Responses of immune organs following cerebral ischemic stroke

(Short Title: Stroke-induced changes in immune organs)

Chengbo Tan1,2,3, Zifeng Wang3, Miao Zheng4, Songji Zhao1, Hideo Shichinohe3,5, Kiyohiro Houkin3

1Advanced Clinical Research Center, Fukushima Global Medical Science Center, Fukushima Medical University, Fukushima, Japan
2Department of Neurosurgery, Shanghai East Hospital, Tongji University School of Medicine, Shanghai, China.
3Department of Neurosurgery, Graduate School of Medicine, Hokkaido University, Sapporo, Japan
4Department of Dermatology, Graduate School of Medicine, Hokkaido University, Sapporo, Japan
5Division of Clinical Research Administration, Hokkaido University Hospital, Sapporo, Japan

Correspondence:

Songji Zhao, MD, PhD
Advanced Clinical Research Center, Fukushima Global Medical Science Center, Fukushima Medical University
1 Hikariga-oka, Fukushima City, Fukushima 960-1295, Japan
Telephone: 81-24-581-5166
Fax: 81-24-581-5170
E-mail: zhao-s@fmu.ac.jp
Abstract

**Background:** Stroke is a leading cause of death and disability worldwide. Recently, brain secondary damage has been hypothesized to be a key aggravating element in an ischemic cascade. However, the interaction between cerebral infarction and immune organs has yet to be fully understood. In this study, we investigated the changes in the rat brain, spleen, thymus, mesenteric lymph node, and liver at 3, 7, and 13 days after transient middle cerebral artery occlusion (tMCAO) by immunohistochemistry.

**Material and methods:** Rat models of stroke were made by tMCAO. Functional assessment was performed 3 h, and 1, 3, 5, 7, 9, 11, and 13 days after MCAO. Rat organs were harvested for 2,3,5-triphenyltetrazolium chloride staining and Immunohistochemistry.

**Results:** The CD8α+ T cells was found to decrease in the spleen, thymus, mesenteric lymph node, and liver, whereas it increased in the brain. Those of Iba1+ and CD68+ macrophages were decreased in the spleen, thymus, and mesenteric lymph node, whereas they were elevated in the brain and liver. Ki67+ cells showed the same characteristics as macrophages, and increased numbers of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) positive apoptotic cells were found in the spleen, mesenteric lymph node, liver, and brain.

**Conclusions:** The present results demonstrated that stroke is a systemic disease, which not only affects the brain, but also induces responses of immune organs. On the basis of these results, a systemic treatment might be a good strategy for clinical stroke care.

**Key Words:** Cerebral ischemic stroke, Inflammation, Immune organ response, Histological analysis
Introduction

According to the World Health Organization, cerebral stroke is the second most common cause of death behind ischemic heart disease. With the occurrence of stroke, inadequate oxygen, low blood flow, and lack of nutrient supply can result in serious nerve damage, which subsequently leads to motor paralysis, lalopathy, unconsciousness, and even death \(^1\). In recent years, the advances in science and technology have greatly improved the lifesaving rate of stroke. However, once the central nervous system (CNS) is damaged, it is difficult for brain tissue to regenerate. Therefore, a large number of patients are suffering from the severe neurological sequelae of stroke in daily living \(^2\).

As a development in research, it has been found that brain damage-derived inflammation plays an important role in the pathogenesis of ischemic stroke. The occurrence of brain damage can immediately induce a series of immune responses, including the activation of brain-resident inflammatory cells and the infiltration of peripheral inflammatory cells \(^3-5\). Nowadays, the secondary attack of ischemic stroke is hypothesized to be a key aggravating element in an ischemic cascade. In Tan’s study, positron emission tomography (PET) with \(^{18}\text{F}\)DPA-714, a ligand of neural inflammatory indicator—translocator protein (TSPO), was used and revealed a high concentration of \(^{18}\text{F}\)DPA-714 in the cerebral infarct area \(^6\). Some studies showed that peripheral T cells may be the major mediators of post-stroke inflammatory responses. After stroke, these cells are activated promptly, migrate to the infarct area within hours, produce cytokines, recruit other inflammatory cells, and exacerbate the development of infarct \(^7,8\). In contrast, T-cell-deficient animals show a reduced infarct size after stroke \(^9\). Similarly, the presence of monocytes/macrophages in the infarct area is also considered to contribute to postischemic inflammation and brain damage. The extent of acute injury positively correlates with the number of monocytes/macrophages \(^10\). Decreasing the number of infiltrating monocytes and
Macrophages can delay injury progression and enhance axonal regeneration with functional benefits \(^{11,12}\). Moreover, accumulating evidence indicates that the levels of inflammatory cytokines, such as tumor necrosis factor (TNF), interleukin (IL)-1, and IL-6, can increase up to 40- to 60-fold in ischemic territories within the first 24 hours after an experimental stroke \(^{13}\). Furthermore, several clinical case reports showed that stroke patients with a systemic inflammatory profile exhibit poorer outcomes \(^{14,15}\).

