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REVIEW

Spleen Derived Immune Cells in Acute Ischemic Brain Injury: A Short Review

Emőke Horváth*, Éva Kiss

George Emil Palade University of Medicine, Pharmacy, Science, and Technology of Targu Mures, Romania

Spleen-derived immune cells are considered to play central role in the progression of ischemic brain damage contributing to both the local and systemic inflammatory response initiated by an ischemic insult in the brain tissue. Brain-spleen communication in acute ischemic brain injury has been studied especially in rodent models of stroke, which mimic the acute focal brain ischemia in humans. Rodent spleens decrease in size after experimentally induced stroke, due mainly by the release of spleen`s immune-cells into the circulation. Splenectomy prior to middle cerebral artery occlusion is protective to the ischemic brain resulting in decreased infarct volume and reduced neuroinflammation. Various therapeutic strategies in clinical use aiming to protect the neural tissue after stroke were found to involve the modulation of splenic activity, altogether indicating that the spleen might be a potential target for therapy in ischemic brain injury. Importantly, the most clinical studies demonstrated that the splenic response in stroke patients is similar to the changes seen in rodent models. Thus, despite the limitations to extrapolate the results of animal experiments to humans, rodent models of stroke represent an important tool for the study and understanding of brain-spleen communication in the pathogenesis of acute brain ischemia.

Keywords: experimental stroke model, splenocytes, neuroinflammation

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Introduction

Stroke, with ischemic stroke accounting for almost 90 % of the cases, is the second leading cause of death in middle-income countries, following ischemic heart disease [1]. Its global public health importance is well reflected by statistical data: in the high-income countries, it is the first cause of long-term disability, significantly increasing health spending worldwide [2].

Acute brain ischemia affects the brain parenchyma generating two different damaged areas: the ischemic core, the central region of the brain area to which blood flow is lost and the penumbra, which retains residual perfusion from collateral blood vessels. The latter covers almost half of the total tissue damage volume during the initial stages of stroke [3]. Rodent models of ischemic stroke using transient or permanent occlusion of the middle cerebral artery (MCAO) mimic the acute focal brain ischemia in humans and reproduce confidently the pathology seen in humans [4].

The penumbra shows a remarkable susceptibility to merge into the ischemic core, therefore represents an important target region for salvage both via post-ischemic and preventive therapy [5]. The consequences of acute brain ischemic injury extend far beyond the brain [6]. A variety of immune cells, not only in the central nervous system (CNS) but also in the periphery is activated soon after a stroke. They play a determinant role in the progression and outcomes after stroke [7]. The ischemic neuronal damage involves various pathways, like anoxic depolarization, perturbed glutamatergic and GABAergic neurotransmission and intracellular calcium signaling as well as excessive formation of reactive oxygen species [8], [9]. This complex process activates the local microglia, regarded as resident immune cells in the central nervous system (CNS). Activated microglia will generate chemotactic signals leading to a significant infiltration of peripheral immune cells into the damaged brain area [10]. The peripheral immune cells migrate through the compromised blood-brain barrier and contribute to the brain damage or repair processes after ischemic stroke [11]. The spleen is the most important immune cell reservoir of the body. High number of experimental studies document that the spleen plays a decisive role in the stroke-induced immune response and neurodegeneration [12,13]. The activation of the sympathetic nervous system following an ischemic insult in the brain results in splenic contraction followed by the mobilization and release of different immune cells from this reservoir contributing to the systemic inflammatory response initiated by the acute brain ischemia [14]. The sequence probably is determined by the nature of the ischemic trigger and by the pattern of secreted cytokines/chemokines [12]. The role of the brain infiltrating immune cells in stroke evolution seems to be a dual one by enhancing neurodegeneration [15], or protecting neurons [16], [17]. The splenic origin of brain infiltrating cells after cerebral ischemia was demonstrated using carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling, a method which enables the following of migrating splenocytes after experimentally induced stroke. These studies evidenced that after experimental stroke immune cells exit the spleen, their number reducing here significantly after 24-48 hours, and migrate into the damaged brain tissue contributing to injury [12,13,18]. However, inflammatory cells have been shown to exert also protective effects, and

