

Splenic Responses in Ischemic Stroke: New Insights into Stroke Pathology

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SUMMARY

In the past decade, the significant contribution of the spleen to ischemic brain damage has gained considerable attention in stroke research. As the largest natural reservoir of immune cells, the spleen establishes critical connections with the ischemic brain during the progression of stroke and mobilizes its cells to the site of injury. Multiple “alarm” signals released from the injured brain are essential for the initiation of brain–spleen communication. Spleen-derived cells, including neutrophils, lymphocytes, and monocytes/macrophages, are known to contribute significantly to ischemic brain damage. Understanding the dynamic splenic responses to stroke will not only provide insights into the evolution of ischemic brain injury but will also identify potential targets for stroke treatment. Here, we review recent studies on the functions of the spleen in ischemic stroke. We have included a discussion of several therapeutic strategies that target splenic responses and reduce acute ischemic brain damage in preclinical studies. Future investigations on the effects of the spleen on long-term stroke recovery are highly warranted.

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Introduction

In the past few decades, the importance of immune responses in the pathobiology of ischemic stroke has been highlighted in the literature [1]. A variety of immune cells, both within the central nervous system (CNS) and in the periphery, are activated soon after stroke onset and contributes significantly to brain damage or repair. The spleen is a major reservoir of blood cells and a key lymph organ that releases immune cells into the circulation for self-defense against infections and injuries, including damage to the CNS [2]. Therefore, splenic responses after stroke and their contribution to ischemic brain damage have gained considerable attention. Early studies in animals revealed that the spleen shrinks dramatically during the acute phase after middle cerebral artery occlusion (MCAO) [3,4]. This morphological change is accompanied by the long-distance trafficking of splenocytes all the way from the spleen into the ischemic area [5]. Remarkably, the MCAO-induced reduction in spleen size is correlated with the extent of ischemic damage [6]. Similarly, recent clinical data

suggest that the size of the spleen is quite dynamic in stroke patients, with an early contraction followed by a subsequent re-expansion. This splenic volume change might be associated with the early release of splenocytes into the circulation, which in turn may contribute to the poststroke inflammatory cascade [7]. Despite our awareness of these splenic alterations, the exact functions of the spleen in ischemic brain injury are not completely clear. Although most animal studies have documented that splenectomy in stroke animals is protective to the ischemic brain, a recent study demonstrated that removal of the spleen after MCAO does not reduce the brain infarct. These discrepancies may be attributed to differences in the timing of spleen removal and in the severity of the stroke (Table 1). Furthermore, these discrepancies probably reflect the complexity and dynamism of splenic functions during the course of ischemic injury. Therefore, a thorough characterization of the evolution of splenic responses to stroke would not only provide insights into the progression of ischemic brain injury but also identify potential targets for stroke treatment.

Table 1 Effect of spleen removal on brain infarct

	Stroke model	Infarct	Time of spleen removal	Species	Duration of occlusion	Time of observation	Citations
1	MCAO	↓	2 weeks before MCAO	Rat	Permanent	96 h	[13]
2	MCAO	↓	2 weeks before MCAO	Rat	Permanent	24 h	[45]
3	MCAO	↓	2 weeks before MCAO	Rat	Permanent	96 h	[12]
4	MCAO	↓	2 weeks before MCAO	Rat	Permanent	48 h	[4]
5	MCAO	↓	2 weeks before MCAO	Rat	Permanent	96 h	[40]
6	MCAO	↓	Splenic irradiation 3, 4, 6, or 8 h after start of cerebral ischemia	Rat	2 h	2 and 7 days	[35]
7	MCAO	↓	2 weeks before MCAO	Mouse	60 min	5 days	[66]
8	MCAO	—	Immediately before MCAO	Mouse	30 min	3 and 7 days	[36]
9	Hypoxia-ischemia (HI)	↓	3 days before HI	Neonatal rat	Permanent followed by 2 h of hypoxia	72 h	[67]

MCAO, middle cerebral artery occlusion.