Although these line of evidence suggest an as yet undetermined role of immune organs in response to brain injury, the underlying process that results in immune activation and brain secondary damage remains unclarified. Therefore, the aim of the present study was to investigate the responses and alterations of systemic immune organs in a rat model of transient middle cerebral artery occlusion (tMCAO) by immunohistochemistry.

**Materials and Methods**

**Rat infarct model**

Thirteen-week-old male F344/NSIc rats (Japan SLC, Inc., Shizuoka, Japan) were used as the tMCAO model. The rats were initially anesthetized with 4% isoflurane in N\(_2\)O/O\(_2\) (70:30) and maintained via spontaneous ventilation with 2% isoflurane in N\(_2\)O/O\(_2\) (70:30). The rectal temperature was maintained at 37°C throughout the surgical procedure using a temperature controller system (NS-TC10, Neuroscience, Inc., Tokyo, Japan).

During the operation, the right common carotid artery (CCA), the external carotid artery (ECA), and internal carotid artery (ICA) were exposed. A silicon rubber-coated monofilament with a tip coating diameter of 0.37 mm (Doccol Corp., Redlands, CA, USA) was inserted into the right ECA and advanced into the ICA to block the origin of the middle cerebral artery (MCA). After 90 min of MCAO,
the suture was carefully removed, and the ECA was coagulated to permit reperfusion \textsuperscript{16,17}. The cerebral blood flow (CBF) in the territory of the MCA was measured by laser doppler flowmetry (OMEGAFL0 FLO-C1; OMEGAWAVE, Tokyo, Japan) before and after MCAO \textsuperscript{17}. Ninety minutes after reperfusion, the 18-point neurological severity score (NSS) was assessed. Rats with a CBF reduction greater than 70\% and an NSS of more than nine were included in this study. The MCAO rats were sacrificed 3, 7, and 13 days after MCAO operation. Sham-operated animals underwent the same surgical procedure without monofilament insertion and were euthanized at 13 days after MCAO. The animals’ brains, spleens, thymuses, mesenteric lymph nodes, and livers were taken out and analyzed by histological assay.

All animal experiments were approved by the Animal Studies Ethical Committee at Hokkaido University Graduate School of Medicine and was conducted in accordance with the Declaration of Helsinki.

**Neurological severity score assessments**

Functional assessment was performed 3 h, and 1, 3, 5, 7, 9, 11, and 13 days after MCAO (n = 13). Briefly, the 18-point NSS system comprises four different domains: (a) motor, (b) sensory, (c) reflex, and (d) balance tests \textsuperscript{18}. In a series of assessments, one point is awarded for the inability to perform a task or for the lack of a reflex test. Thus, a higher score represents a more severe nerve injury \textsuperscript{18}.

**TTC staining and ischemic volume analysis**

Rat brains were harvested for 2,3,5-triphenyltetrazolium chloride (TTC) staining analysis to evaluate infarction volume 3 (n = 10), 7 (n = 13), and 13 days (n = 13) after MCAO. Briefly, six 2 mm thick
serial coronal sections were cut and stained with 2% TTC (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) at 37°C for 15 min. Each section was scanned using a high-resolution scanner (Epson, GT-X820, Nagano, Japan) and quantified using Image J (NIH, Bethesda, MD). Infarction volume was calculated as the percentage volume of the left normal hemisphere using the following formula: (left hemisphere volume – right non-infarct volume) / left hemisphere volume (%) \(^{17,19}\).

