Dynamics of T Lymphocyte Phenotypes Between the Periphery and the Brain From the Acute to the Chronic Phase Following Ischemic Stroke in Mice

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Research

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Abstract

Background

T lymphocytes are involved in infarct size at the early stage of stroke. However, the phenotypes of T lymphocytes and their functions in peripheral immune organs and the brain have not been well-analyzed from the acute to the chronic phase of stroke.

Methods

A 45 min transient middle cerebral artery occlusion mouse model was used. The phenotypes of T lymphocytes in the thymus, spleen, blood, and brain were determined using the neurological severity score (NSS) and body weights during the 6-month follow-up.

Results

Impairment of thymocyte numbers, development, proliferation, and apoptosis was observed for up to 2 weeks. The number of mature T cells in the spleen and blood decreased and showed less interferon-\(\gamma\) production for up to 2 weeks. Increased numbers of CD44+CD62L- effector T cells and CD4-CD8-CD3+ double negative T cells were observed in mouse brains in the early phase of stroke, while interleukin (IL)-10+Foxp3+ regulatory T cell levels increased for 1 week during the chronic phase. These phenotypes were correlated with body weight and the NSS.

Conclusions

The recovery of T lymphocyte numbers and increased IL-10+Foxp3+ regulatory T lymphocytes may be important for the improvement of long-term neurological outcomes. Dynamic changes in T lymphocytes from the acute and chronic phase may play different roles, such as pathological and recovery roles, respectively. This study provides fundamental information regarding the T lymphocyte alterations from the brain to the peripheral immune organs from the acute to the chronic phase of stroke.

Introduction

Stroke is the second most common cause of death and the third most common cause of disability-adjusted life worldwide [1, 2]. Ischemic stroke occurs when the blood flow to the brain becomes blocked. Without oxygen and glucose, neurons undergo cell death. The process of cell death in the brain following a stroke leads to the activation of local immune cells, causing inflammation in brain tissue. Inflammation plays an important role in the pathogenesis of ischemic stroke. Clinically, the susceptibility of patients to stroke and the subsequent prognosis are influenced by systemic inflammatory processes [3, 4]. Patients who undergo stroke with systemic inflammation exhibit clinically poorer outcomes [5, 6].

Peripheral immune cells are involved in systemic inflammation. Animal studies and clinical data consistently reveal a drastic shrinkage in spleen size following stroke, which is accompanied by a
reduced number of splenocytes [7, 8]. Further studies have reported that a splenectomy prior to middle cerebral artery occlusion (MCAO) in both rats and mice decreases infarct volume and improves behavioral deficits up to 96 h after stroke [9, 10], indicating a pathological role of splenocytes in the acute phase of stroke. The spleen contains a wide variety of immune cells, including lymphocytes, monocytes, and natural killer cells. Among them, lymphocyte-deficient Rag1-/- and SCID mice have shown sustained small infarct volumes and improved neurologic deficits within 24 h of MCAO [11, 12]. CD4+ or CD8+ T lymphocyte deficiency decreases the infarct volume after stroke [12] indicating that T lymphocytes play a pathological role in acute ischemic stroke. Most previous studies have focused on the early stages of stroke between 24 and 96 h. A recent study on rats assessed the function of the spleen and its effects on long-term outcomes after stroke for up to 28 d [13]. In this study, the spleen was removed immediately after reperfusion or 3 d after stroke. However, delayed splenectomy failed to provide long-term protection to the ischemic brain or improve functional recovery, such as the recovery of sensorimotor and cognitive functions. The authors suggest that the loss of neuroprotection might be related to the prolonged decrease in the percentage of T cells. These results suggest that T lymphocytes play a detrimental role at the early stage of stroke, and may have a protective role in long-term recovery. However, the phenotypes of T lymphocytes from the acute to chronic phase have not yet been fully elucidated. Most previous studies have focused on the early stages of stroke, rather than all phases of the disease. Therefore, we focused on the pathological phenotype of systemic immune alterations and neurological behavior, especially those of T lymphocytes from day 1 to 6 months after stroke. As mature T lymphocytes are generated from the thymus and stroke induces thymic atrophy [7, 14], we further investigated the impairment and changes in thymocytes after stroke, including T lymphocytes in the brain. This study aimed to provide an understanding of the basis of peripheral (including thymus, spleen, and blood) immune cell alterations following stroke for effective clinical interventions. We aimed to provide a system-based assessment of peripheral responses to cerebral ischemia and show dynamic T cell movement and roles beyond the brain.

