

STROKE AND MICROBIOTA – CURRENT RESEARCH AND CONCEPTS

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SUMMARY

Stroke is a highly prevalent cerebrovascular disease with limited treatment options. Although therapeutic interventions have evolved over the last 30 years, stroke remains the second most common cause of death and the leading cause for long-term disability worldwide. While primarily a disease caused by acute ischaemia to the brain, stroke pathology is characterized by a strong immune component. The intestinal microbiota and gut immune system have recently been linked to the inflammatory processes observed after stroke. It has been shown that intestinal microbiota composition strongly affects the polarization state of immune cells found in Payer's patches and submucosa of the small intestine. This may result in an imbalance of regulatory and effector type T-cells in the gut. Because gut immune cells traffic to the brain after stroke they influence the local immune response triggered by ischaemic injury by damping or increasing inflammation. In the context of stroke, the communication between brain and gut is bidirectional and stroke can impact the composition of intestinal microbiota. In addition, intestinal microbiota can become a source of opportunistic infectious agents and contribute to post-stroke infections thereby increasing morbidity and mortality. In this review I will summarize current concepts on the role of intestinal microbiota and the intestinal immune system in stroke, review the effects stroke and ageing has on the gut microbiome, and describe studies that identified commensal intestinal microbiota as a source of infectious bacteria participating in post-stroke infections of the respiratory tract.

INTRODUCTION

Ischaemic stroke is a highly prevalent and devastating disease with limited therapeutic options (*Henninger et al., 2010*). Most therapeutic efforts have focused on counteracting the intrinsic brain mechanisms leading to ischaemic injury (*Suwanwela and Koroshetz, 2007*). Unfortunately, these efforts have met with limited success in large clinical trials, reflecting, in part, an incomplete understanding of the pathological processes triggered by cerebral ischaemia (*Moskowitz et al., 2010*). In-

flammation is a key component in the pathophysiology of ischaemic stroke (*Iadecola and Anrather, 2011*). Numerous experimental approaches have concentrated on developing the therapeutic potential of immunomodulation (*Macrez et al., 2011*). However, our understanding of the interaction between resident brain cells and peripheral immune cells infiltrating the post-ischaemic brain, and their role in tissue damage and repair is still incomplete (*Macrez et al., 2011*). The peripheral

immune system plays an essential role in the pathophysiology of stroke, involving both innate and adaptive immune cells (*Iadecola and Anrather, 2011*). In turn, neuro-humoral brain signals generated during stroke induce immunosuppression, which contributes to post-stroke morbidity by increasing the risk for infection in stroke patients (*Meisel et al., 2005*). The immune system is forged by the continuous interaction with commensal microbiota that populate the epithelial surfaces and this interaction is essential for immune cell development, maintenance and function (*Mazmanian et al., 2005; Hill and Artis, 2010*). Specifically, intestinal commensal bacteria, the most abundant

symbiotic compartment in the body, have emerged as a potent regulator of the immune system (*Nishio and Honda, 2012*). In particular, the microbiota exerts powerful effects on lymphocyte populations, such as regulatory T-cells (Treg) and $\gamma\delta$ T-cells, which are involved in the mechanisms of cerebral ischaemic injury (*Iadecola and Anrather, 2011*). Conversely, stroke also affects the peripheral immune system and alters the composition of the intestinal microbiome. This sets the stage for complex multidirectional interactions within the microbiota-gut-brain axis that is likely to affect stroke outcome.

EFFECTS OF MICROBIOTA ON IMMUNE CELLS INVOLVED IN STROKE PATHOPHYSIOLOGY

Accumulating evidence suggests that commensal microbes influence the outcome of diseases in which the immune system is involved. For example, germ-free (GF) animals are protected from experimental autoimmune encephalomyelitis (*Ochoa-Repáraz et al., 2009; Berer et al., 2011; Lee et al., 2011*), ankylosing spondylitis (*Sinkorová et al., 2008*), or rheumatoid arthritis (*Wu et al., 2010; Chappert et al., 2013*). Increasing evidence implicates systemic immunity in the pathophysiology of ischaemic brain injury (*Iadecola and Anrather, 2011*). Thus, cells associated with innate and adaptive immunity play a defining role in the outcome of cerebral ischaemia (*Iadecola and Anrather, 2011*). As reviewed below, intestinal microbes are critical for the development, expansion and activity of immune cells implicated in stroke pathophysiology.