**Immunohistochemistry and apoptosis assay**

Immunohistochemistry was performed as previously described \(^3\). The organs were removed and stored in 4% paraformaldehyde for two days. The spleens, thymuses, and mesenteric lymph nodes were embedded in paraffin. On the other hand, the brains and livers were sliced into 2 mm thick sections and paraffinized. Immune organ sections (4 µm thick) were treated with a primary antibody against Ki67 (rabbit monoclonal antibody, 1:200 dilution, Biocare Medical, Pacheco, CA), CD68 (mouse monoclonal antibody, 1:200 dilution, Abcam, Cambridge, UK), or IL-10 (rabbit polyclonal antibody, 1:150 dilution, Bioss Antibodies, Woburn, MA) at 4°C overnight, and then incubated with an Alexa Fluor 594-conjugated goat anti-rabbit antibody, an Alexa Fluor 594-conjugated goat anti-mouse antibody, or an Alexa Fluor 488-conjugated goat anti-rabbit antibody (1:200 dilution, Thermo Fisher Scientific, Waltham, MA) at room temperature for 1 h. A few sections were cotreated with a rabbit polyclonal anti-Iba1 antibody (1:1500 dilution, Wako, Osaka, Japan) and a mouse monoclonal anti-CD8α antibody (mouse monoclonal antibody, 1:150 dilution, EXBIO, Prague, Czech Republic) at 4°C overnight. On the following day, the sections were incubated with Alexa Fluor 594 and Alexa Fluor 488. Brain sections were incubated with an anti-CD8α antibody or an anti-Ki67 antibody or coincubated with an anti-Iba1 antibody and an anti-CD68 antibody at 4°C overnight, and then with
Alexa Fluor 488 and Alexa Fluor 594 on the next day.

Sections of the tissues were dyed using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) Alexa Fluor 647 assay kit (Thermo Fisher Scientific) in accordance with the manufacturer’s instructions. The sections were subsequently stained with an anti-CD8α antibody at room temperature for 1 h and followed by Alexa Fluor 488. Images were obtained using a fluorescence microscope (BZ-X700, KEYENCE, Osaka, Japan).

**Immunohistochemical Quantification**

For each group, the organ sections of five rats were stained and analyzed by an observer blinded to experimental conditions using ImageJ software. Staining was defined by the Mean gray value–Integrated density–Area option and then applied equally to all images. In each section, two random micrographs at a magnification of 100X in regions of interest (ROIs) were taken and calculated by the mean fluorescence intensity (MFI).

**Statistical analysis**

All data were expressed as the mean ± SD. Results of brain infarction volume were compared using one-factor analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. Significance was assumed at $P < 0.05$.

**Results**

**Cerebral infarction causes strong neurological deficits and brain damage**

The NSS demonstrated severe motor deficits 3 h after MCAO (10.15 ± 0.90). The rats showed distinct
paralysis symptoms 13 days after MCAO (6.23 ± 1.79, Fig. 1A), although their motor deficits improved with time. On the other hand, stroke-induced brain lesions were confirmed by TTC staining 3, 7 and, 13 days after MCAO. Cerebral infarction was widely distributed in the affected side of the cerebral cortex and striatum (Fig. 1C). There was a significant infarct and edema in the right cerebral cortex and striatum 3 days after MCAO. Cerebral edema was relieved on the 7th day, and the cavitation and hydrocephalus appeared around the infarct area 13 days after MCAO. The mean infarction volumes were 40.88 ± 9.67%, 37.34 ± 7.56%, and 35.64 ± 8.93% 3, 7, and 13 days after MCAO, respectively (Fig. 1B). There was no significant reduction in these rats, although the infarction volume showed a downward trend.

**Cerebral infarction causes the changes in immune organs**

Compared with sham-operated rats, obvious histological changes in immune organs were found in the cerebral infarction rats. These changes were assessed by measuring the decrease or increase in fluorescence intensity.

**Brain**

Immunohistochemical analysis showed the gradual increase in the number of CD8α+ T cells in the cerebral infarct area after stroke. With aggravation of brain injury, the number of CD8α+ T cells peaked at 13 days after MCAO (Fig. 2A). From the third day after MCAO, Iba1 (a pan-microglia marker in the brain) and CD68 (a marker of the activated phenotype) double-positive inflammatory microglia began to gather around the infarct area, gradually distributed, and increased in number, which remained high level throughout the entire period of injury (Fig. 2B). During the stroke, the number of TUNEL+
apoptotic cells reached the peak in the striatum at 3 days after the injury, and then gradually reduced (Fig. 2C). On the other hand, the Ki67+ proliferating cells were present in the cortex around the injured striatum at 3 days, and peaked at 7 days (Fig. 2D). The MFIs of each marker in brains changed accordingly (Fig. 2E).

