IL-6 than those of spleen in response to antigenic stimulation. *Immunol. Lett.* 141 (1), 109–115.
- Iadecola, C., Anrather, J., 2011. The immunology of stroke: from mechanisms to translation. *Nat. Med.* 17 (7), 796–808.
- Ioannidis, J.P.A., Greenland, S., Hlatky, M.A., Khoury, M.J., Macleod, M.R., Moher, D., et al., 2014. Increasing value and reducing waste in research design, conduct, and analysis. *Lancet* 383 (9912), 166–175.
- Ivanov, I.I., Atarashi, K., Manel, N., Brodie, E.L., Shima, T., Karaoz, U., et al., 2009. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* 139 (3), 485–498.
- Kilkenny, C., Browne, W.J., Cuthill, I.C., Emerson, M., Altman, D.G., 2010. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol.* 8 (6), e1000412.
- Kleinschnitz, C., Schwab, N., Kraft, P., Hagedorn, I., Dreykluft, A., Schwarz, T., et al., 2010. Early detrimental T-cell effects in experimental cerebral ischemia are neither related to adaptive immunity nor thrombus formation. *Blood* 115 (18), 3835–3842.
- Lathrop, S.K., Bloom, S.M., Rao, S.M., Nutsch, K., Lio, C.W., Santacruz, N., et al., 2011. Peripheral education of the immune system by colonic commensal microbiota. *Nature* 478 (7368), U142–250.
- Laukens, D., Brinkman, B.M., Raes, J., De Vos, M., Vandenabeele, P., 2016. Heterogeneity of the gut microbiome in mice: guidelines for optimizing experimental design. *FEMS Microbiol. Rev.* 40 (1), 117–132.
- Liesz, A., Kleinschnitz, C., 2016. Regulatory T cells in post-stroke immune homeostasis. *Transl. Stroke Res.* 7 (4), 313–321.
- Liesz, A., Suri-Payer, E., Veltkamp, C., Doerr, H., Sommer, C., Rivest, S., et al., 2009. Regulatory T cells are key cerebroprotective immunomodulators in acute experimental stroke. *Nat. Med.* 15 (2), 192–199.
- Llovera, G., Hofmann, K., Roth, S., Salas-Perdomo, A., Ferrer-Ferrer, M., Perego, C., et al., 2015. Results of a preclinical randomized controlled multicenter trial (pRCT): Anti-CD49d treatment for acute brain ischemia. *Sci. Transl. Med.* 7 (299), 299ra121.
- Llovera, G., Roth, S., Plesnila, N., Veltkamp, R., Liesz, A., 2014. Modeling stroke in mice: permanent coagulation of the distal middle cerebral artery. *Jove-J. Vis. Exp.* (89).
- McNutt, M., 2014. Journals unite for reproducibility. *Science* 346 (6210), 679.
- Mekada, K., Abe, K., Murakami, A., Nakamura, S., Nakata, H., Moriwaki, K., et al., 2009. Genetic differences among C57BL/6 substrains. *Exp. Anim.* 58 (2), 141–149.
- Na, S.Y., Mracsko, E., Liesz, A., Hunig, T., Veltkamp, R., 2015. Amplification of regulatory T cells using a CD28 superagonist reduces brain damage after ischemic stroke in mice. *Stroke* 46 (1), 212–220.
- Ochoa-Reparaz, J., Mielcarz, D.W., Wang, Y., Begum-Haque, S., Dasgupta, S., Kasper, D.L., et al., 2010. A polysaccharide from the human commensal *Bacteroides fragilis* protects against CNS demyelinating disease. *Mucosal Immunol.* 3 (5), 487–495.
- Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41 (D1), D6–D590.
- Round, J.L., Mazmanian, S.K., 2009. The gut microbiota shapes intestinal immune responses during health and disease. *Nat. Rev. Immunol.* 9 (5), 313–323.
- Schuhmann, M.K., Kraft, P., Stoll, G., Lorenz, K., Meuth, S.G., Wiendl, H., et al., 2015. CD28 superagonist-mediated boost of regulatory T cells increases thromboinflammation and ischemic neurodegeneration during the acute phase of experimental stroke. *J. Cereb. Blood Flow Metab.* 35 (1), 6–10.
- Shichita, T., Sugiyama, Y., Ooboshi, H., Sugimori, H., Nakagawa, R., Takada, I., et al., 2009. Pivotal role of cerebral interleukin-17-producing gamma delta T cells in the delayed phase of ischemic brain injury. *Nat. Med.* 15 (8), U150–946.
- Singh, V., Roth, S., Llovera, G., Sadler, R., Garzetti, D., Stecher, B., et al., 2016. Microbiota dysbiosis controls the neuroinflammatory response after stroke. *J. Neurosci.* 36 (28), 7428–7440.
- Veltkamp, R., Na, S.Y., Liesz, A., 2015. Response to letter regarding article, “amplification of regulatory T cells using a CD28 superagonist reduces brain damage after ischemic stroke in mice”. *Stroke* 46 (2), e52.
- Zurita, E., Chagoyen, M., Cantero, M., Alonso, R., Gonzalez-Neira, A., Lopez-Jimenez, A., et al., 2011. Genetic polymorphisms among C57BL/6 mouse inbred strains. *Transgenic Res.* 20 (3), 481–489.

Suppl. Figure 1

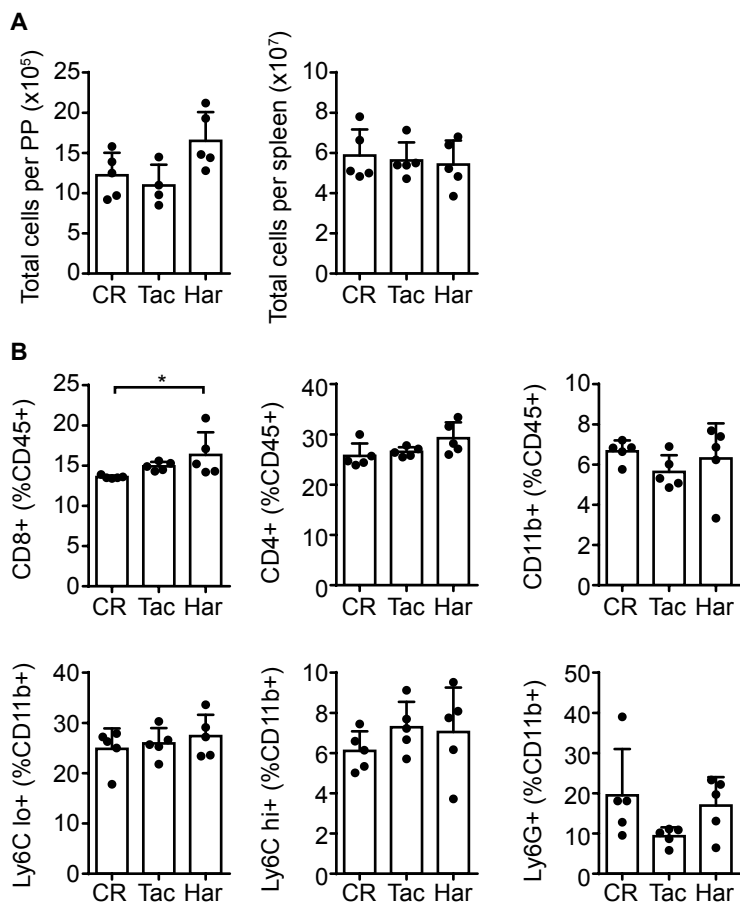
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Supplementary Figure 1. Operational taxonomic units are differentially abundant between different commercial breeders.

(A) Linear discriminant analysis (LDA) was used to compare operational taxonomic units (OTUs) between CR, Tac and Har. An alpha error set to 0.05 and the LDA threshold set to 4.0 (1 independent experiment). CR=Charles River Laboratories, Tac=Taconic Biosciences, Har=Harlan Laboratories. Annotations generated using the SILVA 16S rRNA database.

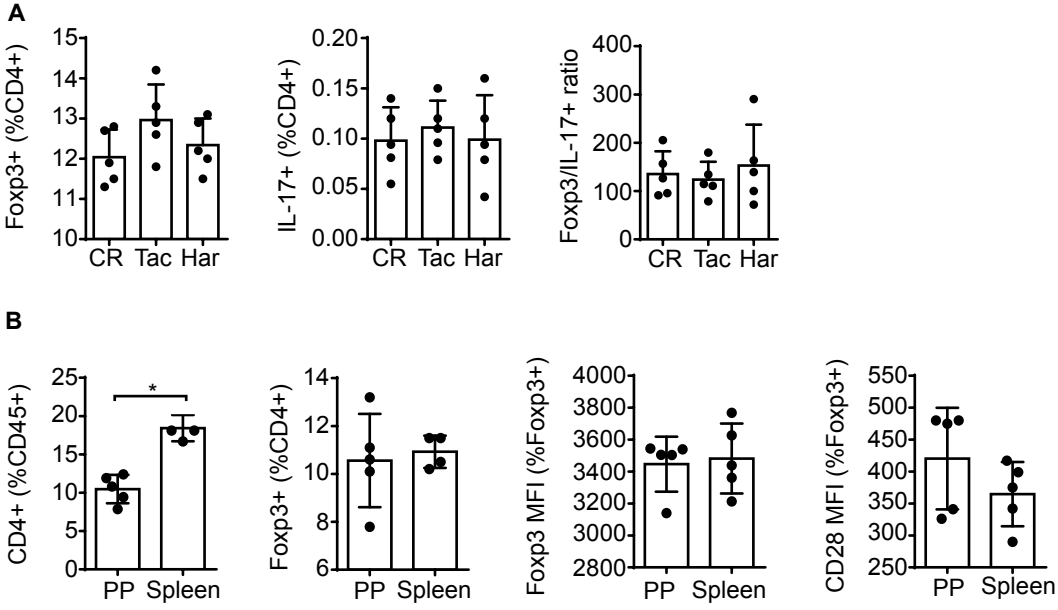
Suppl. Figure 2



Supplementary Figure 2. Mice from different breeders do not differ in basic peripheral immunity.

(A) Total cell counts were determined for Peyer's patches (PP) and spleen in the indicated breeder groups. (B) Leukocyte subpopulations in spleens of the three breeder groups were analyzed for the percentage of $T_{\text{Cytotoxic}}$ cells (CD8), T_{Helper} cells (CD4), monocytes (CD11b), Ly-6C high and low expressing monocyte subpopulations and granulocytes (Ly-6G). Kruskal-Wallis test with Dunn's multiple comparisons test, ($n=5$ per group) (1 independent experiment). **= $p < 0.05$, ***= $p < 0.01$, ****= $p < 0.001$, *****= $p < 0.0001$. CR=Charles River Laboratories, Tac=Taconic Biosciences, Har=Harlan Laboratories.

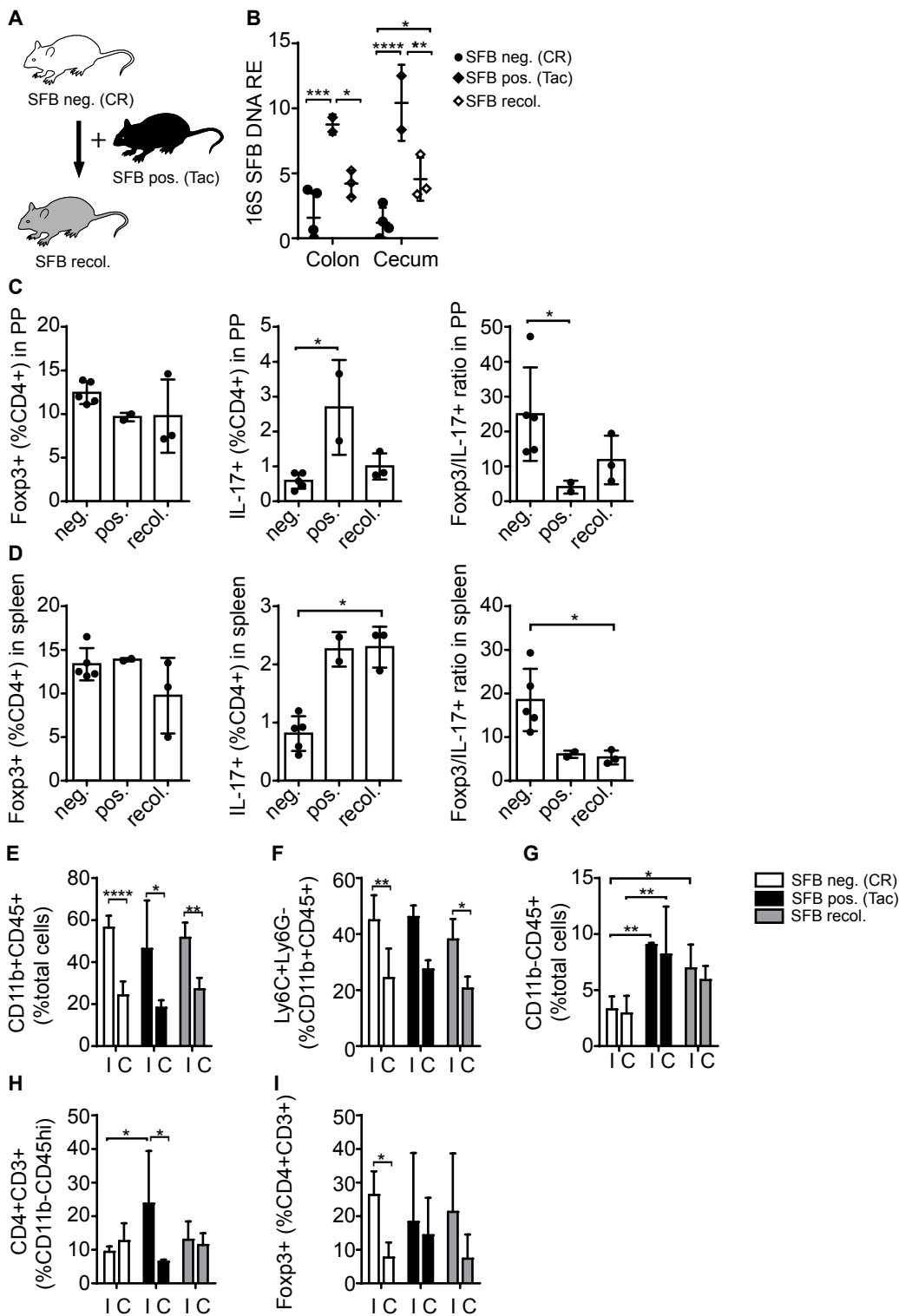
Suppl. Figure 3



Supplementary Figure 3. Characterization of T cell polarization in the periphery.

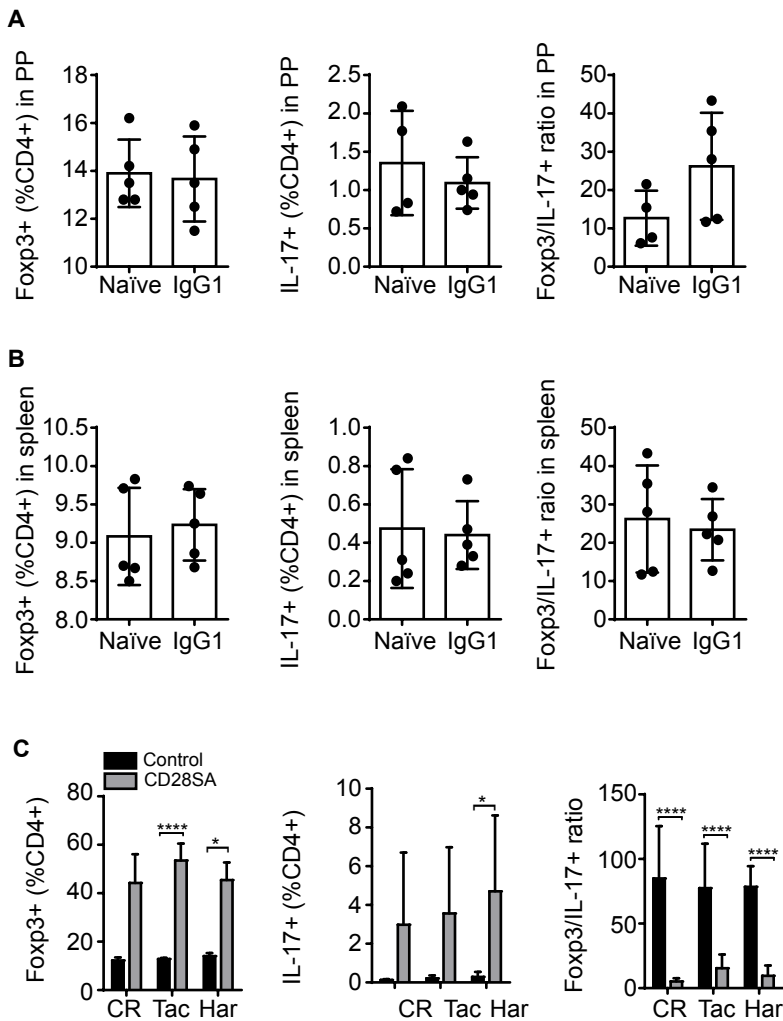
(A) Spleens were collected from naïve mice obtained from the indicated breeder sources and analyzed for the frequency of Treg (Foxp3+) and Th17 (IL-17+) cells within the T_{helper} cell population, in addition to the ratio of Foxp3/IL-17 cells. (n=5 per group) (1 independent experiment). Kruskal-Wallis test with Dunn's multiple comparisons test. (B) Spleens and PP were collected from mice obtained from CR and analyzed for the frequency of T_{helper} (CD4+) and Treg (Foxp3+) cells within the T cell population. Mean fluorescence intensity was analyzed for the Foxp3+ cells for Foxp3 and CD28 (n=5 per group) (1 independent experiment). Mann-Whitney U test, * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001. CR=Charles River Laboratories, Tac=Taconic Biosciences, Har=Harlan Laboratories, PP=Peyer's patches.

Suppl. Figure 4



Supplementary Figure 4. SFB colonization is causally linked to the Foxp3/IL-17 ratio.

For natural recolonization, we housed SFB neg. (CR) mice with SFB pos. (Tac) mice (see Methods section for technical details). **(A)** Schematic illustration of the co-housing experiment and the three investigated mouse groups: SFB neg., SFB pos. and SFB recol. **(B)** Semi-quantitative qPCR of SFB 16S rRNA relative expression in colon and cecum. Indicating efficient SFB recolonization by co-housing in the Cecum (For SFB neg. n=5, SFB pos. n=2, SFB recol. n=3 (1 independent experiment)). Two-way ANOVA with Tukey's multiple comparisons test. **(C)** Flow cytometric analysis of T_{Helper} cell polarization in Peyer's patches and **(D)** spleen according to data shown in Figure 2. SFB recolonization significantly alters T_{Helper} cell homeostasis and Foxp3:IL-17 ratio. Kruskal-Wallis test with Dunn's multiple comparisons test. Brain hemispheres of these mice were processed and flow cytometry for the following immune cell populations was performed. **(E)** CD11b+CD45+ **(F)** Ly6C+Ly6G- **(G)** CD11b-CD45+ **(H)** CD4+CD3+ **(I)** Foxp3+. I= Ipsilateral cortex and C= Contralateral cortex. Two-way ANOVA with Tukey's multiple comparisons test (1 independent experiment). * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001. CR=Charles River Laboratories, Tac=Taconic Biosciences, Har=Harlan Laboratories.



Supplementary Figure 5. Characterization of T cell polarization after isotype control or CD28SA treatment.

Naïve mice were treated with IgG1 isotype control and compared to untreated mice. 5d post injection spleens and PP were taken to assess peripheral T cell polarization. **(A)** Fxp3+, IL-17+ and Fxp3:IL-17 ratio found in the PP. **(B)** Fxp3+, IL-17+ and Fxp3:IL-17 ratio found in the spleen. For the naïve group n=4 for the IgG1 group n=5 (1 independent experiment). Mann-Whitney U test. **(C)** Induction of Treg and Th17 was analyzed in spleens 5d after stroke and CD28SA treatment, then compared to controls. CD28SA administration equally expanded Treg and Th17 cells in all breeder groups, in contrast to the more selective T cell polarization observed in Peyer's patches (see Figure 4). (For control: n=5 per group (1 independent experiment), for CD28SA: CR=6, Tac=8, Har=8 (2 independent experiments)), 2way-ANOVA with Sidak's multiple comparisons test. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001. CR=Charles River Laboratories, Tac=Taconic Biosciences, Har=Harlan Laboratories.

SUMMARY

From the three previous studies it was clear that the interaction between the immune system and microbiota was affecting stroke, and immunotherapies. While in the first study we characterised changes in the gut microbiota, evidence suggested that microbiota alterations were present for much longer time periods after stroke (Swidsinski, Loening-Baucke et al., 2012). For the third aim we wanted to investigate the role of the gut microbial metabolites on long-term stroke recovery.

The SCFA (acetate, propionate and butyrate) are key bioactive metabolites produced from indigestible dietary fibre. We investigated the role of SCFA in post-stroke regeneration using supplementation of SCFA in drinking water. First, automated behavioural analysis demonstrated that SCFA supplementation specifically improved motor deficits at later time points after stroke. This indicated that SCFA could be affecting the plasticity and cortical reorganisation. Using in vivo calcium imaging we saw changes in dynamics of contralateral cortex connectivity following SCFA supplementation. In accordance with the altered connectivity, we saw that excitatory spine and synapse densities were changed under SCFA supplementation. RNA sequencing of the whole cortex indicated the potential involvement of microglia cells. Microglia are indeed the players involved in synaptic pruning. Using an automated morphology analysis of cortical microglia, we found more ramified structures, hinting at reduced microglia activation compared to controls. By flow cytometry, we characterised the immune cell populations within the brain as a potential source of neuroinflammation. SCFA supplementation reduced the cerebral lymphocytes after stroke. This was also evident in the spleens, suggesting SCFA were acting initially in the peripheral compartment. Using Rag^{-/-} mice, we found that in the absence of T and B lymphocytes, microglia did not change morphology under SCFA supplementation. Taken together, these results suggest that microbiota-derived SCFA are important for improved post-stroke recovery. More specifically, cells which are altered by SCFA in the periphery, migrate to the brain after stroke and alter the inflammatory milieu within the brain. The changed inflammatory state within the brain potentially affects microglia and in turn, the synaptic reorganisation after stroke.

REFERENCE

Author contributions: RS, JC, SR, SH, SK, DB, MG and EP performed experiments; RS, JC, SK, BN and AL analysed data, RS and AL wrote the manuscript; MG, AS and LH critically revised the manuscript AL conceived the study and supervised the project.

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Short-chain fatty acids improve post-stroke recovery via immunological mechanisms.

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Conflict of interest

The authors declare no competing financial interests.

721 **Abstract**

722 Recovery after stroke is a multicellular process encompassing neurons, resident immune
723 cells and brain-invading cells. Stroke alters the gut microbiome which in turn has
724 considerable impact on stroke outcome. However, the mechanisms underlying gut-brain
725 interaction and implications for long-term recovery are largely elusive. Here, we tested the
726 hypothesis that short-chain fatty acids (SCFA), key bioactive microbial metabolites, are the
727 missing link along the gut-brain axis and might be able to modulate recovery after
728 experimental stroke. SCFA supplementation in the drinking water significantly improved
729 recovery of affected limb motor function. Using *in vivo* wide-field calcium imaging, we
730 observed that SCFA induced altered contralesional cortex connectivity. This was associated
731 with SCFA-dependent changes in spine and synapse densities. RNA-sequencing of the
732 forebrain cortex indicated a potential involvement of microglial cells in contributing to the
733 structural and functional remodelling. Further analyses confirmed a substantial impact of
734 SCFA on microglial activation, which depended on the recruitment of T cells to the infarcted
735 brain. Our findings identified that microbiota-derived SCFA modulate post-stroke recovery
736 via effects on systemic and brain resident immune cells.

737 **Significance statement**

738 Previous studies have shown a bi-directional communication along the gut-brain axis after
739 stroke. Stroke alters the gut microbiota composition, and in turn, microbiota dysbiosis has a
740 substantial impact on stroke outcome by modulating the immune response. However, until
741 know the mediators derived from the gut microbiome affecting the gut-immune-brain axis
742 and the molecular mechanisms involved in this process have been unknown. Here, we
743 demonstrate that SCFA—fermentation products of the gut microbiome—are potent and
744 proregenerative modulators of post-stroke neuronal plasticity at various structural levels. We
745 identified that this effect was mediated via circulating lymphocytes on microglial polarization.

746 These results identify SCFA as the missing link along the gut-brain axis and as a potential
747 therapeutic to improve recovery after stroke.

748

749 **Introduction**

750 Stroke induces a multiphasic pathophysiological cascade, which consists of an initial
751 excitotoxicity followed by a longer neuroinflammatory phase within the brain (Dirnagl et al.,
752 1999; Iadecola and Anrather, 2011). Moreover, stroke can be regarded as a systemic
753 disease affecting also remote organ function including the lung (Austin et al., 2019) , heart
754 (Bieber et al., 2017), immune system and intestinal function (Singh et al., 2016b) . Recently
755 it has been shown that a dysbiotic gut microbiota is correlated with a worsened outcome in
756 patients (Xia et al., 2019), and that these changes are evident up until 3 weeks after
757 hospitalization (Swidsinski et al., 2012). We have previously demonstrated an important role
758 of the gut microbiome on stroke outcome in proof-of-principle experiments using germ-free
759 (GF) and recolonized mice (Singh et al., 2018). Further experimental studies in rodent stroke
760 models have identified a key role for the immune system—particularly brain-invading
761 lymphocytes originating from the intestinal immune compartment— in mediating along the
762 gut-brain-axis (Benakis et al., 2016; Singh et al., 2016b).

763 The gut microbiome produces a large number of bioactive metabolites which may affect
764 brain function via modulating the immune system or afferent neuronal pathways (Kau et al.,
765 2011; Cryan and Dinan, 2012). In particular, the metabolite group of short-chain fatty acids
766 (SCFA) acetate, butyrate and propionate have been shown to readily cross the blood brain
767 barrier (Frost et al., 2014; Morrison and Preston, 2016) and affect brain function in
768 development, health and disease. For example, SCFA treatment improved the disease
769 course in experimental autoimmune encephalitis by promoting anti-inflammatory
770 mechanisms and reducing axonal damage (Haghikia et al., 2015). In mouse models of
771 chronic stress, mice that received SCFA treatment exhibited significant improvements in
772 antidepressant and anxiolytic behaviours, which was accompanied by reduced plasma
773 corticosterone levels and differential gene regulation (van de Wouw et al., 2018).

774 More recently, the role of SCFA in modulating the immune system has been studied in great
775 detail. Through, these investigations, they have been shown to play a role in the polarization
776 of T cells in the intestinal immune compartment and inducing anti-inflammatory T cell subset
777 (Smith et al., 2013; Tan et al., 2016). Other studies have shown a critical role for
778 microbiotaderived SCFA in the maturation of microglial cells, the brain's resident immune
779 cells (Erny et al., 2015). However, SCFA can have far-reaching pleiotropic effects also
780 beyond the immune system, including a direct effect on neuronal function through their
781 potent function as histone deacetylase inhibitors (HDACi) (Bourassa et al., 2016).
782 Accordingly, the importance of SCFA function has been implicated in neurodegenerative
783 diseases and even in post-ischemic neurogenesis (Chuang et al., 2009; Kim et al., 2009).

