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Responses of Immune Organs after Cerebral Ischemic Stroke

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Background: Stroke is a leading cause of death and disability worldwide. Recently, secondary damage to the brain has been hypothesized as a key aggravating element in the ischemic cascade. However, the interaction between cerebral infarction and immune organs is not fully understood. In this study, we investigated changes in rat brain, spleen, thymus, mesenteric lymph node, and liver at 3, 7, and 13 days after transient middle cerebral artery occlusion (tMCAO) by immunohistochemistry.

Methods: Rat models of stroke were made by tMCAO. Functional assessment was performed at 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 number of $CD8\alpha^+$ T cells decreased in spleen, thymus, mesenteric lymph node, and liver and increased in brain. Numbers of $Iba1^+$ and $CD68^+$ macrophages decreased in spleen, thymus, and mesenteric lymph node and increased in brain and liver. $Ki67^+$ cells exhibited the same characteristics as macrophages, and increased numbers of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL)-positive apoptotic cells were present in spleen, mesenteric lymph node, liver, and brain.

Conclusions: The present results indicate that stroke is a systemic disease that, in addition to affecting the brain, also induces responses in immune organs. These results suggest that systemic treatment might be a good strategy for clinical stroke care. (J Nippon Med Sch 2021; 88: 228–237)

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, after ischemic heart disease. Stroke results in inadequate oxygen, low blood flow, and lack of nutrient supply, which can cause severe nerve damage that may lead to motor paralysis, lalopathy, unconsciousness, and even death[1]. Recent advances in science and technology have greatly improved stroke mortality. However, if the central nervous system (CNS) is damaged, it is difficult for brain tissue to regenerate. Therefore, many people must live with the severe neurological sequelae of stroke[2].

Research indicates that brain damage-derived inflammation is important in the pathogenesis of ischemic stroke. Development of brain damage can immediately induce a series of immune responses, including activation of brain-resident inflammatory cells and infiltration of peripheral inflammatory cells[3–5]. Recently, the secondary attack of ischemic stroke is hypothesized to be a key aggravating element in the ischemic cascade. Using positron emission tomography with [^{18}F]DPA-714, a ligand of neural inflammatory indicator–translocator protein, Tan reported a high concentration of [^{18}F]DPA-714 in the cerebral infarct area[6]. Some studies showed that pe-

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https://doi.org/10.1272/jnms.JNMS.2021_88-308
Journal Website (https://www.nms.ac.jp/sh/jnms/)

ripheral T cells may be the most important mediators of post-stroke inflammatory responses. After stroke, these cells are promptly activated, migrate to the infarct area within hours, produce cytokines, recruit other inflammatory cells, and exacerbate infarct development[7,8]. Indeed, T-cell-deficient animals had smaller infarcts after stroke[9]. Similarly, the presence of monocytes/macrophages in the infarct area might 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 levels of inflammatory cytokines such as tumor necrosis factor (TNF), interleukin (IL)-1, and IL-6 can increase by up to 40- to 60-fold in ischemic regions within the first 24 hours after an experimental stroke[13]. Furthermore, several clinical case reports showed that stroke patients with a systemic inflammatory profile had worse outcomes[14,15].

Although these lines 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 secondary damage to the brain remains unclarified. Therefore, this study investigated responses and changes in systemic immune organs in a rat model of transient middle cerebral artery occlusion (tMCAO) induced immunohistochemically.

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_2O/O_2 (70:30) and maintained via spontaneous ventilation with 2% isoflurane in N_2O/O_2 (70:30). Rectal temperature was maintained at 37°C throughout the surgical procedure by using a temperature controller system (NS-TC10, Neuroscience, Inc., Tokyo, Japan).

During the operation, the right common carotid artery, the external carotid artery (ECA), and internal carotid artery were exposed. A silicone 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 internal carotid artery 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[16,17]. Cerebral blood flow in the territory of the MCA was measured by laser doppler flowmetry (OMEGAFLO FLO-C1; OMEGAWAVE, Tokyo, Japan) before and after MCAO[17]. Ninety minutes after reperfusion, the 18-point neurological severity score (NSS) was assessed. Rats with a cerebral blood flow reduction greater than 70% and an NSS greater than 9 were included in this study. The MCAO rats were sacrificed at 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 removed and analyzed by histological assay.

All animal experiments were approved by the Animal Studies Ethical Committee at Hokkaido University Graduate School of Medicine (Approval Number: 12-0023) and were conducted in accordance with the principles outlined in the Declaration of Helsinki.