Lymphocytes

Th1 and IL-17 secreting T-cells are detrimental in the early phase of cere-

bral ischaemia and lymphocyte-deficient mice are protected in models of focal ischaemia (*Hurn et al., 2007; Kleinschnitz et al., 2010*). The mechanism of T-cell mediated brain injury after ischaemia does not involve classical antigen-mediated T-cell activation and the cytotoxic activity might be related to innate T-cell functions (*Kleinschnitz et al., 2010*). Accordingly, IL-17 secreting $\gamma\delta$ T-cells, that do not undergo classical antigen-dependent T-cell activation, have been shown to contribute to ischaemic injury (*Shichita et al., 2009; Gelderblom et al., 2012*). Peripheral development of T-cells is greatly affected by microbiota (*Kuhn and Stappenbeck, 2013*). Beside Th17 cells (*Ivanov et al., 2006*), intestinal $\gamma\delta$ T-cells residing in the epithelial layer and submucosa (lamina propria) are also a major source for IL 17 (*Roark et al., 2008; Korn and Petermann, 2012; Hou et al., 2013*). IL-17-producing $\gamma\delta$ T-cells are responsible for the colitis seen in Treg deficient mice and the disease is abrogated in $\gamma\delta$ T-

cell-deficient or antibiotic-treated mice, suggesting activation of $\gamma\delta$ T-cells by commensal bacteria (Park et al., 2010). Moreover, intestinal IL-17-producing $\gamma\delta$ T-cells are reduced in antibiotic-treated mice (Duan et al., 2010; Park et al., 2010). While effector T-lymphocytes may contribute to focal ischaemic injury (Yilmaz et al., 2006; Hurn et al., 2007), Treg can have a protective effect by down-regulating post-ischaemic inflammation. Treg appear in the ischaemic tissue after the acute phase and confer neuroprotection by IL-10 secretion, an effect that might be antigen independent (Liesz et al., 2009, 2013; Planas and Chamorro, 2009; Stubbe et al., 2013). Intestinal Treg express high levels of IL-10, which is indispensable for maintaining intestinal homeostasis by suppressing Th17 differentiation (Chaudhry et al., 2009; Huber et al., 2011), $\gamma\delta$ T-cell proliferation (Park et al., 2010), and myeloid cell activation (Takeda et al., 1999). The gut microbiota has a role also in the development of intestinal and extra-intestinal Treg. For example, colonic FoxP3⁺ Treg cells are reduced in GF mice (Round and Mazmanian, 2010; Atarashi et al., 2013). Furthermore, in a mouse model of EAE, the generation of Treg from splenic and lymph node lymphocytes was increased in GF animals (Lee et al., 2011). Therefore, mucosa-microbial interactions influence Treg lymphocytes in the gut as in lymphoid organs.

Myeloid cells

In experimental stroke, as in human stroke, monocytes/macrophages infil-

trate the brain early after ischaemia and persist for several weeks (Schilling et al., 2003; Gelderblom et al., 2009). Inhibition of post-ischaemic monocyte recruitment by interfering with CCR2 and CX3CR1 chemokine receptor utilization is beneficial in experimental stroke (Hughes et al., 2002; Soriano et al., 2002; Denes et al., 2008; Schilling et al., 2009). Monocyte/macrophage development is influenced by signals from intestinal bacteria. Systemic monocyte counts are normal in GF mice, but they are functionally impaired and do not mount an IFN γ response after bacterial stimulation (Ganal et al., 2012). Dendritic cells (DC) have been implicated in stroke pathology (Felger et al., 2010; Gelderblom et al., 2017). Intestinal DC and macrophages are essential to maintain a tolerogenic environment in the gut and are strong inducers of Treg (Coombes et al., 2007; Sun et al., 2007). Neutrophils play a part in post-ischaemic inflammation by limiting tissue perfusion due to intravascular clogging (del Zoppo et al., 1991; Dawson et al., 1996), destabilizing the BBB by releasing MMP9 (Rosell et al., 2008; Ludewig et al., 2013), and by generating ROS and NO (Garcia-Bonilla et al., 2014). Neutrophils isolated from the bone marrow of GF or antibiotic-treated mice exhibit impaired *ex vivo* bacterial killing (Clarke et al., 2010). All together, there is ample evidence that the intestinal environment shapes the activation state of immune cells involved in the immune response to ischaemic brain injury.