* Correspondence to: Emőke Horváth
E-mail: emoke.horvath@umfst.ro

contribute to post-stroke recovery [19], altogether underscoring the multiple facets of the inflammatory process in stroke. Study of the inflammatory pathways, with their variety of cells and mediators involved in the early stages of neuroinflammation in cerebral ischemia, is difficult to carry out in humans. The many structural overlaps in the histology of human and rodent spleen histology [20-22], makes the rodent spleen suitable for studying the splenic response to stroke. Indeed, the results of the last ten years support that several features of brain-spleen communication after stroke described in experimental studies can be extrapolated to human subjects [23,24] and are promising for integration of these results into the therapy targeting the immune system after stroke. In this short review we analyzed previous PubMed indexed experimental and clinical studies investigating the role of spleen-brain communication in the pathophysiology of ischemic stroke. We focused on recent publications providing data on changes in the cellular components of the spleen after ischemic stroke and evaluating the role of spleen derived immune cells in the progression of ischemic brain damage.

Splenic changes after ischemic stroke

The spleen has been proved to be the most pro-inflammatory organ following acute ischemic injury at different sites in the body (brain, liver, intestine, kidney, and heart) [25-28]. The changes in the spleen after stroke include mainly three aspects: spleen morphology, numbers of immune cells derived from the spleen and inflammatory cytokine production by the spleen`s cells [15,29]. The released pro-inflammatory cytokines promote a secondary inflammatory response in the brain contributing to amplification of neural tissue damage [12].

The microscopic examination of the spleen removed after acute cerebral ischemia reveals significant morphological changes compared to the normal spleen. These include depletion of lymphoid tissue with reduced or lack of germinal centers [14], decrease in the number and frequency of apoptotic cell death in splenocytes (4 days after transient ischemic insult MCAO mice show a 90% reduction in splenocyte numbers compared to the sham-operated animals), changes in cellularity and phenotype of lymphoid cells and, in general, splenic atrophy. [30]. The spleen contributes to the systemic inflammatory response and neurodegeneration via peripheral immune cells: splenic leucocytes such as various subsets of T and B cells, Mo/MF, polymorphonuclear neutrophils (PMNs), natural killer cells (NK) and follicular dendritic cells (DCs) [12]. Following stroke, due to the activation of the sympathetic nervous system, production of chemotactic cytokines and antigen presentation by the damaged tissue the splenocytes are released into the circulation and reach the damaged brain tissue [12]. There are still controversies concerning the time course of the recruitment of inflammatory cells into the brain as well as their pathogenic roles in the ischemic brain injury. Studies using CFSE labeling demonstrated that splenocytes appear relatively late in the damaged neural tissue, usually days (48 - 96 h) after ischemic brain injury occurs [18].

Neutrophils

Several studies indicate the neutrophils as the first peripheral cells that infiltrate the brain after ischemic injury (from 30 minutes to 3 days) [11,31,32]. However, recruitment of other inflammatory cells into the brain prior to neutrophil infiltration in response to cerebral ischemia has also been observed [10]. Neutrophils contribute substantially to many aspects of the brain damage occurring after ischemia by releasing ROS, proteases, cytokines and chemokines as summarized in a recent review by Jickling et al [33]. PMNs expressed matrix metalloproteinase 9 (MMP-9/gelatinase B), a member of the family of zinc-dependent proteases has been linked to the disruption of the blood-brain barrier via degradation of the basement membrane and tight junction proteins, followed by edema formation, neuronal death and erythrocyte extravasation [34,35] in animal experiments. High levels of MMP-9 were found in peripheral blood samples of patients with ischemic stroke [36], and an increased number of MMP-9 positive cells was detected in human post mortem ischemic brain tissue, in association with PMNs and activated microglial cells [37]. Quantification of myeloperoxidase (MPO)-labeled neutrophils after permanent MCAO in the damaged brain tissue of rats demonstrated that infarct size significantly correlates with the number of neutrophils around the infarct, larger infarct being accompanied by more neutrophils. In the brain of rats splenectomized 2 weeks before MCAO the number of neutrophils was significantly decreased without significant changes in blood leukocytes, which might contribute to the observed protective effects of splenectomy after ischemic brain injury [38]. Thus, these findings evidence that neutrophils have a negative effect following cerebral acute ischemia and indicate that their inhibition including MMP-9 inhibition might represent a potential therapeutic intervention in stroke [32,39].