Splenic Changes after Stroke

Changes in the Cellular Components of the Spleen after Stroke

Over the course of ischemic injury, the spleen can establish long-distance connections with the brain by mobilizing its cellular constituents. As mentioned above, the total number of cells inside the spleen reduces dramatically within several days after stroke. This reduction probably reflects the increased efflux of immune cells from the spleen into the blood as well as increased cell death within the spleen [3,5]. Many of the released immune cells subsequently infiltrate into the ischemic brain and contribute to brain injury. Using carboxyfluorescein diacetate succinimidyl ester (CFSE) to track splenocyte migration following MCAO, it has been shown that splenocytes such as lymphocytes, monocytes, neutrophils, and NK cells can enter the systemic circulation and migrate into the brain [5]. The reduction of immune cells in the spleen, especially of T lymphocytes and B lymphocytes, can last for several months, resulting in a persistent immunosuppressed status in stroke victims and increasing their susceptibility to post-stroke infections [8].

Interestingly, despite the general reduction of lymphocytes in the spleen, the numbers of several specialized cell populations have been shown to increase after stroke. For example, there is an increase in CD4⁺FoxP3⁺ regulatory T cells (Tregs) in the spleen at 96 h after stroke [3]. Given the evidence in support of protective effects of Tregs in the ischemic brain [9,10], the natural upregulation of the Treg population in the spleen after ischemia might represent an endogenous protective response that mitigates stroke-induced injury.

Cytokine Production in the Spleen after Stroke

Immune cells in the spleen are known to contribute to stroke-induced elevations in the levels of cytokines in the blood and subsequently in the brain. For example, spleen cells collected from stroke-afflicted mice display a higher capacity to secrete inflammatory cytokines upon activation, including tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), monocyte chemoattractant

protein-1 (MCP-1), and interferon γ (IFN γ), compared with splenocytes collected under physiological conditions [11]. Furthermore, many of these splenocyte-derived inflammatory cytokines and chemokines, such as IFN γ and interferon-inducible protein-10 (IP-10), have been shown to be critical for stroke-induced neurodegeneration [12,13].

How are Splenic Responses Triggered after Stroke?

Although the exact mechanisms underlying the initiation of splenocyte responses to the ischemic brain have yet to be identified, several events, including chemokine/chemokine receptor interactions, autonomic nervous system activation, and the release of CNS antigen have been documented to be essential for efficient brain-spleen communication.

CNS Antigens

In response to cerebral ischemic injury, the damaged brain may secrete a variety of antigens that activate adaptive immune responses and recruit immune cells from the spleen. Neo-antigens, such as microtubule-associated protein 2 (MAP2), NMDA receptor subunit NR-2A, myelin basic protein (MBP), and myelin oligodendrocyte glycoprotein (MOG), can all be released into the periphery in acute stroke patients and captured by antigen-presenting cells (APCs), especially dendritic cells (DCs) and macrophages. This response is thought to eventually initiate the activation of T-cell-dependent adaptive immune responses in the T-cell zone [14,15].

Chemokine/Chemokine Receptor System

A variety of chemokines, including the chemokine (C-C motif) ligand 2 (CCL2), CCL3, CCL5, CX3CL1, CXCL8, CXCL12, etc., are secreted by damaged CNS cells to recruit inflammatory cells into the injured brain (Figure 1). During this process, the corresponding chemokine receptors are also upregulated in splenocytes after stroke [3].