**Spleen**

In the spleens of sham-operated rats, CD8α+ T cells mainly resided in the white pulp, whereas Iba1+ and CD68+ macrophages mainly existed principally in the red pulp. Ki67+ proliferating cells widely distributed in the spleen and mainly accumulated in the red pulp. After the stroke, the numbers of CD8α+ T cells (Fig. 3A), Iba1+ macrophages (Fig. 3A), CD68+ macrophages (Fig. 3B), and Ki67+ proliferating cells (Fig. 3D) all decreased. Among these cells, CD8α+ T cells decreased markedly in number, were even depleted. In addition, the number of TUNEL+ apoptotic cells markedly increased in number and peaked at 7 days (Fig. 3C). The MFIs of each marker in spleens changed accordingly (Fig. 3E).

**Thymus**

As the production and storage site of T cells, the thymus showed high fluorescence intensities of CD8α (Fig. 4A) and Ki67 (Fig. 4D) in the cortex. In addition, numerous Iba1+ macrophages (Fig. 4A) and CD68+ (Fig. 4B) macrophages accumulated in the medulla. Following the stroke, the thymus gradually shrank with the reduction in the number of CD8α+, Iba1+, CD68+, Ki67+, even TUNEL+ cells (Fig. 4C). The MFIs of each marker in thymuses changed accordingly (Fig. 4E).
**Mesenteric lymph node**

The cell changes in the mesenteric lymph nodes were essentially the same as those in the spleen. The numbers of CD8α⁺ T cells (Fig. 5A), Iba1⁺ macrophages (Fig. 5A), CD68⁺ macrophages (Fig. 5B), and Ki67⁺ proliferating cells (Fig. 5D) gradually decreased during the period of injury, and the number of TUNEL⁺ apoptotic cells (Fig. 5C) peaked at 7 days. The MFIs of each marker in mesenteric lymph nodes changed accordingly (Fig. 5E).

**Liver**

In the liver, the number of CD8α⁺ T cells decreased and that of TUNEL⁺ apoptotic cells (Fig. 6C) increased after MCAO (Fig. 6A). However, different from the other immune organs, the Iba1⁺ macrophages (Fig. 6A), CD68⁺ macrophages (Fig. 6B), Ki67⁺ proliferating cells (Fig. 6D) and IL-10⁺ anti-inflammatory cells (Fig. 6E) showed increasing fluorescence intensities after the stroke. The MFIs of each marker in livers changed accordingly (Fig. 6F).

The summary of alterations in numbers of cells in systemic immune organs after stroke was shown in Fig. 7.

**Discussion**

Inflammation plays an important role in the pathogenesis of ischemic stroke. Previous studies have indicated that ischemic stroke can induce rapid and drastic activation of microglia around the peri-infarct area in the early phase, resulting in the release of microglia-mediated inflammatory substances such as TNF-α, prostaglandin E2, IL-1β, IL-6, and free radicals.²⁰⁻²¹ During the acute inflammatory process, these substances can trigger an inflammatory cascade reaction that aggravates and prolongs...
brain edema and nerve injury, and ultimately leads to a wider range of brain damage \textsuperscript{13,22}.

Although the interaction between cerebral ischemic stroke and systemic repercussions in immune organs remains unclarified, some researchers have proposed that with the breach of the blood brain barrier (BBB) after stroke, myelin-reactive antigens leak out and are exposed to the peripheral immune system, which recognizes the antigens as foreign matters and induces autoaggressive immune responses to facilitate the infiltration of immune cells into the brain \textsuperscript{7,23}. More recently, several studies have shown that individuals who suffer from stroke have higher titers of antibodies to CNS antigens such as portions of the \textit{N}-methyl\textit{D}-aspartate (NMDA) receptor and neurofilaments \textsuperscript{24,25}.