784 Despite the key contribution of the gut microbiome to stroke outcome and the identification of
785 SCFA as one of the microbiome's primary bioactive mediators, the role of SCFA and their
786 potential therapeutic use for post-stroke recovery in the chronic phase after brain ischemia
787 has not been investigated. In this study, we comprehensively investigated the effect of SCFA
788 administration on post-stroke recovery using advanced behavior analyses, *in vivo* wide-field
789 calcium imaging, transcriptomic studies and histological analyses to study and link
790 SCFA-mediated recovery mechanisms from the molecular level up to behavior.

791 **Materials and methods**

792 *Animals and treatment.* All experimental protocols were approved by the responsible
793 governmental committees (Regierung von Oberbayern, Munich, Germany and Institutional
794 Animal Care and Use Committee, UT Southwestern, Dallas, Texas, USA). SPF C57BL/6J
795 female and male mice were purchased from Charles River Laboratories (Germany) or
796 Jackson Laboratories (USA). On the day of arrival mice were aged 6-8 weeks. Mice were
797 given SCFA (25.9mM sodium propionate, 40mM sodium butyrate and 67.5mM sodium
798 acetate) or salt-matched control (133.4mM sodium chloride) (9265.1, ROTH) in drinking

799 water ad libitum for 4 weeks as previously reported (Smith et al., 2013; Erny et al., 2015).
800 For antibiotic treatment, mice received Metronidazol 0.5mg/ml, (Sigma, #46461) Neomycin
801 0.5mg/ml, (Sigma, # N1142), Ampicillin 0.5mg/ml (Sigma, #31591), and Vancomycin
802 0.5mg/ml (Sigma, #V8138) and with 5% sucrose in drinking water. All drinking water
803 solutions were prepared and changed twice a week and blinded for experimenters.

804 *Experimental stroke model.*

805 For the photothrombotic stroke model (PT), mice were anaesthetized with isoflurane,
806 delivered in a mixture of 30% O₂ and 70% N₂O. Mice were placed into a stereotactic frame,
807 and throughout the surgical procedure body temperature was maintained at 37°C with a
808 mouse warming pad. Dexpanthenol eye ointment was applied to both eyes. A skin incision
809 was used to expose the skull. Bregma was located and using the laser the lesion location
810 was marked in the left hemisphere (1.5mm lateral and 1.0mm rostral to bregma). Mice were
811 then injected intraperitoneally with Rosa Bengal (Sigma, #198250). Shielding was placed on
812 skull allowing a 2mm diameter circular exposure over the lesion area. After 10 minutes, the
813 laser (Cobolt Jive, 561nm, 25mV output power with fiber collimation at f=7.66mm) was
814 applied to the lesion area for 17 minutes.

815 *For the distal, permanent occlusion of the middle cerebral artery (dMCAo),* the distal middle
816 cerebral artery was permanently electro coagulated as previously described (Llovera et al.,
817 2014). In brief, under isoflurane anesthesia mice were placed in the lateral position. A skin
818 incision was made to expose the skull. Using a drill, a burr hole into the skull revealed the
819 bifurcated middle cerebral artery. Using electric forceps, the artery was occluded, and
820 checked for no blood flow. Mice were then sutured across the skull incision.

821 *For the filament, transient occlusion of the middle cerebral artery (fMCAo),* the internal
822 carotid artery was transiently occluded for 60min as previously described (Singh et al.,
823 2016b). In brief, mice were anaesthetized with isoflurane and an incision was made to

824 expose the temporal bone. A laser doppler probe was affixed to the middle cerebral artery
825 territory to determine blood flow. At the neck an incision to the skin exposed the common
826 carotid and external carotid artery, which were ligated. The filament was inserted into the
827 internal carotid artery for 60 minutes. Mice were sutured across the neckline.

828 After all surgeries, mice were recovered in a nursing box at 32°C for 15 minutes and then
829 returned to home cages.

830 *Widefield calcium imaging.* *In vivo* widefield calcium imaging was performed as previously
831 published (Cramer et al., 2018). Briefly, we used Thy1GCaMP6s heterozygous reporter
832 mice. Mice were scalped and transparent dental cement was placed upon the intact skull, at
833 least 3 days before start of the experiment. Resting state *in vivo* imaging was performed
834 under mild anesthesia (0.5mg/kg body weight of Medetomidin with 0.75% isoflurane
835 inhalation). Mice were placed in a stereotactic frame below a customized macroscopic
836 imaging set-up and the mouse cortex was illuminated with 450nm blue LED light. We
837 recorded for 4min in resting-state with a CCD camera at 25Hz frame rate. Functional
838 connectivity was computed for seed-based analysis with a seed pixel time series centered in
839 the right caudal forelimb cortex (rCFL), contralesional to the infarcted cortex. Pearson's
840 correlation between the time course of this seed and any other signal time course within the
841 masked area was computed, then Fisher's z transformed and topographically displayed.
842 Analogous functional connectivity between seed pairs was calculated as correlation between
843 signal time courses of two seeds within the sensorimotor cortex. Seeds were defined as
844 previously described (Cramer et al., 2018). Mean correlation per group was calculated for
845 each time point. We used two sample T test and Bonferroni correction for statistical testing.

846 *Automated skilled reaching.* Behavioral training and assessments were measured using an
847 automated reach task previously described (Becker et al., 2016). Mice are trained to pull an
848 isometric lever using the contralesional forelimb, with each successful application of force
849 criteria rewarded by a drop of peanut oil. For the first week, mice were trained in groups of

850 23 littermates for six hours. During this time, spontaneous peanut oil deliveries occurred at
851 randomly spaced time intervals ranging from 30sec to 5min. Additionally, the isometric
852 handle was positioned approximately 0.5cm from the inner edge of the chamber during
853 these six-hour sessions. The force criterion necessary to trigger a 4 μ l peanut oil droplet, was
854 set just above electrical noise so that any slight touch could trigger peanut oil dispersion. For
855 the next three weeks, mice were trained individually for 2-hour sessions. The handle was
856 positioned further back at 1cm from the inner edge of the chamber. The criterion for success
857 during training sessions was changed based on a customized, adaptive MATLAB program.
858 The adaptive program initially set the criterion for success at a specified value of at least one
859 gram. After the first 15 pulls, the criterion was adjusted to the median force value. The
860 adjustments continued for the duration of the session unless the median reached the
861 maximum force requirement of 20g. Finally, we fixed the criterion at 20g to measure baseline
862 motor function for three consecutive sessions. After stroke, assessments of reaching
863 behaviors were measured weekly for six weeks.

864 *SCFA quantification.* Mice were saline perfused, and organs extracted. For feces, colon
865 content was collected and for plasma, cardiac puncture blood was centrifuged at 4°C at
866 3000rpm and the plasma removed. All samples were frozen immediately on dry ice. SCFA
867 (acetic, propionic and butyric acid) were determined according to published protocols, using
868 gas chromatography – mass spectrometry (Hoving et al., 2018). Briefly, for plasma 10 μ l
869 were used, feces were homogenized with LC-MS grade water in a bead beater. Samples
870 were spiked with deuterated internal standards for each SCFA. Subsequently, SCFA were
871 derivatized using pentafluorobenzyl bromide and analyzed using gas chromatography –
872 electron capture negative ionization in single ion monitoring mode. Quantification was
873 carried out against external calibration lines.

874 *Infarct volumetry.* After saline perfusion, the brain was removed from the skull and placed
875 immediately on dry ice. Microscopy slides were mounted with 20 μ m thick coronal

876 cryosections cut at 400 μ m intervals for dMCAo and fMCAo lesions, and at 120 μ m intervals
877 for PT lesions. Sections were then stained with cresyl violet solution. For the infarct volume,
878 all slides were scanned using 600dpi. The area of infarct tissue was measured using the
879 ImageJ software (NIH) and integrated for calculation of infarct volume.

880 *Spine density analysis.* Following saline perfusion, mice were perfused with aldehyde fixative
881 solution (Bioenno, #003780). Brains were then carefully removed and placed in fixative
882 solution at 4°C overnight. Brains were then sliced at 100 μ m thick vibratome sections and
883 immersed in impregnation solution (Bioenno, sliceGolgi Kit, #003760) for 5 days. Further
884 staining was performed as described by the manufacturer (Bioenno). Images of dendrites
885 were obtained at the same anatomical level for all brains, 400 μ m from the lesion perimeter in
886 cortical layer 2/3. In total, 5 dendrites from 5 neurons each in both hemispheres were
887 imaged (100x brightfield). Dendrites from the images were then reconstructed using Imaris
888 x64 (version 8.4.0, Bitplane).

889 *Immunohistochemistry.* Mice were perfused with saline and then 4% paraformaldehyde
890 (PFA). Brains were removed and post-fixed in 4% PFA at 4°C overnight. Brains were then
891 dehydrated in 30% sucrose in 1x PBS. Microglia staining was performed on 100 μ m thick
892 sections using rabbit anti-iba1 (1:200, Wako, #019-19741), and then goat anti-rabbit 594
893 (1:200, Thermo Fisher Scientific, #A-11012). Automated analysis of microglial cell counts
894 and morphology was performed using a MATLAB automated morphology protocol as
895 previously described (Heindl et al., 2018). For synaptic staining, sections were incubated
896 with guinea pig anti-VGlu1 (1:1000, Millipore, #AB5905) and chicken anti-Homer1 (1:2000,
897 Synaptic Systems, #160006) for 3d at 4°C. Then counterstained with goat anti-guinea pig
898 488 (1:500, Invitrogen, #A11073) and goat anti-chicken 647(1:500, Invitrogen, #A21449) for
899 18h at RT. For synaptic puncta analysis a z-stack of 3 slices at 0.33 μ m were used as
900 previously described (Au - Ippolito and Au - Eroglu, 2010) using FIJI with the
901 SynapseCounter plugin (Schindelin et al., 2012).

902 *Flow cytometric analysis.* Following saline perfusion in deep anesthesia, the entire spleen
903 and both brain hemispheres were dissected. Spleens were immediately placed on ice cold
904 1x PBS, were passed through a 40µm cell strainer and treated with red blood cell lysis
905 buffer. Cell surface markers were stained using the following antibodies: Anti-CD3 FITC
906 (clone 17A2), Anti-CD45 eFluor450 (clone 30-F11). Stained cells were analyzed on a BD
907 FACSVerse flow cytometer (BD Biosciences) and analysis performed by FlowJo software
908 (version 10.0).

909 For brain, hemispheres were isolated and cells isolated by mechanical dissociation.
910 Mononuclear cells were enriched using discontinuous Percoll gradient centrifugation by
911 standard protocols and then with 30µm cell strainer, as described previously (Benakis et al.,
912 2016). Cell staining for flow cytometry was performed by first pre-incubation of cells with Fc
913 receptor blocker (1:100, Invitrogen, 14-91-61-73) for 10 min and then staining with the
914 following monoclonal antibodies: Anti-CD3 FITC (clone 17A2), anti-CD45 eFluor450 (clone
915 30-F11) and anti-CD11b Pe-Cy7 (clone M1/70). Stained cells were analyzed on a BD
916 FACSVerse flow cytometer (BD Biosciences) and analysis performed by FlowJo software
917 (version 10.0).

918 *RNA sequencing and data analysis.* Fourteen days post stroke, brains were extracted from
919 the skulls and placed into a stainless-steel brain matrix. Brains were sliced coronally at 2mm
920 distance caudally and rostrally away from the lesion. The cortex was removed, frozen on dry
921 ice and the mRNA isolated using the RNeasy Mini Kit (Qiagen, #74109). For library
922 preparation 100ng of total RNA were fragmented and processed using the Ovation Human
923 FFPE RNA-Seq Library Systems (Nugen) according to the instructions of the manufacturer.
924 Barcoded libraries were quantified using the Library Quantification Kit - Illumina/Universal
925 (KAPA Biosystems). Cluster generation was performed with a concentration of 10nM using a
926 cBot (Illumina). Sequencing of 2x 100bp paired-end reads was performed with a HiScanSQ
927 sequencing platform (Illumina) using version 3 chemistry at the sequencing core facility of

928 the IZKF Leipzig (Faculty of Medicine, University Leipzig). Raw reads were mapped to the
929 reference genome mm10 using split-read mapping algorithm implemented in segemehl
930 (Hoffmann et al., 2009). Mapped reads were counted using featureCounts (Liao et al., 2014)
931 according to RefSeq annotation. Differential expression was computed using DESeq2
932 algorithm (Love et al., 2014). Raw data have been deposited at Gene Expression Omnibus
933 database (Accession number GSE131788).

934 *RT-PCR.* mRNA from brain tissue around the lesion was isolated as described above and
935 transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems,
936 #4368814) with RNasin Plus RNase Inhibitor (Promega, #N2611).

937 The following primers were used: BDNF (forward: CGGCGCCCATGAAAGAAGTA and
938 reverse: AGACCTCTCGAACCTGCCCT), TrkB (forward: ACTTCGCCAGCAGTAGCAG and
939 reverse: ACCTCAGGGCTGGGGAG), Synaptophysin (forward:
940 AGTACCCATTCAGGCTGCAC and reverse: CCGAGGAGGAGTAGTCACCA), EphrinA5
941 (forward: CTGGTGCTCTGGATGTGTGT and reverse: CCCTCTGGAATCTGGGGTTG) and
942 PPIA (forward: ACACGCCATAATGGCACTGG and reverse:
943 ATTTGCCATGGACAAGATGCC). The QuantiNova SYBR Green PCR Kit (Qiagen,
944 #208052) was used with a LightCycler 480 II (Roche). All gene expression was expressed
945 relative to the PPIA house keeping gene and calculated using the relative standard curve
946 method.

947 *Experimental Design and statistical analysis.* All statistical analyses were performed using
948 GraphPad Prism software (GraphPad, version 6.0). Sample size was chosen based on
949 comparable experiments from previous experiments (Singh et al., 2016b). For experimental
950 design details on sample size, please refer to manuscript results and figure legends. A p
951 value of <0.05 was regarded as statistically significant.

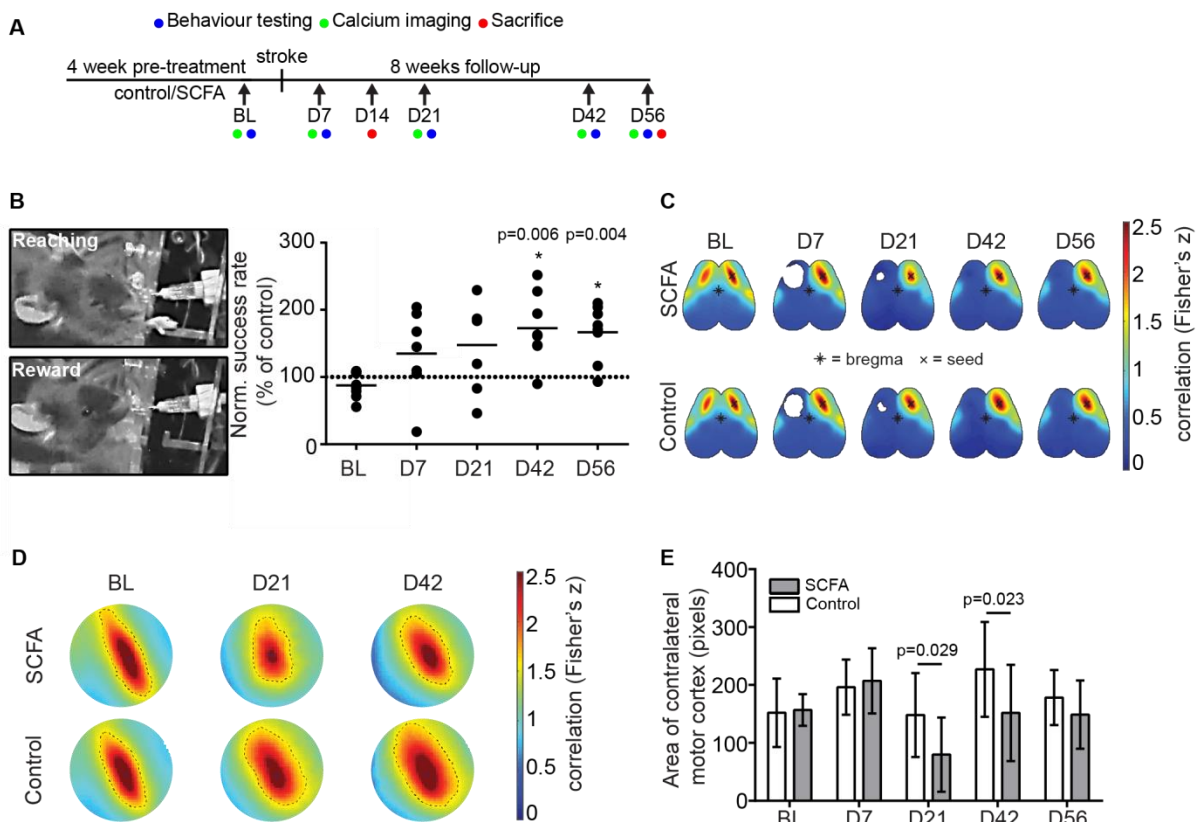
952 **Results**

953 SCFA supplementation improves recovery and cortical reorganization after stroke

954 We previously showed that stroke induces dysbiosis of the gut microbiome with the hallmark
955 of reduced bacterial diversity (Singh et al., 2016b). In order to test the impact of post-stroke
956 gut dysbiosis on the metabolic function of the microbiome, we performed targeted analysis of
957 plasma samples for SCFA concentrations after stroke and sham surgery in mice by mass
958 spectrometry. This analysis revealed significantly reduced plasma SCFA concentrations 3
959 days after stroke surgery compared to sham-operated mice (Fig. 1-1 A). Therefore, we
960 hypothesized that supplementation of SCFA would increase circulating SCFA
961 concentrations, and potentially induce therapeutic effects within the chronic post-stroke
962 recovery period. In order to test this hypothesis, we supplemented mice for 4 weeks with
963 drinking water containing either a mix of acetate, butyrate and propionate (see methods
964 section for details) or control drinking water with matched sodium chloride concentration.
965 SCFA supplementation did not affect body weight (Fig. 1-1 B) or overt behavior of the
966 animals. We performed photothrombotic stroke surgery after four weeks of SCFA
967 supplementation and assessed post-stroke motor deficits of the affected forelimb with an
968 automated lever pull test (Fig. 1A, B). Mice receiving SCFA supplementation performed
969 significantly better in the chronic post-stroke phase at days 42 and 56 (Fig. 1B). In order to
970 analyze cortical network plasticity as the morphological surrogate of behavioral recovery, we
971 used Thy1-GCaMP6s mice and performed resting state in vivo calcium to record cortical
972 widefield fluorescence from a neuronal based calcium reporter. Using the homotypical
973 contralesional region of the cortex, we performed seed-based correlation analysis, indicating
974 the connectivity strength (z-score) of this area to every other pixel in the cortex (Fig. 1C).
975 Previous research using fMRI in stroke patients has indicated that the homotypical
976 contralesional region receives less neuronal inhibition from the stroked hemisphere, leading
977 to disinhibition of the contralesional hemisphere (Rehme and Grefkes, 2013). To analyze the

978 size of highly connected homotypical contralesional regions (i.e. the contralesional motor
 979 cortex), we measured the area of pixels with a z-score above 2.25 (Fig. 1D). We observed a
 980 significantly reduced area of the contralesional motor cortex in SCFA- compared to
 981 control-treated mice at D21 and D42 (Fig. 1E). In contrast, SCFA treatment did not
 982 significantly affect the primary infarct area by *in vivo* imaging (Fig. 1-1 C) and histological
 983 analyses with infarct volumetry in 3 different focal stroke models after stroke (Fig. 2-1 E).
 984 These results indicated that while SCFA supplementation did not affect the initial lesion
 985 development, SCFA improved behavioral stroke outcome and modulated cortical network
 986 plasticity at later stages after stroke.

987



988

989 **Figure 1. SCFA supplementation improves recovery after stroke.** (A) Schematic
 990 diagram illustrating the timeline of SCFA supplementation and analysis timepoints. (B)
 991 Representative images obtained during the lever pull test of trained mouse successfully

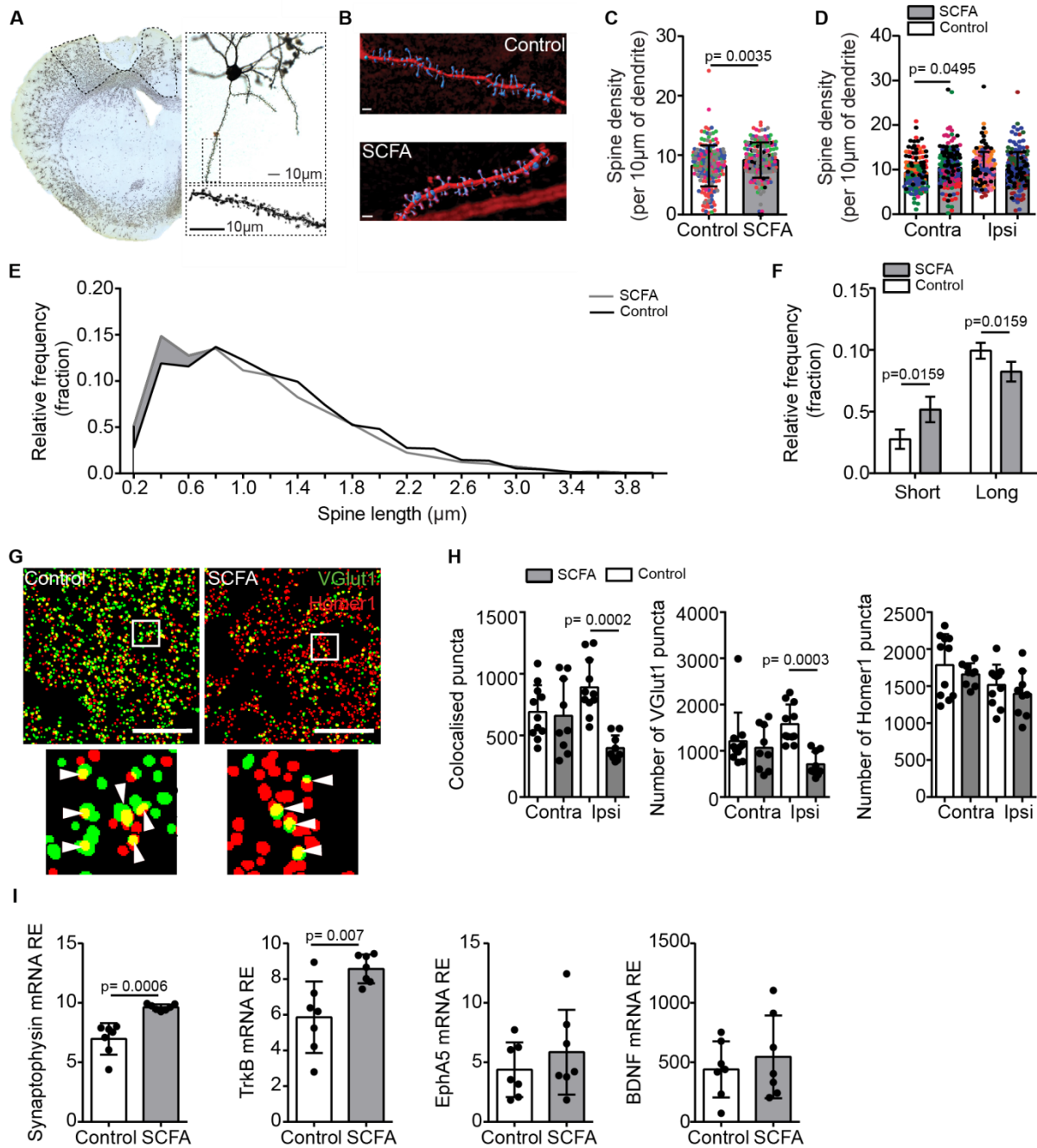
992 reaching for the lever (left, above) and obtaining the peanut oil reward (left, below). Right
993 panel: normalized success rate for lever pulls by the affected (contralesional) forelimb.
994 Relative values are shown per time point normalized to the mean of the control group. $N=6$
995 per group, horizontal line indicates mean. **(C)** Topographic depiction of seed-based
996 functional connectivity of both hemispheres at indicated time points of SCFA and
997 control-treated mice. Seed is placed in the homotypic contralesional region to the ipsilesional
998 lesion area (i.e. the contralesional motor cortex). Colour code indicates Fischer's z
999 correlation between the seed and every other pixel in the cortex. **(D)** Enlarged images of the
1000 contralesional motor cortex (region homotypic to the infarct lesion). Area highlighted with
1001 dotted line indicates the highly connected functional motor cortex area (pixels with Fischer's
1002 z values >2.25). **(E)** Quantification of highly-correlated (Fischer's $z >2.25$) area of the
1003 contralesional motor cortex in control (open bars) and SCFA treated mice (grey bars). $N=15$
1004 per group. Statistical tests in B and E have been performed using multiple T tests per time
1005 point with Holm-Sidak's correction for multiple testing.

1006 SCFA supplementation modulates post-stroke synaptic plasticity.

1007 After an ischemic brain injury, the entire cortex undergoes rapid functional and morphological
1008 reorganization including neuronal dendritic plasticity which allows adult neurons to form new
1009 connections. This process is correlated with improved functional connectivity after a cortical
1010 lesion (Jones and Schallert, 1994; Biernaskie et al., 2004). In order to analyze underlying
1011 morphological plasticity of the observed functional recovery in behavior and cortical
1012 connectivity, we performed Golgi-Cox staining of brain sections to investigate the effects of
1013 SCFA supplementation on dendritic spine density of pyramidal cells (Fig. 2A, B). We initially
1014 performed this analysis in the brains of naive animals that received either SCFA or control
1015 treatment, which revealed a higher pyramidal cortical spine density in the SCFA-treated mice
1016 (Fig. 2C). Next, we quantified brains at 14 days after stroke to capture a time-period before
1017 the behavioral and cortical connectivity improvements were evident. Correspondingly, we

1018 observed that after stroke SCFA supplementation was associated with a significantly higher
1019 spine density in the homotypic contralesional region, however, these differences were not
1020 observed in the ipsilesional hemisphere (Fig. 2D) and were independent of infarct volume
1021 (Fig. 2-1). Moreover, the analysis of spine length distribution revealed that SCFA treatment
1022 induced a shift towards shorter spine lengths, specifically in the ipsi- but not contralesional
1023 hemisphere (Fig. 2E, F, Fig. 2-1 F, G). In order to further investigate synaptic plasticity under
1024 control of SCFA supplementation, we assessed synaptic density using co-staining for VGlut1
1025 (pre-synaptic) and Homer1 (postsynaptic) (Fig. 2G). We detected a significant reduction of
1026 the number of synapses (i.e. colocalized VGlut1 and Homer1 puncta) in the peri-lesional
1027 cortex of SCFA-supplemented mice (Fig. 2H). Interestingly, the difference in synapse counts
1028 was exclusively driven by the number of pre-synaptic VGlut1 puncta while Homer1-positive
1029 puncta remained unaffected.

