A focused update on stroke neuroimmunology: current progress in preclinical and clinical research and recent mechanistic insight

Johannes Boltze, MD, PhD[1,*]; Miguel A. Perez-Pinzon, PhD[2,*]

[1]School of Life Sciences, University of Warwick, Coventry CV4 7AL, United Kingdom

[2]Peritz Scheinberg Cerebral Vascular Disease Research Laboratory, Department of Neurology, University of Miami Miller School of Medicine, Miami, FL 33136, United States

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***Correspondence**

Miguel A. Perez-Pinzon, PhD

Peritz Scheinberg Cerebral Vascular Disease Research Laboratory

Department of Neurology, University of Miami Miller School of Medicine

Miami, FL 33136, United States

Email: perezpinzon@med.miami.edu

Orcid ID: 0000-0001-6555-8935

Or

Johannes Boltze, MD, PhD

School of Life Sciences, University of Warwick

Coventry CV4 7AL, United Kingdom

Email: johannes.boltze@warwick.ac.uk

Orcid ID: 0000-0003-3956-4164

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Abstract

Local and systemic inflammation contributes significantly to stroke risk factors as well as determining stroke impact and outcome. Previously being considered as an immuno-privileged domain, the central nervous system is now recognized for multiple and complex interactions with the immune system in health and disease. The sterile inflammatory response emerging after ischemic stroke is a major pathophysiological hallmark and considered to be a promising therapeutic target. Even (mal-)adaptive immune responses following stroke, potentially contributing to long-term impact and outcome, are increasingly discussed. However, the complex interaction between the central nervous and the immune system are only partially understood, placing neuroimmunological investigations at the forefront of preclinical and clinical research. This focused update summarizes current knowledge in stroke neuroimmunology across all relevant disciplines and discusses major advances as well as recent mechanistic insights. Specifically, neuroimmunological processes and neuroinflammation following ischemic are discussed in the context of blood brain barrier dysfunction, microglia activation, thromboinflammation, and sex differences in post-stroke neuroimmunological responses. The focused update further highlights advances in neuroimaging and experimental treatments to visualize and counter neuroinflammatory consequences of ischemic stroke.

Keywords: adaptive immunity, blood-brain barrier, cell therapies, cerebral ischemia, innate immunity, neuroimmunology, microglia, neuroinflammation, sex differences, stem cells, stroke, thromboinflammation

Non-standard abbreviations

BBB	blood-brain barrier
CIDS	central nervous system injury-induced immunodepression syndrome
CNS	central nervous system
DAMPs	danger-associated molecular patterns
FXII	coagulation factor XII
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GP	glycoprotein
KKS	kallikrein-kinin system
MCAO	middle cerebral artery occlusion
MMP	matrix metalloproteinase
NIH	National Institute of Health
NVU	neurovascular unit
PK	plasmakallikrein
SAP	stroke-associated pneumonia
STAIR	Stroke Treatment Academic Industry Roundtable
TNF	tumor necrosis factor
SPAN	Stroke Preclinical Assessment Network
tPA	tissue plasminogen activator
vWF	von Willebrand factor

Introduction

Ischemic stroke is the most important reason for permanent disabilities and a major burden for affected patients, relatives, care takers, and healthcare systems. Contrasting its massive socioeconomic and medical relevance, treatment options remain limited. Although the breakthrough in mechanical recanalization[1-3] and progress in pharmacological thrombolysis has brought new optimism to the field, recanalization options are still limited by relatively narrow therapeutic time windows, numerous contraindications, and restricted availability. This results in an urgent need for additional treatment options, ideally being compatible or even acting synergistically with state-of-the-art stroke treatments.