NSS Assessment

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

2,3,5-Triphenyltetrazolium Chloride Staining and Ischemic Volume Analysis

Rat brains were harvested for 2,3,5-triphenyltetrazolium chloride (TTC) staining to evaluate infarction volume at 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 with a high-resolution scanner (Epson, GT-X820, Nagano, Japan) and quantified with Image J software (NIH, Bethesda, MD). Infarction volume was calculated as the percentage volume of the left normal hemisphere using the formula: (left hemisphere volume - right non-infarction 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 2 days. The spleens, thymuses, and mesenteric lymph nodes were embedded in paraffin. In contrast, the brains and livers were sliced into 2-mm-thick sections and paraffinized. Immune organ sections (thickness, 4 μm) were treated with a primary antibody

against Ki67 (rabbit monoclonal antibody, 1:200 dilution, Biocare Medical, Pacheco, CA, USA), CD68 (mouse monoclonal antibody, 1:200 dilution, Abcam, Cambridge, UK), or IL-10 (rabbit polyclonal antibody, 1:150 dilution, Bioss Antibodies, Woburn, MA, USA) 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, USA) at room temperature for 1 h. A few sections were cotreated with a rabbit polyclonal anti-Iba1 antibody (1:1,500 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.

Tissue sections were dyed using a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate 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 with a fluorescence microscope (BZ-X700, KEYENCE, Osaka, Japan).

Immunohistochemical Quantification

For each group, the organ sections of five rats were stained and analyzed with ImageJ software by an observer blinded to experimental conditions. 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 100× in regions of interest were obtained, and mean fluorescence intensity (MFI) was calculated.

Statistical Analysis

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

Cerebral Infarction Causes Changes in Immune Organs

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

Brain

Immunohistochemical analysis showed a gradual increase in the number of $CD8\alpha^+$ T cells in the cerebral infarct area after stroke. With aggravation of brain injury, the number of $CD8\alpha^+$ 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. The numbers of these markers remained high throughout the injury period (**Fig. 2B**). During the stroke, the number of $TUNEL^+$ apoptotic cells reached a peak in the striatum at 3 days after injury, and then gradually reduced (**Fig. 2C**). $Ki67^+$ 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 changed accordingly in brain tissue (**Fig. 2E**).

Spleen

In the spleens of sham-operated rats, $CD8\alpha^+$ T cells were mainly present in the white pulp, whereas $Iba1^+$ and $CD68^+$ macrophages were mostly present in the red pulp. $Ki67^+$ proliferating cells were widely distributed in