**Methods**

**Animals**

Male C57BL/6 mice (10–12 weeks old) were used in this study (Orient, Seongnam, South Korea). Mice were housed in a specific pathogen-free barrier facility at Inha University. All animal experiments were approved by our Institutional Animal Care and Use Committee (INHA 170908-513-1).

**Transient MCAO (tMCAO) stroke model**

The tMCAO stroke model was induced via MCAO using a 6-0 nylon monofilament suture (Doccol Corporation, Sharon, MA, USA). The mice were anesthetized with 2% isoflurane (Kyongbo Pharmaceutical Co. Ltd, Asan, South Korea) in 30% oxygen and 70% nitrous oxide using a face mask. The mice were placed on a heat pad throughout the procedure. After anesthetization, a midline neck incision was made. The left common carotid artery (CCA), internal carotid artery (ICA), and external carotid artery (ECA) were
exposed. One knot was made in the CCA, and two were made in the ECA. After cutting between the two knots in the ECA, the suture with a round, coated tip (filament size 6-0, diameter 0.09-0.11 mm, length 20 mm; diameter of coated tip 0.25+/- 0.02 mm, length 5-6 mm, Cat# 602556PK10Re; Doccol Corporation, Sharon, MA, USA) was inserted into the ECA lumen and then gently advanced into the ICA lumen to block MCA blood flow and left there for 45 min until reperfusion. In sham-operated mice, after incision of the skin and the exposure of blood vessels (as in tMCAO), a knot was made in the CCA. After 10 min, this knot was removed and the skin was sealed. Mice were euthanized at various time points.

**Neurological severity score (NSS)**

The NSS was determined and recorded using the following four parameters:

**Zea-longa: 5 score**

The Zea-longa score was determined by observing the movement of mice in and out of the cage. Score 0: mice had no neurological deficit. Score 1: mice could not fully extend the left forepaw. Score 2: mice circled to the left while walking. Score 3: Mice fell to the left orlimped. Score 4: mice were unable to walk spontaneously. Score 5: death [15].

**Prehensile traction: 4 score**

The ability of the mice to grab and hold on to a 0.5 cm-diameter wire elevated at a height of 70 cm from a surface was tested. Performance was evaluated on a four-grade score. Score 0: mice could hang on the wire for over 5 s with the hind legs placed on the wire. Score 1: mice could hang on the wire for 5 s. Score 2: mice could hang on the wire for 3–4 s. Score 3: mice could hang on the wire for 0–2 s. In scores 1–3, the mice were unable to place their hind legs on the wire [16].

**Circling: 5 score**

Circling behavior was determined by observing and scoring the ability of the mice to remain in contact with the floor with their forelegs. Score 0: mice had no observable deficit. Score 1: mice exhibited flexion of the contralateral torso. Score 2: mice circled clockwise. Score 3: mice continuously spun clockwise. Score 4: there were no movements or reactions [17].

**Forelimb: 5 score**

The mice were held by their tail, and the bending of the torso or forelimbs in the air was observed and scored. Score 0: normal reaction. Score 1: mice occasionally exhibited flexion of the asymmetric forelimb. Score 2: asymmetric forelimb flexion. Score 3: mice exhibited flexion of the asymmetric forelimb and torso. Score 4: there was no motor activity or reaction [17].