Inflammatory processes are not only recognized as an important element of major stroke risk factors such as atherosclerosis or hypertension but also contribute to primary and secondary brain damage following ischemic stroke. Consequently, neuroimmunological research became a major discipline within translational and clinical stroke research and may provide links to stroke sequelae such as cognitive decline or depression[4-6]. Importantly, neuroimmunological responses after stroke extend into the subacute and even chronic stages, providing numerous promising therapeutic targets. However, the field of stroke neuroimmunology is complex and we still lack a detailed understanding of underlying mechanisms and interactions, particular on a cellular and molecular level. This focused update summarizes the current knowledge in the field of stroke neuroimmunology, focusing on both preclinical and clinical research and highlighting recent progress in related areas spanning from experimental therapies to novel neuroimaging approaches. It also reviews the increasing body of preliminary evidence for (mal-)adaptive immune responses following ischemic stroke. Thus, this focus update was created by leading experts in the field to serve as a concise yet comprehensive update on stroke immunology for basic and translational stroke researchers as

well as clinician-scientists. The following paragraphs will summarize highlights from this article collection and puts its content into a wider research framework.

Post-stroke immunodepression and infections

Neuroinflammation in stroke is initiated by danger-associated molecular patterns (DAMPs) released from ischemically challenged brain tissue and triggering proinflammatory cytokine signaling. However, the inflammatory response is not restricted to the activation of local microglia. DAMPs and proinflammatory cytokines reaching the peripheral circualtion initiate a cascade of innate and, potentially, adaptive immune responses that emerge almost directly after disruption of cerebral blood flow[7,8]. The peripheral immune responses can cause significant additional brain damage in the acute stage[9] as leukocytes are attracted across the compromised blood-brain barrier (BBB).

The reflex-like central nervous system (CNS) injury-induced immunodepression syndrome (CIDS) strongly suppresses the peripheral immune system in subsequent subacute and early chronic stages[10]. The immunodepression in CIDS is mainly characterized by lymphopenia, a shift from T helper 1 cell to T helper 2 cell activity, and reduced production of proinflammatory cytokines such as tumor necrosis factor (TNF)-α. Thus, CIDS is considered as an attempt to rebalance the immunological situation after stroke but has detrimental consequences on its own. The immunodepression in CIDS may mitigate excessive neuroinflammation by reducing leukocyte egress from the circulation into the ischemic brain but at the same time causes a high susceptibility to infectious pathogens. Indeed, around 15% (up to 65% in individual studies) of all stroke patients suffer from concomitant infections[11,12]. The majority of these are urinary tract and pulmonary infections such as pneumonia, the latter being facilitated by dysphagia, a frequent stroke consequence. Increased infectious disease incidence after stroke is a major problem because these infections impede functional recovery,

prolong hospital admissions, and are associated with a higher risk of long-term dependency or death[13]. Preventive antibiotic screening can reduce urinary tract infections but neither prevents pneumonia nor does it contribute to a better functional outcome.[14] It is also inefficient against viral infections.

The review by Westendorp at al. provides a focused yet comprehensive update on contemporary knowledge of post-stroke immunodepression, related infections, and their impact on functional outcome. A major focus in set on stroke-associated pneumonia (SAP). Next to providing recent information on SAP epidemiology, diagnosis, and microbiology, it also focuses on clinically relevant aspects such risk factors, prognosis, and state-of the art treatment. As such, this work is a useful clinical compendium, but goes beyond that by covering pathophysiological and basic research aspects. For instance, the review provides explanations for poor outcome of strokes complicated by infection and explains the signaling cascades of CIDS in which the hypothalamic-pituitary-adrenal axis plays a central role (Fig. 1A).[15] The review eventually discusses experimental treatment options including immunomodulatory strategies to prevent or mitigate CIDS and thereby stroke-associated infections. In fact, it has been shown that timely administration of hematopoietic cytokines such as granulocyte colony-stimulating factor (G-CSF)[16] or granulocyte-macrophage colony-stimulating factor (GM-CSF)[17] (Fig. 1B, C) may preserve or restore peripheral immune cell levels and functions after stroke. Identifying such potential therapeutic mechanisms is highly relevant since CIDS also occurs after other forms of brain damage such as traumatic brain injury or intracerebral hemorrhage. Nevertheless, additional research is required because improper dose translation or timing of such cytokine treatments may have detrimental effects.[18]

The role of thromboinflammation in stroke pathophysiology

The complex interaction between thrombotic and inflammatory processes, summarized under the umbrella term thromboinflammation, are increasingly recognized as a major pathophysiological element in stroke. Treatment options for ischemic stroke are still limited causing a strong demand for additional therapeutic options, ideally being compatible with established recanalization approaches[19,20]. Thromboinflammation, linking blood vessel occlusion with acute and subacute brain tissue damage, is therefore an ideal therapeutic target candidate. However, thromboinflammation is not a singular process but includes different pathways, i.e., thromboinflammatory platelet activity, the contact-kinin pathway, and activation of immune responses by the thromboinflammatory micro-milieu. The latter comprise both innate and adaptive immune system elements. The comprehensive review by De Meyer and colleagues discusses state-of-the-art knowledge on thromboinflammation pathophysiology obtained from preclinical and clinical research.