In the present study, histological assessment showed that the number of CD8\textalpha\textsuperscript{+} cytotoxic T cells rapidly decreased in all immune organs after the stroke. On the other hand, large numbers of these cells appeared in the brain infarct area. It is considered that CD8\textalpha\textsuperscript{+} T cells are released from peripheral organs into the circulation and settle around the brain injury area to contribute to the secondary inflammatory cascade in the brain and induce more severe damage. After the reduction in the number of CD8\textalpha\textsuperscript{+} T cells in the immune organs, the regions in which they were originally abundant, such as the white pulp of the spleen and the cortex of thymus began to shrink. At the same time, the proportions of Iba1\textsuperscript{+} and CD68\textsuperscript{+} macrophages increased correspondingly. However, when we observed the histological changes over 13 days after the stroke, we found that the number of macrophages were also gradually decreased in the spleen, thymus, and mesenteric lymph node. Considering the large number of macrophages accumulated around the infarct area after the stroke, a proportion of activated macrophages were released into the peripheral blood and subsequently aggravated brain damage.

On the other hand, stroke-associated infection (SAI) frequently occurs in patients with severe cerebral ischemic stroke \textsuperscript{26,27}. SAI is related to the decreased consciousness, abnormal brainstem
reflexes, or the application of invasive maneuvers. However, other clinical clues suggest that SAI could also result from CNS-induced immunodepression\textsuperscript{28,29}. In the TUNEL analysis of this study, large numbers of cells, especially those residing in the red pulp of the spleen—a reservoir of monocytes—entered the apoptosis phase during the period of injury. Moreover, the number of Ki67\textsuperscript{+} proliferating cells also decreased in the red pulp. Taking the marked decrease in the number of CD8α\textsuperscript{+} T cells in immune organs together, we believe that the body might regulate the apoptosis of cells in immune organs after severe post-ischemic inflammation. However, the underlying signal process that results in widespread immunosuppression is not well understood. Some researchers proposed that the apoptosis of immune cells results from an overactivation of the sympathetic nervous system, inducing the alterations of lymphocytes and monocytes\textsuperscript{22,30}. This vicious cascade could lead to the collapse of the immune system in the advanced stage of stroke injury, causing the atrophy of immune organs and cell depletion, and ultimately resulting in the development of pneumonia and other serious fatal infections.

The liver is the most important organ for metabolism and detoxification in the body. It contains a unique population of macrophages called Kupffer cells function in immunity. In this study, unlike in other immune organs, the number of macrophages in the liver increased after the stroke. Furthermore, an increasing number of cells expressed IL-10 in the liver. Because Kupffer cells are primarily immunosuppressive\textsuperscript{31,32}, they can mediate suppression through their synthesis of nitric oxide, secretion of IL-10, and induction of CD4\textsuperscript{+} T-cell apoptosis\textsuperscript{33-35}. We consider that when stroke-induced necrotic cell fragments and toxic substances reach the liver, these proinflammatory mediators could activate Kupffer cells to induce an anti-inflammatory repercussion. Thus, the liver might play an immunosuppression role in the inflammatory response to stroke.

The limitation of our study was STAIR and STEPS criteria recommend a surveillance period of
at least 1 month for behavioral phenotyping. However, in this study, neurological severity score (NSS) was used for showing the stroke severity of the rats which were sacrificed for TTC staining 13 days after MCAO. Therefore, the NSS was only monitored up to 13 days. And we should perform more extensive studies in the future.

Conclusions

In conclusion, we demonstrated that cerebral ischemic stroke can induce systemic immune responses. Intense responses not only aggravate brain damage, but also deplete immune cells. The collapse of the immune system could eventually cause fatal infections. On the basis of these results, a systemic treatment might be a good strategy for cerebral stroke care.

Competing interests

The authors declare no potential competing interest with respect to the research, authorship, and/or publication of this article.