1030 Additionally, this pattern was also mirrored in the mean size of VGlut1 and Homer1 puncta
1031 (Fig. 2-1 H). Finally, we analyzed the transcriptional regulation of key factors involved in
1032 synaptic plasticity (Fig. 2I). We observed that SCFA significantly increased the expression of
1033 the presynaptic vesicle molecule synaptophysin and the BDNF (brain-derived neurotrophic
1034 factor) receptor TrkB. BDNF itself, or the receptor tyrosine kinase EphA2 was not affected by
1035 SCFA supplementation. These results indicate effects of SCFA on morphological, synaptic
1036 plasticity which could potentially precede the effects observed on functional recovery at later
1037 time points after stroke.



1038

1039 **Figure 2. Post-stroke neuronal plasticity is altered by SCFA treatment.** (A)
 1040 Representative images of Golgi-Cox stained brain sections 14d after stroke. Dotted area in
 1041 overview image indicates peri-lesional cortical area used for spine analysis. Magnifications
 1042 show representative pyramidal neuron and high-magnification image as used for spine
 1043 analysis. Top right shows a cortical pyramidal neuron. Lower right example of spines

1044 identified on dendrite. Scale bar = 10 μ m. **(B)**. 3D reconstruction for a dendrite section with
1045 spines as used for further quantification of spine densities and lengths, scale bar = 2 μ m. **(C)**.
1046 Quantification of pyramidal spine density per 10 μ m of dendrite in the cortex of control and
1047 SCFA treated naïve mice (no stroke induction). Each colour represents a different mouse
1048 and each dot a different dendrite. 5 neurons per hemisphere analysed in total for 4/5 mice
1049 per group (Mann-Whitney U test). **(D)**. Quantification of spine density per 10 μ m of dendrite in
1050 the perilesional and contralesional cortex at 14d after stroke (Kruskal-Wallis test with Dunn's
1051 correction for multiple comparisons. **(E)** Histogram of the relative frequency (fraction) of
1052 spines found at different lengths 14d after photothrombotic lesion in the perilesional cortex.
1053 Bin width is 0.2 μ m **(F)**. Quantification of short (0.2 μ m) and long (1.4 μ m) spines in control
1054 (open bars) and SCFA (grey bars) treated mice. N=4/5 per group, Mann-Whitney U test. **(G)**.
1055 Representative particle images of presynaptic terminals by VGlut1 (green), postsynaptic
1056 densities by Homer1 (red) and nuclei with DAPI (blue) of the cortex from control and SCFA
1057 treated mice (scale bar = 20 μ m), as used for quantification of co-localized pre- and
1058 postsynaptic particles (puncta). Arrowheads indicate co-localized (yellow) puncta. **(H)**.
1059 Synapse counts were quantified as co-localised VGlut1 and Homer1 puncta. Quantification
1060 for co-localization (left and for single markers (middle and right) revealed significantly
1061 changed synapse counts as a result of the reduced number of VGlut1+ terminals in the
1062 perilesional cortex (contra: contralesional hemisphere; ipsi: ipsilesional hemisphere). N=3/4
1063 mice (3 sections per mouse), statistical analysis performed with Kruskal-Wallis test with
1064 Dunn's multiple comparison correction. **(I)**. Relative expression (RE) of mRNA for key
1065 molecules involved in synaptic plasticity (left) synaptophysin, (left middle) TrkB, (right
1066 middle) EphA5, (right) BDNF from the peri-lesional cortex in control (open bars) and SCFA
1067 (grey bars) treated mice. N= 7 per group, Mann-Whitney U test.

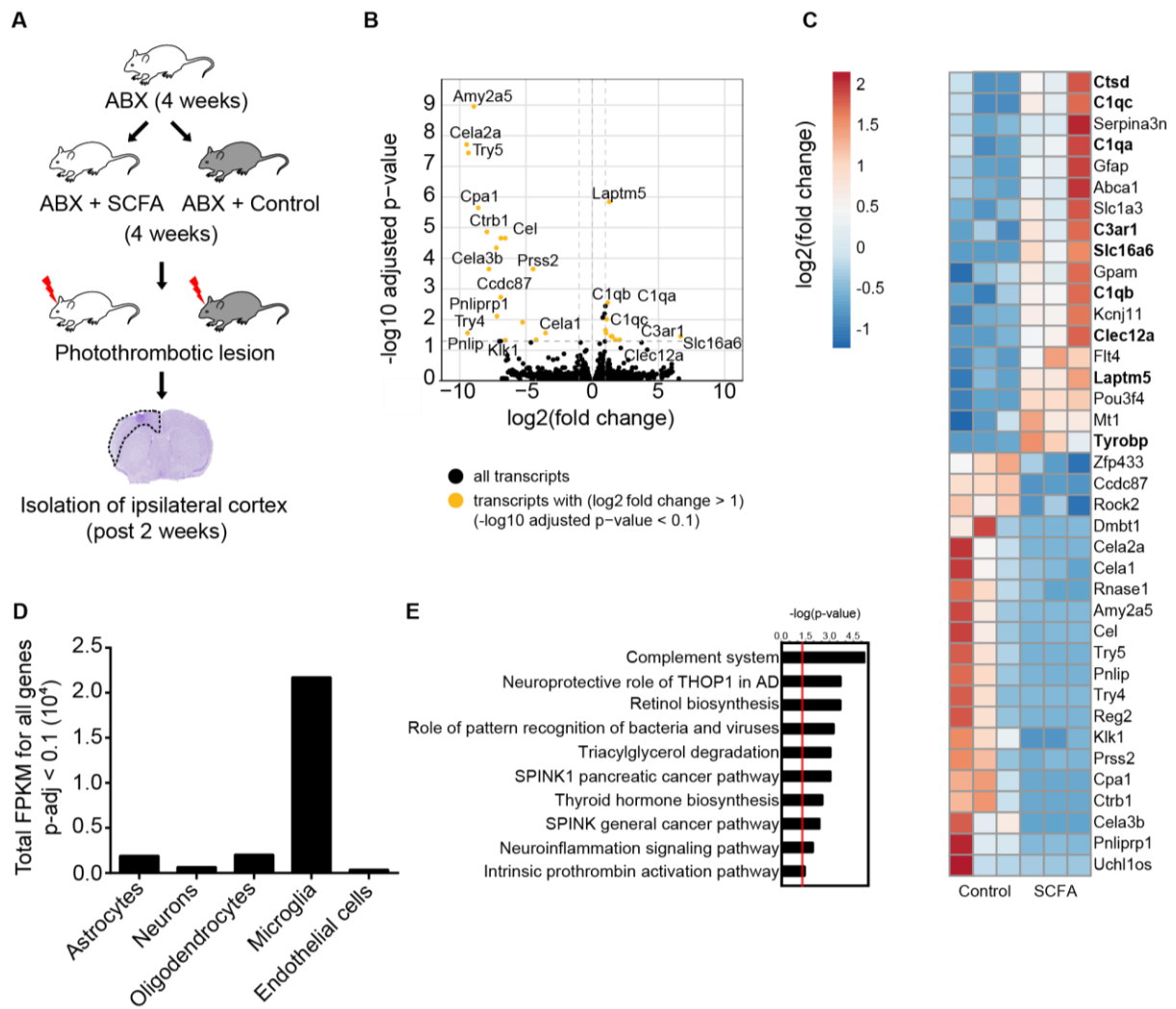
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1070 Brain transcriptomic analysis indicates microglia as the main cellular target of SCFA.

1071 In order to determine whether the observed effects of SCFA were either directly mediated on
1072 neuronal function or affecting other cerebral cell populations, we took an unbiased approach
1073 to investigate the effects of SCFA on gene expression in the peri-infarct cortex. For this, we
1074 first depleted the gut microbiome of mice as the main SCFA source by administration of
1075 antibiotics, followed by supplementation of SCFA (Fig. 3A). Plasma GC-MS quantification
1076 confirmed an increase of SCFA after supplementation in drinking water (Fig. 3-1). Fourteen
1077 days post-surgery the infarct and peri-infarct region were isolated for RNA sequencing,
1078 identifying 18 upregulated and 20 downregulated genes in SCFA supplemented mice (Fig.
1079 3B, C).

1080 The list of the top upregulated genes (Fig. 3C) hinted at a role for microglia as we found
1081 numerous genes that have previously been reported to be involved in microglial function
1082 and/or activation such as *Ctsd*, various complement molecules, *Tyrobp* and *Laptn5* (Zhong
1083 et al., 2018; Li et al., 2019). In order to ascertain which cell type SCFA supplementation was
1084 mainly regulating in the brain, we took the 38 differentially regulated genes and compared
1085 them to an existing RNA-Seq database which lists the fragments per kilobase of exon model
1086 per million reads (FPKM) found in astrocytes, neurons, oligodendrocytes, microglia and
1087 endothelial cells (brainrnaseq.org) (Zhang et al., 2014). From the total number of FPKM
1088 values of the 38 differentially regulated genes (Suppl. Table 1), we discovered that the vast
1089 majority of gene reads from the significantly regulated genes were associated with microglial
1090 cells (Fig. 3D). Additionally, we performed an ingenuity pathway analysis on the 38
1091 differentially regulated genes, and found that the complement system was the top pathway
1092 and highly upregulated in mice supplemented with SCFA (Fig. 3E). Within the brain the
1093 complement pathway is critical for microglia activation and has been associated with
1094 synaptic pruning by microglia (Schafer et al., 2012; Stephan et al., 2012; Wu et al., 2015).



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Figure 3. SCFA supplementation affects microglial gene signature after stroke. (A). Schematic diagram illustrating antibiotic treatment regimen and followed by supplementation with SCFA or control saline in drinking water. Delineated area on histological image illustrate perilesional cortex isolated for mRNA sequencing. **(B).** Volcano plot of regulated transcripts (SCFA/control) in the perilesional cortex 14d after stroke, $n=3$ per group. All gene transcripts are in black, and gene transcripts with fold change (\log_2) >1 and $-\log_{10}$ adjusted p -value < 0.1 are in orange. **(C).** Heatmap of fold change (\log_2) for significantly regulated genes with an adjusted p value < 0.1. Each column represents one individual mouse. **(D).** FPKM abundance and association per cerebral cell type of all significantly regulated genes (panel C) was performed as detailed in the methods section, revealing the strongest association of the significantly regulated genes with microglial cells. **(E).** Ingenuity pathway analysis

1107 *showing the top networks regulated by SCFA supplementation in comparison to control*
1108 *treatment.*

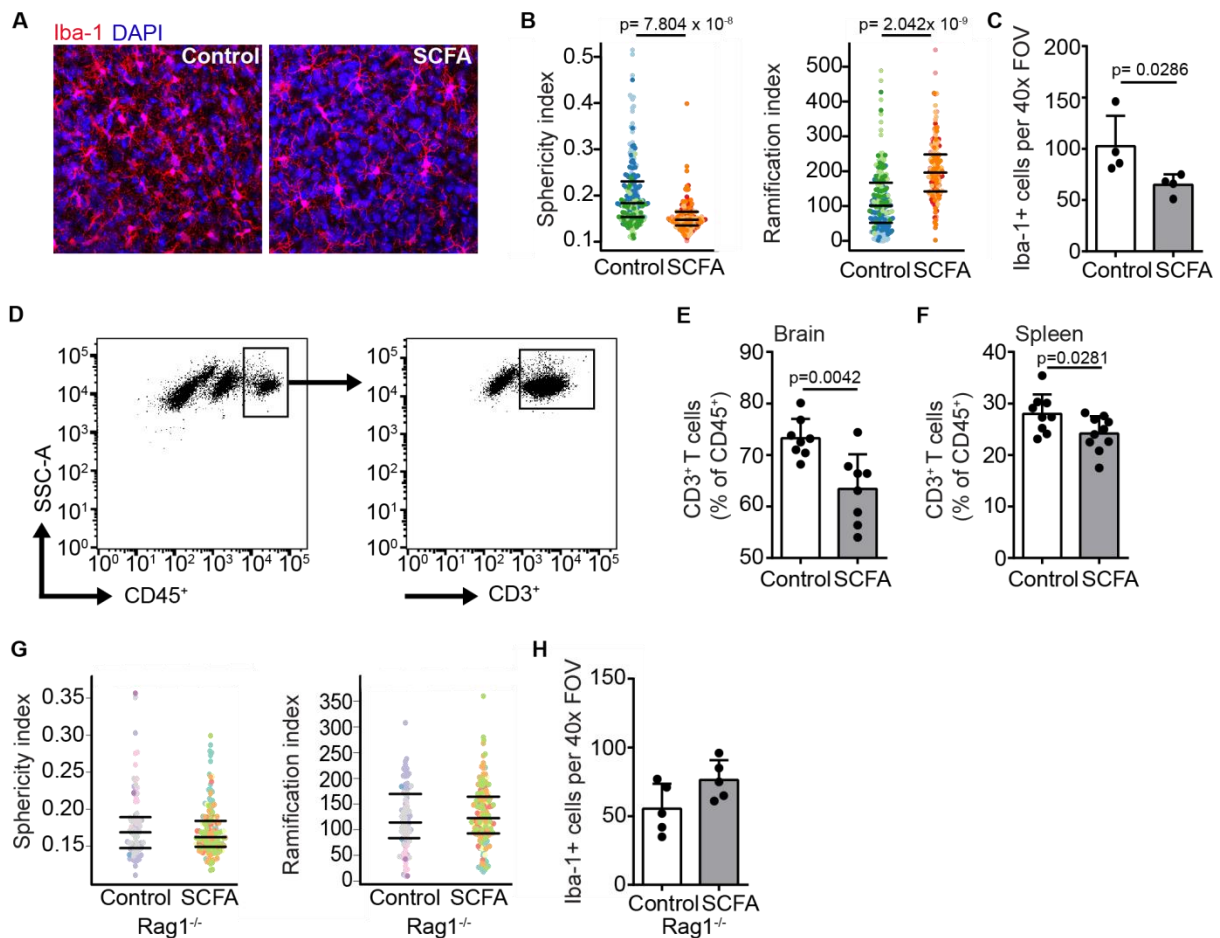
1109 SCFA modulate microglial activation and immune cell composition.

1110 Based on the transcriptomic data's indication that microglia are the effector of SCFA
1111 mediated post-stroke recovery, we performed more in-depth analyses of microglial activation
1112 and the inflammatory response to stroke. As a surrogate of microglia activation we
1113 performed Iba-1 immunohistochemistry of the cortex (Fig. 4A) and assessed microglia
1114 morphology using automated analysis (Heindl et al., 2018). 14d after stroke, cortical
1115 microglia from SCFA supplemented mice displayed a significantly more ramified and less
1116 spherical (i.e. less activated) morphology in comparison to controls (Fig. 4B). Additionally,
1117 the total number of microglia were significantly reduced in SCFA- compared to control-treated
1118 mice (Fig. 4C). These results indicate a modulation of the microglial response to stroke by
1119 SCFA. Aside from evoking a microglial response, stroke induces invasion of peripheral
1120 immune cells into the brain parenchyma, which can deteriorate stroke outcome (Iadecola
1121 and Anrather, 2011; Liesz et al., 2015; Neumann et al., 2015; Selvaraj and Stowe, 2017). In
1122 particular, lymphocyte counts in brains after photothrombosis are still elevated 14 days after
1123 surgery (Feng et al., 2017). Additionally, cytokines secreted by brain-invading lymphocytes
1124 can modulate microglial activation (Liesz et al., 2009; Meng et al., 2019). Therefore, we
1125 performed flow cytometry of brain homogenates (Fig. 4D) and observed a significant
1126 reduction in cerebral lymphocyte invasion in SCFA-treated animals compared to controls
1127 (Fig. 4E).

1128 The invasion of peripheral lymphocytes to the ischemic brain depends on the number of
1129 circulating lymphocytes, the expression of cerebral endothelial adhesion molecules and the
1130 chemokine gradient derived from the injured brain tissue (Ransohoff and Engelhardt, 2012).
1131 In accordance with our non-targeted transcriptomic analysis (see results in Fig. 3) which did
1132 not reveal significant regulation of chemokines or adhesion molecules by SCFA, also a

1133 targeted PCR analysis of key adhesion molecules and tight junction proteins involved in
1134 post-stroke lymphocyte recruitment at the blood-brain barrier did not show a significant
1135 regulation by SCFA (Fig. 4-1). In contrast, we detected a significant reduction of systemic T
1136 cells counts by SCFA supplementation in the spleen (Fig. 4F), a secondary lymphatic organ
1137 which well characterizes the systemic immune response after stroke (Offner et al., 2006;
1138 Liesz et al., 2013; Roth et al., 2018).

1139 These findings suggest that SCFA may primarily affect lymphocytes already in the peripheral
1140 immune compartments which then might secondarily mediate changes in the cerebral
1141 immune milieu after brain invasion. Therefore, we next aimed to test this hypothesis by
1142 investigating the effects of SCFA on microglia in the absence of lymphocytes. For this we
1143 used lymphocyte-deficient Rag1^{-/-} mice which were given SCFA supplementation or control
1144 drinking water. In contrast to lymphocyte-competent WT mice, SCFA supplementation in
1145 Rag1^{-/-} did neither affect microglia morphology nor did it reduce microglial cell counts (Fig.
1146 4G, H), thus indicating a key role of lymphocytes for mediating the SCFA effects on
1147 microglia.



1148

1149 **Figure 4. Modulation of post-stroke neuroinflammation by SCFA depends on**
 1150 **peripheral lymphocytes.** (A). Representative maximum intensity projections of microglial
 1151 staining using Iba-1 (red) and DAPI (blue) in the ipsilesional hemisphere 14d after stroke
 1152 control (left) and SCFA (right) treated mice. (B). Microglial morphology was analyzed in 3D
 1153 using an automated analysis algorithm in the ipsilesional cortex of mice 14d post-stroke,
 1154 which revealed significantly reduced sphericity (left) and increased number of branch nodes
 1155 (right) as markers of reduced microglial activation by SCFA compared to control treatment.
 1156 Each symbol represents one microglia, different colors group together microglia from the
 1157 same mouse. (C). Number of microglia found per one high power (40x) field of view (FOV) in
 1158 the perilesional cortex. (D). Representative gating strategy for flow cytometric analysis of T
 1159 cells (CD45⁺CD3⁺). SCFA supplementation significantly decreased the frequency of T cells
 1160 in (E) brains and (F) spleens 14d post-stroke. N=9 per group. Quantification of (G) sphericity

1161 (left) and ramification index (right) and (H) absolute cell counts of microglia 14d post-stroke
1162 in the peri-lesional cortex of *Rag1^{-/-}* mice. In contrast to WT mice (compare to panel B, C),
1163 SCFA treatment did not affect microglia activation in lymphocyte-deficient *Rag1^{-/-}* mice. All
1164 statistical analyses in this figure were performed using the Mann-Whitney U test.

1165 Discussion

1166 This study demonstrates that SCFA, critical metabolites derived from the gut microbiome,
1167 are capable of improving post-stroke recovery via the modulating effects of brain-invading
1168 lymphocytes on microglial function. Results from our study support this conclusion through
1169 several key findings. First, stroke lowers blood SCFA concentrations. Second, SCFA
1170 supplementation combats the deleterious effects of this post-stroke response which we
1171 demonstrated by associated SCFA supplementation with improved behavioral recovery,
1172 changes in cortical network connectivity which are generally associated with improved stroke
1173 outcomes and changes in histological markers of synaptic plasticity. Third, we indicated a
1174 potential mechanism for the observed improvements in the recovery of SCFA-treated
1175 animals by finding changes in microglial function which were dependent on circulating
1176 lymphocytes. Consequently, these findings indicate that SCFA affect peripheral
1177 lymphocytes—maturation or egress from primary lymphatic tissue—and lymphocytes then
1178 indirectly mediate the SCFA effects on the brain micromilieu either by their overall reduction
1179 in cerebral invasion or polarization of the secreted cytokine profile.

1180 These novel findings introduce SCFA as the most likely missing link in the
1181 pathophysiological function of the gut-brain axis in stroke and post-stroke recovery, for which
1182 the microbiota-derived effector molecules were so far unknown (Benakis et al., 2016;
1183 Houlden et al., 2016; Singh et al., 2016b). Our use of a novel imaging modality, *in vivo*
1184 widefield calcium imaging, provided a highly sensitive tool for assessing cortical network
1185 plasticity after stroke (Cramer et al., 2018). This tool allowed us to perform analyses of
1186 network plasticity by comparable analytical approaches to functional MRI in patients, with the

1187 exception of using a genetically encoded reporter for direct neuronal activation instead of the
1188 blood flow surrogate marker (BOLD) used in MRI (Cramer et al., 2018). The analysis of
1189 connectivity within the cortical network provides information about the dynamic changes in
1190 defined cortical areas under resting-state conditions, which gave us a unique ability to
1191 measure even the more subtle effects of SCFA supplementation on post-stroke plasticity.

1192 In human stroke patients, interhemispheric resting-state connectivity is significantly
1193 weakened following stroke. Specifically, it is thought that inhibitory projections from the
1194 lesioned hemisphere to the homotypic contralesional hemisphere is attenuated, leading to a
1195 disinhibition of the homotypic area in the contralesional hemisphere (Rehme and Grefkes,
1196 2013). We confirmed this effect with our optogenetic imaging approach, by observing an
1197 increase in the contralesional motor cortex area as an indicator of reduced transhemispheric
1198 inhibition. This “blooming” effect of the contralesional motor cortex was significantly
1199 ameliorated by the SCFA treatment at chronic time points after stroke, which could indicate
1200 an improvement of interhemispheric connectivity and thereby reestablished inhibition of the
1201 contralesional hemisphere. However, the further in-depth exploration of such
1202 interhemispheric inhibition is impeded by several technical limitations of our imaging tool
1203 such as autofluorescent artefacts in the peri-lesional territory and the lack of directional
1204 information of the interhemispheric connections. This would require *in vivo*
1205 electrophysiological studies on connectivity between the recovering peri-lesional tissue and
1206 the homotypic contralesional brain area which are currently not yet established in stroke
1207 research. The functional relevance for the changes within connectivity were ultimately
1208 confirmed by the corresponding beneficial effects of SCFA on recovery of motor functions.
1209 Behavioral recovery was assessed in a highly sensitive, rater-blinded and high-throughput
1210 automated test, specifically analyzing motor deficits of the affected forelimb. Hence,
1211 providing a reliable readout post-stroke recovery, even in the chronic phase. Something
1212 which most conventional behavior tests lose their test sensitivity towards (Zausinger et al.,
1213 2000; Li et al., 2004; Manwani et al., 2011; Rosell et al., 2013).

1214 As an independent line of evidence for the effects of SCFA on post-stroke recovery, we
1215 detected significant changes in dendritic spine densities and synaptic counts based on
1216 histological analysis. Previous studies have demonstrated changes in dendritic spine density
1217 of the peri-lesional cortex as well as contralateral hemisphere and have been identified as a
1218 hallmark of post-stroke tissue remodeling and marker of synaptic plasticity (Brown et al.,
1219 2008; Huang et al., 2018). After stroke, it has been shown that there is a gradual increase of
1220 spine density in the contralateral cortex (Huang et al., 2018) while spine density in the
1221 perinfarct region is reduced by about 38% within the acute phase (Brown et al., 2008).
1222 Accordingly, we observed significantly increased spine densities in the contralateral motor
1223 cortex of SCFA supplemented mice, suggesting that these microbial metabolites could aid
1224 recovery by promoting dendritic spine plasticity.

1225 A key limitation of the various analysis readouts used in this study (calcium imaging, spine
1226 density, synapse count) is their exclusive reflection of processes in excitatory neurons. The
1227 expression of GCamp in Thy1-positive neurons, analysis of dendritic spines on pyramidal
1228 neurons and the synapse count of VGlut1-positive (glutamatergic) synapses all limit the
1229 analysis to the excitatory system of the forebrain cortex and does not account for potential
1230 alterations in subcortical structures or the complex integration of information by (inhibitory)
1231 interneurons within the cortex (Markram et al., 2004). However, the focus on the excitatory
1232 cortical system across the different methodological approaches allowed us to thoroughly
1233 investigate the impact of SCFA from a global network perspective (widefield imaging) to
1234 synaptic (sub-)structures within the same system. This approach revealed a striking
1235 alignment of results across different functional levels from synapse to network plasticity and
1236 behavior that strongly support the impact of SCFA on post-stroke recovery. Despite this,
1237 future studies need to address the intricate regulation of cortical microcircuits underlying this
1238 effect and particularly the role of the various interneuron types.

1239 The transcriptomic analysis performed in this study indicated several genes which have
1240 previously been associated with microglial activity and phagocytic function to be regulated by
1241 SCFA in the post-stroke recovery phase. Therefore, we hypothesized that the SCFA effect
1242 on post-stroke recovery and neuronal plasticity might be indirectly mediated via microglial
1243 activity. Indeed, microglia are key players involved in synaptic pruning and dendritic
1244 remodelling (Salter and Stevens, 2017). Upon pro-inflammatory stimuli, microglial activation
1245 is associated with a change in cell morphology. While homeostatic microglia are highly
1246 ramified, activated cells acquire a more amoeboid shape. We observed after stroke an
1247 increase in cell ramification by the SCFA supplementation, indicating reduced or altered
1248 microglial activation state. Interestingly, microglial activation after stroke has previously been
1249 associated with reduced phagocytic capacities (Faustino et al., 2011). Hence, modification of
1250 microglial activation by SCFA could have an impact on synapse elimination and thereby
1251 microglia-dependent synaptic plasticity (Salter and Stevens, 2017). However, microglial
1252 elimination (“pruning”) of dendritic synapses is vice versa associated with synaptic activity,
1253 i.e. less active pre-synaptic inputs are more likely to be phagocytosed. Reduced synaptic
1254 activity in the peri-lesional cortex due to the tissue injury or in the contralesional cortex due
1255 to loss of transcallosal innervation from the injured cortex, could both induce excessive
1256 (pathological) dendrite pruning (Brown et al., 2008; Riccomagno and Kolodkin, 2015; Salter
1257 and Stevens, 2017). This would suggest that dendritic spine remodelling and microglial
1258 activation is a reciprocal, bi-directional interaction of neurons and microglia in the recovering
1259 cortex after stroke. While the results of RNASeq analysis indicate that SCFA modulate
1260 primarily microglial activation in this process, the detailed interaction of neurons and
1261 microglia as well as the directionality of their association will require further experimental
1262 studies. Finally, microglia activation has been associated with neuronal plasticity and
1263 function beyond morphological reorganization and spine remodelling. We have recently
1264 demonstrated, that microglia monitor neuronal function at the cell soma and that this somatic
1265 interaction is altered during pathology after stroke (Cserép et al., 2019) a process that could

1266 likely contribute to the functional changes observed in cortical calcium imaging and
1267 behavioural outcomes. However, the contribution of SCFA to this somatic cell-cell interaction
1268 is so far unknown.