Platelets play a central role in thromboinflammation, but platelet adhesion and activation, rather than aggregation, is the primary regulator of thromboinflammation. Platelet adhesion is mediated by glycoprotein (GP) VI and integrin α2β1 both binding to the GPIb-V-IX complex which in turn interacts with von Willebrand factor (vWF). vWF, which is present in the blood plasma but can also be produced by endothelial cells, is immobilized in areas of endothelial damage. Interaction with platelets leads to platelet adhesion and, eventually, platelet activation. The process also fosters platelet-leukocyte interaction, contributing to local neuroinflammation. Transgenic mice lacking CD69, an endothelial vWF release inhibitor, suffer from aggravated brain damage[21] whereas animal slacking vWF exhibit less brain damage after stroke. A therapeutic approach potentially being feasible for clinical application is the use of recombinant ADAMTS13, a vWF-cleaving enzyme. ADAMTS13 also limits thrombus formation. A constitutively active ADAMTS13 variant with fivefold biological activity,

exerting strong anti-inflammatory and thrombolytic effects as well as improving regional cerebral blood flow became available recently.[22]

The contact-kinin pathway is initiated by coagulation factor XII (FXII) which is not required for hemostasis and intrinsic coagulation under physiological conditions.[23] In turn, FXII-deficient animals are protected from stroke sequelae[24] without exhibiting an increased risk for hemorrhagic transformation. However, activated FXII triggers the kallikrein-kinin system (KKS) by converting plasmaprekallikrein into its active form, plasmakallikrein (PK). PK cleaves kininogen which releases bradykinin, a proinflammatory peptide that contributes to BBB damage and thus promotes edema formation. Inhibition of the KKS mitigates ischemic stroke impact while blocking PK and kininogen actions prevents intracerebral thrombosis and limits both BBB damage and local neuroinflammation.[25,26] De Meyer et al. provide exciting insights how this may also be relevant in thrombolytic treatments as tissue plasminogen activator (tPA) increases bradykinin formation what in turn induces kininogen cleavage. Experimental data suggest that this process requires PK and FXII, so blocking those pharmacologically may help to counter secondary damage after stroke. Since increased bradykinin generation by tPA and associated BBB damage may also explain the higher risk for malignant edema formation and hemorrhagic transformation when tPA is applied late.

A particular highlight of the review by De Meyer and co-workers is the thorough review of ongoing clinical trials evaluating the impact of novel strategies to modulate neuroinflammation. This comprehensive presentation of both experimental and clinical data clearly adds much translational value. Currently, platelets and platelet modulation are promising approaches although recent studies revealed mixed results. Moreover, there is the concept of limiting neuroinflammation by modulating both immune cell egress from the circulation into the brain and residual immune cell (microglia) activation, approaches also under clinical investigation for primary inflammatory CNS diseases such as Multiple Sclerosis.

Neuroinflammation after ischemic stroke and the blood-brain barrier

One of the main features of the pathological mechanisms of ischemic stroke is the disruption of the BBB integrity. Brain homeostasis is highly dependent on a healthy BBB. The BBB is structurally complex, made of many different cell types, comprising of the neurovascular unit (NVU). Many years of research have uncovered many mechanisms that lead to BBB disruption following cerebral ischemia. But more recently, the role of neuroinflammation has become prominent in the disruption of the BBB after ischemic stroke. The article by Candelario-Jalil and colleagues is a comprehensive review of the pathological mechanisms of neuroinflammation on the BBB. Two important sections of this review examine the role of reactive oxygen species on BBB damage and then establish ROS mediated links to disruptions on matrix metalloproteinases (MMPs), which have been implicated on BBB disruptions of its basal lamina and tight junctions. Next, the authors examine the role of inflammatory mediators released by microglia and peripheral immune cells on disruptions to the BBB.