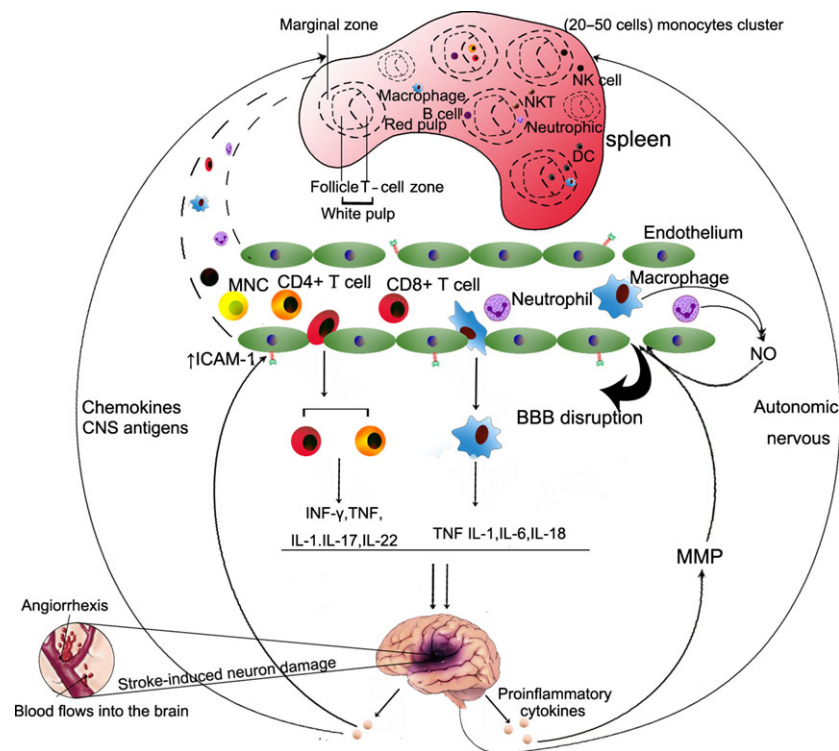


Figure 1 Spleen–brain communication after stroke. The ischemic brain stimulates the autonomic nervous system and releases chemotactic factors or CNS antigens to trigger the efflux of immune cells from the spleen to the site of brain injury. At the site of injury, inflammatory cells migrate through the compromised blood–brain barrier and enter the brain with a large amount of chemokines/cytokines, free radicals, and other inflammatory mediators. These factors are thought to exacerbate brain injury in the acute phases of stroke.

CCL2 is a potent mediator of monocyte/macrophage and neutrophil infiltration in cerebral ischemic injury [16]. The deficiency of CCR2 expression resulted in reduced monocyte and neutrophil infiltration into the ischemic brain and subsequent reduction in cerebral inflammation and brain infarct. Furthermore, it is suggested that the CCL2–CCR2 interaction might be important for the deployment of monocyte from the spleen and their movement to the injured brain [17]. The inhibition of CCR2 expression on monocytes by moxifloxacin treatment significantly attenuates infarct size after stroke.

The CXCR4–CXCL12 axis is implicated in the pathology of ischemic stroke. Cerebral ischemia results in a prompt and long-lasting elevation of CXCL12 in the ischemic penumbra. Transplanted GFP+ bone marrow cells are recruited in proximity to these CXCL12+ vessels and display the characteristics of activated microglial cells. These results suggest that CXCL12 is important in the homing of bone marrow-derived monocytes, which transform into microglia at the site of ischemic injury [18]. Interestingly, a recent study demonstrated that the blocking of CXCR4–CXCL12 interaction by CXCR4 antagonist AMD3100 not only reduce cerebral inflammation and brain infarct, but also prevent the poststroke spleen atrophy, suggesting a role of CXCR4–CXCL12 in regulation splenic responses after stroke [19].

Many other cytokines have been shown to be important for the recruitment of immune cells to the ischemic brain. For example,

CCL3 is associated with monocyte and neutrophil accumulation in injured brain tissue [20,21]. CCL5 is involved in leukocyte infiltration after stroke [22]. CX3CR1 knockout mice display reduced neuroinflammation after focal cerebral ischemia, suggesting that CX3CL1 promotes inflammation after stroke, an effect that may be related to chemotaxis in monocyte, T cells and NK cells [23–25]. CXCL8 is thought to be an important chemoattractant for neutrophils after ischemic stroke [26]. The functions of these cytokines on the mobilization of splenocytes warrant to be further investigated.