1269 A large part of previous experimental studies with SCFA have focused on their effect on
1270 circulating immune cells. SCFA modulate the polarization of lymphocyte subsets such as the
1271 ratio of anti-inflammatory regulatory T cells (Treg) to pro-inflammatory T_H17 cells (Arpaia et
1272 al., 2013; Haghikia et al., 2015; Park et al., 2015; Asarat et al., 2016), as well as their
1273 migratory behaviour between organs (Nakamura et al., 2017). Importantly, lymphocyte
1274 invasion to the ischemic brain is a hallmark of post-stroke neuroinflammation (Iadecola and
1275 Anrather, 2011). It is therefore likely, that SCFA may not exclusively act directly on resident
1276 microglia per se, but also via the peripheral effects of SCFA which change lymphocyte
1277 function. The altered peripheral lymphocytes later invade the injured brain and indirectly
1278 influence the cerebral inflammatory milieu. Indeed, T cells have been consistently identified
1279 in several reports as the invading leukocyte subpopulation with the largest impact on stroke
1280 outcome with partially divergent functions of T cell subsets (Kleinschnitz et al., 2010). On
1281 one hand, immunosuppressive Treg cells provide a neuroprotective function by suppressing
1282 an overshooting inflammatory reaction to the brain infarct (Liesz et al., 2015), on the other
1283 hand the pro-inflammatory T_H1 and T_H17 cells induce secondary neurotoxicity and lead to
1284 infarct expansion with worsened functional outcome (Shichita et al., 2009; Gelderblom et al.,
1285 2012).

1286

1287 Surprisingly, despite this obviously pronounced effect of lymphocytes in post-stroke
1288 pathology, their role in activating or inhibiting microglial activation has so far barely been
1289 investigated. We have observed indirect evidence for T cell effects on microglial activity in
1290 previous experiments, in which depletion of circulating Treg cells affected microglial
1291 activation and cytokine release (Liesz et al., 2009). Additional indirect evidence comes from
1292 studies demonstrating T cell interaction and particularly the influence of T cell-secreted

1293 cytokines on multiple other cell types in the healthy and injured brain such as T cell-derived
1294 Interferon-gamma on animal behavior (Walsh et al., 2015; Filiano et al., 2016). Yet, to our
1295 knowledge, the role of T cells as indirect cellular mediators of microbiota-derived SCFA on
1296 the brain has so far not been studied. In this study, we unequivocally demonstrate for the
1297 first time that T cells are crucial to mediate the immunomodulatory effects of SCFA on
1298 brainresident microglia because in lymphocyte-deficient mice this effect became abolished.
1299 This finding is also in accordance with our previous proof-of-concept experiments showing
1300 that circulating T cells were key in facilitating the impact of the gut microbiome on stroke
1301 outcome in GF vs. recolonized animals as well as in mice with a healthy versus a dysbiotic
1302 microbiome (Singh et al., 2016a; Singh et al., 2018). These previous reports by us and
1303 independent reports by others have also consistently identified a translocation of
1304 lymphocytes from the intestinal immune compartment to the post-stroke brain, providing a
1305 cellular link by this circulating and highly motile cells across the gut-brain axis (Benakis et al.,
1306 2016; Singh et al., 2016b).

1307

1308 Taken together, this study identified SCFA as critical metabolites derived from the gut
1309 microbiome affecting T cell function and thereby indirectly modulating the neuro-regenerative
1310 milieu. This expands our current understanding of the mechanisms along the gut-brain axis
1311 in acute brain injury and recovery where post-stroke dysbiosis affects the production of key
1312 microbiota-derived metabolites, their impact on immunological homeostasis and finally the
1313 capability for efficient functional recovery. The efficacy of SCFA for promoting recovery in an
1314 experimental stroke model on a functional as well as morphological level, opens up novel
1315 therapeutic possibilities for improving recovery of human stroke patients. Future studies
1316 should validate the pro-regenerative effect of SCFA on post-stroke recovery before further
1317 translational development. However, based on the findings from this study and indications
1318 for efficacy and similar modes of actions in primary autoimmune brain disorders (Melbye et

1319 al., 2019), it is well-conceivable that SCFA supplementation could be used as a safe and
1320 practical add-on therapy to stroke rehabilitation.

1321 **References**

- 1322 Arpaia N, Campbell C, Fan XY, Dikiy S, van der Veecken J, deRoos P, Liu H, Cross JR, Pfeffer K, Coffey PJ,
1323 Rudensky AY (2013) Metabolites produced by commensal bacteria promote peripheral
1324 regulatory T-cell generation. *Nature* 504:451-+.
- 1325 Asarat M, Apostolopoulos V, Vasiljevic T, Donkor O (2016) Short-Chain Fatty Acids Regulate
1326 Cytokines and Th17/Treg Cells in Human Peripheral Blood Mononuclear Cells in vitro.
1327 *Immunological Investigations* 45:205-222.
- 1328 Au - Ippolito DM, Au - Eroglu C (2010) Quantifying Synapses: an Immunocytochemistry-based Assay
1329 to Quantify Synapse Number. *JoVE*:e2270.
- 1330 Austin V, Ku JM, Miller AA, Vlahos R (2019) Ischaemic stroke in mice induces lung inflammation but
1331 not acute lung injury. *Scientific Reports* 9:3622.
- 1332 Becker AM, Meyers E, Sloan A, Rennaker R, Kilgard M, Goldberg MP (2016) An automated task for
1333 the training and assessment of distal forelimb function in a mouse model of ischemic stroke.
1334 *Journal of Neuroscience Methods* 258:16-23.
- 1335 Benakis C, Brea D, Caballero S, Faraco G, Moore J, Murphy M, Sita G, Racchumi G, Lingo L, Pamer EG,
1336 Iadecola C, Anrather J (2016) Commensal microbiota affects ischemic stroke outcome by
1337 regulating intestinal gamma delta T cells. *Nature Medicine* 22:516-523.
- 1338 Bieber M, Werner RA, Tanai E, Hofmann U, Higuchi T, Schuh K, Heuschmann PU, Frantz S, Ritter O,
1339 Kraft P, Kleinschnitz C (2017) Stroke-induced chronic systolic dysfunction driven by
1340 sympathetic overactivity. *Annals of Neurology* 82:729-743.
- 1341 Biernaskie J, Chernenko G, Corbett D (2004) Efficacy of rehabilitative experience declines with time
1342 after focal ischemic brain injury. *Journal of Neuroscience* 24:1245-1254.
- 1343 Bourassa MW, Alim I, Bultman SJ, Ratan RR (2016) Butyrate, neuroepigenetics and the gut
1344 microbiome: Can a high fiber diet improve brain health? *Neuroscience Letters* 625:56-63.
- 1345 Brown CE, Wong C, Murphy TH (2008) Rapid morphologic plasticity of peri-infarct dendritic spines
1346 after focal ischemic stroke. *Stroke* 39:1286-1291.
- 1347 Chuang DM, Leng Y, Marinova Z, Kim HJ, Chiu CT (2009) Multiple roles of HDAC inhibition in
1348 neurodegenerative conditions. *Trends in Neurosciences* 32:591-601.
- 1349 Cramer JV, Gesierich B, Roth S, Dichgans M, Duering M, Liesz A (2018) In vivo widefield calcium
1350 imaging of the mouse cortex for analysis of network connectivity in health and brain disease.
1351 *bioRxiv*:459941.
- 1352 Cryan JF, Dinan TG (2012) Mind-altering microorganisms: the impact of the gut microbiota on brain
1353 and behaviour. *Nature Reviews Neuroscience* 13:701-712.
- 1354 Cserép C et al. (2019) Microglia monitor and protect neuronal function via specialized somatic
1355 purinergic junctions. *bioRxiv*:606079.
- 1356 Dirnagl U, Iadecola C, Moskowitz MA (1999) Pathobiology of ischaemic stroke: an integrated view.
1357 *Trends in Neurosciences* 22:391-397.
- 1358 Erny D, de Angelis ALH, Jaitin D, Wieghofer P, Staszewski O, David E, Keren-Shaul H, Mhlahoi T,
1359 Jakobshagen K, Buch T, Schwierzeck V, Utermohlen O, Chun E, Garrett WS, McCoy KD,
1360 Diefenbach A, Staeheli P, Stecher B, Amit I, Prinz M (2015) Host microbiota constantly
1361 control maturation and function of microglia in the CNS. *Nature Neuroscience* 18:965-+.

1362 Faustino JV, Wang X, Johnson CE, Klivanov A, Derugin N, Wendland MF, Vexler ZS (2011) Microglial
1363 Cells Contribute to Endogenous Brain Defenses after Acute Neonatal Focal Stroke. *Journal of*
1364 *Neuroscience* 31:12992-13001.

1365 Feng Y, Liao SW, Wei CJ, Jia DM, Wood K, Liu Q, Wang XY, Shi FD, Jin WN (2017) Infiltration and
1366 persistence of lymphocytes during late-stage cerebral ischemia in middle cerebral artery
1367 occlusion and photothrombotic stroke models. *Journal of Neuroinflammation* 14.

1368 Filiano AJ, Xu Y, Tustison NJ, Marsh RL, Baker W, Smirnov I, Overall CC, Gadani SP, Turner SD, Weng
1369 ZP, Peerzade SN, Chen H, Lee KS, Scott MM, Beenhakker MP, Litvak V, Kipnis J (2016)
1370 Unexpected role of interferon-gamma in regulating neuronal connectivity and social
1371 behaviour. *Nature* 535:425-+.

1372 Frost G, Sleeth ML, Sahuri-Arisoylu M, Lizarbe B, Cerdan S, Brody L, Anastasovska J, Ghourab S,
1373 Hankir M, Zhang S, Carling D, Swann JR, Gibson G, Viardot A, Morrison D, Thomas EL, Bell JD
1374 (2014) The short-chain fatty acid acetate reduces appetite via a central homeostatic
1375 mechanism. *Nature Communications* 5.

1376 Gelderblom M, Weymar A, Bernreuther C, Velden J, Arunachalam P, Steinbach K, Orthey E,
1377 Arumugam TV, Leyboldt F, Simova O, Thom V, Friese MA, Prinz I, Holscher C, Glatzel M, Korn
1378 T, Gerloff C, Tolosa E, Magnus T (2012) Neutralization of the IL-17 axis diminishes neutrophil
1379 invasion and protects from ischemic stroke. *Blood* 120:3793-3802.

1380 Haghikia A et al. (2015) Dietary Fatty Acids Directly Impact Central Nervous System Autoimmunity via
1381 the Small Intestine. *Immunity* 43:817-829.

1382 Heindl S, Gesierich B, Benakis C, Llovera G, Duering M, Liesz A (2018) Automated Morphological
1383 Analysis of Microglia After Stroke. *Frontiers in Cellular Neuroscience* 12.

1384 Hoffmann S, Otto C, Kurtz S, Sharma CM, Khaitovich P, Vogel J, Stadler PF, Hackermuller J (2009) Fast
1385 mapping of short sequences with mismatches, insertions and deletions using index
1386 structures. *PLoS Comput Biol* 5:e1000502.

1387 Houlden A, Goldrick M, Brough D, Vizi ES, Lenart N, Martinecz B, Roberts IS, Denes A (2016) Brain
1388 injury induces specific changes in the caecal microbiota of mice via altered autonomic
1389 activity and mucoprotein production. *Brain Behavior and Immunity* 57:10-20.

1390 Hoving LR, Heijink M, van Harmelen V, van Dijk KW, Giera M (2018) GC-MS Analysis of Short-Chain
1391 Fatty Acids in Feces, Cecum Content, and Blood Samples. *Methods Mol Biol* 1730:247-256.

1392 Huang SY, Chang CH, Hung HY, Lin YW, Lee EJ (2018) Neuroanatomical and electrophysiological
1393 recovery in the contralateral intact cortex following transient focal cerebral ischemia in rats.
1394 *Neurological Research* 40:130-138.

1395 Iadecola C, Anrather J (2011) The immunology of stroke: from mechanisms to translation. *Nature*
1396 *Medicine* 17:796-808.

1397 Jones TA, Schallert T (1994) USE-DEPENDENT GROWTH OF PYRAMIDAL NEURONS AFTER
1398 NEOCORTICAL DAMAGE. *Journal of Neuroscience* 14:2140-2152.

1399 Kau AL, Ahern PP, Griffin NW, Goodman AL, Gordon JI (2011) Human nutrition, the gut microbiome
1400 and the immune system. *Nature* 474:327-336.

1401 Kim HJ, Leeds P, Chuang DM (2009) The HDAC inhibitor, sodium butyrate, stimulates neurogenesis in
1402 the ischemic brain. *Journal of Neurochemistry* 110:1226-1240.

1403 Kleinschnitz C, Schwab N, Kraft P, Hagedorn I, Dreykluft A, Schwarz T, Austinat M, Nieswandt B,
1404 Wiendl H, Stoll G (2010) Early detrimental T-cell effects in experimental cerebral ischemia
1405 are neither related to adaptive immunity nor thrombus formation. *Blood* 115:3835-3842.

1406 Li QY, Cheng ZL, Zhou L, Darmanis S, Neff NF, Okamoto J, Gulati G, Bennett ML, Sun LO, Clarke LE,
1407 Marschallinger J, Yu GQ, Quake SR, Wyss-Coray T, Barres BA (2019) Developmental
1408 Heterogeneity of Microglia and Brain Myeloid Cells Revealed by Deep Single-Cell RNA
1409 Sequencing. *Neuron* 101:207-+.

1410 Li XL, Blizzard KK, Zeng ZY, DeVries AC, Hurn PD, McCullough LD (2004) Chronic behavioral testing
1411 after focal ischemia in the mouse: functional recovery and the effects of gender.
1412 *Experimental Neurology* 187:94-104.

1413 Liao Y, Smyth GK, Shi W (2014) featureCounts: an efficient general purpose program for assigning
1414 sequence reads to genomic features. *Bioinformatics* 30:923-930.

1415 Liesz A, Hu XM, Kleinschnitz C, Offner H (2015) Functional Role of Regulatory Lymphocytes in Stroke
1416 Facts and Controversies. *Stroke* 46:1422-1430.

1417 Liesz A, Suri-Payer E, Veltkamp C, Doerr H, Sommer C, Rivest S, Giese T, Veltkamp R (2009)
1418 Regulatory T cells are key cerebroprotective immunomodulators in acute experimental
1419 stroke. *Nature Medicine* 15:192-199.

1420 Liesz A, Zhou W, Na SY, Hammerling GJ, Garbi N, Karcher S, Mracsko E, Backs J, Rivest S, Veltkamp R
1421 (2013) Boosting Regulatory T Cells Limits Neuroinflammation in Permanent Cortical Stroke.
1422 *Journal of Neuroscience* 33:17350-17362.

1423 Llovera G, Roth S, Plesnila N, Veltkamp R, Liesz A (2014) Modeling Stroke in Mice: Permanent
1424 Coagulation of the Distal Middle Cerebral Artery. *Jove-Journal of Visualized Experiments*.

1425 Love MI, Huber W, Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq
1426 data with DESeq2. *Genome Biol* 15:550.

1427 Manwani B, Liu FD, Xu Y, Persky R, Li J, McCullough LD (2011) Functional recovery in aging mice after
1428 experimental stroke. *Brain Behavior and Immunity* 25:1689-1700.

1429 Markram H, Toledo-Rodriguez M, Wang Y, Gupta A, Silberberg G, Wu CZ (2004) Interneurons of the
1430 neocortical inhibitory system. *Nature Reviews Neuroscience* 5:793-807.

1431 Melbye P, Olsson A, Hansen TH, Sondergaard HB, Oturai AB (2019) Short-chain fatty acids and gut
1432 microbiota in multiple sclerosis. *Acta Neurologica Scandinavica* 139:208-219.

1433 Meng HL, Zhao HR, Cao X, Hao JW, Zhang H, Liu Y, Zhu MS, Fan LZ, Weng LH, Qian L, Wang XY, Xu Y
1434 (2019) Double-negative T cells remarkably promote neuroinflammation after ischemic
1435 stroke. *Proceedings of the National Academy of Sciences of the United States of America*
1436 116:5558-5563.

1437 Morrison DJ, Preston T (2016) Formation of short chain fatty acids by the gut microbiota and their
1438 impact on human metabolism. *Gut Microbes* 7:189-200.

1439 Nakamura YK, Janowitz C, Metea C, Asquith M, Karstens L, Rosenbaum JT, Lin P (2017) Short chain
1440 fatty acids ameliorate immune-mediated uveitis partially by altering migration of
1441 lymphocytes from the intestine. *Scientific Reports* 7.

1442 Neumann J, Riek-Burchardt M, Herz J, Doepfner TR, König R, Hutten H, Etemire E, Mann L, Klingberg
1443 A, Fischer T, Gortler M, Heinze HJ, Reichardt P, Schraven B, Hermann DM, Reymann KG,
1444 Gunzer M (2015) Very-late-antigen-4 (VLA-4)-mediated brain invasion by neutrophils leads
1445 to interactions with microglia, increased ischemic injury and impaired behavior in
1446 experimental stroke. *Acta Neuropathologica* 129:259-277.

1447 Offner H, Subramanian S, Parker SM, Afentoulis ME, Vandenbark AA, Hurn PD (2006) Experimental
1448 stroke induces massive, rapid activation of the peripheral immune system. *Journal of*
1449 *Cerebral Blood Flow and Metabolism* 26:654-665.

1450 Park J, Kim M, Kang SG, Jannasch AH, Cooper B, Patterson J, Kim CH (2015) Short-chain fatty acids
1451 induce both effector and regulatory T cells by suppression of histone deacetylases and
1452 regulation of the mTOR-S6K pathway. *Mucosal Immunology* 8:80-93.

1453 Ransohoff RM, Engelhardt B (2012) The anatomical and cellular basis of immune surveillance in the
1454 central nervous system. *Nature Reviews Immunology* 12:623-635.

1455 Rehme AK, Grefkes C (2013) Cerebral network disorders after stroke: evidence from imaging-based
1456 connectivity analyses of active and resting brain states in humans. *Journal of*
1457 *Physiology* London 591:17-31.

1458 Riccomagno MM, Kolodkin AL (2015) Sculpting Neural Circuits by Axon and Dendrite Pruning. Annual
1459 Review of Cell and Developmental Biology, Vol 31 31:779-805.

1460 Rosell A, Agin V, Rahman M, Morancho A, Ali C, Koistinaho J, Wang XY, Vivien D, Schwaninger M,
1461 Montaner J (2013) Distal Occlusion of the Middle Cerebral Artery in Mice: Are We Ready to
1462 Assess Long-Term Functional Outcome? Translational Stroke Research 4:297-307.

1463 Roth S, Singh V, Tiedt S, Schindler L, Huber G, Geerlof A, Antoine DJ, Anfray A, Orset C, Gauberti M,
1464 Fournier A, Holdt LM, Harris HE, Engelhardt B, Bianchi ME, Vivien D, Haffner C, Bernhagen J,
1465 Dichgans M, Liesz A (2018) Brain-released alarmins and stress response synergize in accelerating
1466 atherosclerosis progression after stroke. Science Translational Medicine 10:11. Salter MW, Stevens B
1467 (2017) Microglia emerge as central players in brain disease. Nature Medicine 23:1018-1027.

1468 Schafer DP, Lehrman EK, Kautzman AG, Koyama R, Mardinly AR, Yamasaki R, Ransohoff RM,
1469 Greenberg ME, Barres BA, Stevens B (2012) Microglia Sculpt Postnatal Neural Circuits in an
1470 Activity and Complement-Dependent Manner. Neuron 74:691-705.

1471 Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C,
1472 Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A
1473 (2012) Fiji: an open-source platform for biological-image analysis. Nature Methods
1474 9:676682.

1475 Selvaraj UM, Stowe AM (2017) Long-term T Cell Responses in the Brain After an Ischemic Stroke.
1476 Discovery Medicine 24:323-333.

1477 Shichita T, Sugiyama Y, Ooboshi H, Sugimori H, Nakagawa R, Takada I, Iwaki T, Okada Y, Iida M, Cua
1478 DJ, Iwakura Y, Yoshimura A (2009) Pivotal role of cerebral interleukin-17-producing gamma
1479 delta T cells in the delayed phase of ischemic brain injury. Nature Medicine 15:946-U150.

1480 Singh V, Sadler R, Heindl S, Llovera G, Roth S, Benakis C, Liesz A (2018) The gut microbiome primes a
1481 cerebroprotective immune response after stroke. Journal of Cerebral Blood Flow &
1482 Metabolism 38:1293-1298.

1483 Singh V, Roth S, Llovera G, Sadler R, Garzetti D, Stecher B, Dichgans M, Liesz A (2016a) Microbiota
1484 Dysbiosis Controls the Neuroinflammatory Response after Stroke. Journal of Neuroscience
1485 36:7428-7440.

1486 Singh V, Roth S, Llovera G, Sadler R, Garzetti D, Stecher B, Dichgans M, Liesz A (2016b) Microbiota
1487 Dysbiosis Controls the Neuroinflammatory Response after Stroke. The Journal of
1488 neuroscience : the official journal of the Society for Neuroscience 36:7428-7440.

1489 Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly-Y M, Glickman JN, Garrett WS
1490 (2013) The Microbial Metabolites, Short-Chain Fatty Acids, Regulate Colonic T-reg Cell
1491 Homeostasis. Science 341:569-573.

1492 Stephan AH, Barres BA, Stevens B (2012) The Complement System: An Unexpected Role in Synaptic
1493 Pruning During Development and Disease. Annual Review of Neuroscience, Vol 35
1494 35:369389.

1495 Swidsinski A, Loening-Baucke V, Krüger M, Kirsch S (2012) - Central Nervous System and the Colonic
1496 Bioreactor: Analysis of Colonic Microbiota in Patients with Stroke Unravels Unknown
1497 Mechanisms of the Host Defense after Brain Injury. - 10:- 342.

1498 Tan J, McKenzie C, Vuillermin PJ, Goverse G, Vinuesa CG, Mebius RE, Macia L, Mackay CR (2016)
1499 Dietary Fiber and Bacterial SCFA Enhance Oral Tolerance and Protect against Food Allergy
1500 through Diverse Cellular Pathways. Cell Reports 15:2809-2824.

1501 van de Wouw M, Boehme M, Lyte JM, Wiley N, Strain C, O'Sullivan O, Clarke G, Stanton C, Dinan TG,
1502 Cryan JF (2018) Short-chain fatty acids: microbial metabolites that alleviate stress-induced
1503 brain-gut axis alterations. Journal of Physiology-London 596:4923-4944.

1504 Walsh JT et al. (2015) MHCII-independent CD4(+) T cells protect injured CNS neurons via IL-4. Journal
1505 of Clinical Investigation 125:699-714.

1506 Wu YW, Dissing-Olesen L, MacVicar BA, Stevens B (2015) Microglia: Dynamic Mediators of Synapse
1507 Development and Plasticity. *Trends in Immunology* 36:605-613.

1508 Xia G-H, You C, Gao X-X, Zeng X-L, Zhu J-J, Xu K-Y, Tan C-H, Xu R-T, Wu Q-H, Zhou H-W, He Y, Yin J
1509 (2019) Stroke Dysbiosis Index (SDI) in Gut Microbiome Are Associated With Brain Injury and
1510 Prognosis of Stroke. *Frontiers in Neurology* 10.

1511 Zausinger S, Hungerhuber E, Baethmann A, Reulen HJ, Schmid-Elsaesser R (2000) Neurological
1512 impairment in rats after transient middle cerebral artery occlusion: a comparative study
1513 under various treatment paradigms. *Brain Research* 863:94-105.

1514 Zhang Y, Chen KN, Sloan SA, Bennett ML, Scholze AR, O'Keefe S, Phatnani HP, Guarnieri P, Caneda C,
1515 Ruderisch N, Deng SY, Liddelow SA, Zhang CL, Daneman R, Maniatis T, Barres BA, Wu JQ
1516 (2014) An RNA-Sequencing Transcriptome and Splicing Database of Glia, Neurons, and
1517 Vascular Cells of the Cerebral Cortex. *Journal of Neuroscience* 34:11929-11947.

1518 Zhong SJ, Zhang S, Fan XY, Wu Q, Yan LY, Dong J, Zhang HF, Li L, Sun L, Pan N, Xu XH, Tang FC, Zhang
1519 J, Qiao J, Wang XQ (2018) A single-cell RNA-seq survey of the developmental landscape of
1520 The human prefrontal cortex. *Nature* 555:524+.

Figure 1-1

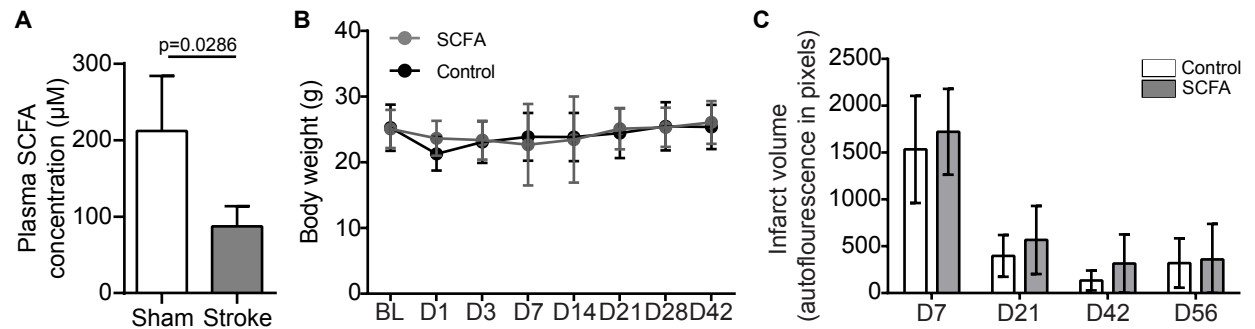


Figure 1-1. SCFA concentrations are reduced after stroke.

(A) Plasma concentration (μM) showing a decrease of total SCFA (sum of acetate, propionate and butyrate) in mice 3 days after fMCAo stroke surgery (grey bars) compared to sham (open bars). $N=4$ per group (Mann-Whitney U test). **(B)** No difference of body weight in grams at baseline (BL) until D42 post PT in mice supplemented with SCFA (grey line) or control (black line). $N=14/15$ per group (2way ANOVA with Holm-Sidak correction for multiple testing). **(C)** Lesion size as measured in pixels did not differ between SCFA (grey bar) and control mice (open bar) at any time points. $N=11/10$ and statistical test with multiple T tests per time point and Holm-Sidak method correction for multiple testing.