A crucial section of this article is a comprehensive review of imaging markers for neuroinflammation, especially emphasizing medical imaging modalities such as computed tomography, magnetic resonance imaging, and positron emission tomography. These techniques will not only allow pre-clinical studies to study neuroinflammation non-invasively on disruptions of the BBB, but also establish diagnostic tools to be used in future clinical trials, which the authors discuss at length.

Neuroinflammation and stem cells after ischemic stroke

Stem cell therapy has been a promising therapeutic approach for stroke. Many years of research have provided multiple paths by which stem cells can be beneficial for stroke recovery. With the advent of thrombectomy and tPA therapy which are extending the

therapeutic period, stem cell therapy fits well with prolonged treatments. New discoveries into stem cell mechanisms of action now include a robust anti-inflammatory component. Anthony and colleagues review a novel approach in which stem cells can diminish stroke-induced inflammation in both the brain and spleen, suggesting a paradigm-shift from a traditionally brain-focused therapy to treating stroke as a neurological disorder with a significant peripheral pathology. This article provides an in-depth review of the brain-spleen axis of inflammation. This axis arises from the peripheral immune response to stroke. For example, the authors specifically review splenic immune response to stroke. In previous studies, splenic contraction follows middle cerebral artery occlusion (MCAO) with a concomitant elevation of monocytes into the brain[27], and splenectomy prior to MCAO mitigated infarct volume.[28] Another peripheral immune response to stroke emerges from cervical lymph nodes. Ischemic stroke instills a rapid vascular endothelial growth factor C release from these nodes with a consequent activation of macrophages.[29] Among other targets reviewed in this article, the authors suggest that these peripheral immune responses warrant in-depth investigations and may provide novel therapeutic targets. The authors then round up their review by defining how stem cells may target some of the peripheral responses to ischemic stroke, for example by modifying the spleen response.

Sex differences in neuroinflammation after ischemic stroke

Translation of experimental stroke treatments into clinical routine procedures has been unsuccessful for decades since promising efficacy findings could not be reproduced in clinical trials. A relatively low quality of preclinical studies has previously been considered as a major reason for this lack of progress, and recommendations such as the Stroke Treatment Academic Industry Roundtable (STAIR) guidelines have been published to define minimum quality assurance criteria for preclinical and translational research.[30] Indeed, the quality of preclinical

stroke studies has been largely increased since then, and the field is now considered to be ahead of others in term of study quality.[31,32] However, recent systematic analyses revealed striking design differences between preclinical and clinical studies. For instance, an investigation in the field of cellular therapeutics found that all clinical trials performed so far enrolled patients of both sexes whereas only 0.3% of preclinical studies used male and female animals.[33] The influence of sex on stroke impact and outcome is well known in general, but we lack a detailed understanding how exactly sex may influence individual pathophysiological elements and responses contributing to stroke impact and outcome - as is the case for neuroinflammation. Two complementary review articles in this issue focus on neuroinflammation, stroke, and sex differences.

The first one by Banerjee and McCullough provides a comprehensive review of sex, immunity, and aging. They first provide clinical evidence of sex differences in stroke incidence at different ages. For example, they cite studies that show that stroke incidence in women is higher due to their increased longevity[34], and review literature indicating that stroke outcomes are worse in women than men in the elderly, but when younger women are compared with age-matched men, women had better outcomes. This epidemiological evidence points to the importance of an in-depth assessment of the sex differences on the neuroinflammatory cascade following stroke. To this complex subject, the authors then review the aging component, reviewing concepts such as the 'inflammaging', which is at an enhanced state in aging. With this baseline reference, the authors delve into reviewing the literature on the innate and adaptive immunity in stroke. For example, they document how genes involved in the toll-like receptor-mediated signaling linked to immune activation are encoded in the X chromosome[35].