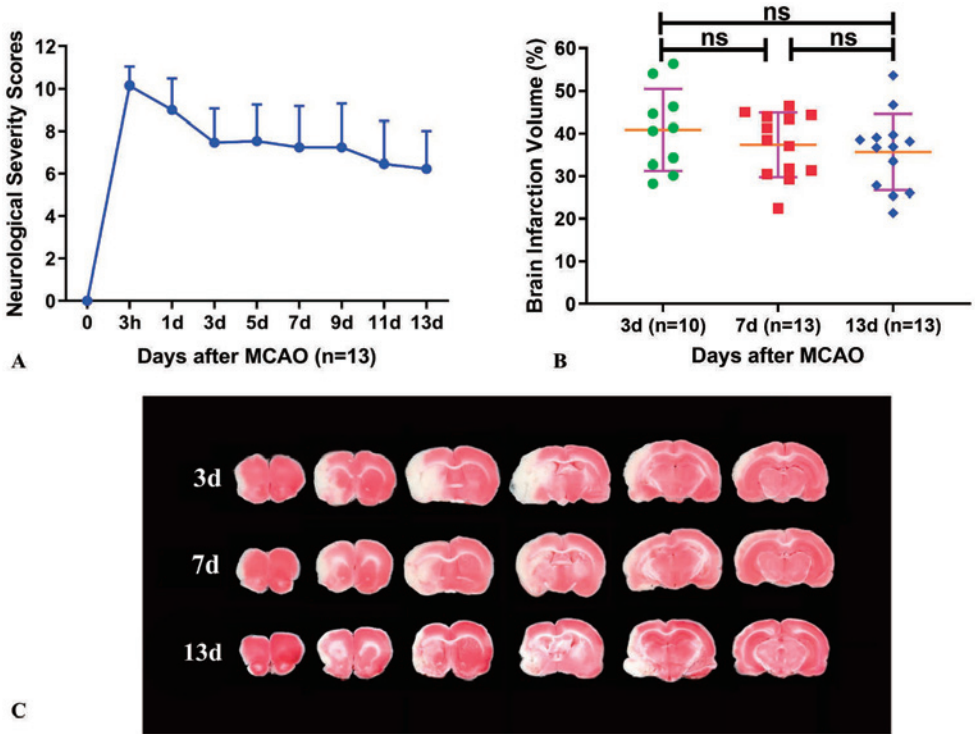

Fig. 1 Changes in rats after cerebral infarction. Panel A shows the neurological severity scores. Panel B shows the brain infarction volume. Panel C shows representative TTC staining images of rat brains. ns, no significance; 3d vs. 7d/13d, 7d vs. 13d.

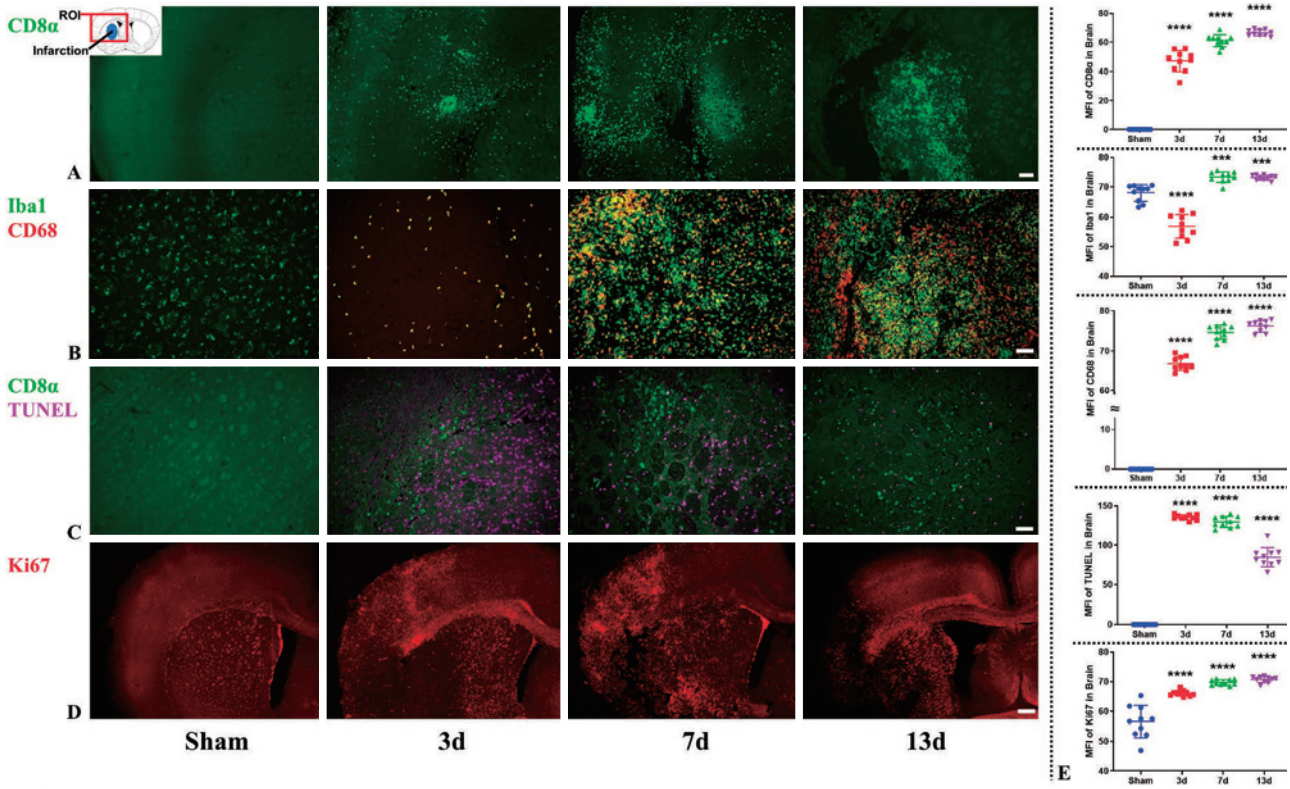

Fig. 2 Histology of brains. Panel A shows photomicrographs of CD8α$^+$ cells (green). Panel B shows Iba1$^+$ (green), and CD68$^+$ cells (red). Panel C shows the CD8α$^+$ cells (green) and TUNEL$^+$ cells (purple). Panel D shows the Ki67$^+$ cells (red). 3d, 7d, and 13d indicate the days after MCAO. Scale bars, 200 μm (A, D), 100 μm (B, C). Panel E shows the MFIs of each marker in brains. ***P<0.001 and ****P<0.0001; sham vs. 3d/7d/13d.