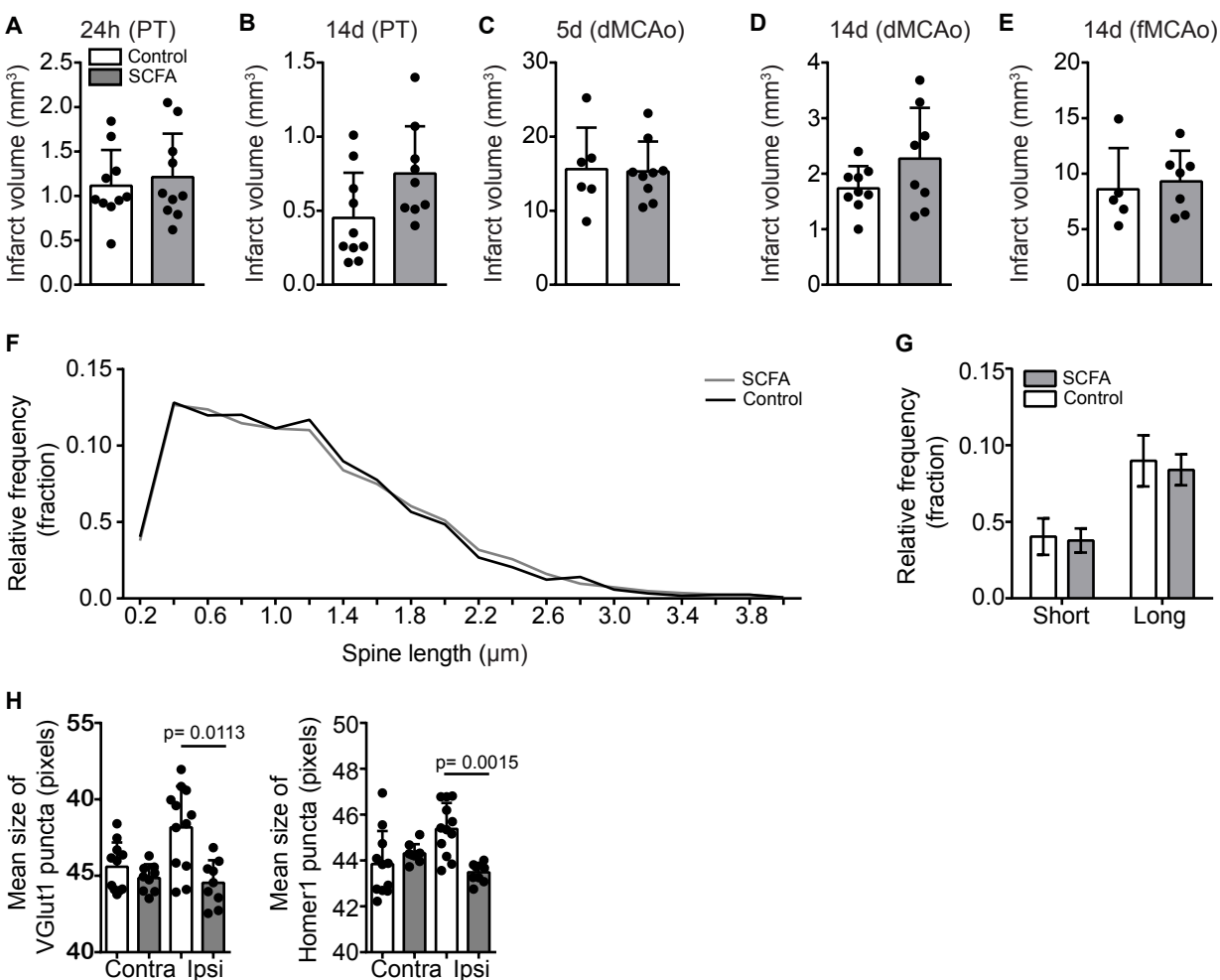
Figure 2-1

Figure 2-1. SCFA supplementation does not alter tissue injury in 3 focal stroke models. Infarct volume from Nissl staining in mice supplemented with control mix (open bar) or SCFA (grey bar). **(A)** 24h after PT, N=10 per group; **(B)** 14d after PT, 10/9 per group; **(C)** 5d after dMCAo, N=6/9 per group; **(D)** 14d after dMCAo, N=9/8 per group; and **(E)** 14d after fMCAo, N=5/7 per group. Statistical analyses were performed using the Mann-Whitney U test. **(F)** As in Figure 2E, histogram of the relative frequency (fraction) of spines found at different lengths 14d after PT in the contralateral cortex (bin width = 0.2µm). **(G)** Quantification of short (0.2µm) and long (1.4µm) spines in control (open bars) and SCFA (grey bars) treated mice. N=4/5 per group, Mann-Whitney U test. **(H)** The size in pixels of synapses stained with VGlut1 (left) and Homer1 (right). Quantification revealed larger synapse areas in the peri-lesional cortex which was significantly ameliorated with SCFA supplementation. Contra: contralateral hemisphere; ipsi: ipsilateral hemisphere, n=3/4 mice (3 sections per mouse), statistical analysis performed with Kruskal-Wallis test with Dunn's multiple comparison correction.

Figure 3-1

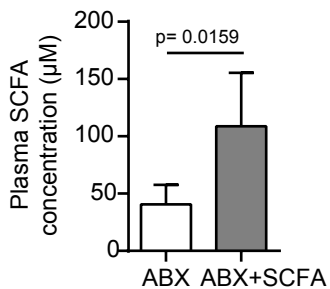


Figure 3-1. SCFA supplementation increases plasma concentrations.

Mice were pre-treated with antibiotic mix for 4 weeks, and then additionally given control (ABX, open bars) or SCFA supplementation (ABX+SCFA, grey bars). Total plasma concentration (µM) of total SCFA (acetate, propionate and butyrate) showing significant increase after SCFA supplementation compared to control treated mice, N=4/5 per group (Mann-Whitney U test).

Figure 4-1

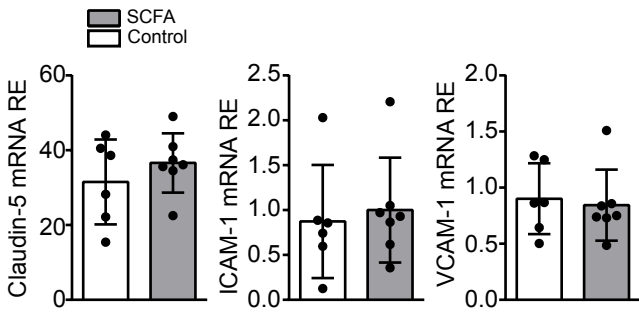


Figure 4-1. SCFA supplementation does not affect cerebral endothelial expression. Relative mRNA expression (RE) for the tight-junction and adhesion molecules Claudin-5 (left), ICAM-1 (middle) and VCAM-1 (right) from the peri-lesional cortex in control (open bars) and SCFA (grey bars) treated mice (Mann-Whitney U test).

DISCUSSION

The major findings of this thesis were: 1) there is a bidirectional interaction between the gut microbiota and stroke; changes in the gut microbiota composition can alter stroke outcome, and stroke itself induces a dysbiosis of the gut microbiome. 2) By modulation of the peripheral immune system, the gut microbiome is able to influence the neuroinflammatory response to stroke. 3) The gut microbiota metabolites are altered after stroke, and modulation of these can improve long-term recovery.

We found that the fMCAo (larger lesions) induced substantial changes in the gut microbiota composition. This was not evident within mice that underwent dMCAo (smaller lesions) and suggested that a greater dysbiosis was associated with larger stroke lesions. In other studies, a mathematical algorithm was designed for stroke patients which positively correlated the gut microbiome dysbiosis to the outcome of stroke. I.e. the greater the dysbiotic index, the worse the stroke outcome. Instead of causally correlating the infarct size with dysbiosis as performed in our mouse study, they assessed neurological patient's scores. The results suggested that patients with a greater gut microbiome dysbiosis index had poorer stroke outcome (Xia et al., 2019). Our mouse study revealed that post stroke dysbiosis was causally linked to a slowing of the gut motility. In patients, care-givers or stroke patients themselves reported a 30% increase in bowel dysfunction after stroke. The umbrella term bowel dysfunction was used to categorise: diminished frequency of bowel movements, lumpy or hard stools, intestinal constipation, straining, incomplete evacuation, and incontinence (Engler, Dourado et al., 2014, Olsen, Hetz et al., 2013). A possible mechanism for how the reduced motility and thereby gut microbiome dysbiosis is induced after stroke, is via the elevated levels of catecholamines in the blood. The hypothalamus-pituitary-adrenal axis is responsible for the elevated levels of cortisol after stroke which is causally correlated to worse stroke outcome (Anne, Juha et al., 2007). The catecholamines have been shown to interact with the ENS, reducing gut motility and altering gut microbiota composition (Bailey et al., 2011). In our study, we saw elevated catecholamine levels after stroke. This suggested that larger strokes, with greater amounts of catecholamine release, have reduced gastrointestinal transit and a greater gut microbiota dysbiosis.

From our study we observed that stroke-induced dysbiosis altered the polarisation of T cells in the spleen and PP. Utilising GF mice, we employed FMT techniques to investigate the intestinal immune cell priming from dysbiotic stroke gut microbiota. In general, we saw that when FMT was given more Tregs and fewer IL-17⁺ cells were present, and stroke outcome was improved. This is concurrent with

studies showing the neuroprotective role of Tregs and proinflammatory role of IL-17 producing cells in stroke (Gelderblom, Weymar et al., 2012, Kleinschnitz et al., 2010, Liesz et al., 2009, Shichita et al., 2009). However, the novel connection linking the gut microbiome to the immune system in stroke has only recently become elucidated. A key study in this field showed that after stroke, the altered microbiota composition led to an imbalance of Treg to IL-17⁺ cells in the intestinal immune compartment, resulting in differences of stroke outcome (Benakis et al., 2016b). Whilst it is clear that the gut microbiome interacts with the local immune system, the contribution of intestinal immune cells to the neuroinflammation was unclear. In our study, we saw that 25% of all T cells located in the brain after stroke originated from the intestinal immune compartment, as shown with injection of CFSE dye in the PP. Furthermore, Benakis et al. used Kikume Green-Red transgenic mice, allowing illuminated cells to be photoconverted specifically in the intestinal compartment. The photoconverted T cells were then tracked to show immune cells clustering at the meninges after stroke. It has been shown that the cytokine release from T cells indirectly modulates neurodegeneration and microglia in the brain (Appel, 2009, Liesz, Zhou et al., 2011). In our study and in Benakis et al., we saw that the proinflammatory cytokine levels in the brain were reduced when more Tregs were observed in the intestinal immune compartment. Our studies have identified the immune system as a key mediator along the gut-brain axis. However, at this point it is important to acknowledge the other potential routes of brain-axis communication. The vagus nerve acts as a direct link for the gut to the brain with 80% afferent and 20% efferent fibres (Agostoni et al., 1957). The afferent fibres do not sense the gut microbiota directly and mainly detect metabolites or mediators produced by the bacteria which may influence enteroendocrine cells. For example, butyrate and LPS have been shown to directly interact with afferent terminals (Hosoi, Okuma et al., 2005, Lal et al., 2001). The efferent fibres work to alter intestinal permeability and reduce inflammation (Zhou, Liang et al., 2013). Acetylcholine (ACh) released from the distal end of the vagus nerve inhibits TNF α release by macrophages through the α 7nicotinic ACh receptors (Wang, Yu et al., 2003). In studies which investigated the neural route for gut-brain axis communication in stroke, the authors showed that vagotomy or vagal nerve stimulation exhibited neuroprotective effects with reductions of proinflammatory cytokine TNF α in brain and in liver – potentially due to downregulation of microglial α 7nicotinic ACh receptors (Jiang, Li et al., 2014, Ottani, Giuliani et al., 2009). Whilst this study did not focus on the gut microbiota, but mainly the connection between the brain and the gut itself, interestingly it appears that both the immune and neural route work to alter stroke outcome by primarily modulating the inflammatory milieu in the brain.

Our earlier studies and others have already clearly identified that modulation of the gut microbiome alters neuroinflammation and stroke outcome mainly within the acute phase via the immune system.

The peak infiltration of immune cells in the brain after experimental stroke is 3-5 days (Gelderblom et al., 2009). Therefore, investigation of the gut microbiome-priming of the immune system after stroke has mainly been focused within these acute time-frames. In other studies there is evidence to suggest that T cells infiltrate into the brain up to at least 1 month after stroke (Xie, Li et al.). This indicated that gut microbiome-priming of the immune system could play a role in the recovery phase after stroke. In our mouse models, we showed that the gut microbiota composition was altered in the larger fMCAo model up until D3 after stroke. So far there has been no mouse studies which investigate the long-term effects of stroke on the gut microbiome. However, in patients, a longitudinal study showed that the stroke induced dysbiotic microbiome was evident until day 20 after stroke and then began to normalise (Swidsinski, Loening-Baucke et al., 2012b). Within our studies, instead of investigating the longitudinal changes to the gut microbiota after stroke, we focused on the metabolites produced by the gut microbiome. We found that the key bioactive metabolites named SCFA were low in concentration after stroke compared to sham. Using pre-supplementation of SCFA in drinking water we investigated the role of these bacterial derived metabolites on the recovery after stroke. Other studies using this treatment of SCFA have revealed neuro-protective properties (Bourassa, Alim et al., 2016). For example, in the multiple sclerosis mouse model, EAE, pre-treatment with SCFA was shown to reduce axonal damage and induce an anti-inflammatory immune system (Haghikia, Jorg et al., 2015). For the first time, our results show that the gut microbiota metabolites alter the recovery phase after stroke indirectly by the immune system. Different techniques must be employed when analysing the stroke outcome within the recovery phase. Conventional methods used in the acute phase, such as infarct volumetry do not provide accurate representation of stroke severity, i.e. it is difficult to quantify lesion volume due to missing or scarring to tissue. For this reason, behavioural tests which measure functional outcome are often used as a readout. Our study utilised an automated, rater-blinded behavioural test specifically assessing motor improvement of the affected forelimb (Becker, Meyers et al., 2016). Assays must be extremely sensitive to detect differences in post stroke recovery phase, as small changes in metabolites produced by the gut bacteria are likely to result in subtle differences. It is important to have a behavioural test which discriminates between these less obvious changes after stroke which limits rater-dependant effects (Li, Blizzard et al., 2004, Manwani, Liu et al., 2011, Rosell, Agin et al., 2013, Zausinger, Hungerhuber et al., 2000).

The recovery phase after stroke entails reorganisation of cortical networks which requires multicellular process encompassing neuronal plasticity, resident immune cells and brain-invading cells. In our study we saw SCFA altered the dynamics of cortical connectivity after stroke. Stroke patient fMRI has shown that interhemispheric resting-state connectivity is significantly weakened

following ischemic stroke. Specifically, it is thought that inhibitory activity from the stroked hemisphere to the homotypic contralateral hemisphere is attenuated, leading to a disinhibition or a more active homotypic contralateral hemisphere (Rehme & Grefkes, 2013(Grefkes, Nowak et al., 2008, Grefkes, Nowak et al., 2010, Rehme, Eickhoff et al., 2011)). During the recovery phase after stroke, it has been shown that the strength of interhemispheric connectivity increases, and is associated with motor improvements (Golestani, Tymchuk et al., 2013, Park, Chang et al., 2011, Wang, Yu et al., 2010). Within our study, we used widefield *in vivo* calcium instead of fMRI to analyse network connectivity. One limitation of the technique is that unlike fMRI, the assessment of the whole brain is not possible. *In vivo* calcium imaging is spatially and temporally more sensitive, allowing characterisation of the different topographically located functional areas across the cortex (Cramer, Gesierich et al., 2018). In accordance with studies performed in patients, our *in vivo* calcium imaging revealed an increase of the contralesional motor area after stroke. This was significantly ameliorated by the SCFA treatment during the recovery phase. This implied that the stroked ipsilateral hemisphere in SCFA mice had re-established interhemispheric connectivity providing stronger inhibition of homotypic contralateral regions compared to controls. There is existing evidence, which supports the concept of inhibitory interneurons altering post stroke recovery (Clarkson, Huang et al., 2010, Zeiler, Gibson et al., 2013). However, in our study, one main limitation was the lack of information on directionality, and inhibitory connections found between the two hemispheres after stroke. Due to the techniques used within this study, (GCamp in Thy1-positive neurons, analysis of dendritic spines on pyramidal neurons and the synapse count of VGlut1-positive (glutamatergic) synapses) assessment of excitatory connections was only possible. On one hand, our findings in the contralateral hemisphere may only reflect changes in the functional connectivity as shown with calcium imaging. On the other hand, our results may relate to changes in physical trans-callosal connectivity between hemispheres. In previous studies, interhemispheric connections after stroke have been assessed with neuronal tracers. Tracers such as wheat-germ agglutinin horse radish peroxidase visualised with immunohistochemistry, and manganese visualised with MRI, are injected into the perilesional area and show the pathway of neuronal processes. It was shown that at 4-10 weeks after stroke tracers were significantly increased in the contralateral hemisphere due to an enhancement of interhemispheric connectivity at these time points (van der Zijden, Bouts et al., 2008). This is in line with our study where we saw a decrease in area of the highly active contralateral seed at 3- and 6- weeks post stroke. One could hypothesise that the interhemispheric neuronal connections from the ipsilateral cortex were restored. It is thought that after stroke the inhibitory connections are diminished between hemispheres within the acute phase (Grefkes et al., 2008, Grefkes et al., 2010, Rehme et al., 2011, Rehme & Grefkes, 2013). Disinhibition is when the contralateral hemisphere does not receive inhibitory input. This concurs with our study where we

see a “blooming” of the contralateral motor seed. Hypothetically at chronic time points the restoration of interhemispheric connections could be mainly inhibitory resulting in a reduction of excitatory activity as seen in our results. This would therefore suggest that SCFA promote the interhemispheric connections between hemispheres after stroke, as we see significantly smaller contralateral motor seeds compared to controls. However further studies need to be performed to clarify the interhemispheric neuronal connections after stroke specifically after SCFA supplementation.

From our study we saw higher spine density in mice supplemented with SCFA compared to controls. This was evident in sham operated mice and within the contralateral hemisphere after stroke surgery. A sign of cortical recovery after stroke has been associated with a gradual increase in contralateral cortical spine density (Huang, Chang et al., 2018). The higher spine density in the contralesional cortex after stroke was linked to neuroprotection in SCFA supplemented mice. In another brain disease, autism spectrum disorder (ASD), elevated levels of faecal SCFA have been reported (Wang, Christophersen et al., 2012). Additionally, it was also shown that the SCFA levels in ASD were associated with increased spine density of cortical pyramidal neurons (Hutsler & Zhang, 2010). Although ASD and stroke have both a completely different pathophysiology, taken together in general it suggests that SCFA may be able to regulate spine density in the brain. Spine density may be altered via either molecular cues, guidance molecules, self-destruction, or synapse elimination (Riccomagno & Kolodkin, 2015). The mechanisms of how SCFA increased neuronal spine density remain still to be elucidated. Our study demonstrated that SCFA supplementation resulted in increased molecular cues such as Tropomyosin receptor kinase B (TrkB) and Synaptophysin. TrkB is a receptor for BDNF and upon activation leads to plasticity, dendrite outgrowth, proliferation, differentiation and survival (Yoshii & Constantine-Paton, 2010). Synaptophysin is a presynaptic vesicle marker which has shown to be decreased where reduced spine density is present (Karson, Mrak et al., 1999). This is concurrent with our results where we see higher spine density with and greater TrkB and Synaptophysin mRNA expression. Therefore, in our studies TrkB is likely to be a better method for indication of synaptic outgrowth after SCFA treatment, whereas Synaptophysin represents a second indicator for spine density and that synapses are likely to be functioning (Tarsa & Goda, 2002). SCFA could alter TrkB and Synaptophysin gene expression via epigenetic modifications, due to their ability to function as histone deacetylase inhibitors (HDACs). Bacterial derived SCFA have a clear role in modulating epigenetic markers in CD4+ T cells at the Foxp3 gene locus (Arpaia et al., 2013, Furusawa et al., 2013), CD8+ T cells (Luu, Weigand et al., 2018) and in general lymphocytes (Luu, Pautz et al., 2019). Furthermore there is evidence that shows the HDAC function of SCFA are able to alter transcription within brain resident cells such as microglia and neurons (Patnala, Varumugam et al., 2017, Sharma, Taliyan et al., 2015). SCFA could influence the transcriptional

profile of neuronal molecular cues, and therefore be a possible route of synaptic modulation. Another explanation for differences in spine density is altered synaptic pruning by microglia and astrocytes (Chen, Jalabi et al., 2014, Chung, Clarke et al., 2013, Ji, Akgul et al., 2013). In our RNA sequencing we saw that SCFA supplementation resulted in an increase in cortical complement and phagocytic genes which were lysosomal associated. Complement based synaptic pruning of spines has been shown in microglia and found to be independent of astrocytes (Chung et al., 2013, Stevens, Allen et al., 2007). In turn we observed altered microglia morphology under SCFA supplementation. Specifically, we saw that microglia were more ramified with SCFA, hinting that microglia were resting compared to controls. Other studies have also shown that specifically microglia are affected by SCFA supplementation whilst other brain resident cells were unaffected (Erny, de Angelis et al., 2015). However, further investigations need to pinpoint mechanism of how SCFA affect spines; indirectly by microglia as hypothesised in our study, or directly alter the molecules cues in neurons. This could potentially be solved with SCFA supplementation in combination with TrkB inhibitors (Shimada, Mason et al., 1998) or via blockade of synapse elimination via microglia i.e. with C1q^{-/-} mice (Ma, Ramachandran et al., 2013).

In our study we observed that SCFA supplementation was associated with reduced number of T cells in the brain and ramified microglia. We then supplemented mice in the absence of T and B lymphocytes (Rag^{-/-} mice), and saw that microglia cells were unaffected by the SCFA. The cross-talk between microglia and T cells has been shown to affect the activation state of microglia after stroke (Wang, Zhang et al., 2016). Our suggests that in general, the lower number of T cells present in the brain found under SCFA supplementation, led to lower levels of neuroinflammation and reduced microglia activation. Previously it has been shown that different subsets of T cells influence different effects on the microglia. Th1 and Th17 cells induce a proinflammatory microglia phenotype via TNF- α , IFN- γ and IL-17 release (Chabot et al., 2001, Shichita et al., 2009). Th2 and Tregs induce an anti-inflammatory microglia phenotype via IL-4, IL-10 and TGF β (Gonzalez & Pacheco, 2014, Liesz et al., 2009). Therefore, the infiltration of T cells into the brain can highly influence microglia activation. SCFA have previously been reported to change the polarization states of T cells in the intestinal immune compartment, inducing Tregs (Smith et al., 2013, Tan, McKenzie et al., 2016). However, in our study there was no clear change in the intestinal immune compartment of the Tregs (not reported). Recent studies investigating the impact of SCFA in an uveitis model also showed that prolonged treatment (4 weeks) of SCFA in drinking water was sufficient to reduce Treg counts in the intestinal immune compartment (Nakamura, Janowitz et al., 2017). This therefore suggested that changes in microglia activation after SCFA supplementation was not due to different T cells subsets infiltrating into the brain. Other studies using antibiotic treatment to alter the microbiome, and thus SCFA reduction, showed that more T cells entered the CNS in the EAE model of MS (Kadowaki, Saga

et al., 2019). One could hypothesise that the decrease in SCFA resulted in more T cell recruitment to the brain. This agrees with our study where we found high levels of SCFA reduced T cells entering the brain. After stroke microglia secrete chemokines which recruit T cells to the brain (Mirabelli-Badenier et al., 2011, Ramesh, MacLean et al., 2013) and local chemokines home T cells to the gut (Kunkel, Campbell et al., 2003). Chemokines located in the gut or the brain could also influence the number of T cells recruited to the brain, and thus the interaction with microglia after stroke. Further studies must be done to elucidate the role of SCFA on T cell migration into the brain, and thus the effects of microglia activation.

In our studies we have used Rag^{-/-} mice to validate the effect of SCFA in the absence of T cells and B cells on stroke outcome. While, we have mainly focused on the role of T cells in our studies, potentially B cells could also play a role in post stroke outcome. The research of B cells in stroke outcome is somewhat controversial (Javidi & Magnus, 2019). On the one hand there is evidence to suggest B cells do not play a role in stroke outcome. Transfer of B cells in Rag^{-/-} mice (Kleinschnitz et al., 2010), and depletion of B cells (Schuhmann, Langhauser et al., 2017) did not alter stroke outcome. On the other hand, others suggest a role for B cells in stroke; IL-10 from B cells and B cell administration improves stroke outcome (Chen, Bodhankar et al., 2012, Ren, Akiyoshi et al., 2011). Importantly B cell counts have been shown to be elevated at much later at around 7-12 weeks post stroke, in particular IgA production is elevated in the brain (Doyle, Quach et al., 2015) and systemically in stroke patients at day 90 (Tsai, Berry et al., 2019). This shows that B cells could potentially play a role in the regeneration phase after stroke. Furthermore, SCFA have been shown to alter the function of B cells; promoting IL-10 release (Austin, Ku et al., 2019), and reducing the gut IgG, IgA and IgE responses (Sanchez, Gan et al., 2018). To what extent the B cells altered by SCFA in the periphery change the stroke outcome, is yet to be elucidated, as lower rates of migration to the brain was seen in B cells in comparison to T cells (Benakis, Brea et al., 2016a). In our studies we have clearly focused on the effects of T cells on post stroke regeneration following SCFA supplementation. However, from all of our studies utilising Rag^{-/-} mice, there is scope to additionally investigate the role of the gut microbiome on B cells, particularly in the recovery phase after stroke.

In order to investigate the impact of the gut microbiota on the host system after stroke, one can eradicate or deplete the gut microbiota from the host. In the studies discussed in this thesis, we have demonstrated two methods; the use of GF mice (as shown in the 1st and 2nd manuscript) and the use of antibiotic treatment (as shown 4th manuscript). Both of these methods however have limitations. GF mice are expensive, difficult to maintain GF, have developmental defects, and an altered immune system. However, they are sterile and allow for colonisation with defined microbes. Antibiotic treated mice are not completely bacteria free, may have off target neuroprotective affects, but can

be utilised in many transgenic models and have no developmental defects (Hooks, Konsman et al., 2018, Kennedy et al., 2018, Lundberg, Toft et al., 2016, Round & Mazmanian, 2009). In order to account for unwanted changes observed between these two methods in our studies we carefully compared the appropriate treatment and mouse model. The comparison of GF mice with GF recolonised was to account for any developmental defects and immunological differences. Antibiotic treatment was then used to compare control supplementation or SCFA supplementation, as a tool to show reduction and restoration of SCFA concentrations. Whilst the use of GF mice in stroke research is a cleaner approach, methodologically, stroke experiments are difficult to perform under GF conditions. The use of antibiotics as a model to research stroke, is easier however has led to somewhat variable results across different institutes. First, mice are likely to be purchased from different breeders, which, as we learnt from our third study resulted in polarised T cells induced by different gut microbiota composition. Second there is no standardised antibiotic protocol for dosage or for antibiotic type. This has led to various doses and antibiotic cocktails, which are different in their effectiveness for eradicating different bacterial populations. Therefore, investigation of antibiotics on stroke, and not as a tool for microbiota depletion has led to different outcomes. In one study, the authors used a broad spectrum of antibiotics (ampicillin, vancomycin, ciprofloxacin, imipenem, and metronidazole) and reported that depletion of gut did not alter stroke outcome (Winek et al., 2016). On the contrary, Benakis et al. reported that stroke outcome was improved following antibiotic (vancomycin) treatment. Benakis et al. raised mice to have a specific bacterial gut microbiome which was resistant to amoxicillin and clavulanic acid antibiotics and compared this to mice which had a microbiome which was sensitive to antibiotics. The results revealed that antibiotic resistant mice had a worse stroke outcome with more IL-17 cells and less Treg cells compared to the antibiotic sensitive mice. The results while different, in general both studies claimed that the gut microbiota composition altered the immune system and in turn stroke outcome.