The authors then continue to review microglia within the brain parenchyma and the distinct immune-CNS compartments with the presence of neutrophils, dendritic cells, lymphocytes, and other infiltrating immune cells, which are all activated by ischemia. They

continue to provide a comprehensive review of the role of sex-specific host immune responses in both acute ischemic stroke and stroke outcomes long term, and the potential consequences of sex differences.

Complementary to this review, a second review by Ugidos and coworkers presents results of a highly interesting analysis focusing on sex differences in microglial responses after ischemic stroke. Interestingly, there seems to be a sexual dimorphism in the distribution of microglial in the healthy rodent brain. Ugidos et al. provide preliminary evidence from rodent studies showing a higher density of microglia in the female hippocampus and prefrontal cortex.[36,37] Moreover, there seem to be morphological differences between female and male subjects, with more ramified microglia being observed in the female prefrontal cortex and the male hippocampus. This is important because morphology can indicate major functional difference in glial cell populations, in particular after ischemic stroke.[38,39] However, results are not uniform throughout the literature, and it cannot be excluded that observed differences may also stem from species and age differences in the respective studies. These preliminary findings are nevertheless invaluable as they may help to derive proper working hypotheses for future experimental studies.

Interesting data also exist for sex differences in microglial activation after ischemic stroke. Preclinical studies tend to report higher stroke volumes in male than female animals during their respective reproductive ages. Whereas the number of microglia after stroke seems to be indifferent between sexes, a larger proportion of proinflammatory microglia phenotypes in males may indicate a stronger microglial activation following stroke.[40] That activation indeed seems to emerge from different responses to stroke, rather than sex-specific pre-stroke differences. Similar differences were observed in pre- and perinatal ischemia[41], i.e., in a developmental stage before sexual maturation. This suggests that hereditary differences between sexes are at least partly responsible for these different activation patterns. Sexual

maturation of the brain during very early stages of individual development may explain those differences. For instance, there is a higher expression of pro-inflammatory molecules such as prostaglandin E2 in the developing male brain, and microglia seem to be both a source of and a responder to those.[42] It is not unreasonable to assume that the resulting differences persist into adult hood although this hypothesis requires further investigation. The review by Ugidos et al. further highlights influences of circulating sex hormones and the X chromosome, particularly during ageing, on microglia activation following stroke.

Eventually, sex differences in microglia phenotype and activation may become an attractive therapeutic target contributing to personalized medicine approaches. The number of studies addressing this is currently very limited, potentially because respective investigations require relatively large study populations in preclinical and clinical trials to achieve sufficient statistical power for proper discrimination of therapeutic and sex-related effects. Apart from pilot exploratory studies, these approaches may be a domain of large-scale confirmative preclinical trials such as the NIH Stroke Preclinical Assessment Network (SPAN) network or well-orchestrated international collaborations[43].

Conclusions

This focused update provides an extensive overview of the molecular, cellular, systemic, and physiological effects of neuroinflammatory processes following ischemic strokes. This focused update is timely because of the significant advancements in the field and the complexity of the inflammatory cascade that ensues following strokes. This in-depth overview of the field should benefit both pre-clinical and clinical researchers to gather the main concepts of neuroinflammation and see future directions from some of the leaders in the field. Nevertheless, advancements on neuroinflammation move at a rapid pace and this area will need continued updates.

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Figure 1. The central nervous system injury-induced immunodepression syndrome and potential treatments.

(A) The central nervous system injury-induced immunodepression syndrome (CIDS) is the major reason for higher susceptibility to infection after stroke. It is triggered by sterile inflammation after stroke or other brain damage. Lacking peripheral pro-inflammatory signaling causes the resulting post-stroke immunodepression. (B) Granulocyte colony-stimulating factor (G-CSF) can partially reconstitute depleted peripheral immune cell counts. Immune cell counts were obtained in male spontaneously hypertensive rats treated with 50 µg/kg G-CSF or saline for 5 consecutive days after stroke, starting 24 hours after stroke induction. Gray areas represent physiological immune cell counts in these animals. Similar results were reported for granulocyte-macrophage colony-stimulating factor (GM-CSF). (C) Treatment with G-CSF and GM-CSF may rebalance the peripheral immune system after stroke. (A) and (C) were modified from[10], data shown in (B) was previously published in Stroke.[16]