References

1. Szeto V, Chen NH, Sun HS, Feng ZP. The role of KATP channels in cerebral ischemic stroke and diabetes. *Acta Pharmacol Sin.* 2018;39(5):683-694.
2. Donnan GA, Fisher M, Macleod M, Davis SM. Stroke. *Lancet.* 2008;371(9624):1612-1623.
3. Tan C, Shichinohe H, Abumiya T, et al. Short-, middle- and long-term safety of superparamagnetic iron oxide-labeled allogeneic bone marrow stromal cell transplantation in rat model of lacunar infarction. *Neuropathology.* 2015;35(3):197-208.
4. Iadecola C, Anrather J. The immunology of stroke: from mechanisms to translation. *Nat Med.* 2011;17(7):796-808.
5. Acosta SA, Tajiri N, Hoover J, Kaneko Y, Borlongan CV. Intravenous Bone Marrow Stem Cell Grafts Preferentially Migrate to Spleen and Abrogate Chronic Inflammation in Stroke. *Stroke.* 2015;46(9):2616-2627.
Tan C, Zhao S, Higashikawa K, et al. [18F]DPA-714 PET imaging shows immunomodulatory effect of intravenous administration of bone marrow stromal cells after transient focal ischemia. EJNMMI Research. 2018;8(1):35.

Dziennis S, Mader S, Akiyoshi K, et al. Therapy with recombinant T-cell receptor ligand reduces infarct size and infiltrating inflammatory cells in brain after middle cerebral artery occlusion in mice. Metab Brain Dis. 2011;26(2):123-133.

Jin R, Yang G, Li G. Inflammatory mechanisms in ischemic stroke: role of inflammatory cells. J Leukoc Biol. 2010;87(5):779-789.

Yilmaz G, Arumugam TV, Stokes KY, Granger DN. Role of T lymphocytes and interferon-gamma in ischemic stroke. Circulation. 2011;123(2):123-133.

Chen Y, Hallenbeck JM, Ruetzler C, et al. Overexpression of monocyte chemoattractant protein 1 in the brain exacerbates ischemic brain injury and is associated with recruitment of inflammatory cells. J Cereb Blood Flow Metab. 2003;23(6):748-755.

Popovich PG, Guan Z, Wei P, Huitinga I, van Rooijen N, Stokes BT. Depletion of hematogenous macrophages promotes partial hindlimb recovery and neuroanatomical repair after experimental spinal cord injury. Exp Neurol. 1999;158(2):351-365.

Ajami B, Bennett JL, Krieger C, McNagny KM, Rossi FM. Infiltrating monocytes trigger EAE progression, but do not contribute to the resident microglia pool. Nat Neurosci. 2011;14(9):1142-1149.

Lambertsen KL, Biber K, Finsen B. Inflammatory cytokines in experimental and human stroke. J Cereb Blood Flow Metab. 2012;32(9):1677-1698.

Elkind MS, Cheng J, Rundek T, Boden-Albala B, Sacco RL. Leukocyte count predicts outcome after ischemic stroke: the Northern Manhattan Stroke Study. J Stroke Cerebrovasc Dis. 2004;13(5):220-227.

McColl BW, Allan SM, Rothwell NJ. Systemic infection, inflammation and acute ischemic stroke. Neuroscience. 2009;158(3):1049-1061.

Kurisu K, Abumiya T, Ito M, et al. Transarterial regional hypothermia provides robust neuroprotection in a rat model of permanent middle cerebral artery occlusion with transient collateral hypoperfusion. Brain Res. 2016;1651:95-103.

Kurisu K, Abumiya T, Nakamura H, et al. Transarterial Regional Brain Hypothermia Inhibits Acute Aquaporin-4 Surge and Sequential Microvascular Events in Ischemia/Reperfusion Injury. Neurosurgery. 2016;79(1):125-134.

Chen J, Li Y, Wang L, et al. Therapeutic benefit of intravenous administration of bone marrow stromal cells after cerebral ischemia in rats. Stroke. 2000;32(4):1005-1011.

Yasuda H, Shichinohe H, Kuroda S, Ishikawa T, Iwasaki Y. Neuroprotective effect of a heat shock protein inducer, geranylgeranylacetone in permanent focal cerebral ischemia. Brain Res. 2005;1032(1-2):176-182.

Kawabori M, Yenari MA. The role of the microglia in acute CNS injury. Metab Brain Dis. 2014.

Zheng LT, Hwang J, Ock J, Lee MG, Lee WH, Suk K. The antipsychotic spiperone attenuates inflammatory response in cultured microglia via the reduction of proinflammatory cytokine expression and nitric oxide production. J Neurochem. 2008;107(5):1225-1235.

Offner H, Subramanian S, Parker SM, et al. Splenic atrophy in experimental stroke is accompanied by increased regulatory T cells and circulating macrophages. J Immunol. 2006;176(11):6523-6531.