Autonomic Nervous System

Recent studies have demonstrated a link between autonomic nervous system activity and the peripheral immune response (Figure 1) [27,28]. The sympathetic nervous system is one of the major pathways whereby the CNS communicates with the immune system [29]. Shortly after an ischemic insult, there is an increase in norepinephrine and epinephrine in the systemic circulation. Both norepinephrine and epinephrine have been shown to induce significant splenic atrophy [30]. Blockade of α - and β -adrenergic receptors significantly inhibits the reduction in spleen size and reduces infarct volume, while α 1-receptor antagonism blocks the shrinkage of the spleen. These findings indicate that activation of both α - and β -adrenergic receptors mediates the splenic response to stroke and that both

receptor subtypes are potential targets for therapeutic treatments of ischemic injury [4].

Other Factors

Lymphocytes are important players in the pathophysiology of acute ischemic stroke. Sphingosine-1-phosphate (S1P) and S1P-receptor-1 (S1PR1) are vital for lymphocyte egress from white to red pulp and back into the circulation [31]. Furthermore, the S1PR1 modulator FTY720 has been shown to block the egress of lymphocytes from lymphoid organs, resulting in reduced ischemic neurodegeneration [32].

The Spleen Contributes to Neurodegeneration after Stroke

Various immune cells of the innate and adaptive immune systems contribute to the pathogenesis of ischemic injury and profoundly affect stroke outcomes [33]. After the initial ischemic injury, alarm signals are released that promote an influx of peripheral leukocytes into the brain [34]. The trafficking of peripheral immune cells into the brain is thought to exacerbate the local brain inflammatory response, thereby enhancing neurodegeneration. Dramatic changes in splenic cellular components after stroke have long suggested that the splenic response may play a pathological role in ischemic injury. Splenic leukocytes such as various subsets of T and B cells, DCs, and macrophages may participate in the systemic inflammatory response to stroke and contribute to neurodegeneration. Removal of the largest pool of peripheral immune cells before MCAO or neonatal hypoxia-ischemia by splenectomy has been shown to reduce brain infarct volume in rodents. As expected, the splenectomy-induced protection is accompanied by a decrease in the number of immune cells in brain tissue (Table 1). Poststroke splenic irradiation can also significantly reduce brain infarct size and inflammation after MCAO [35]. Thus far, the majority of studies are in agreement that inhibition of splenic function prior to or soon after MCAO is neuroprotective and significantly reduces neuroinflammation during the acute phases of stroke. However, Kim *et al.* [36] recently observed that splenectomy does not alter stroke-induced acute brain injury when performed immediately before 30 min of MCAO. This finding contradicts other studies of the beneficial effects of splenectomy (Table 1). The discrepancy may be due to the differences in the timing of spleen removal and the severity of the stroke insult. Further investigations are needed to explain the outcome differences using different stroke models and systematically changing the timing of spleen removal.

Immune Cells from the Spleen are Involved in the Progression of Ischemic Brain Damage

As summarized above, the spleen exerts a profound influence on the ischemic brain by mobilizing its resident cells, including lymphocytes, monocytes, neutrophils, and natural killer cells into the brain. In this section, we discuss the roles of immune

cells from the spleen in the pathogenesis of ischemic brain damage.

Neutrophils

Neutrophils are the first responders to ischemic injury and infiltrate into the injured brain within a few hours. Neutrophil infiltration peaks at 1–3 days after cerebral ischemia in animal models of stroke [37]. In patients with stroke, neutrophils are recruited within 24 h of symptom onset [38]. A number of studies indicate that neutrophil infiltration may contribute to postischemic brain injury [38–40]. Moreover, neutrophils may cause the breakdown of the BBB by releasing matrix metalloproteinase-9 (MMP-9), resulting in the infiltration of even more leukocytes and the exacerbation of neuroinflammation [41,42]. As expected, the protective effects of splenectomy 2 weeks before stroke are associated with a reduction in the number of neutrophils in the injured area [40].