Lymphocytes

The pathophysiological importance of lymphocyte accumulation and their interaction with PMNs into the damaged brain tissue following stroke is not clearly defined [40]. T lymphocytes are considered central players in the development of a sustained inflammatory response after stroke. The massive reduction of splenic immune cells, especially B and T lymphocytes and the concomitant activation of the sympathetic nervous system are considered the main causes leading to a persistent immunosuppressed status of stroke patients, responsible for the increased susceptibility of these patients to post-stroke infections [14]. Although the number of blood lymphocytes declines early after an ischemic insult, the most studies indicate that they appear relatively late in the brain, usually days after the onset of brain injury. Some studies in rodent models demonstrated

accumulation of T cells in the brain already within the first 24 h after focal cerebral ischemia influencing the evolution of brain injury [41,42]. There is a time shift in the distribution of different T cell subsets, which play differential roles in response to cerebral ischemia. The early appearing T-cell subsets after stroke (day 3 to 7) are represented by helper $CD4^+$ (day 3 to 7) and cytotoxic $CD8^+$ T-cells (10). Experimental studies connect the key role of helper $CD4^+$ Th1 cells in the pathogenesis of stroke to the release of proinflammatory cytokines (e.g. interleukins, such as IL-2, IL-12, IFN-γ, tumor necrosis factor -TNF-α) promoting brain damage, however some cytokines (e.g. IFN-γ) are critical for the prevention of post-stoke infections [43,44]. $CD4^+$ Th2 cells may play a protective role through production of anti-inflammatory cytokines (IL-4, IL-5, IL-10, and IL-13) [45,46]. Neo-antigens originating from the neural cells detritus, such as microtubule-associated protein 2 (MAP 2), NMDA receptor subunit NR-2A, myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG), released into circulation are captured by antigen-presenting cells, especially DCs and macrophages. It is thought, that these stimuli finally trigger the activation of T-cell-dependent adaptive immune responses in the T-cell zone of the spleen [47]. In parallel to the decrease in the total number of splenic immune cells in the early days after stroke, increased number of CD4+FoxP3+ Tregs has been observed in the ischemic brain. Several studies suggested Tregs to exert beneficial effects on stroke evolution. Depletion of Tregs increased tissue loss and worsened neurological functions, probably due to reduced IL-10 production [12,48,49]. In contrast, other studies reported that Treg depletion did not affect stroke infarct volume or even reduced infarct size and improved neurologic function after MCAO, indicating a detrimental Treg effect after experimental stroke [50]. The summary of preclinical studies though indicates an overall neuroprotective effect of Treg-targeted therapies (adoptive transfer of purified polyclonal Treg) in models of stroke, making it a potential candidate for therapy in a specific group of patients with ischemic stroke [51]. Tregs are known to suppress, modulate the activity of other immune cells, including CD4+ and CD8+ T-cells, B-cells, NK cells and circulatory CD11b+ monocytes [52]. Findings revealed that γδT cells, a small subset of T cells that bear a distinct TCR on their surface might also be involved in the pathogenesis of ischemic stroke, TCR-γδ knockout mice, as well as mice treated with TCR-γδ–specific antibody presenting decreased infarct volume [53]. The complex role of lymphocytes in the immune response to stroke is highlighted also by the study of Chen et al. Using flow-cytometric analysis, this group documented that remote ischemic preconditioning of a limb (reported as a protective method against ischemic stroke) followed by MCAO significantly is associated by reduced brain infiltration of $CD8^+$ T cells and NKT cells, increased splenic volume and elevated lymphocyte number in the spleen, including B lymphocytes [54]. The role of B-lymphocytes, the major component of splenic withe pulp [55] in ischemic brain injury is poorly investigated. The low number of reports on this topic provided discrepant results indicating beneficial or neutral effects of the B lymphocyte infiltrate on ischemic brain injury [56,57]. A recent study using pharmacological B cell depletion, B cell transgenic mice, and adoptive B cell transfer experiments disclosed that B cells did not influence infarct volume and functional behavior in mice after acute ischemic stroke [58]. Moreover, Doyle et al. observed B-lymphocyte infiltration of the injured brain, which could contribute to the intensification of the cognitive deficits after stroke [59]. Performing immunostainings of human postmortem tissue, the same group detected B lymphocytes also in the brain of some patients with stroke supporting a B lymphocyte response to stroke also in humans [59]. In contrast, Bregs secreting IL10 had a protective role in ischemia/reperfusion injury in mice due probably by post-stroke immunosuppression [60].