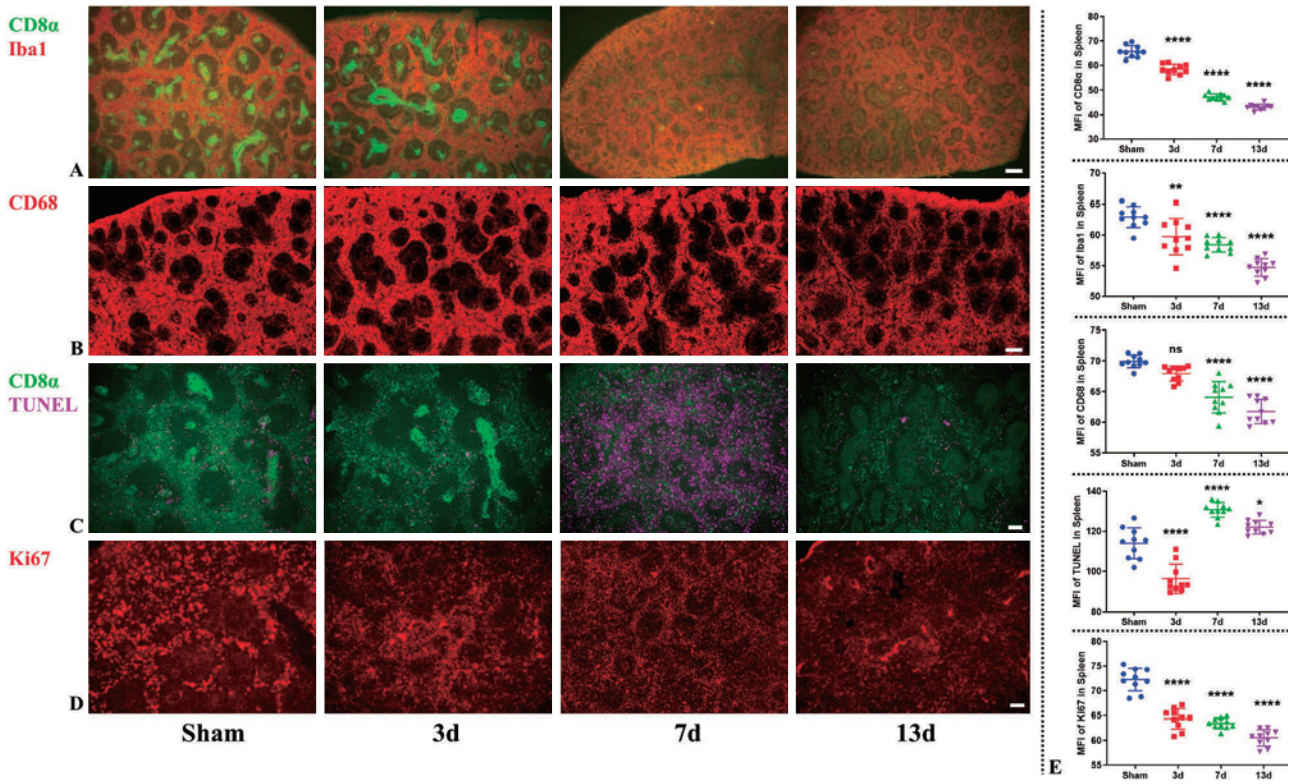

Fig. 3 Histology of spleens. Panel A shows photomicrographs of Iba1$^+$ (red) and CD8α$^+$ cells (green). Panel B shows CD68$^+$ cells (red). Panel C shows the CD8α$^+$ cells (green) and TUNEL$^+$ cells (purple). Panel D shows Ki67$^+$ cells (red). Scale bars, 500 μm (A, B), 200 μm (C, D). Panel E shows the MFIs of each marker in spleens. *P<0.05, **P<0.01, ****P<0.0001 and ns, no significance; Sham vs. 3d/7d/13d.

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. The number of CD8α$^+$ T cells decreased markedly, to the point of depletion. In addition, the number of TUNEL$^+$ apoptotic cells markedly increased and peaked at 7 days (**Fig. 3C**). The MFIs of each marker changed accordingly in spleen tissue (**Fig. 3E**).