Monocyte/Macrophages

Peripheral monocytes/macrophages (MMs) have been shown to infiltrate the ischemic brain in a CCL-2 dependent manner and contribute to poststroke cerebral inflammation and brain injury [17]. Interestingly, both pro-inflammatory Ly-6C^{high} and anti-inflammatory Ly-6C^{low} monocyte subsets are mobilized from the spleen and migrate toward the brain. A recent study showed that complete removal of spleen-derived MMs by splenectomy does not provide any protection to the ischemic brain [36]. Therefore, selective depletion of pro-inflammatory Ly-6C^{high} and anti-inflammatory Ly-6C^{low} MM subsets would be useful to determine the contribution of individual subsets to stroke outcomes.

Lymphocytes

Several studies have confirmed that T lymphocytes play a detrimental role in ischemia/reperfusion injury [43,44]. The protective effects of splenectomy on the brain are accompanied by reductions in the number of T cells in the injured brain [45]. Interestingly, recent studies suggest the some specific T-cell subsets, such as Tregs, may exert beneficial roles in stroke [9,10]. The number of Tregs in the spleen is known to increase after stroke [3], which might reflect an endogenous protective mechanism.

B cells are a major type of splenic lymphocyte. However, there are few studies investigating the role of B lymphocytes in cerebral ischemia/reperfusion injury. Mice lacking only B cells fail to show improvements against ischemic injury, suggesting that endogenous B cells do not play a detrimental role following cerebral ischemia. Furthermore, a special population of regulatory B lymphocytes secretes IL10 and is protective in cerebral ischemia/reperfusion injury [46]. Further studies are warranted to verify whether these protective B cells are released from the spleen after stroke.

Natural Killer Cells

Natural killer (NK) cells, which also travel from the spleen into the ischemic brain [5], are a type of cytotoxic cell and form part of

the innate immune system. Recent studies have shown that ischemic neuron-derived chemokines recruit NK cells into the brain and that these NK cells subsequently promote brain injuries [25,47].

Dendritic Cells

Splenic T lymphocytes are known to be activated by antigen-presenting cells (APCs), especially DCs. An increase in the number of DCs is observed in the brain in animal models of permanent/transient focal cerebral ischemia [48]. Immature DCs patrol the blood and invade injured tissues, where they pick up antigens and acquire the ability to stimulate T cells in lymphoid tissues, such as the lymph nodes and spleen [49,50]. Thus, DCs may present antigens to T lymphocytes in the spleen to activate adaptive immunity after stroke. The exact role of splenic DCs on stroke outcome remains to be explored.

Therapeutic Strategies that Impact Spleen Responses to Stroke

Although splenectomy soon after ischemic stroke might weaken the pro-inflammatory immunologic response and reduce cerebral injury, it is obviously not a clinically practical approach for stroke treatment. Permanent loss of the spleen will significantly impair immune defense against infections and may be catastrophic over the long term. Therefore, alternative approaches have to be developed to target splenic responses after stroke. Recent promising studies have shown that some treatment strategies can indeed modulate splenic responses and ameliorate stroke-induced brain injury.

Stem Cells

Human Umbilical Cord Blood Cells

Human umbilical cord blood (HUCB) cells have been shown to reduce brain infarcts after stroke and improve functional outcomes [51–53]. In addition to their capacity to differentiate into neuronal cells [51], HUCB cells can protect the ischemic brain through immunomodulation. Transplantation of HUCB cells after stroke can reduce the number of infiltrating immune cells in the ischemic areas and inhibit the increase in inflammatory cytokines [54]. Moreover, some studies have shown that HUCB cells move from the site of the injection to the spleen, altering immune cell profiles and cytokine production in the spleen [6]. Specifically, HUCB cell transplantation reduces splenic atrophy and increases the number of CD8⁺ T cells in the spleen after MCAO. By increasing IL-10 and decreasing IFN- γ release, HUCB cell treatment can inhibit the proliferation of MCAO-associated T cells [6]. These studies suggest that HUCB cells directly target the spleen and may achieve therapeutic effects in stroke victims at least partially by modulating immune cell activation inside the spleen.