Recently many researchers have highlighted the gut microbiota as potential source of variability in the reproducibility conversation (Begley & Ioannidis, 2015, Franklin & Ericsson, 2017, Knight, Vrbanc et al., 2018, Velazquez et al., 2019). Inconsistent findings which derive from differences in microbiota composition, demonstrate the impact on various diseases, infections (Velazquez et al., 2019), and even immunotherapies as shown in our study. In 2016 the participating members of the DFG Priority Program 1656 “Intestinal Microbiota” released a study showing the extent of variability between C57BL/6 mice across Germany (Rausch, Basic et al., 2016). Not only did they find a huge variability across institutions within the country, but additionally differences within the same facility. The authors suggested that animal husbandry was the main cause for this variability, with deviations in food content, bedding, circadian rhythms and housing conditions. As shown in our study between

commercial breeders, the differences were evident up until phylum level, and the largest changes were at species level. Our study revealed that mice purchased from CR were absent in SFB. This fits with previous studies which showed mice from the Jackson Laboratory (JL) were also absent from SFB (Ivanov et al., 2009). Interestingly, it also favours the concept that some bacterial species are inherited, as CR mice were originally from JL (Faith, Colomel et al., 2015). In our experiments, we also investigated whether the changes in gut microbiota composition from different commercial breeders could partly contribute to the reproducibility crisis within the stroke field. We saw that mice with small changes in gut microbiota composition had a different stroke outcome following CD28 superagonist (SA) treatment. CD28 is a co-stimulatory receptor found on T cells which encourages proliferation and expansion. The immunotherapy named CD28SA was designed to theoretically boost the immune system, with specific preferential expansion of the immunosuppressive Treg population (Hünig, 2007, Lin & Hünig, 2003). Experimental research showed that expansion of the Treg population was neuroprotective in stroke (Liesz et al., 2009). However, two studies using CD28SA in stroke published controversial results on the one hand exacerbating stroke (Schuhmann et al., 2015) and the other improving outcome (Na et al., 2015). The mice in these two studies were purchased from two different commercial breeders CR and Har. Interestingly this is in accordance with our results which showed CD28SA resulted in exacerbated stroke outcome dependent on the gut microbiome composition. Other studies have also reported outcomes of immunotherapies or disease models affected by the gut microbiome from different commercial breeders, for example in bone density immunotherapies (McCabe et al., 2013) and infection susceptibility to salmonella (Velazquez et al., 2019).

In this thesis we have clearly shown that stroke is affected by the gut microbiome composition and that modification of the gut microbiome can improve recovery. We used GF mice and Ex-GF to show that the presence of the gut microbiota was protective in stroke. Using other GF mice models, others have also elucidated the importance of gut microbiota in brain disease. In a study which investigated the multiple sclerosis mouse model EAE, they showed that spontaneous EAE did not occur in GF mice and that gut microbiota was essential for diseases onset (Berer et al., 2011). On one hand this implies that general concepts about brain disease and gut microbiota cannot be compared; GF models of EAE are protective, whilst in stroke we saw worsened outcome. On the other hand, the impact of brain disease on gut dysbiosis has seemingly similar consequences. For example, variations of certain phyla such as the Bacteroides was not only found to alter in our experimental stroke model but also was altered in multiple sclerosis (Tremlett, Fadrosch et al., 2016). Treatment by FMT to reverse the effects of dysbiosis is currently a very attractive therapy for many brain diseases. There are over 292 clinical trials registered for FMT, however currently Clostridium difficile infection is the only disease which

has FDA approval. Within these 292 clinical trials, 17 of those are investigating the use of FMT to specifically treat brain diseases such as epilepsy, bipolar disorder and Parkinson’s disease. In order for stroke to reach FMT clinical trials many more investigations in the patient populations must be completed, to compare experimental findings in rodents to stroke patients. Presently, clinical trials for stroke and gut microbiota are ongoing (GUTSTROKE, NCT02008604). The focus of this trial is to characterise the gut microbiota differences after stroke and correlate this to peripheral immune system.

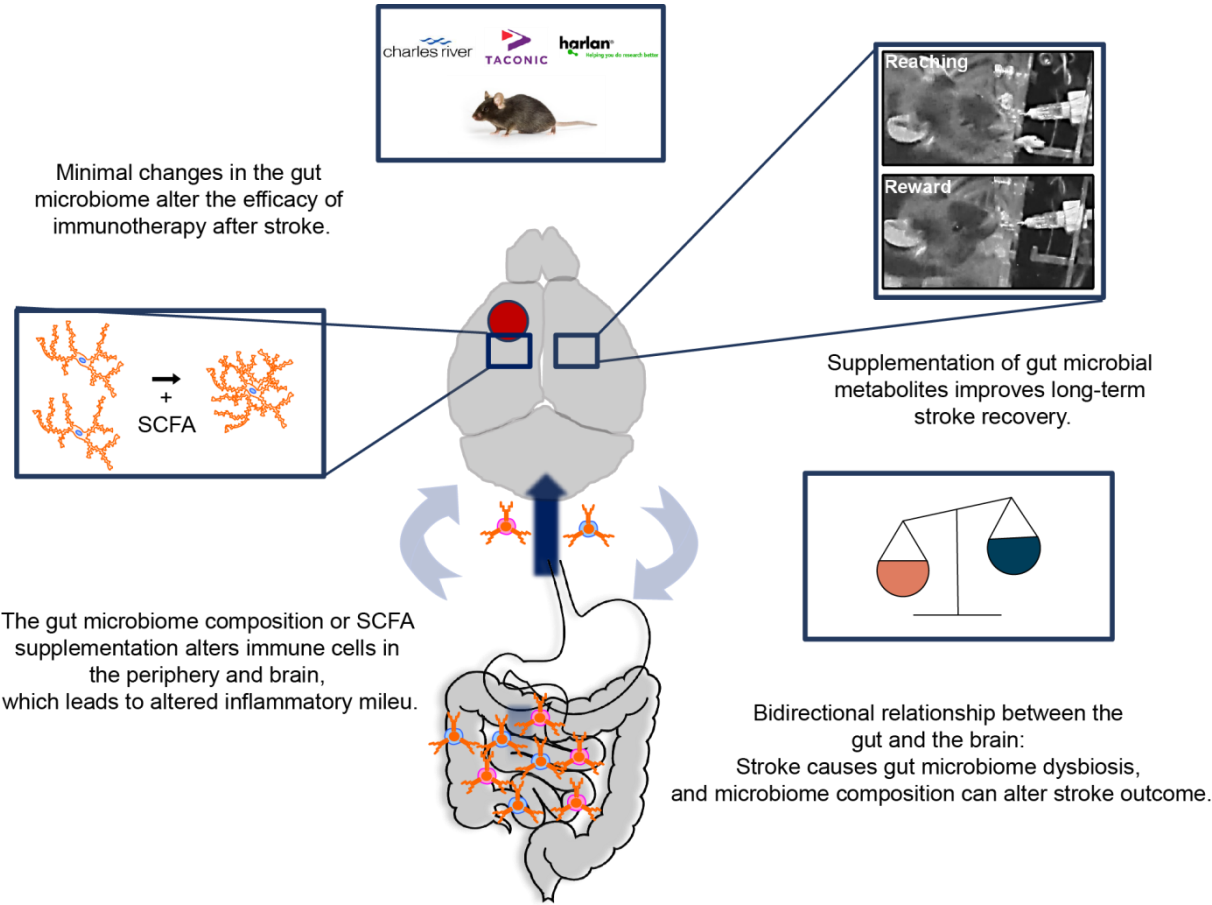


Figure 6. Schematic representation of key concepts identified. From the four papers contributing to this thesis work, there have been key concepts about the gut microbiome and stroke established. Stroke can alter microbiome composition, and likewise, modulation of the microbiome composition can alter stroke outcome. The dysbiotic gut microbiome induced after stroke changes the polarisation of immune cells in the periphery, which migrate to the brain and alter the inflammatory milieu. Mice from different commercial breeders with the same genetic strain hold small differences in their gut microbiome composition. This in turn effects how immunotherapy performs after stroke. Supplementations of microbial metabolites improve functional post stroke recovery.

CONCLUSIONS AND OUTLOOK

The four publications presented within this thesis have focused on the role of the gut microbiota in stroke. We have demonstrated a bi-directional interaction between the gut microbiota and stroke. We have shown that the presence of the gut microbiota or FMT treatment post-surgery is neuroprotective in experimental stroke models. We have observed that mice from different commercial breeders had a different gut microbiota composition, which resulted in altered response to immunotherapy after stroke. Finally, we discovered that the gut microbial metabolites, SCFA were beneficial within the post-stroke recovery phase. The questions answered within the thesis have opened up further experimental questions, however it is clear that gut microbial and dietary intervention could improve stroke outcome. I believe this thesis provides outlook to begin therapeutic research within patients, investigating an easy and practical metabolite or bacterial-based add-on alongside existing stroke recovery therapies.

REFERENCES

- Agostoni E, Chinnock JE, Daly MDB, Murray JG (1957) FUNCTIONAL AND HISTOLOGICAL STUDIES OF THE VAGUS NERVE AND ITS BRANCHES TO THE HEART, LUNGS AND ABDOMINAL VISCERA IN THE CAT. *Journal of Physiology-London* 135: 182-&
- An DD, Oh SF, Olszak T, Neves JF, Avci FY, Erturk-Hasdemir D, Lu X, Zeissig S, Blumberg RS, Kasper DL (2014) Sphingolipids from a Symbiotic Microbe Regulate Homeostasis of Host Intestinal Natural Killer T Cells. *Cell* 156: 123-133
- Anne M, Juha K, Timo M, Mikko T, Olli V, Kyosti S, Heikki H, Vilho M (2007) Neurohormonal activation in ischemic stroke: Effects of acute phase disturbances on long-term mortality. *Current Neurovascular Research* 4: 170-175
- Appel SH (2009) CD4(+) T cells mediate cytotoxicity in neurodegenerative diseases. *Journal of Clinical Investigation* 119: 13-15
- Arpaia N, Campbell C, Fan XY, Dikuy S, van der Veeken J, deRoos P, Liu H, Cross JR, Pfeffer K, Coffey PJ, Rudensky AY (2013) Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature* 504: 451-+
- Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, Fernandes GR, Tap J, Bruls T, Batto JM, Bertalan M, Borruel N, Casellas F, Fernandez L, Gautier L, Hansen T, Hattori M, Hayashi T, Kleerebezem M, Kurokawa K et al. (2011) Enterotypes of the human gut microbiome. *Nature* 473: 174-180
- Arumugam TV, Granger DN, Mattson MP (2005) Stroke and T-cells. *Neuromolecular Medicine* 7: 229-242
- Asensio VC, Campbell IL (1999) Chemokines in the CNS: plurifunctional mediators in diverse states. *Trends in Neurosciences* 22: 504-512
- Atarashi K, Tanoue T, Ando M, Kamada N, Nagano Y, Narushima S, Suda W, Imaoka A, Setoyama H, Nagamori T, Ishikawa E, Shima T, Hara T, Kado S, Jinnohara T, Ohno H, Kondo T, Toyooka K, Watanabe E, Yokoyama S et al. (2015) Th17 Cell Induction by Adhesion of Microbes to Intestinal Epithelial Cells. *Cell* 163: 367-380
- Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, Cheng GH, Yamasaki S, Saito T, Ohba Y, Taniguchi T, Takeda K, Hori S, Ivanov, II, Umesaki Y, Itoh K, Honda K (2011) Induction of Colonic Regulatory T Cells by Indigenous Clostridium Species. *Science* 331: 337-341
- Austin V, Ku JM, Miller AA, Vlahos R (2019) Ischaemic stroke in mice induces lung inflammation but not acute lung injury. *Scientific Reports* 9: 3622
- Bailey MT, Dowd SE, Galley JD, Hufnagle AR, Allen RG, Lyte M (2011) Exposure to a social stressor alters the structure of the intestinal microbiota: Implications for stressor-induced immunomodulation. *Brain Behavior and Immunity* 25: 397-407
- Baumler AJ, Sperandio V (2016) Interactions between the microbiota and pathogenic bacteria in the gut. *Nature* 535: 85-93
- Becker AM, Meyers E, Sloan A, Rennaker R, Kilgard M, Goldberg MP (2016) An automated task for the training and assessment of distal forelimb function in a mouse model of ischemic stroke. *Journal of Neuroscience Methods* 258: 16-23
- Begley CG, Ioannidis JPA (2015) Reproducibility in Science Improving the Standard for Basic and Preclinical Research. *Circulation Research* 116: 116-126
- Benakis C, Brea D, Caballero S, Faraco G, Moore J, Murphy M, Sita G, Racchumi G, Ling L, Pamer EG, Iadecola C, Anrather J (2016a) Commensal microbiota affects ischemic stroke outcome by regulating intestinal gammadelta T cells. *Nat Med* 22: 516-23
- Benakis C, Brea D, Caballero S, Faraco G, Moore J, Murphy M, Sita G, Racchumi G, Lingo L, Pamer EG, Iadecola C, Anrather J (2016b) Commensal microbiota affects ischemic stroke outcome by regulating intestinal gamma delta T cells. *Nature Medicine* 22: 516-523
- Bercik P, Park AJ, Sinclair D, Khoshdel A, Lu J, Huang X, Deng Y, Blennerhassett PA, Fahnstock M, Moine D, Berger B, Huizinga JD, Kunze W, McLean PG, Bergonzelli GE, Collins SM, Verdu EF (2011) The anxiolytic effect of *Bifidobacterium longum* NCC3001 involves vagal pathways for gut-brain communication. *Neurogastroenterology and Motility* 23: 1132-E544
- Berer K, Mues M, Koutrolos M, Al Rasbi Z, Boziki M, Johnner C, Wekerle H, Krishnamoorthy G (2011) Commensal microbiota and myelin autoantigen cooperate to trigger autoimmune demyelination. *Nature* 479: 538-U266
- Biagi E, Nylund L, Candela M, Ostan R, Bucci L, Pini E, Nikkila J, Monti D, Satokari R, Franceschi C, Brigidi P, De Vos W (2010) Through Ageing, and Beyond: Gut Microbiota and Inflammatory Status in Seniors and Centenarians. *Plos One* 5

- Bogousslavsky J, Victor SJ, Salinas EO, Pallay A, Donnan GA, Fieschi C, Kaste M, Orgogozo JM, Chamorro A, Desmet A (2002) Fibrinogen (trafermin) in acute stroke: Results of the European-Australian phase II/III safety and efficacy trial. *Cerebrovascular Diseases* 14: 239-251
- Bourassa MW, Alim I, Bultman SJ, Ratan RR (2016) Butyrate, neuroepigenetics and the gut microbiome: Can a high fiber diet improve brain health? *Neuroscience Letters* 625: 56-63
- Braak H, Braak E (1991) NEUROPATHOLOGICAL STAGING OF ALZHEIMER-RELATED CHANGES. *Acta Neuropathologica* 82: 239-259
- Bravo JA, Forsythe P, Chew MV, Escaravage E, Savignac HM, Dinan TG, Bienenstock J, Cryan JF (2011) Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proceedings of the National Academy of Sciences of the United States of America* 108: 16050-16055
- Brown AJ, Goldsworthy SM, Barnes AA, Eilert MM, Tcheang L, Daniels D, Muir AI, Wigglesworth MJ, Kinghorn I, Fraser NJ, Pike NB, Strum JC, Stepkowski KM, Murdock PR, Holder JC, Marshall FH, Szekeres PG, Wilson S, Ignar DM, Foord SM et al. (2003) The orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. *Journal of Biological Chemistry* 278: 11312-11319
- Brown CE, Wong C, Murphy TH (2008) Rapid morphologic plasticity of peri-infarct dendritic spines after focal ischemic stroke. *Stroke* 39: 1286-1291
- Brus-Ramer M, Carmel JB, Martin JH (2009) Motor Cortex Bilateral Motor Representation Depends on Subcortical and Interhemispheric Interactions. *Journal of Neuroscience* 29: 6196-6206
- Buma F, Kwakkel G, Ramsey N (2013) Understanding upper limb recovery after stroke. *Restorative Neurology and Neuroscience* 31: 707-722
- Campbell BCV, Donnan GA, Mitchell PJ, Davis SM (2016) Endovascular thrombectomy for stroke: current best practice and future goals. *Stroke and Vascular Neurology* 1: 16
- Carmichael ST, Archibeque I, Luke L, Nolan T, Momiy J, Li SL (2005) Growth-associated gene expression after stroke: Evidence for a growth-promoting region in peri-infarct cortex. *Experimental Neurology* 193: 291-311
- Cattaneo A, Cattane N, Galluzzi S, Provasi S, Lopizzo N, Festari C, Ferrari C, Guerra UP, Paghera B, Muscio C, Bianchetti A, Volta GD, Turla M, Cotelli MS, Gennuso M, Prella A, Zanetti O, Lussignoli G, Mirabile D, Bellandi D et al. (2017) Association of brain amyloidosis with pro-inflammatory gut bacterial taxa and peripheral inflammation markers in cognitively impaired elderly. *Neurobiology of Aging* 49: 60-68
- Chabot S, Charlet D, Wilson TL, Yong VW (2001) Cytokine production consequent to T cell-microglia interaction: the PMA/IFN gamma-treated U937 cells display similarities to human microglia. *Journal of Neuroscience Methods* 105: 111-120
- Chan PH (2001) Reactive oxygen radicals in signaling and damage in the ischemic brain. *Journal of Cerebral Blood Flow and Metabolism* 21: 2-14
- Chang PV, Hao LM, Offermanns S, Medzhitov R (2014) The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. *Proceedings of the National Academy of Sciences of the United States of America* 111: 2247-2252
- Chen YX, Bodhankar S, Murphy SJ, Vandenberg AA, Alkayed NJ, Offner H (2012) Intrastriatal B-cell administration limits infarct size after stroke in B-cell deficient mice. *Metabolic Brain Disease* 27: 487-493
- Chen ZH, Jalabi W, Hu WW, Park HJ, Gale JT, Kidd GJ, Bernatowicz R, Gossman ZC, Chen JT, Dutta R, Trapp BD (2014) Microglial displacement of inhibitory synapses provides neuroprotection in the adult brain. *Nature Communications* 5
- Chen ZQ, Mou RT, Feng DX, Wang Z, Chen G (2017) The role of nitric oxide in stroke. *Medical Gas Research* 7: 194-203
- Chung WS, Clarke LE, Wang GX, Stafford BK, Sher A, Chakraborty C, Joung J, Foo LC, Thompson A, Chen CF, Smith SJ, Barres BA (2013) Astrocytes mediate synapse elimination through MEGF10 and MERTK pathways. *Nature* 504: 394-+
- Clarkson AN, Huang BS, MacIsaac SE, Mody I, Carmichael ST (2010) Reducing excessive GABA-mediated tonic inhibition promotes functional recovery after stroke. *Nature* 468: 305-U193
- Craig LE, Middleton S, Hamilton H, Cudlip F, Swatzell V, Alexandrov AV, Lightbody E, Watkins DC, Philip S, Cadilhac DA, McInnes E, Dale S, Alexandrov AW (2019) Does the Addition of Non-Approved Inclusion and Exclusion Criteria for rtPA Impact Treatment Rates? Findings in Australia, the UK, and the USA. *Interventional Neurology* 8: 1-12
- Cramer JV, Benakis C, Liesz A (2019) T cells in the post-ischemic brain: Troopers or paramedics? *Journal of Neuroimmunology* 326: 33-37

- Cramer JV, Gesierich B, Roth S, Dichgans M, Duering M, Liesz A (2018) In vivo widefield calcium imaging of the mouse cortex for analysis of network connectivity in health and brain disease. *bioRxiv*: 459941
- Cramer SC (2015) Drugs to Enhance Motor Recovery After Stroke. *Stroke* 46: 2998-3005
- Cryan JF, Dinan TG (2012) Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nature Reviews Neuroscience* 13: 701-712
- Cummings JH, Pomare EW, Branch WJ, Naylor CPE, Macfarlane GT (1987) SHORT CHAIN FATTY-ACIDS IN HUMAN LARGE-INTESTINE, PORTAL, HEPATIC AND VENOUS-BLOOD. *Gut* 28: 1221-1227
- den Besten G, van Eunen K, Groen AK, Venema K, Reijngoud DJ, Bakker BM (2013) The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *Journal of Lipid Research* 54: 2325-2340
- Dennis M, Forbes J, Graham C, Hackett M, Hankey GJ, House A, Lewis S, Lundstrom E, Sandercock P, Mead G, Anderson R, Buchanan D, Deary A, Drever J, Fraser R, Innes K, McGill C, McGrath A, Perry D, Walker P et al. (2019) Effects of fluoxetine on functional outcomes after acute stroke (FOCUS): a pragmatic, double-blind, randomised, controlled trial. *Lancet* 393: 265-274
- Dethlefsen L, Relman DA (2011) Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. *Proceedings of the National Academy of Sciences of the United States of America* 108: 4554-4561
- Dijkhuizen RM, Ren JM, Mandeville JB, Wu ON, Ozdag FM, Moskowitz MA, Rosen BR, Finklestein SP (2001) Functional magnetic resonance imaging of reorganization in rat brain after stroke. *Proceedings of the National Academy of Sciences of the United States of America* 98: 12766-12771
- Dirnagl U, Iadecola C, Moskowitz MA (1999) Pathobiology of ischaemic stroke: an integrated view. *Trends in Neurosciences* 22: 391-397
- Doyle KP, Quach LN, Sole M, Axtell RC, Nguyen TVV, Soler-Llavina GJ, Jurado S, Han J, Steinman L, Longo FM, Schneider JA, Malenka RC, Buckwalter MS (2015) B-Lymphocyte-Mediated Delayed Cognitive Impairment following Stroke. *Journal of Neuroscience* 35: 2133-2145
- Droste JHJ, Wieringa MH, Weyler JJ, Nelen VJ, Vermeire PA, Van Bever HP (2000) Does the use of antibiotics in early childhood increase the risk of asthma and allergic disease? *Clinical and Experimental Allergy* 30: 1547-1553
- Engler TMNdM, Dourado CC, Amâncio TG, Farage L, de Mello PA, Padula MPC (2014) Stroke: bowel dysfunction in patients admitted for rehabilitation. *The open nursing journal* 8: 43-47
- Erny D, de Angelis ALH, Jaitin D, Wieghofer P, Staszewski O, David E, Keren-Shaul H, Mhlahkoi T, Jakobshagen K, Buch T, Schwierzeck V, Utermohlen O, Chun E, Garrett WS, McCoy KD, Diefenbach A, Staeheli P, Stecher B, Amit I, Prinz M (2015) Host microbiota constantly control maturation and function of microglia in the CNS. *Nature Neuroscience* 18: 965-+
- Faith JJ, Colombel J-F, Gordon JI (2015) Identifying strains that contribute to complex diseases through the study of microbial inheritance. *Proceedings of the National Academy of Sciences of the United States of America* 112: 633-640
- Falk PG, Hooper LV, Midtvedt T, Gordon JI (1998) Creating and Maintaining the Gastrointestinal Ecosystem: What We Know and Need To Know from Gnotobiology. *Microbiology and Molecular Biology Reviews* 62: 1157
- Farkas AM, Panea C, Goto Y, Nakato G, Galan-Diez M, Narushima S, Honda K, Ivanov, II (2015) Induction of Th17 cells by segmented filamentous bacteria in the murine intestine. *Journal of Immunological Methods* 421: 104-111
- Faustino JV, Wang X, Johnson CE, Klibanov A, Derugin N, Wendland MF, Vexler ZS (2011) Microglial Cells Contribute to Endogenous Brain Defenses after Acute Neonatal Focal Stroke. *Journal of Neuroscience* 31: 12992-13001
- Ford G, Bhakta B, Cozens A, Hartley S, Holloway I, Meads D, Ruddock S, Santorelli G, Sackley C, Walker M, Farrin A (2015) DARS (Dopamine Augmented Rehabilitation in Stroke): Longer-term results for a randomised controlled trial of Co-careldopa in addition to routine occupational and physical therapy after stroke. *International Journal of Stroke* 10: 6-7
- Franklin CL, Ericsson AC (2017) Microbiota and reproducibility of rodent models. *Lab Animal* 46: 114-122
- Frijns CJM, Kappelle LJ (2002) Inflammatory cell adhesion molecules in ischemic cerebrovascular disease. *Stroke* 33: 2115-2122
- Frost G, Sleeth ML, Sahuri-Arisoylu M, Lizarbe B, Cerdan S, Brody L, Anastasovska J, Ghourab S, Hankir M, Zhang S, Carling D, Swann JR, Gibson G, Viardot A, Morrison D, Thomas EL, Bell JD (2014) The short-chain fatty acid acetate reduces appetite via a central homeostatic mechanism. *Nature Communications* 5
- Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, Nakanishi Y, Uetake C, Kato K, Kato T, Takahashi M, Fukuda NN, Murakami S, Miyauchi E, Hino S, Atarashi K, Onawa S, Fujimura Y, Lockett T, Clarke

- JM et al. (2013) Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature* 504: 446-+
- Gelderblom M, Leypoldt F, Steinbach K, Behrens D, Choe C-U, Siler DA, Arumugam TV, Orthey E, Gerloff C, Tolosa E, Magnus T (2009) Temporal and Spatial Dynamics of Cerebral Immune Cell Accumulation in Stroke. *Stroke* 40: 1849-1857
- Gelderblom M, Weymar A, Bernreuther C, Velden J, Arunachalam P, Steinbach K, Orthey E, Arumugam TV, Leypoldt F, Simova O, Thom V, Friese MA, Prinz I, Holscher C, Glatzel M, Korn T, Gerloff C, Tolosa E, Magnus T (2012) Neutralization of the IL-17 axis diminishes neutrophil invasion and protects from ischemic stroke. *Blood* 120: 3793-3802
- Gensollen T, Iyer SS, Kasper DL, Blumberg RS (2016) How colonization by microbiota in early life shapes the immune system. *Science* 352: 539-544
- Gill SR, Pop M, DeBoy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, Gordon JI, Relman DA, Fraser-Liggett CM, Nelson KE (2006) Metagenomic analysis of the human distal gut microbiome. *Science* 312: 1355-1359
- Girard S, Murray KN, Rothwell NJ, Metz GAS, Allan SM (2014) Long-term functional recovery and compensation after cerebral ischemia in rats. *Behavioural Brain Research* 270: 18-28
- Gliem M, Mausberg AK, Lee JI, Simiantonakis I, van Rooijen N, Hartung HP, Jander S (2012) Macrophages prevent hemorrhagic infarct transformation in murine stroke models. *Annals of Neurology* 71: 743-752
- Gonzalez CLR, Gharbawie OA, Williams PT, Kleim JA, Kolb B, Whishaw IQ (2004) Evidence for bilateral control of skilled movements: ipsilateral skilled forelimb reaching deficits and functional recovery in rats follow motor cortex and lateral frontal cortex lesions. *European Journal of Neuroscience* 20: 3442-3452
- Gonzalez H, Pacheco R (2014) T-cell-mediated regulation of neuroinflammation involved in neurodegenerative diseases. *Journal of Neuroinflammation* 11: 11
- Goto Y, Panea C, Nakato G, Cebula A, Lee C, Diez MG, Laufer TM, Ignatowicz L, Ivanov, II (2014) Segmented Filamentous Bacteria Antigens Presented by Intestinal Dendritic Cells Drive Mucosal Th17 Cell Differentiation. *Immunity* 40: 594-607
- Goverman J, Woods A, Larson L, Weiner LP, Hood L, Zaller DM (1993) TRANSGENIC MICE THAT EXPRESS A MYELIN BASIC PROTEIN-SPECIFIC T-CELL RECEPTOR DEVELOP SPONTANEOUS AUTOIMMUNITY. *Cell* 72: 551-560
- Grefkes C, Nowak DA, Eickhoff SB, Dafotakis M, Kust J, Karbe H, Fink GR (2008) Cortical connectivity after subcortical stroke assessed with functional magnetic resonance imaging. *Annals of Neurology* 63: 236-246
- Grefkes C, Nowak DA, Wang LE, Dafotakis M, Eickhoff SB, Fink GR (2010) Modulating cortical connectivity in stroke patients by rTMS assessed with fMRI and dynamic causal modeling. *Neuroimage* 50: 233-242
- Gulke E, Gelderblom M, Magnus T (2018) Danger signals in stroke and their role on microglia activation after ischemia. *Therapeutic Advances in Neurological Disorders* 11
- Haghikia A, Jorg S, Duscha A, Berg J, Manzel A, Waschbisch A, Hammer A, Lee DH, May C, Wilck N, Balogh A, Ostermann AI, Schebb NH, Akkad DA, Grohme DA, Kleinewietfeld M, Kempa S, Thone J, Demir S, Muller DN et al. (2015) Dietary Fatty Acids Directly Impact Central Nervous System Autoimmunity via the Small Intestine. *Immunity* 43: 817-829
- Hankey GJ (2017) Stroke. *Lancet* 389: 641-654
- Heindl S, Gesierich B, Benakis C, Llovera G, Duering M, Liesz A (2018) Automated Morphological Analysis of Microglia After Stroke. *Frontiers in Cellular Neuroscience* 12
- Hess DC, Wechsler LR, Clark WM, Savitz SI, Ford GA, Chiu D, Yavagal DR, Uchino K, Liebeskind DS, Auchus AP, Sen S, Sila CA, Vest JD, Mays RW (2017) Safety and efficacy of multipotent adult progenitor cells in acute ischaemic stroke (MASTERS): a randomised, double-blind, placebo-controlled, phase 2 trial. *Lancet Neurology* 16: 360-368
- Hinnebusch BF, Meng SF, Wu JT, Archer SY, Hodin RA (2002) The effects of short-chain fatty acids on human colon cancer cell phenotype are associated with histone hyperacetylation. *Journal of Nutrition* 132: 1012-1017
- Hooks KB, Konsman JP, O'Malley MA (2018) Microbiota-gut-brain research: a critical analysis. *Behavioral and Brain Sciences*: 1-40
- Hosoi T, Okuma Y, Matsuda T, Nomura Y (2005) Novel pathway for LPS-induced afferent vagus nerve activation: Possible role of nodose ganglion. *Autonomic Neuroscience-Basic & Clinical* 120: 104-107
- Hoverstad T, Midtvedt T (1986) SHORT-CHAIN FATTY-ACIDS IN GERM-FREE-MICE AND RATS. *Journal of Nutrition* 116: 1772-1776
- Huang SY, Chang CH, Hung HY, Lin YW, Lee EJ (2018) Neuroanatomical and electrophysiological recovery in the contralateral intact cortex following transient focal cerebral ischemia in rats. *Neurological Research* 40: 130-138