NK cells

NK cells, an important part of the innate immune system, have cytotoxic properties. Following stroke, these cells migrate from the spleen into the infarcted area of the brain together with T lymphocytes and monocytes [18]. The protective effects of splenectomy before acute brain ischemia probably imply also the reduction of NK cells in the damaged area of the brain [61].

Monocytes/macrophages (Mo/MF)

The local microglia and peripheral macrophages are among the first responders to cell damage in the CNS and are mobilized to the site of injury within hours [62]. The spleen is the main source of monocyte intake in ischemic injury [13]. The structure of the spleen`s marginal zone (MZ) plays an important role in the distribution of the spleen macrophage population. The MZ outer ring contains resident MZ macrophages (CD209b+) that present processed antigens to MZ B cells. The inner rim of the MZ is lined to CD169+ metallophylic macrophages that transfer captured antigen to DCs for activation of the cytotoxic (CD8+) T cells. Macrophages are also present in the red pulp that are F4/80hi and help maintain blood homeostasis by phagocytosis of senescent erythrocytes and blood-borne particulates and their phenotype differ from that of macrophages associated with MZ [20,22].

The spleen contraction after stroke is accompanied by a decreased number of macrophage subsets in the spleen. The displacement of these macrophage subsets from the spleen was found to temporally coincide with increases of the respective macrophage subsets in the ischemic brain [13]. Research over the past decade has evidenced that spleen-derived mouse monocytes can be divided into two distinct populations, each having a different effect on ischemia outcome: the Ly6Chi / CCR2+ subset is pro-inflammatory and the Ly6Clow / CCR2- subset has anti-inflammatory