ALTERED INTESTINAL IMMUNE STATUS AND ITS EFFECTS ON ISCHAEMIC STROKE

The role of altered intestinal microbial composition on stroke outcome has been investigated in mice undergoing

transient focal ischaemia by intraluminal obstruction of the middle cerebral artery (MCA) (Benakis et al.,

2016). In this study mice were treated with amoxicillin (amoxicillin/clavulanic acid; AC) to alter the microbiome resulting in elimination of *Clostridia* and *Bacteroidetes* with concomitant expansion of *Proteobacteria*. The authors observed remarkable neuroprotection both on the anatomical and functional level. Because protection was not observed after one week of AC treatment, a time point when microbial alterations were already present, let the authors to hypothesize that changes in the immune system leading to an altered inflammatory response after stroke might be at the basis of the observed neuroprotection. Analysis of the intestinal immune system revealed an increase in Treg and a decrease in IL17⁺ $\gamma\delta$ T-cells, whereas Th17 cells were not affected. In addition, the authors could show that DC isolated from AC treated animals showed higher expression of the “tolerogenic” marker protein CD103 and were more potent in inducing Treg when co-cultured with naïve CD4⁺ T-cells. After MCA occlusion IL17⁺ $\gamma\delta$ T-cells were increased in the meninges of control but not AC-treated mice indicating that the deleterious IL17 response was blocked in mice with altered microbiota. Accordingly, the protective effect of AC treat-

ment was absent in IL17 deficient mice. Similar alterations in T-cell homeostasis resulting in reduced Treg and increased Th1 and Th17 cells were observed after ischaemic stroke in mice and post-stroke immune changes could be prevented by faecal matter transplants from healthy donors (Singh et al., 2016). It was also shown that T-cells trafficked from the intestine to the brain after stroke indicating that the polarization state of intestinal T-cells might determine the course of post-ischaemic inflammation in the brain (Benakis et al., 2016; Singh et al., 2016). A recent study by Singh and co-workers (Singh et al., 2018) compared GF animals with GF animals recolonized with conventional SPF flora in a permanent focal ischaemia model in mice. Interestingly, GF animals showed increased infarct volume and decreased inflammatory response to cerebral ischaemia. Using Rag1^{-/-} mice that lack T and B cells, the authors found that ischaemic lesions in GF Rag1^{-/-} mice were not different from lesions of recolonized Rag^{-/-} mice, albeit the lesions were smaller than in wild-type mice. These studies identified disturbances of the intestinal immune homeostasis as an important contributor to ischaemic brain injury.

EFFECTS OF STROKE ON THE INTESTINAL MICROBIOME

The relationship between microbiota and stroke is complex, given that ischaemic brain injury has been found to alter gut microbiota composition. After stroke, up to 50% of patients experience gastrointestinal symptoms, including dysphagia, gastrointestinal bleeding, and constipation. Stroke is likely to alter the intestinal microbial environment by altering intestinal epithelial permeability, motility, mucus biosynthesis and the immune system. Large

ischaemic infarcts in mice after transient MCA occlusion altered the gut microbiome partially by inducing intestinal paralysis leading to reduced microbial diversity and overgrowth of *Bacteroidetes* species (Singh et al., 2016). Another study found that after stroke, relative abundance of *Peptococcaceae* increased in the mouse caecum, whereas the proportion of *Prevotellaceae* decreased (Houlden et al., 2016). This alteration was paralleled by aug-

mented release of noradrenaline and the reduction of mucoprotein-producing goblet cells. Investigating the mucosa-associated microbiome it was found that microbial communities within the mucosa were significantly different between sham-operated and post-stroke mice at 24 hours following surgery (Stanley et al., 2018). Microbi-

ota composition was substantially different in all sections of the gastrointestinal mucosa. The main changes in mucosal microbiota composition were due to increased abundance of *Akkermansia muciniphila* and clostridial species while operational taxonomic units (OTUs) potentially belonging to the *Barnesiella* genus were reduced.