- Hurn PD, Subramanian S, Parker SM, Afentoulis ME, Kaler LJ, Vandembark AA, Offner H (2007) T- and B-cell-deficient mice with experimental stroke have reduced lesion size and inflammation. *Journal of Cerebral Blood Flow and Metabolism* 27: 1798-1805
- Hutsler JJ, Zhang H (2010) Increased dendritic spine densities on cortical projection neurons in autism spectrum disorders. *Brain Research* 1309: 83-94
- Huttenhower C, Gevers D, Knight R, Abubucker S, Badger JH, Chinwalla AT, Creasy HH, Earl AM, FitzGerald MG, Fulton RS, Giglio MG, Hallsworth-Pepin K, Lobos EA, Madupu R, Magrini V, Martin JC, Mitreva M, Muzny DM, Sodergren EJ, Versalovic J et al. (2012) Structure, function and diversity of the healthy human microbiome. *Nature* 486: 207-214
- Hünig T (2007) Manipulation of Regulatory T-Cell Number and Function with CD28-Specific Monoclonal Antibodies. In *Advances in Immunology*, pp 111-148. Academic Press
- Iadecola C, Anrather J (2011) The immunology of stroke: from mechanisms to translation. *Nature Medicine* 17: 796-808
- Ivanov II, Frutos RD, Manel N, Yoshinaga K, Rifkin DB, Sartor RB, Finlay BB, Littman DR (2008) Specific Microbiota Direct the Differentiation of IL-17-Producing T-Helper Cells in the Mucosa of the Small Intestine. *Cell Host & Microbe* 4: 337-349
- Ivanov II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, Wei D, Goldfarb KC, Santee CA, Lynch SV, Tanoue T, Imaoka A, Itoh K, Takeda K, Umesaki Y, Honda K, Littman DR (2009) Induction of Intestinal Th17 Cells by Segmented Filamentous Bacteria. *Cell* 139: 485-498
- Jablonska JA, Witte OW, Kossut M (2007) Photothrombotic infarct impairs experience-dependent plasticity in neighboring cortex. *Neuroreport* 18: 165-169
- Javidi E, Magnus T (2019) Autoimmunity After Ischemic Stroke and Brain Injury. *Frontiers in Immunology* 10
- Ji K, Akgul G, Wollmuth LP, Tsirka SE (2013) Microglia Actively Regulate the Number of Functional Synapses. *Plos One* 8
- Jiang Y, Li LL, Liu B, Zhang YH, Chen Q, Li CQ (2014) Vagus Nerve Stimulation Attenuates Cerebral Ischemia and Reperfusion Injury via Endogenous Cholinergic Pathway in Rat. *Plos One* 9
- Jickling GC, Liu DZ, Ander BP, Stamova B, Zhan XH, Sharp FR (2015) Targeting neutrophils in ischemic stroke: translational insights from experimental studies. *Journal of Cerebral Blood Flow and Metabolism* 35: 888-901
- Jin KL, Sun YJ, Xie L, Childs J, Mao XO, Greenberg DA (2004) Post-ischemic administration of heparin-binding epidermal growth factor-like growth factor (HB-EGF) reduces infarct size and modifies neurogenesis after focal cerebral ischemia in the rat. *Journal of Cerebral Blood Flow and Metabolism* 24: 399-408
- Johnson CO, Nguyen M, Roth GA, Nichols E, Alam T, Abate D, Abd-Allah F, Abdelalim A, Abraha HN, Abu-Rmeileh NM, Adebayo OM, Adeoye AM, Agarwal G, Agrawal S, Aichour AN, Aichour I, Aichour MTE, Alahdab F, Ali R, Alvis-Guzman N et al. (2019) Global, regional, and national burden of stroke, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet Neurology* 18: 439-458
- Josefowicz SZ, Niec RE, Kim HY, Treuting P, Chinen T, Zheng Y, Umetsu DT, Rudensky AY (2012) Extrathymically generated regulatory T cells control mucosal T(H)2 inflammation. *Nature* 482: 395-U1510
- Kadowaki A, Saga R, Lin Y, Sato W, Yamamura T (2019) Gut microbiota-dependent CCR9+CD4+ T cells are altered in secondary progressive multiple sclerosis. *Brain* 142: 916-931
- Kalladka D, Sinden J, Pollock K, Haig C, McLean J, Smith W, McConnachie A, Santosh C, Bath PM, Dunn L, Muir KW (2016) Human neural stem cells in patients with chronic ischaemic stroke (PISCES): a phase 1, first-in-man study. *Lancet* 388: 787-796
- Kamp F, Hamilton JA (2006) How fatty acids of different chain length enter and leave cells by free diffusion. *Prostaglandins Leukotrienes and Essential Fatty Acids* 75: 149-159
- Karson CN, Mrak RE, Schluterman KO, Sturmer WQ, Sheng JG, Griffin WST (1999) Alterations in synaptic proteins and their encoding mRNAs in prefrontal cortex in schizophrenia: a possible neurochemical basis for 'hypofrontality'. *Molecular Psychiatry* 4: 39-45
- Katsura K, Kristian T, Siesjo BK (1994) ENERGY-METABOLISM, ION HOMEOSTASIS, AND CELL-DAMAGE IN THE BRAIN. *Biochemical Society Transactions* 22: 991-996
- Kawamata T, Dietrich WD, Schallert T, Gotts JE, Cocke RR, Benowitz LI, Finklestein SP (1997) Intracisternal basic fibroblast growth factor enhances functional recovery and up-regulates the expression of a molecular marker of neuronal sprouting following focal cerebral infarction. *Proceedings of the National Academy of Sciences of the United States of America* 94: 8179-8184
- Keita AV, Soderholm JD (2010) The intestinal barrier and its regulation by neuroimmune factors. *Neurogastroenterology and Motility* 22: 718-733

- Keller JJ, Wang J, Hwang YL, Chou CC, Wang LH, Hsu JL, Bai CH, Chiou HY (2015) Increased risk of stroke among patients with Crohn's disease: a population-based matched cohort study. *International Journal of Colorectal Disease* 30: 645-653
- Kennedy EA, King KY, Baldrige MT (2018) Mouse Microbiota Models: Comparing Germ-Free Mice and Antibiotics Treatment as Tools for Modifying Gut Bacteria. *Frontiers in Physiology* 9
- Kim M, Qie YQ, Park J, Kim CH (2016) Gut Microbial Metabolites Fuel Host Antibody Responses. *Cell Host & Microbe* 20: 202-214
- Kim N, Yun M, Oh YJ, Choi HJ (2018) Mind-altering with the gut: Modulation of the gut-brain axis with probiotics. *Journal of Microbiology* 56: 172-182
- Kleinschnitz C, Schwab N, Kraft P, Hagedorn I, Dreykluft A, Schwarz T, Austinat M, Nieswandt B, Wiendl H, Stoll G (2010) Early detrimental T-cell effects in experimental cerebral ischemia are neither related to adaptive immunity nor thrombus formation. *Blood* 115: 3835-3842
- Knight R, Vrbanac A, Taylor BC, Aksenov A, Callewaert C, Debelius J, Gonzalez A, Kosciolk T, McCall LI, McDonald D, Melnik AV, Morton JT, Navas J, Quinn RA, Sanders JG, Swafford AD, Thompson LR, Tripathi A, Xu ZJZ, Zaneveld JR et al. (2018) Best practices for analysing microbiomes. *Nature Reviews Microbiology* 16: 410-422
- Kunkel EJ, Campbell DJ, Butcher EC (2003) Chemokines in lymphocyte trafficking and intestinal immunity. *Microcirculation* 10: 313-323
- Lal S, Kirkup AJ, Brunnsden AM, Thompson DG, Grundy D (2001) Vagal afferent responses to fatty acids of different chain length in the rat. *American Journal of Physiology-Gastrointestinal and Liver Physiology* 281: G907-G915
- Lamas B, Richard ML, Leducq V, Pham HP, Michel ML, Da Costa G, Bridonneau C, Jegou S, Hoffmann TW, Natividad JM, Brot L, Taleb S, Couturier-Maillard A, Nion-Larmurier I, Merabtene F, Seksik P, Bourrier A, Cosnes J, Ryffel B, Beaugerie L et al. (2016) CARD9 impacts colitis by altering gut microbiota metabolism of tryptophan into aryl hydrocarbon receptor ligands. *Nature Medicine* 22: 598-+
- Lambertsen KL, Biber K, Finsen B (2012) Inflammatory cytokines in experimental and human stroke. *Journal of Cerebral Blood Flow and Metabolism* 32: 1677-1698
- Lander ES, Int Human Genome Sequencing C, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Dewar K, Doyle M, FitzHugh W, Funke R, Gage D, Harris K, Heaford A, Howland J, Kann L, Lehoczky J, LeVine R, McEwan P et al. (2001) Initial sequencing and analysis of the human genome. *Nature* 409: 860-921
- Le Poul E, Loison C, Struyf S, Springael JY, Lannoy V, Decobecq ME, Brezillon S, Dupriez V, Vassart G, Van Damme J, Parmentier M, Detheux M (2003) Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. *Journal of Biological Chemistry* 278: 25481-25489
- Leblhuber F, Egger M, Schuetz B, Fuchs D (2018) Commentary: Effect of Probiotic Supplementation on Cognitive Function and Metabolic Status in Alzheimer's Disease: A Randomized, Double-Blind and Controlled Trial. *Frontiers in Aging Neuroscience* 10
- Li XL, Blizzard KK, Zeng ZY, DeVries AC, Hurn PD, McCullough LD (2004) Chronic behavioral testing after focal ischemia in the mouse: functional recovery and the effects of gender. *Experimental Neurology* 187: 94-104
- Liesz A, Suri-Payer E, Veltkamp C, Doerr H, Sommer C, Rivest S, Giese T, Veltkamp R (2009) Regulatory T cells are key cerebroprotective immunomodulators in acute experimental stroke. *Nature Medicine* 15: 192-199
- Liesz A, Zhou W, Mracsko E, Karcher S, Bauer H, Schwarting S, Sun L, Bruder D, Stegemann S, Cerwenka A, Sommer C, Dalpke AH, Veltkamp R (2011) Inhibition of lymphocyte trafficking shields the brain against deleterious neuroinflammation after stroke. *Brain* 134: 704-720
- Lin C-H, Hünig T (2003) Efficient expansion of regulatory T cells in vitro and in vivo with a CD28 superagonist. *European Journal of Immunology* 33: 626-638
- Lin DJ, Finklestein SP, Cramer SC (2018) New Directions in Treatments Targeting Stroke Recovery. *Stroke* 49: 3107-3114
- Llovera G, Hofmann K, Roth S, Salas-Perdomo A, Ferrer-Ferrer M, Perego C, Zanier ER, Mamrak U, Rex A, Party H, Agin V, Fauchon C, Orset C, Haelewyn B, De Simoni MG, Dirnagl U, Grittner U, Planas AM, Plesnila N, Vivien D et al. (2015) Results of a preclinical randomized controlled multicenter trial (pRCT): Anti-CD49d treatment for acute brain ischemia. *Science Translational Medicine* 7
- Llovera G, Liesz A (2016) The next step in translational research: lessons learned from the first preclinical randomized controlled trial. *Journal of Neurochemistry* 139: 271-279
- Lo EH (2008) A new penumbra: transitioning from injury into repair after stroke. *Nature Medicine* 14: 497

- Lundberg R, Toft MF, August B, Hansen AK, Hansen CHF (2016) Antibiotic-treated versus germ-free rodents for microbiota transplantation studies. *Gut microbes* 7: 68-74
- Luu M, Pautz S, Kohl V, Singh R, Romero R, Lucas S, Hofmann J, Raifer H, Vachharajani N, Carrascosa LC, Lamp B, Nist A, Stiewe T, Shaul Y, Adhikary T, Zaiss MM, Lauth M, Steinhoff U, Visekruna A (2019) The short-chain fatty acid pentanoate suppresses autoimmunity by modulating the metabolic-epigenetic crosstalk in lymphocytes. *Nature Communications* 10
- Luu M, Weigand K, Wedi F, Breidenbend C, Leister H, Pautz S, Adhikary T, Visekruna A (2018) Regulation of the effector function of CD8(+) T cells by gut microbiota-derived metabolite butyrate. *Scientific Reports* 8
- Lycke NY, Bemark M (2017) The regulation of gut mucosal IgA B-cell responses: recent developments. *Mucosal Immunology* 10: 1361-1374
- Lyte M (2011) Probiotics function mechanistically as delivery vehicles for neuroactive compounds: Microbial endocrinology in the design and use of probiotics. *Bioessays* 33: 574-581
- Ma YY, Ramachandran A, Ford N, Parada I, Prince DA (2013) Remodeling of dendrites and spines in the C1q knockout model of genetic epilepsy. *Epilepsia* 54: 1232-1239
- Macfarlane S, Macfarlane GT (2003) Regulation of short-chain fatty acid production. *Proceedings of the Nutrition Society* 62: 67-72
- Macpherson AJ, Gatto D, Sainsbury E, Harriman GR, Hengartner H, Zinkernagel RM (2000) A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. *Science* 288: 2222-+
- Macpherson AJ, Uhr T (2004) Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. *Science* 303: 1662-1665
- Mangalam A, Shahi SK, Luckey D, Karau M, Marietta E, Luo NL, Choung RS, Ju J, Sompallae R, Gibson-Corley K, Patel R, Rodriguez M, David C, Taneja V, Murray J (2017) Human Gut-Derived Commensal Bacteria Suppress CNS Inflammatory and Demyelinating Disease. *Cell Reports* 20: 1269-1277
- Manwani B, Liu FD, Xu Y, Persky R, Li J, McCullough LD (2011) Functional recovery in aging mice after experimental stroke. *Brain Behavior and Immunity* 25: 1689-1700
- McCabe LR, Irwin R, Schaefer L, Britton RA (2013) Probiotic use decreases intestinal inflammation and increases bone density in healthy male but not female mice. *Journal of Cellular Physiology* 228: 1793-1798
- McNutt M (2014) Journals unite for reproducibility. *Science* 346: 679-679
- Mead GE, Hsieh CF, Lee R, Kutlubaev M, Claxton A, Hankey GJ, Hackett M (2013) Selective Serotonin Reuptake Inhibitors for Stroke Recovery A Systematic Review and Meta-analysis. *Stroke* 44: 844-+
- Minato T, Maeda T, Fujisawa Y, Tsuji H, Nomoto K, Ohno K, Hirayama M (2017) Progression of Parkinson's disease is associated with gut dysbiosis: Two-year follow-up study. *Plos One* 12
- Mirabelli-Badenier M, Braunersreuther V, Viviani GL, Dallegrì F, Quercioli A, Veneselli E, Mach F, Montecucco F (2011) CC and CXC chemokines are pivotal mediators of cerebral injury in ischaemic stroke. *Thrombosis and Haemostasis* 105: 409-420
- Miyauchi S, Gopal E, Fei YJ, Ganapathy V (2004) Functional identification of SLC5A8, a tumor suppressor down-regulated in colon cancer, as a Na⁺-coupled transporter for short-chain fatty acids. *Journal of Biological Chemistry* 279: 13293-13296
- Morrison DJ, Preston T (2016) Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. *Gut Microbes* 7: 189-200
- Morton AM, Sefik E, Upadhyay R, Weissleder R, Benoist C, Mathis D (2014) Endoscopic photoconversion reveals unexpectedly broad leukocyte trafficking to and from the gut. *Proceedings of the National Academy of Sciences of the United States of America* 111: 6696-6701
- Mosmann TR, Sad S (1996) The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunology Today* 17: 138-146
- Murphy TH, Corbett D (2009) Plasticity during stroke recovery: from synapse to behaviour. *Nature Reviews Neuroscience* 10: 861-872
- Na S-Y, Mracsko E, Liesz A, Huenig T, Veltkamp R (2015) Amplification of Regulatory T Cells Using a CD28 Superagonist Reduces Brain Damage After Ischemic Stroke in Mice. *Stroke* 46: 212-+
- Nakamura YK, Janowitz C, Metea C, Asquith M, Karstens L, Rosenbaum JT, Lin P (2017) Short chain fatty acids ameliorate immune-mediated uveitis partially by altering migration of lymphocytes from the intestine. *Scientific Reports* 7
- Natividad JMM, Verdu EF (2013) Modulation of intestinal barrier by intestinal microbiota: Pathological and therapeutic implications. *Pharmacological Research* 69: 42-51
- Neufeld KAM, Mao YK, Bienenstock J, Foster JA, Kunze WA (2013) The microbiome is essential for normal gut intrinsic primary afferent neuron excitability in the mouse. *Neurogastroenterology and Motility* 25: 183-+

- Noyce AJ, Bestwick JP, Silveira-Moriyama L, Hawkes CH, Giovannoni G, Lees AJ, Schrag A (2012) Meta-Analysis of Early Nonmotor Features and Risk Factors for Parkinson Disease. *Annals of Neurology* 72: 893-901
- Ochoa-Reparaz J, Mielcarz DW, Ditrio LE, Burroughs AR, Foureau DM, Haque-Begum S, Kasper LH (2009) Role of Gut Commensal Microflora in the Development of Experimental Autoimmune Encephalomyelitis. *Journal of Immunology* 183: 6041-6050
- Offner H, Subramanian S, Parker SM, Wang CH, Afentoulis ME, Lewis A, Vandenbark AA, Hurn PD (2006) Splenic atrophy in experimental stroke is accompanied by increased regulatory T cells and circulating macrophages. *Journal of Immunology* 176: 6523-6531
- Olsen AB, Hetz RA, Xue H, Aroom KR, Bhattarai D, Johnson E, Bedi S, Cox CS, Uray K (2013) Effects of traumatic brain injury on intestinal contractility. *Neurogastroenterology and Motility* 25
- O'Neill LAJ, Kaltschmidt C (1997) NF-kappa B: A crucial transcription factor for glial and neuronal cell function. *Trends in Neurosciences* 20: 252-258
- Ottani A, Giuliani D, Mioni C, Galantucci M, Minutoli L, Bitto A, Altavilla D, Zaffe D, Botticelli AR, Squadrito F, Guarini S (2009) Vagus nerve mediates the protective effects of melanocortins against cerebral and systemic damage after ischemic stroke. *Journal of Cerebral Blood Flow and Metabolism* 29: 512-523
- Patnala R, Varumugam T, Gupta N, Dheen ST (2017) HDAC Inhibitor Sodium Butyrate-Mediated Epigenetic Regulation Enhances Neuroprotective Function of Microglia During Ischemic Stroke. *Molecular Neurobiology* 54: 6391-6411
- Penfield W, Boldrey E (1937) Somatic motor and sensory representation in the cerebral cortex of man as studied by electrical stimulation. *Brain* 60: 389-443
- Planas AM (2018) Role of Immune Cells Migrating to the Ischemic Brain. *Stroke* 49: 2261-2267
- Planas AM, Soriano MA, Berruezo M, Justicia C, Estrada A, Pitarch S, Ferrer I (1996) Induction of Stat3, a signal transducer and transcription factor, in reactive microglia following transient focal cerebral ischaemia. *European Journal of Neuroscience* 8: 2612-2618
- Ploughman M, Windle V, MacLellan CL, White N, Dore JJ, Corbett D (2009) Brain-Derived Neurotrophic Factor Contributes to Recovery of Skilled Reaching After Focal Ischemia in Rats. *Stroke* 40: 1490-1495
- Ramesh G, MacLean AG, Philipp MT (2013) Cytokines and Chemokines at the Crossroads of Neuroinflammation, Neurodegeneration, and Neuropathic Pain. *Mediators of Inflammation*
- Rausch P, Basic M, Batra A, Bischoff SC, Blaut M, Clavel T, Glasner J, Gopalakrishnan S, Grass GA, Gunther C, Haller D, Hirose M, Ibrahim S, Loh G, Mattner J, Nagel S, Pabst O, Schmidt F, Siegmund B, Strowig T et al. (2016) Analysis of factors contributing to variation in the C57BL/6J fecal microbiota across German animal facilities. *International Journal of Medical Microbiology* 306: 343-355
- Rehme AK, Eickhoff SB, Wang LE, Fink GR, Grefkes C (2011) Dynamic causal modeling of cortical activity from the acute to the chronic stage after stroke. *Neuroimage* 55: 1147-1158
- Rehme AK, Grefkes C (2013) Cerebral network disorders after stroke: evidence from imaging-based connectivity analyses of active and resting brain states in humans. *Journal of Physiology-London* 591: 17-31
- Ren XF, Akiyoshi K, Dziennis S, Vandenbark AA, Herson PS, Hurn PD, Offner H (2011) Regulatory B Cells Limit CNS Inflammation and Neurologic Deficits in Murine Experimental Stroke. *Journal of Neuroscience* 31: 8556-8563
- Reshef R, Kudryavitskaya E, Shani-Narkiss H, Isaacson B, Rimmerman N, Mizrahi A, Yirmiya R (2017) The role of microglia and their CX3CR1 signaling in adult neurogenesis in the olfactory bulb. *Elife* 6
- Riccomagno MM, Kolodkin AL (2015) Sculpting Neural Circuits by Axon and Dendrite Pruning. *Annual Review of Cell and Developmental Biology, Vol 31* 31: 779-805
- Rinninella E, Raoul P, Cintoni M, Franceschi F, Miggiano GAD, Gasbarrini A, Mele MC (2019) What is the Healthy Gut Microbiota Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases. *Microorganisms* 7
- Rosell A, Agin V, Rahman M, Morancho A, Ali C, Koistinaho J, Wang XY, Vivien D, Schwaninger M, Montaner J (2013) Distal Occlusion of the Middle Cerebral Artery in Mice: Are We Ready to Assess Long-Term Functional Outcome? *Translational Stroke Research* 4: 297-307
- Rosenberg GA (2002) Matrix metalloproteinases in neuroinflammation. *Glia* 39: 279-291
- Rothwell NJ, Hopkins SJ (1995) Cytokines and the nervous system II: actions and mechanisms of action. *Trends in Neurosciences* 18: 130-136
- Round JL, Mazmanian SK (2009) The gut microbiota shapes intestinal immune responses during health and disease. *Nature Reviews Immunology* 9: 313-323
- Round JL, Mazmanian SK (2010) Inducible Foxp3(+) regulatory T-cell development by a commensal bacterium of the intestinal microbiota. *Proceedings of the National Academy of Sciences of the United States of America* 107: 12204-12209

Ruscher K, Isaev N, Trendelenburg G, Weih M, Iurato L, Meisel A, Dirnagl U (1998) Induction of hypoxia inducible factor 1 by oxygen glucose deprivation is attenuated by hypoxic preconditioning in rat cultured neurons. *Neuroscience Letters* 254: 117-120

Sampson TR, Debelius JW, Thron T, Janssen S, Shastri GG, Ilhan ZE, Challis C, Schretter CE, Rocha S, Gradinaru V, Chesselet MF, Keshavarzian A, Shannon KM, Krajmalnik-Brown R, Wittung-Stafshede P, Knight R, Mazmanian SK (2016) Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson's Disease. *Cell* 167: 1469-+

Sanchez HN, Gan HQ, Moroney JB, Daw CC, Taylor J, Zan H, Casali P (2018) B cell-intrinsic epigenetic modulation of local and systemic antibody response by gut microbiota through catabolic short-chain fatty acids. *Journal of Immunology* 200

Sandvig I, Augestad IL, Haberg AK, Sandvig A (2018) Neuroplasticity in stroke recovery. The role of microglia in engaging and modifying synapses and networks. *European Journal of Neuroscience* 47: 1414-1428

Sano T, Huang W, Hall JA, Yang Y, Chen A, Gavzy SJ, Lee J-Y, Ziel JW, Miraldi ER, Domingos AI, Bonneau R, Littman DR (2015) An IL-23R/IL-22 Circuit Regulates Epithelial Serum Amyloid A to Promote Local Effector Th17 Responses. *Cell* 163: 381-393

Santa-Cecilia FV, Leite CA, Del-Bel E, Raisman-Vozari R (2019) The Neuroprotective Effect of Doxycycline on Neurodegenerative Diseases. *Neurotoxicity Research* 35: 981-986

Schabitz WR, Schwab S, Spranger M, Hacke W (1997) Intraventricular brain-derived neurotrophic factor reduces infarct size after focal cerebral ischemia in rats. *Journal of Cerebral Blood Flow and Metabolism* 17: 500-506

Schafer DP, Lehrman EK, Kautzman AG, Koyama R, Mardinly AR, Yamasaki R, Ransohoff RM, Greenberg ME, Barres BA, Stevens B (2012) Microglia Sculpt Postnatal Neural Circuits in an Activity and Complement-Dependent Manner. *Neuron* 74: 691-705