effects. The Ly6Chi/CCR2+ monocyte subset is specifically recruited in acute ischemic conditions by the monocyte chemoattractant protein-1 (MCP-1), secreted by the cells of the inflamed tissue, and will become classically activated M1 macrophages, with pro-inflammatory phenotype. The Ly6Clow / CCR2 - subset is recruited to the normal tissue and develops into resident M2 macrophages, which have host defense and repair functions after injury [63]. In a previous study CCR2-null mice were protected against cerebral inflammation following brain ischemia, suggesting the important role of CCR2 in stroke-induced brain injury [64]. The polarization of macrophages in a classic pro-inflammatory (M1) or alternative anti-inflammatory (M2) phenotype thus depend on specific environmental signals that induce these different polarization states and consequently determine the function of microglia and Mo/MF [65]. The factors that drive the activation of microglia/macrophages include cytokines, chemokines, released degradation products, and extravasated molecules [63]. Studies document that toll-like receptors (TLRs) are essential players in the process of macrophage activation [63]. Interestingly, it has been found that TLRs play a role in the inflammatory response to ischemic injury even in the absence of infection. More specifically, stimulation of TLRs through TLR ligands and INF-γ induces classical M1 activation either 1) by inducing NF-ƙB, which in turn upregulates pro-inflammatory cytokines (TNFα, IL-12, suppressor of cytokine signaling-3 (SOCS3)) and hypoxia inducible factor 1a (HIF-1a) to promote inducible nitric oxide synthase (iNOS) synthesis, or 2) by inducing interferon regulatory factor 3 (IRF-3), one of the main transcriptional regulators initiating M1 polarization (via signal transducer and activator of transcription 1 (STAT1)) and M2 gene silencing. Against it, IL-4/IL-13 stimulation favors alternative M2 activation. Recently, the triggering receptor expressed on myeloid cells 2 (TREM2), an anti-inflammatory receptor was suggested as an important player in controlling microglial M1/M2-like phenotypes, its deficiency exacerbating ischemic damage in experimental stroke [66]. Many markers (e.g. CD68, CD200, F4/80, CD14, HLA-DR, TLRs, heat shock protein (Hsp)-70, C3b/iC3b, CR3, sodium-calcium exchanger (NCX)1 antigen) are used to denote the reactive status of the macrophages, but they do not define whether macrophages have toxic or protective functions. The classification of macrophages into M1 or M2 subgroups is once again difficult because of the overlap between the antigenic structures. For example, major histocompatibility complex (MHC) class II (involved in antigen presentation to immune cells) and CD86 (functions as a co-stimulatory signal for T-cell activation) do not clearly belong to the M1 or the M2 phenotype. However, despite the antigenic heterogeneity, generally is accepted, that M1 phenotype markers include CD16, CD32, CD86, and inducible nitric oxide synthase (iNOS), while the M2 phenotype expresses arginase-1 (Arg1), CD163, and CD206 antigens [67]. A growing number of published data based on conventional molecular analysis-immunostaining, real-time PCR, western blots and morphological data confirms the importance of macrophage polarization in stroke physiopathology [68,69]. In an experimental model of stroke, we found, that following ischemia/reperfusion the number of mononuclear cells is increased in the MZ of the spleen, and the Arg1/iNOS2 expression ratio on macrophages of marginal zone/red pulp interface is significantly shifted in the sham group compared to ischemic group in the favour of iNOS2 expressing cells (in press). Correspondingly, in the brain sections of the same animals we detected an overall reduced number of CD68-positive macrophages/microglia in the early (24h) infiltrates of ischemic cerebral tissue. These cells were present in a higher number in the penumbra than in the central core. The expression of cellular markers of macrophage polarization, iNOS2, and Arg1 was also higher in the penumbra than in the core and evidenced a significant a significant M1-phenotype dominance [70]. Thus, the modulation of microglia/macrophage polarization represent another promising therapeutic possibility for stroke.

Conclusion

Acute cerebral ischemia triggers a prompt neuroinflammatory response involving resident microglia as well as peripheral immune cells. The various immune cells contribute significantly to both brain damage and repair processes making unfeasible a categorization of stroke-related inflammatory processes as either exclusively beneficial or detrimental. The spleen plays a central role in the coordination of inflammatory responses in stroke although the exact mechanisms underlying the splenic responses after stroke are not fully identified. The different subsets of splenic cells seemingly play distinct roles in different stages of the stroke especially through the release of various cytokines\chemokines affecting both the local and systemic inflammation after stroke. Moreover, stroke-induced immune response is increasingly recognized to influence the neuropathological outcome after ischemic brain injury. Thus, further studies focusing on the spleen-brain cross-talk with their variety of cells and signaling molecules already in the early stages of neuroinflammation in acute brain ischemia are necessary 1) to provide new insights into the role of immune response in the pathogenesis of ischemic brain injury and 2) to answer the question if in addition to the traditional surgery and thrombolytic therapy immunomodulatory interventions targeting the spleen could represent a complementary and wider therapeutic time window strategy, especially for improvement of the long-term prognosis in stroke patients.

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Conflict of interes

The author has no conflicts of interest to declare.

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