**Cresyl violet staining**
Brains were fixed in 4% paraformaldehyde (PFA; Biosesang, Seongnam, South Korea) and the vibratome sections were stained with 0.1% cresyl violet (Sigma-Aldrich, St. Louis, MO, USA) in distilled water. The infarct areas were measured using ImageJ software (NIH, Bethesda, MD, USA).

**Flow cytometric analysis of cell populations in the blood, spleen, and thymus**

After anesthesia, blood was collected via retro-orbital bleeding using sterile capillary tubes (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany). The blood samples (50 µL) were stained using different antibodies at 4°C for 20 min and then the red blood cells (RBCs) were removed with RBC lysis buffer (Sigma-Aldrich). The samples were washed once with fluorescence-activated cell sorting (FACS) buffer [phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA) and 20 mM ethylenediaminetetraacetic acid] and fixed with 4% PFA. Lymphocytes (1 x 10⁶) from the spleen and thymus were incubated with FACS antibodies at 4°C for 20 min. The cells were rinsed once with FACS buffer and fixed with 4% PFA. Cells were then analyzed via flow cytometry (BD FACS Verse™; BD Biosciences, San Jose, CA, USA), and FlowJo software (BD Biosciences, San Jose, CA, USA) was used for data analysis. FACS antibodies anti-CD3 (145-2C11), CD4 (RM4-5, GK1.5), CD8 (53-6.7), CD44 (IM7), CD25 (3C7), CD45R/B220 (RA3-6B2), and T-cell receptor (TCR)-β (H57-597) were purchased from BD Biosciences. Anti-CD3 (17A2), was purchased from BioLegend (San Diego, CA, USA). Anti-CD8 (5H10) and CD62L (MEL-14) were purchased from Invitrogen (Carlsbad, CA, USA).

**Thymocyte proliferation assay in vivo using BrdU**

One day after tMCAO, 150 µl of BrdU (10 mg/mL) was injected intraperitoneally into the tMCAO and sham mice. The thymus was removed 2 h later and BrdU-positive cells were analyzed using a BrdU assay kit, according to the manufacturer's protocol (BD Biosciences).

**Cell culture and cytokine analysis using enzyme-linked immunosorbent assay (ELISA)**

First, 1 x 10⁶ lymphocytes from the spleen were stimulated with 0.1 µg/mL of anti-CD3 (145-2C11) and anti-CD28 (37.51) antibodies (BioGems, Westlake Village, CA, USA) in Roswell Park Memorial Institute (RPMI) 1640 (HyClone, GE Healthcare Life Science, PA, USA) culture medium containing 0.1% 2-mercaptoethanol (Thermo Fisher Scientific, MA, USA), 10% heat-inactivated fetal bovine serum (FBS; Gibco®, Australia origin, Thermo Fisher Scientific), and 1% antibiotic-antimycotic (Thermo Fisher Scientific) in a 24-well plate and incubated at 37°C in a 5% CO₂ incubator. After 24 and 48 h of stimulation, interleukin (IL)-2, IL-4, IL-13, and interferon (IFN)-γ levels in the culture medium were quantified using ELISA kits according to the manufacturer's protocol (BD Biosciences).

**Isolation of T lymphocytes from the brain**

T lymphocytes from the brain were isolated as described previously, with some modifications [18]. A Percoll gradient protocol was used. The stock isotonic Percoll (SIP) was prepared by mixing nine parts of Percoll (Sigma-Aldrich) with one part of 10× PBS without calcium chloride or magnesium sulfate. Each
hemisphere of the brain was minced using a homogenizer in a 1.5 mL tube with RPMI 1640 containing
0.1% 2-mercaptoethanol, 10% FBS, and 1% antibiotic-antimycotic. Digestive enzymes, including
collagenase Type I (2 mg/mL, Sigma-Aldrich) and DNase Type II (40 μg/mL, Sigma-Aldrich) in 1× DPBS
without calcium chloride or magnesium sulfate (GenDEPOT, Texas, USA) were added after
homogenization and incubated at 37°C for 45 min. The cell suspension was then passed through a 70
μm cell strainer and rinsed with 1× DPBS, and the volume was made up to 7 mL. Next, 3 mL of SIP was
added to the cell suspension to obtain a final concentration of 30% SIP. The cell suspension (10 mL in
30% SIP) was layered on top of 2 mL of 70% SIP in a 15 mL conical tube to make a total volume of 12
mL. After centrifugation at 2000 rpm for 20 min continuously at room temperature (RT), 3–4 mL of the
white layer at the 70–30% interface was collected into a new tube. After further centrifugation at 1500
rpm for 3 min, the cells were rinsed once with FACS buffer or 1× PBS.