Other Stem Cells

Other stem cells, such as mesenchymal stromal cells (MSCs), embryonic stem cells (ESCs), induced pluripotent stem cells

(iPSs), neural stem cells (NSCs), and human amniotic epithelial cells (hAECs), have all been applied toward the treatment of experimental stroke. The mechanism of protection by these cell types may all be related to immune modulation [55]. For example, MSCs have been shown to protect the brain against ischemic injury by modulating inflammatory responses, as manifested in reduced cytokine/chemokine levels in the blood and mitigated glial activation in the brain [56]. Whether these stem cells can target the spleen is still unknown.

Gamma Ray Radiation

Immune cells derived from spleen are highly sensitive to irradiation. A low dose of Gamma ray (Gy) can induce cell apoptosis in the spleen [57]. Ostrowski and colleagues have used gamma irradiation with 8 Gy of Cobalt 60 to irradiate the splenic area at different time points after cerebral ischemia. The cerebral infarct was significantly reduced when the spleen was irradiated at 3 or 4 h after the onset of ischemia. The authors also observed reduction of microglial activation, T-cell infiltration, and neuronal apoptosis [35]. Irradiation at later time points failed to elicit protection of the ischemic brain, suggesting that the therapeutic temporal window for strategies targeting splenic inflammatory responses is fairly narrow.

Catecholamines and Other Products Targeting the Sympathetic Nervous System

As mentioned above, catecholamine levels in the spleen are raised by activation of the sympathetic nervous system after MCAO. Catecholamines can modulate the splenic response to stroke by binding to splenic α - and β -adrenoreceptors. Ajmo and colleagues have observed that carvedilol, an α - and β -adrenergic receptor repressor, significantly reduces spleen atrophy and brain infarct volume. Their findings demonstrate that activation of both α - and β -adrenergic receptors by catecholamines can regulate the splenic response to stroke and represents a promising target for stroke therapies [4].

Agmatine is an α 2- adrenergic and imidazoline receptor agonist and an *N*-methyl-*D*-aspartate receptor antagonist [58]. Uranchimeg *et al.* [59] have shown that agmatine ameliorates immune responses in the spleen after brain ischemia, as shown by reduced contraction of the white pulp and decreases in the number of macrophages inside the spleen. As a consequence, agmatine can reduce the size of the cerebral infarct and lower the risk for post-stroke infection [59].

Statins

Preclinical studies have shown that statins are beneficial against ischemic brain injury [60,61]. For example, pretreatment with simvastatin remarkably reduces infarct size and improves neurological recovery after stroke [62]. Delayed simvastatin treatment until 24 h after stroke fails to decrease infarct volume, but still improves neurological recovery [63]. Clinical studies have also confirmed that statins exert protective effects on patients with ischemic stroke [64]. The protective effect of statins is associated

with their antiinflammatory properties [65]. Simvastatin can reduce spleen atrophy and inhibit splenocyte apoptosis after MCAO through an antiapoptotic mechanism involving increased expression of Bcl-2 and decreased expression of Bax [66]. Simvastatin treatment also inhibits IFN- γ , which may be critical for Simvastatin-afforded neuroprotection [66].

Conclusions

The past decade has witnessed significant achievements in our understanding of the splenic response after ischemic stroke. The release and migration of immune cells from the spleen into the circulation and their subsequent infiltration into the ischemic brain is thought to exacerbate brain damage during the acute stages of stroke. However, there are still many unsolved questions in this area. For example, the direct interaction between infiltrated splenocyte and CNS cells and how such interaction impact the pathogenesis of stroke have not been addressed. The advances in cell labeling and two-photon imaging techniques would help to address this question in stroke animals. In addition, it is still not clear whether and how the spleen contributes to brain repair after

stroke. Given the evidence favoring an increase in the release and the numbers of neuroprotective immune cells such as Tregs from the spleen after stroke, we can certainly envision potentially protective roles for the spleen in long-term recovery from stroke. Although some experimental strategies that target splenic responses have shown therapeutic effects during acute phases of stroke models, translating these studies into the clinic for the long term will be far more challenging and demand a more comprehensive understanding of splenic responses in stroke patients.

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Conflict of Interest

The authors declare no conflicts of interest.

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