ROLE OF INTESTINAL MICROBIOTA IN POST-STROKE INFECTIONS

Secondary infections, including pneumonia and urinary tract infections, are major complications in stroke patients. Pneumonia occurring in up to 20% of all stroke patients is the most common serious medical complication in stroke care resulting in a 2.5-fold increased mortality rate (Meisel et al., 2005). Post-stroke immunodepression has been recognized as a key factor in facilitating infections. Immunodepression in stroke patients manifests itself in reduced peripheral blood lymphocyte counts and impaired T- and natural killer (NK)-cell activity (Meisel et al., 2005). Experimental stroke in rodents induced a rapid and extensive apoptotic loss of lymphocytes in lymphoid organs and peripheral blood (Prass et al., 2003). The effects were linked to increased noradrenalin and glucocorticoid secretion through overactivation of the hypothalamic-pituitary-adrenal axis. Moreover, stroke also reduced intestinal lymphocyte counts likely re-

sulting in suppression of the intestinal immune system and possibly facilitating bacterial invasion (Schulte-Herbrüggen et al., 2009). Further it has been shown that the majority of bacteria detected in stroke patients who developed infections were common commensal bacteria that normally reside in the intestinal tract including *Escherichia coli*, *Enterococcus spp.*, and *Morganella morganii* (Stanley et al., 2016). Using the transient MCA occlusion model in mice, the same study found that loss of intestinal epithelial barrier function, which was observed as early as 3 hours after stroke, facilitated the translocation of facultative pathogenic bacteria into the blood stream from where they may have colonized the respiratory tract. Using bioinformatic algorithms it was predicted that the small intestine and liver were the most likely origins of microbial communities present in the lung of post-stroke mice.

STROKE, MICROBIOME AND AGEING

Age has a strong effect on stroke outcome and the composition of the intestinal microbiome. Aged (18-20 month) mice show increased *Firmicutes* and reduced *Bacteroidetes* (Spsychala et al., 2018). The ratio of *Firmicutes* to *Bac-*

teroidetes (F:B) increased 9-fold compared to young (8-12 week) mice. Interestingly, stroke increased the F:B ratio in aged as well young mice. When young mice were transplanted with faecal microbiota harvested from aged

mice, stroke outcome was worse. On the other hand, aged mice transplanted with a “young microbiome” performed better than control mice. It was also found that short chain fatty acids, potential mediators of neuroprotective effects afforded by commensal microbiota, were decreased in faecal matter of aged mice and levels could be recovered after faecal transplantation. Aged mice are also more likely to develop sepsis after cerebral ischaemia than young animals (Crapser et al., 2016).

While high bacterial burden was detected in the mesenteric lymph nodes and spleens of both young and aged mice after stroke, substantial bacterial colonization of liver and lung was found only in aged stroke mice and not in those from young. Increased bacterial dissemination in aged mice was associated with increased gut permeability, lymphopenia, and increased plasma levels of inflammatory markers as compared to young mice.

CONCLUSIONS

Increasing evidence points to a role of intestinal microbiota in stroke pathophysiology and outcome. Recent studies have identified a gut-brain immune axis as an essential component of the inflammatory response to stroke. The microbiota-gut-brain axis is complex and shows a strong bi-directional interaction. Stroke disturbs intestinal motility and epithelial barrier function and alters the composition of intestinal microbiota. Therefore, it is likely that stroke itself induced changes of the mi-

crobial environment contribute to stroke pathology in the subacute and chronic stages of the disease. The current research also evokes many questions. Is the presence of certain microbiota a risk factor for stroke? Could one predict stroke outcome based on gut microbial composition? Besides the immune axis, are there humoral or neural pathways engaged in microbiota-brain communications that will affect stroke? Future studies are likely to address some of these questions.

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