Immunohistochemistry

Mice were transcardially perfused with saline solution containing 0.5% sodium nitrate and heparin (10
U/mL), and then fixed with 4% PFA dissolved in 0.1 M phosphate buffer. Brain tissues were dissected
from the skull, post-fixed overnight in buffered 4% PFA at 4°C, and stored in a 30% sucrose solution for
24–48 h at 4°C until they were settled, frozen, and sectioned on a cryostat in 30 μm-thick coronal
sections. Tissue sections were washed in cold PBS three times for 10 min and blocked with universal
blocking solution (0.3% Triton™ X-100, 1% BSA, 0.05% Tween 20, and 0.05% sodium azide in PBS) for 1 h
at RT. Sections were then incubated with the following primary antibodies: rat anti-Foxp3 (1:100,
Invitrogen) and anti-IL-10 (1:90, R&D system) for 2 h at RT. Thereafter, the tissues were rinsed and
incubated with Alexa Fluor 488 donkey anti-rabbit IgG (1:400, Invitrogen) and Alexa Fluor 594 goat anti-
mouse IgG (1:400, Invitrogen) for 1 h. After washing with PBS, coverslips were mounted on glass slides
using mounting media with DAPI (Vector Laboratories), and were analyzed using a confocal microscope
(LSM 800, Carl Zeiss). For CD4 staining, tissues were stained with rabbit anti-CD4 (1:400, Santa Cruz) at
4°C overnight. The following day, tissues were rinsed and incubated with Cy3-conjugated-anti-rabbit IgG
(1:800, Millipore) for 1 h. Labeled tissue sections were mounted on gelatin-coated slides and analyzed
under a confocal microscope (LSM 700, Carl Zeiss).

Statistical analysis

Student’s t-test was used to compare two independent groups of data that were properly distributed.
Values of *p ≤ 0.05, ** p ≤ 0.01, and ***p ≤ 0.001 were considered significant.

Results

Evaluation of infarct area and neurological impairment from day 1 up to 6 months in tMCAO mice

First, we evaluated infarct volume from day 1 up to 6 months. Brain damage after tMCAO was measured
using cresyl violet staining. The extent of brain damage was evaluated as a percentage of the infarct area
in the acute phase (1 d and 1 week) and as atrophy in the chronic phase (2 weeks, 2 months, and 6
As stroke causes atrophy in the chronic phase, the brain volume representing non-injured tissue (NI), ischemic scar tissue (IS), remaining total ipsilateral tissue T (NI + IS), and resorbed tissue (estimated infarct: EI) was analyzed according to the methods of a previously reported study [19]. We found that cerebral ischemia caused infarction that peaked at 1 week (Fig. 1A and 1B). In addition, the neurological impairment status of tMCAO mice was assessed via NSS at different time points. Compared to the sham-operated mice, the tMCAO mice showed decreased body weight and neurological dysfunction in four parameters of NSS up to 2 weeks (Fig. 1C and 1D). Neuronal atrophy was observed between 2 weeks and 6 months. Improvement of animal body weight and behavior was observed after 2 weeks. Next, we evaluated the alterations in immune organs from the acute to the chronic phases after stroke.