Frenkel D, Huang Z, Maron R, et al. Nasal vaccination with myelin oligodendrocyte glycoprotein reduces stroke size by inducing IL-10-producing CD4+ T cells. J Immunol. 2003;171(12):6549-6555.

Dambinova SA, Khounteev GA, Izykenova GA, Zavolokov IG, Ilyuhina AY, Skoromet AA. Blood test detecting autoantibodies to N-methyl-D-aspartate neuroreceptors for evaluation of patients with transient ischemic attack and stroke. Clin Chim. 2003;49(10):1752-1762.
25. Becker K. Autoimmune responses to brain following stroke. *Transl Stroke Res.* 2012;3(3):310-317.

26. Johnston KC, Li JY, Lyden PD, et al. Medical and neurological complications of ischemic stroke: experience from the RANTTAS trial. RANTTAS Investigators. *Stroke.* 1998;29(2):447-453.

27. Weimar C, Roth MP, Zillessen G, et al. Complications following acute ischemic stroke. *Eur Neurol.* 2002;48(3):133-140.

28. Aslanyan S, Weir CJ, Diener HC, et al. Pneumonia and urinary tract infection after acute ischaemic stroke: a tertiary analysis of the GAIN International trial. *Eur J Neurol.* 2004;11(1):49-53.

29. Urra X, Obach V, Chamorro A. Stroke induced immunodepression syndrome: from bench to bedside. *Curr Mol Med.* 2009;9(2):195-202.

30. Meyer S, Strittmatter M, Fischer C, Georg T, Schmitz B. Lateralization in autonomic dysfunction in ischemic stroke involving the insular cortex. *Neuroreport.* 2004;15(2):357-361.

31. Crispe IN. The liver as a lymphoid organ. *Annu Rev Immunol.* 2009;27:147-163.

32. Knolle P, Schlaak J, Uhrig A, Kempf P, Meyer zum Buschenfelde KH, Gerken G. Human Kupffer cells secrete IL-10 in response to lipopolysaccharide (LPS) challenge. *J Hepatol.* 1995;22(2):226-229.

33. Roland CR, Walp L, Stack RM, Flye MWJIoJ. Outcome of Kupffer cell antigen presentation to a cloned murine Th1 lymphocyte depends on the inducibility of nitric oxide synthase by IFN-gamma. 1994;153(12):5453-5464.

34. Knolle P, Schlaak J, Uhrig A, Kempf P, Kh MZB, Gerken GJJoH. Human Kupffer cells secrete IL-10 in response to lipopolysaccharide (LPS) challenge. 1995;22(2):226-229.

35. Miyagawa-Hayashino A, Tsuruyama T, Egawa H, et al. FasL Expression in Hepatic Antigen-Presenting Cells and Phagocytosis of Apoptotic T Cells by FasL + Kupffer Cells Are Indicators of Rejection Activity in Human Liver Allografts. 2007;171(5):1499-1508.
Fig. 1
Fig. 2
**Fig. 3**

A: CD8α
B: CD68
C: CD8α TUNEL
D: Ki67

E: MFI of CD8α in Spleen
   - Sham
   - 3d
   - 7d
   - 13d

E: MFI of CD68 in Spleen
   - Sham
   - 3d
   - 7d
   - 13d

E: MFI of TUNEL in Spleen
   - Sham
   - 3d
   - 7d
   - 13d

E: MFI of Ki67 in Spleen
   - Sham
   - 3d
   - 7d
   - 13d
|       | CD8a | Iba1 | CD68 | TUNEL | Ki67 | IL-10 |
|-------|------|------|------|-------|------|-------|
| Spleen| ↓↓↓↓ | ↓↓↓  | ↓↓↓  | ↑↑↑↑  | ↓↓   |
| Thymus| ↓↓↓↓ | ↓↓↓↓ | ↓↓↓  | ↓↓↓   | ↓↓   |
| Lymph node| ↓↓   | ↓    | ↓    | ↑     | ↓    |
| Liver | ↓    | ↑↑↑  | ↑    | ↑     | ↑    | ↑↑    |
| Brain | ↑↑   | ↑↑↑↑ | ↑↑↑↑ | ↑↑↑↑   | ↑↑   |

Fig. 7