Thymus

As the site of T cell production and storage, 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. After the stroke, the thymus gradually shrank as the numbers of CD8α$^+$, Iba1$^+$, CD68$^+$, Ki67$^+$, and even TUNEL$^+$ cells decreased (**Fig. 4C**). The MFIs of each marker changed accordingly in thymus tissue (**Fig. 4E**).

Mesenteric lymph node

Cell changes in mesenteric lymph nodes were essentially the same as those in 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 changed accordingly in mesenteric lymph node tissue (**Fig. 5E**).

Liver

In liver, the number of CD8α$^+$ T cells decreased and the number of TUNEL$^+$ apoptotic cells (**Fig. 6C**) increased after MCAO (**Fig. 6A**). However, unlike in the other immune organs, 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 changed accordingly in liver tissue (**Fig. 6F**).

Figure 7 summarizes changes in the numbers of cells in systemic immune organs after stroke.

Discussion

Inflammation plays an important role in the pathogenesis of ischemic stroke. Previous studies indicate 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 inflamma-

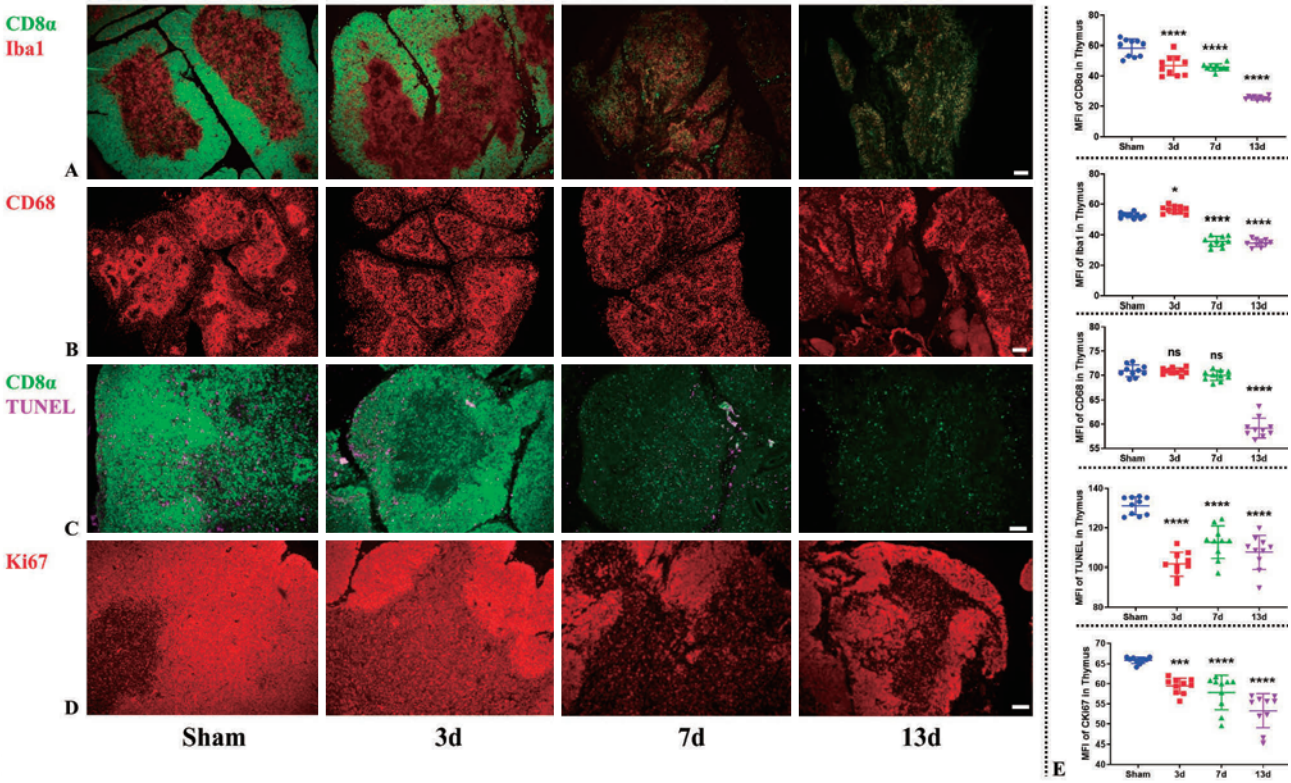

Fig. 4 Histology of thymuses. Panel A shows photomicrographs of Iba1+ (red) and CD8α+ cells (green). Panel B shows CD68+ cells (red). Panel C shows CD8α+ cells (green) and TUNEL+ cells (purple). Panel D shows Ki67+ cells (red). Scale bars, 200 μm (A, B), 100 μm (C, D). Panel E shows the MFIs of each marker in thymuses. *P<0.05, ***P<0.001, ****P<0.0001 and ns, no significance; Sham vs. 3d/7d/13d.