Scheidtmann K, Fries W, Muller F, Koenig E (2001) Effect of levodopa in combination with physiotherapy on functional motor recovery after stroke: a prospective, randomised, double-blind study. *Lancet* 358: 787-790

Schetters STT, Gomez-Nicola D, Garcia-Vallejo JJ, Van Kooyk Y (2018) Neuroinflammation: Microglia and T cells Get Ready to Tango. *Frontiers in Immunology* 8

Schuhmann MK, Kraft P, Stoll G, Lorenz K, Meuth SG, Wiendl H, Nieswandt B, Sparwasser T, Beyersdorf N, Kerkau T, Kleinschnitz C (2015) CD28 superagonist-mediated boost of regulatory T cells increases thromboinflammation and ischemic neurodegeneration during the acute phase of experimental stroke. *Journal of Cerebral Blood Flow and Metabolism* 35: 6-10

Schuhmann MK, Langhauser F, Kraft P, Kleinschnitz C (2017) B cells do not have a major pathophysiologic role in acute ischemic stroke in mice. *Journal of Neuroinflammation* 14

Seifert HA, Hall AA, Chapman CB, Collier LA, Willing AE, Pennypacker KR (2012) A Transient Decrease in Spleen Size Following Stroke Corresponds to Splenocyte Release into Systemic Circulation. *Journal of Neuroimmune Pharmacology* 7: 1017-1024

Seifert HA, Vandenbark AA, Offner H (2018) Regulatory B cells in experimental stroke. *Immunology* 154: 169-177

Sender R, Fuchs S, Milo R (2016) Revised Estimates for the Number of Human and Bacteria Cells in the Body. *Plos Biology* 14

Sharma S, Taliyan R, Singh S (2015) Beneficial effects of sodium butyrate in 6-OHDA induced neurotoxicity and behavioral abnormalities: Modulation of histone deacetylase activity. *Behavioural Brain Research* 291: 306-314

Shichita T, Sugiyama Y, Ooboshi H, Sugimori H, Nakagawa R, Takada I, Iwaki T, Okada Y, Iida M, Cua DJ, Iwakura Y, Yoshimura A (2009) Pivotal role of cerebral interleukin-17-producing gamma delta T cells in the delayed phase of ischemic brain injury. *Nature Medicine* 15: 946-U150

Shimada A, Mason CA, Morrison ME (1998) TrkB signaling modulates spine density and morphology independent of dendrite structure in cultured neonatal Purkinje cells. *Journal of Neuroscience* 18: 8559-8570

Singh V, Roth S, Llovera G, Sadler R, Garzetti D, Stecher B, Dichgans M, Liesz A (2016) Microbiota Dysbiosis Controls the Neuroinflammatory Response after Stroke. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 36: 7428-40

Siopi E, Saha S, Moigneu C, Bigot M, Lledo P-M (2019) Gut microbiota requires vagus nerve integrity to promote depression. *bioRxiv*: 547778

Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly-Y M, Glickman JN, Garrett WS (2013) The Microbial Metabolites, Short-Chain Fatty Acids, Regulate Colonic T-reg Cell Homeostasis. *Science* 341: 569-573

- Spychala MS, Venna VR, Jandzinski M, Doran SJ, Durgan DJ, Ganesh BP, Ajami NJ, Putluri N, Graf J, Bryan RM, McCullough LD (2018) Age-related changes in the gut microbiota influence systemic inflammation and stroke outcome. *Annals of Neurology* 84: 23-36
- Steinberg GK, Kondziolka D, Wechsler LR, Lunsford LD, Coburn ML, Billigen JB, Kim AS, Johnson JN, Bates D, King B, Case C, McGrogan M, Yankee EW, Schwartz NE (2016) Clinical Outcomes of Transplanted Modified Bone Marrow-Derived Mesenchymal Stem Cells in Stroke A Phase 1/2a Study. *Stroke* 47: 1817-1824
- Stevens B, Allen NJ, Vazquez LE, Howell GR, Christopherson KS, Nouri N, Micheva KD, Mehalow AK, Huberman AD, Stafford B, Sher A, Litke AM, Lambris JD, Smith SJ, John SWM, Barres BA (2007) The classical complement cascade mediates CNS synapse elimination. *Cell* 131: 1164-1178
- Stockinger B, Di Meglio P, Gialitakis M, Duarte JH (2014) The Aryl Hydrocarbon Receptor: Multitasking in the Immune System. *Annual Review of Immunology, Vol 32* 32: 403-432
- Strandwitz P (2018) Neurotransmitter modulation by the gut microbiota. *Brain Research* 1693: 128-133
- Stubbe T, Ebner F, Richter D, Engel OR, Klehmet J, Royl G, Meisel A, Nitsch R, Meisel C, Brandt C (2013) Regulatory T cells accumulate and proliferate in the ischemic hemisphere for up to 30 days after MCAO. *Journal of Cerebral Blood Flow and Metabolism* 33: 37-47
- Swidsinski A, Loening-Baucke V, Krüger M, Kirsch S (2012) Central Nervous System and the Colonic Bioreactor: Analysis of Colonic Microbiota in Patients with Stroke Unravels Unknown Mechanisms of the Host Defense after Brain Injury. In
- Szalay G, Martinecz B, Lenart N, Kornyei Z, Orsolits B, Judak L, Csaszar E, Fekete R, West BL, Katona G, Rozsa B, Denes A (2016) Microglia protect against brain injury and their selective elimination dysregulates neuronal network activity after stroke. *Nature Communications* 7
- Tan J, McKenzie C, Vuillermin PJ, Goverse G, Vinuesa CG, Mebius RE, Macia L, Mackay CR (2016) Dietary Fiber and Bacterial SCFA Enhance Oral Tolerance and Protect against Food Allergy through Diverse Cellular Pathways. *Cell Reports* 15: 2809-2824
- Tarsa L, Goda Y (2002) Synaptophysin regulates activity-dependent synapse formation in cultured hippocampal neurons. *Proceedings of the National Academy of Sciences of the United States of America* 99: 1012-1016
- Thayer JF, Sternberg EM (2009) Neural concomitants of immunity-Focus on the vagus nerve. *Neuroimage* 47: 908-910
- Tremlett H, Fadrosch DW, Faruqi AA, Hart J, Roalstad S, Graves J, Spencer CM, Lynch SV, Zamvil SS, Waubant E, Ctr USNPM (2016) Associations between the gut microbiota and host immune markers in pediatric multiple sclerosis and controls. *Bmc Neurology* 16
- Tsai AS, Berry K, Beneyto MM, Gaudilliere D, Ganio EA, Culos A, Ghaemi MS, Choisy B, Djebali K, Einhaus JF, Bertrand B, Tanada A, Stanley N, Fallahzadeh R, Baca Q, Quach LN, Osborn E, Drag L, Lansberg MG, Angst MS et al. (2019) A year-long immune profile of the systemic response in acute stroke survivors. *Brain* 142: 978-991
- Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI (2007) The Human Microbiome Project. *Nature* 449: 804-810
- Turrigiano GG, Nelson SB (2004) Homeostatic plasticity in the developing nervous system. *Nature Reviews Neuroscience* 5: 97-107
- van der Zijden JP, Bouts M, Roeling TAP, Bleys R, van der Toorn A, Dijkhuizen RM (2008) Manganese-enhanced MRI of brain plasticity in relation to functional recovery after experimental stroke. *Journal of Cerebral Blood Flow and Metabolism* 28: 832-840
- Velazquez EM, Nguyen H, Heasley KT, Saechao CH, Gil LM, Rogers AWL, Miller BM, Rolston MR, Lopez CA, Litvak Y, Liou MJ, Faber F, Bronner DN, Tiffany CR, Byndloss MX, Byndloss AJ, Bäumlér AJ (2019) Endogenous Enterobacteriaceae underlie variation in susceptibility to Salmonella infection. *Nature Microbiology*
- Viale L, Catoira NP, Di Girolamo G, Gonzalez CD (2018) Pharmacotherapy and motor recovery after stroke. *Expert Review of Neurotherapeutics* 18: 65-82
- Waldecker M, Kautenburger T, Daumann H, Busch C, Schrenk D (2008) Inhibition of histone-deacetylase activity by short-chain fatty acids and some polyphenol metabolites formed in the colon. *Journal of Nutritional Biochemistry* 19: 587-593
- Wang H, Yu M, Ochani M, Amella CA, Tanovic M, Susarla S, Li JH, Wang HC, Yang H, Ulloa L, Al-Abed Y, Czura CJ, Tracey KJ (2003) Nicotinic acetylcholine receptor alpha 7 subunit is an essential regulator of inflammation. *Nature* 421: 384-388
- Wang J, Xie L, Yang C, Ren C, Zhou K, Wang B, Zhang Z, Wang Y, Jin K, Yang G-Y (2015) Activated regulatory T cell regulates neural stem cell proliferation in the subventricular zone of normal and ischemic mouse brain through interleukin 10. *Frontiers in Cellular Neuroscience* 9

- Wang L, Christophersen CT, Sorich MJ, Gerber JP, Angley MT, Conlon MA (2012) Elevated Fecal Short Chain Fatty Acid and Ammonia Concentrations in Children with Autism Spectrum Disorder. *Digestive Diseases and Sciences* 57: 2096-2102
- Wang S, Huang MQ, You X, Zhao JY, Chen LL, Wang L, Luo YJ, Chen Y (2018) Gut microbiota mediates the anti-obesity effect of calorie restriction in mice. *Scientific Reports* 8
- Wang SW, Zhang H, Xu Y (2016) Crosstalk between microglia and T cells contributes to brain damage and recovery after ischemic stroke. *Neurological Research* 38: 495-503
- Wikoff WR, Anfora AT, Liu J, Schultz PG, Lesley SA, Peters EC, Siuzdak G (2009) Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. *Proceedings of the National Academy of Sciences of the United States of America* 106: 3698-3703
- 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 (2016) Depletion of Cultivable Gut Microbiota by Broad-Spectrum Antibiotic Pretreatment Worsens Outcome After Murine Stroke. *Stroke* 47: 1354-1363
- Wolf SL, Winstein CJ, Miller JP, Taub E, Uswatte G, Morris D, Giuliani C, Light KE, Nichols-Larsen D, Investigators E (2006) Effect of constraint-induced movement therapy on upper extremity function 3 to 9 months after stroke - The EXCITE randomized clinical trial. *Jama-Journal of the American Medical Association* 296: 2095-2104
- Wu RQ, Zhang DF, Tu E, Chen QM, Chen WJ (2014) The mucosal immune system in the oral cavity-an orchestra of T cell diversity. *International Journal of Oral Science* 6: 125-132
- Wu YW, Dissing-Olesen L, MacVicar BA, Stevens B (2015) Microglia: Dynamic Mediators of Synapse Development and Plasticity. *Trends in Immunology* 36: 605-613
- Xia G-H, You C, Gao X-X, Zeng X-L, Zhu J-J, Xu K-Y, Tan C-H, Xu R-T, Wu Q-H, Zhou H-W, He Y, Yin J (2019) Stroke Dysbiosis Index (SDI) in Gut Microbiome Are Associated With Brain Injury and Prognosis of Stroke. *Frontiers in Neurology* 10
- Xie L, Li W, Hersh J, Liu R, Yang S-H Experimental ischemic stroke induces long-term T cell activation in the brain. *Journal of Cerebral Blood Flow & Metabolism* 0: 0271678X18792372
- Yano JM, Yu K, Donaldson GP, Shastri GG, Ann P, Ma L, Nagler CR, Ismagilov RF, Mazmanian SK, Hsiao EY (2015) Indigenous Bacteria from the Gut Microbiota Regulate Host Serotonin Biosynthesis. *Cell* 161: 264-276
- Yin J, Liao SX, He Y, Wang S, Xia GH, Liu FT, Zhu JJ, You C, Chen Q, Zhou L, Pan SY, Zhou HW (2015) Dysbiosis of Gut Microbiota With Reduced Trimethylamine-N-Oxide Level in Patients With Large-Artery Atherosclerotic Stroke or Transient Ischemic Attack. *Journal of the American Heart Association* 4
- Yoshii A, Constantine-Paton M (2010) Postsynaptic BDNF-TrkB Signaling in Synapse Maturation, Plasticity, and Disease. *Developmental Neurobiology* 70: 304-322
- Zausinger S, Hungerhuber E, Baethmann A, Reulen HJ, Schmid-Elsaesser R (2000) Neurological impairment in rats after transient middle cerebral artery occlusion: a comparative study under various treatment paradigms. *Brain Research* 863: 94-105
- Zeiler SR, Gibson EM, Hoesch RE, Li MY, Worley PF, O'Brien RJ, Krakauer JW (2013) Medial Premotor Cortex Shows a Reduction in Inhibitory Markers and Mediates Recovery in a Mouse Model of Focal Stroke. *Stroke* 44: 483-489
- Zelante T, Iannitti RG, Cunha C, De Luca A, Giovannini G, Pieraccini G, Zecchi R, D'Angelo C, Massi-Benedetti C, Fallarino F, Carvalho A, Puccetti P, Romani L (2013) Tryptophan Catabolites from Microbiota Engage Aryl Hydrocarbon Receptor and Balance Mucosal Reactivity via Interleukin-22. *Immunity* 39: 372-385
- Zhang JF, Malik A, Choi HB, Ko RWY, Dissing-Olesen L, MacVicar BA (2014) Microglial CR3 Activation Triggers Long-Term Synaptic Depression in the Hippocampus via NADPH Oxidase. *Neuron* 82: 195-207
- Zhao Q, Elson CO (2018) Adaptive immune education by gut microbiota antigens. *Immunology* 154: 28-37
- Zheng P, Zeng BH, Liu ML, Chen JJ, Pan JX, Han Y, Liu YY, Cheng K, Zhou CJ, Wang HY, Zhou XY, Gui SW, Perry SW, Wong ML, Licinio J, Wei H, Xie P (2019) The gut microbiome from patients with schizophrenia modulates the glutamate-glutamine-GABA cycle and schizophrenia-relevant behaviors in mice. *Science Advances* 5
- Zhou H, Liang H, Li ZF, Xiang H, Liu W, Li JG (2013) VAGUS NERVE STIMULATION ATTENUATES INTESTINAL EPITHELIAL TIGHT JUNCTIONS DISRUPTION IN ENDOTOXEMIC MICE THROUGH alpha 7 NICOTINIC ACETYLCHOLINE RECEPTORS. *Shock* 40: 144-151

Rebecca Sadler

Personal information

Nationality British

Education

- 2015 – 2019 **Institute for Stroke and Dementia Research**, *Graduate School of Systemic Neurosciences*, München, Germany.
PhD in Neuroscience
- 2014 – 2015 **Ludwig-Maximilians-Universität**, München, Germany.
Master of Biology
- 2010 – 2014 **University of Manchester**, England.
Bachelor of Science with Honours in Neuroscience with Professional Experience
- 2005 – 2009 **Northallerton College**, North Yorkshire, England.
Secondary School and Sixth Form
- 2002 – 2005 **Allertonshire School**, North Yorkshire, England.
Secondary School

Professional & Research Experience

- Oct 2015 – Sept 2019 **Institute of Stroke and Dementia Research (Prof. M. Dichgans), Ludwig-Maximilians-Universität**, München, Germany.
Doctorate Thesis in PD Dr. Arthur Liesz's group "The role of gut microbiota-derived metabolites in stroke outcome."
Activities: Germ-free mouse handling, fecal microbiota transfer, advanced flow cytometry (8-colour FACS), immunohistology, RNA extraction, RT-PCR, experimental stroke mouse models and behavioural testing.
- Apr 2015 – Sep 2015 **Institute of Stroke and Dementia Research, Ludwig-Maximilians-Universität**, München, Germany.
Master's Thesis in PD Dr. Arthur Liesz's group "T-cell priming in by environmental mediators after acute brain injury"
Activities: Advanced flow cytometry (8-colour FACS), immunohistology, RT-PCR, Stroke mouse models and behavioural testing.
- Oct 2014 – Apr 2015 **Institute of Stroke and Dementia Research, Ludwig-Maximilians-Universität**, München, Germany.
Hilfswissenschaftler for PD Dr. Arthur Liesz's group.
Activities: Cryosectioning, manuscript language editing, immune-histology, RT-PCR.
- Nov 2014 – Feb 2015 **The Department of Cell and Developmental Biology (Prof. B. Conradt), Ludwig-Maximilians-Universität**, München, Germany.
Research Course on Mitochondrial Dynamics in Professor Barbara Conradt's group.
Activities: Using *C.Elegans* to investigate the interaction of peroxisome genes and the mitochondria unfolded protein response.

- Feb 2014 – Mar 2014 **Faculty of Life Sciences (Prof. M. Humphries), University of Manchester, England.**
 Bachelor Thesis: “Effects of Th-1 mediated trichuris muris infection on AD-related pathology and neuroinflammation in a transgenic mouse model of Alzheimer’s disease.”
 Activities: Pathological and immunofluorescence analysis of hippocampus, amygdala and cortex amyloid and microglial pathology, in triple transgenic mice and WT mice, infected and uninfected.
- Jul 2013 – Aug 2013 **Institute of Physiology, (Prof. M. Götz), Ludwig-Maximilians-Universität, München, Germany.**
 Summer internship in Professor Stephan Kröger’s group with grant awarded by The Pathological Society of Great Britain & Ireland.
 Activities: Analysis of the Dystroglycan and Dystrophin localisation in muscle spindles of Duchenne Muscular Dystrophy mouse models (mdx) and wt mice.
- Aug 2012 – Jun 2013 **Department of Medical Biochemistry and Biophysics, (Prof. Ernest Arenas), Karolinska Institutet, Stockholm, Sweden.**
 Year in professional experience in Professor Patrik Ernfors and Ulrika Marklund’s group.
 Activities: Analysis of the enteric nervous system in mouse aged E16.5-E18.5 in mutants for proneural gene Mash1, to find genes which cause sub-specification of the Calbindin, TH, NOS and VIP neuronal phenotypes. This research was based on a previously performed microarray.

Awards, Prizes, Memberships & Fellowships

- 2019 SyNergy Travel Grant
- 2019 Neurowind e.V. Travel Grant
- 2019 Society for Neuroscience - Student membership
- 2019 British Neuroscience Association - Student membership
- 2017 Early Career Investigator Travel Bursary for the BRAIN and BRAIN PET meeting
- 2013 Undergraduate Bursary, *The Pathological Society of Great Britain & Ireland*
- 2012 Shadow-student stipend, *Karolinska Institutet*

Scientific Reviewer

Journal of Neuroinflammation
 Circulation Research
 Frontiers Neurology
 Stroke

Publications

- 1 Singh V, **Sadler R**, Heindl S, Llovera G, Roth S, Benakis C, Liesz A. *The gut microbiome primes a cerebroprotective immune response after stroke.* **J Cereb Blood Flow Metab.** 2018 Aug;38(8):1293-1298
- 2 Memic F, Knoflach V, Morarach K, **Sadler R**, Laranjeira C, Hjerling-Leffler J, Sundström E, Pachnis V, Marklund U. *Transcription and Signaling Regulators in Developing Neuronal Subtypes of Mouse and Human Enteric Nervous System.* **Gastroenterology.** 2018 Feb;154(3):624-636.
- 3 **Sadler R**, Singh V, Benakis C, Garzetti D, Brea D, Stecher B, Anrather J, Liesz A. *Microbiota differences between commercial breeders impacts the post-stroke immune response.* **Brain Behav Immun.** 2017 Nov;66:23-30

- 4 Friedländer F, Bohmann F, Brunkhorst M, Chae JH, Devraj K, Köhler Y, Kraft P, Kuhn H, Lucaciu A, Luger S, Pfeilschifter W, **Sadler R**, Liesz A, Scholtyschik K, Stolz L, Vutukuri R, Brunkhorst R. *Reliability of infarct volumetry: Its relevance and the improvement by a software-assisted approach.* **J Cereb Blood Flow Metab.** 2017 Aug;37(8):3015-3026.
- 5 Singh V, Roth S, Llovera G, **Sadler R**, Garzetti D, Stecher B, Dichgans M, Liesz A. *Microbiota Dysbiosis Controls the Neuroinflammatory Response after Stroke.* **J Neurosci.** 2016 Jul 13;36(28):7428-40.
- 6 Memic F, Knoflach V, **Sadler R**, Tegerstedt G, Sundström E, Guillemot F, Pachnis V, Marklund U. *Ascl1 Is Required for the Development of Specific Neuronal Subtypes in the Enteric Nervous System.* **J Neurosci.** 2016 Apr 13;36(15):4339-50.

Abstracts

- 2019 **R. Sadler**, J. Cramer, S. Heindl, D.M. Betz, M. Giera, A.M. Stowe, and A. Liesz. Abstract: *The role of short-chain fatty acids in post-stroke regeneration.* Neuroscience 2019, Chicago, U.S.A, **Poster**
- 2018 **R. Sadler**, J. Cramer, C. Benakis, S. Roth, S. Kostid, A. Becker, M. Giera, A. Stowe and A. Liesz. Abstract: *The role of short-chain fatty acids in post stroke regeneration.* Neuroprotection and Neurorepair, Dresden, Germany, **Oral presentation**
- 2017 **R. Sadler**, V. Singh, C. Benakis, D. Brea-Lopez, T. Hünig, J. Anrather, B. Stecher and A. Liesz. Abstract: *Minimal microbiota differences between commercial breeders impacts post-stroke immunity.* BRAIN Meeting, Berlin, Germany, **Poster**
- 2016 **R. Sadler**, V. Singh, C. Benakis, D. Brea-Lopez, T. Hünig, J. Anrather, B. Stecher and A. Liesz. Abstract: *Minimal microbiota differences between commercial breeders impacts post-stroke immunity.* 8th Neurowind Meeting, Motzen, Germany, **Oral presentation**
- 2016 **R. Sadler**, V. Singh, C. Benakis, D. Brea-Lopez, T. Hünig, J. Anrather, B. Stecher and A. Liesz. Abstract: *Minimal microbiota differences between commercial breeders impacts post-stroke immunity.* 16th Eibsee Meeting on cellular mechanisms of neurodegeneration, Grainau, Germany, **Flash talk and poster**
- 2016 **R. Sadler**, V. Singh, C. Benakis, D. Brea-Lopez, T. Hünig, J. Anrather, B. Stecher and A. Liesz. Abstract: *Minimal microbiota differences between commercial breeders impacts post-stroke immunity.* Neuroprotection and Neurorepair, Leipzig, Germany, **Oral presentation**

LIST OF PUBLICATIONS

1. The gut microbiome primes a cerebroprotective immune response after stroke.

Singh V, Sadler R, Heindl S, Llovera G, Roth S, Benakis C, Liesz A.

J Cereb Blood Flow Metab. 2018 Aug;38(8):1293-1298. doi: 10.1177/0271678X18780130. Epub 2018 May 30.

2. Transcription and Signaling Regulators in Developing Neuronal Subtypes of Mouse and Human Enteric Nervous System.

Memic F, Knoflach V, Morarach K, Sadler R, Laranjeira C, Hjerling-Leffler J, Sundström E, Pachnis V, Marklund U.

Gastroenterology. 2018 Feb;154(3):624-636. doi: 10.1053/j.gastro.2017.10.005. Epub 2017 Oct 12.

3. Microbiota differences between commercial breeders impacts the post-stroke immune response.

Sadler R, Singh V, Benakis C, Garzetti D, Brea D, Stecher B, Anrather J, Liesz A.

Brain Behav Immun. 2017 Nov;66:23-30. doi: 10.1016/j.bbi.2017.03.011. Epub 2017 Mar 24.

4. Reliability of infarct volumetry: Its relevance and the improvement by a software-assisted approach.

Friedländer F, Bohmann F, Brunkhorst M, Chae JH, Devraj K, Köhler Y, Kraft P, Kuhn H, Lucaciu A, Luger S, Pfeilschifter W, Sadler R, Liesz A, Scholtyschik K, Stolz L, Vutukuri R, Brunkhorst R.

J Cereb Blood Flow Metab. 2017 Aug;37(8):3015-3026. doi: 10.1177/0271678X16681311. Epub 2016 Jan 1.

5. Microbiota Dysbiosis Controls the Neuroinflammatory Response after Stroke.

Singh V, Roth S, Llovera G, Sadler R, Garzetti D, Stecher B, Dichgans M, Liesz A.

J Neurosci. 2016 Jul 13;36(28):7428-40. doi: 10.1523/JNEUROSCI.1114-16.2016.

6. Ascl1 Is Required for the Development of Specific Neuronal Subtypes in the Enteric Nervous System.

Memic F, Knoflach V, Sadler R, Tegerstedt G, Sundström E, Guillemot F, Pachnis V, Marklund U.

J Neurosci. 2016 Apr 13;36(15):4339-50. doi: 10.1523/JNEUROSCI.0202-16.2016.

AFFIDAVIT

Eidesstattliche Versicherung/Affidavit

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation **“Microbiota, metabolomics and stroke”** selbstständig angefertigt habe, mich außer der angegebenen keener weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

I hereby confirm that the dissertation **“THE GUT MICROBIOME MODULATES POST STROKE OUTCOME”** is the result of my own work and that I have only used sources or materials listed and specified in the dissertation.

München, den
Munich, 06.06.2019

Rebecca Katie Sadler

DECLARATION OF AUTHOR CONTRIBUTIONS

Microbiota Dysbiosis Controls the Neuroinflammatory Response after Stroke.

Singh V, Roth S, Llovera G, Sadler R, Garzetti D, Stecher B, Dichgans M, Liesz A.

J Neurosci. 2016 Jul 13;36(28):7428-40. doi: 10.1523/JNEUROSCI.1114-16.2016.

Author contributions: A.L. designed research; V.S., S.R., G.L., R.S., and A.L. performed research; V.S., S.R., D.G., B.S., and A.L. analysed data; B.S., M.D., and A.L. wrote the paper. V.S. and S.R. contributed equally to this work.

The gut microbiome primes a cerebroprotective immune response after stroke.

Singh V, Sadler R, Heindl S, Llovera G, Roth S, Benakis C, Liesz A. J Cereb Blood Flow Metab. 2018 Aug;38(8):1293-1298. doi: 10.1177/0271678X18780130. Epub 2018 May 30.

Author contributions: VS, RS, SH, GL, SR, CB performed experiments; VS, RS, CB and AL analyzed data, VS and AL wrote the manuscript; AL conceived the study and supervised the project.

Microbiota differences between commercial breeders impacts the post-stroke immune response.

Sadler R, Singh V, Benakis C, Garzetti D, Brea D, Stecher B, Anrather J, Liesz A.

Brain Behav Immun. 2017 Nov;66:23-30. doi: 10.1016/j.bbi.2017.03.011. Epub 2017 Mar 24.

Author contributions: RS, VS, CB and DB performed experiments; RS, VS, CB, DG, DB and AL analyzed data, RS and AL wrote the manuscript; BS and JA critically revised the manuscript; AL conceived the study and supervised the project.

Short-chain fatty acids improve post-stroke recovery via immunological mechanisms.

Author contributions: RS, JC, SR, SH, SK, DB, MG and EP performed experiments; RS, JC, SK, BN and AL analyzed data, RS and AL wrote the manuscript; MG, AS and LH critically revised the manuscript AL conceived the study and supervised the project.

The first supervisor hereby confirms the listed contributions of Rebecca Sadler to the publications included in this thesis.

Date: 06.06.2019

PD Dr. Arthur Liesz

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