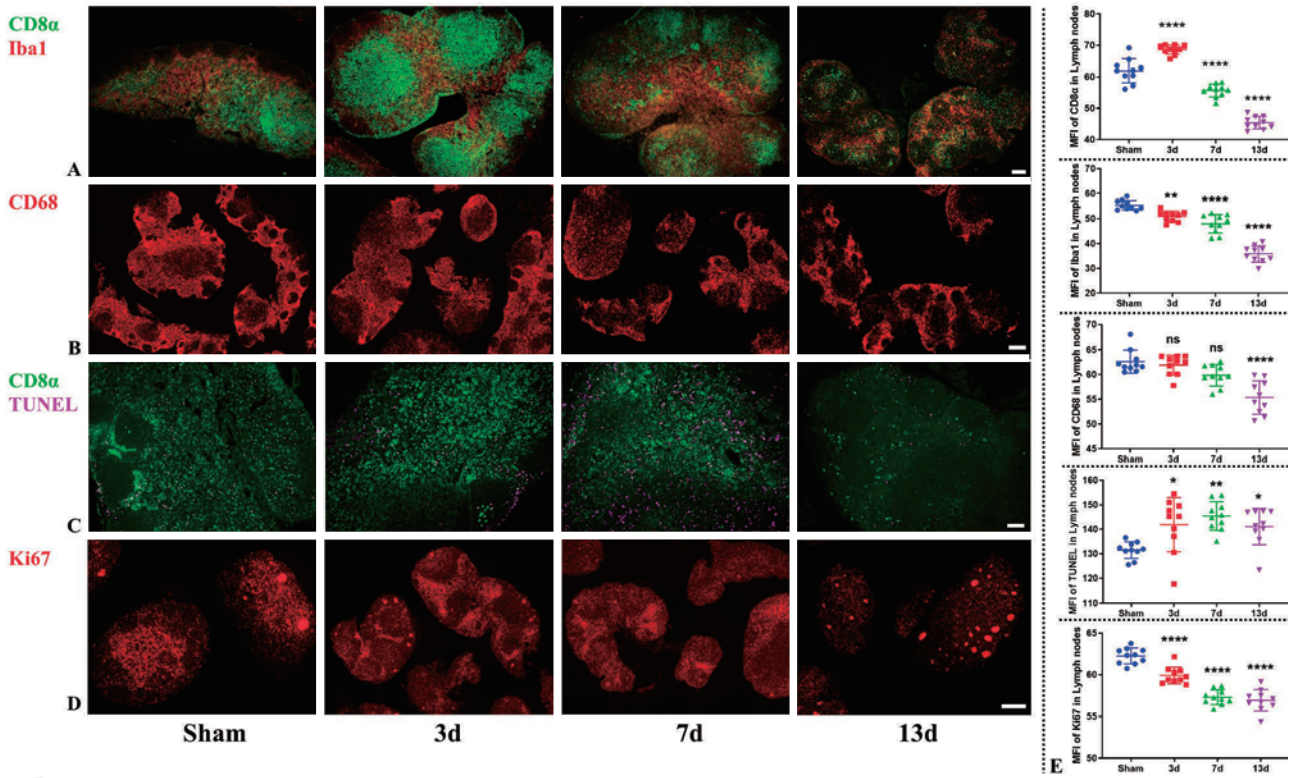

Fig. 5 Histology of mesenteric lymph nodes. Panel A shows photomicrographs of Iba1+ (red) and CD8α+ cells (green). Panel B shows CD68+ cells (red). Panel C shows CD8α+ cells (green) and TUNEL+ cells (purple). Panel D shows Ki67+ cells (red). Scale bars, 200 μm (A, B), 100 μm (C, D). Panel E shows the MFIs of each marker in mesenteric lymph nodes. *P<0.05, **P<0.01, ****P<0.0001 and ns, no significance; Sham vs. 3d/7d/13d.

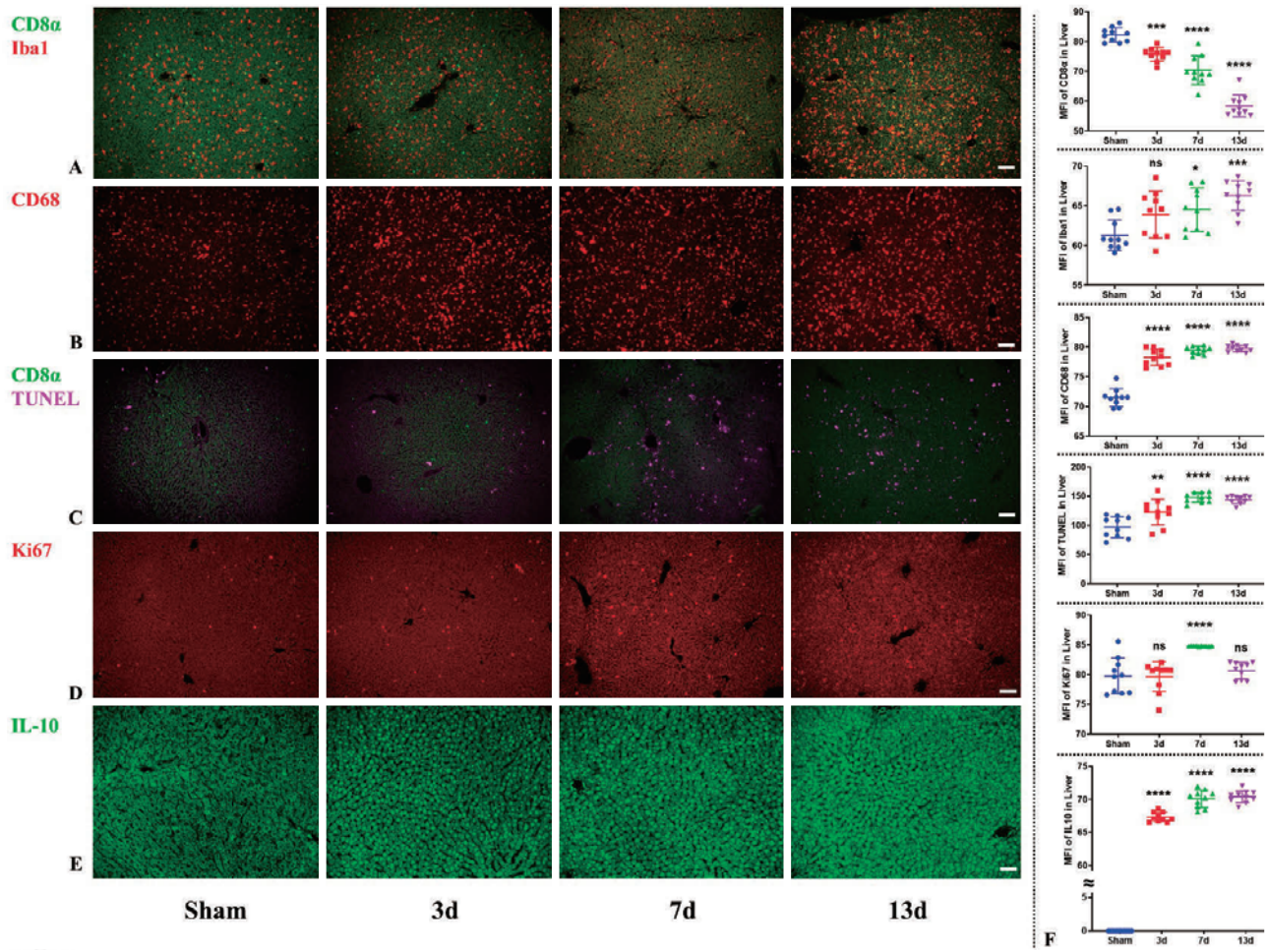

Fig. 6 Histology of livers. Panel A shows photomicrographs of Iba1$^+$ (red) and CD8α$^+$ cells (green). Panel B shows CD68$^+$ cells (red). Panel C shows CD8α$^+$ cells (green) and TUNEL$^+$ cells (purple). Panel D shows Ki67$^+$ cells (red). Panel E shows IL10$^+$ cells (green). Scale bars, 100 μm (A-D), 50 μm (E). Panel F shows the MFIs of each marker in livers. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 and ns, no significance; Sham vs. 3d/7d/13d.

	CD8a	Iba1	CD68	TUNEL	Ki67	IL-10
Spleen	↓↓↓	↓↓	↓↓	↑↑↑	↓↓	
Thymus	↓↓↓	↓↓↓	↓↓	↓↓	↓↓	
Lymph node	↓↓	↓	↓	↑	↓	
Liver	↓	↑↑	↑	↑	↑	↑↑
Brain	↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑	

Fig. 7 Summary of alterations in numbers of cells in systemic immune organs after stroke. ↑ ↑ ↑ , markedly increased; ↑ ↑ , increased; ↑ slightly increased. ↓ ↓ ↓ , markedly reduced; ↓ ↓ , reduced; ↓ ; slightly reduced.

tory 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 greater range of brain damage[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 and induces autoaggressive immune responses to facilitate infiltration of immune cells into the brain[7,23]. More recently, several studies reported that persons with stroke have higher titers of antibodies to CNS antigens such as portions of the *N*-methy1-*D*-aspartate (NMDA) receptor and neurofilaments[24,25].

The present histological assessment showed that the number of CD8α$^+$ cytotoxic T cells rapidly decreased in all immune organs after stroke. In contrast, large numbers of these cells appeared in the brain infarct area. CD8 α$^+$ T cells are likely released from peripheral organs into the circulation and accumulate around the area of brain injury to contribute to the secondary inflammatory cascade in the brain and induce more severe damage. After the number of CD8α$^+$ T cells in the immune organs decreases, the regions in which they were originally abundant, such as the white pulp of the spleen and cortex of thymus began to shrink. Proportions of Iba1$^+$ and CD68$^+$ macrophages increase correspondingly. However, our observation of histological changes during the 13 days after stroke showed that the number of macrophages also gradually decreased in spleen, thymus, and mesenteric lymph node. The large number of macrophages accumulated around the infarct area after the stroke suggests that activated macrophages were released into peripheral blood and later worsened brain damage.

Stroke-associated infection (SAI) frequently develops after severe cerebral ischemic stroke[26,27]. SAI is associated with decreased consciousness, abnormal brainstem reflexes, and use of invasive maneuvers. However, other clinical clues suggest that SAI could also result from CNS-induced immunodepression[28,29]. In the present TUNEL analysis, large numbers of cells, especially those present 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$^+$ proliferating cells also decreased in the red pulp. This, along with the marked decrease in the number of CD8α$^+$ T cells in immune organs, suggests that the body might regulate cell apoptosis in immune organs after severe post-ischemic inflammation. However, the underlying signal process that results in widespread immunosuppression is not well understood. Some researchers have proposed that apoptosis of immune cells results from overactivation of the sympathetic nervous system, which induces alterations of lymphocytes and monocytes[22,30]. This vicious cascade could lead to collapse of the immune system in the advanced stage of stroke injury, thereby causing atrophy of immune organs, cell depletion, and, ultimately, pneumonia and other serious fatal infections.

The liver is the most important organ in metabolism and detoxification in the body. It contains a unique population of macrophages called Kupffer cells, which have a role in immunity. In this study, the number of macrophages increased in liver after the stroke, which was not the case in other immune organs. Furthermore, an increasing number of cells expressed IL-10 in liver. Because Kupffer cells are primarily immunosuppressive[31,32], they can mediate suppression through their synthesis of nitric oxide, secretion of IL-10, and induction of CD4$^+$ T-cell apoptosis[33–35]. We hypothesize that when stroke-induced necrotic cell fragments and toxic substances reach the liver, these proinflammatory mediators activate Kupffer cells, thus inducing anti-inflammatory effects. Therefore, the liver might play an immunosuppressive role in the inflammatory response to stroke.

A limitation of our study was that STAIR and STEPS criteria recommend a surveillance period of at least 1 month for behavioral phenotyping. However, in this study, NSS was used to indicate stroke severity in rats that were sacrificed for TTC staining at 13 days after MCAO. Therefore, NSS was monitored only until day 13. We expect to 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-they also deplete immune cells. The collapse of the immune system could eventually cause fatal infection. These results suggest that systemic treatment might be a good strategy for cerebral stroke care.

Fundings: This study was supported in part by National Natural Science Foundation of China (NSFC) No. 81901780

and JSPS KAKENHI Grant Number JP20H03626.

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

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(Received, September 8, 2019)
(Accepted, July 31, 2020)
(J-STAGE Advance Publication, August 31, 2020)