**Impairment of thymocyte development in tMCAO mice**

Although thymus shrinkage occurs in the early stages of stroke [7, 14], their phenotypes have not been well-analyzed long-term in tMCAO mice. Here, we evaluated the phenotypes of thymocytes from day 1 up to 6 months after stroke. Strong thymic shrinkage and decreased thymocyte numbers peaked at 1 week after stroke and then gradually recovered (Fig. 2A and 2B). The number of thymocytes was restored to the level observed in sham-operated mice after 2 months (Fig. 2B). The thymocyte sub-population is well-defined according to CD4 and CD8 expression; double-negative (DN: CD4-CD8-), double-positive (DP: CD4+CD8+), and single-positive (CD4+CD8- or CD4-CD8+). The percentage of the DP subpopulation dramatically decreased until 1 week after stroke (Fig. 2C). The absolute cell number in each subpopulation was significantly reduced until 2 weeks after stroke (Fig. 2D). Although stroke induces apoptosis by releasing stress hormones [14], we found that this may not be the only explanation for thymic shrinkage. The thymocyte development process was identified based on the expression of specific cell surface markers, such as CD4, CD8, CD44, and CD25. The DN population was divided into four stages; DN1 (CD44+CD25-), DN2 (CD44+CD25+), DN3 (CD44-CD25+), and DN4 (CD44-CD25-). The percentage of DN1 increased, whereas the percentage of DN4 decreased until 1 week after stroke (Fig. 3A and 3B). These results suggest that stroke may arrest development from DN1 to DN2. As the thymus produces thymocytes daily, and a certain number mature and circulate in the periphery, we analyzed whether stroke inhibited thymocyte proliferation. BrdU was injected into the mice 1 d after MCAO, and BrdU-incorporated thymocytes were analyzed via flow cytometry. As seen in figure 3C, stroke completely blocks the proliferation of thymocytes. Furthermore, we found that stroke increased the percentage of TCR-β+ thymocytes in the DN stage up to 1 week after stroke (Fig. 3D and 3E). Thymocytes that do not undergo appropriate TCR gene rearrangement undergo apoptosis, whereas those that express appropriate TCRs interact with peptides, determining the positive and negative selection essential for thymocyte differentiation [20, 21]. This increased inappropriate TCR-β+ population in the DN stage might be a reason for cell death. As CD4-CD8-CD3+ (DN CD3+ T cells) and CD4+Foxp3+CD3+ thymus-derived natural regulatory T cells (nTregs) play a role in the pathogenesis of the brain in stroke [22, 23], we analyzed these cells in the thymus. Interestingly, both populations increased in the thymus 1 week after stroke (Fig. 3F). Inappropriate thymus development was observed until 2 weeks after stroke. This time
frame is correlated with the recovery of the NSS and body weight. It is likely that appropriate thymic development is important for long-term recovery.

**Immunosuppression of T lymphocytes in the spleen of tMCAO mice**

Consistent with the decreased total thymocyte numbers in the tMCAO mice, the spleen size and total number of splenocytes were significantly reduced until 1 week after stroke (Fig. 4A-C). To determine whether stroke induced the activation of T lymphocytes, splenocytes were analyzed with CD44 and CD62L antibodies using flow cytometry. The percentage of CD44+CD62L- (effector-type) T lymphocytes in the spleen was significantly decreased in both CD4+ and CD8+ populations 1 week after stroke (Fig. 4D and 4E). In addition, to test whether ischemic stroke downregulates T lymphocyte functions, splenocytes were stimulated with anti-CD3 and -CD28 antibodies. After 48 h, the expression level of IFN-γ decreased at day 1 and week 1 (Suppl. Fig. 1A). Consistently, the fluorescence intensity of CD25+ significantly decreased 1 week after stroke in both CD4+ and CD8+ T lymphocytes compared to that in sham-operated mice (Suppl. Fig. 1B). This suggests that stroke induced the immunosuppressive function of T cells in the spleen at the early stage.

**Increased CD44+CD62L- T lymphocytes in the blood of tMCAO mice**

Circulating lymphocytes in whole blood were examined. In particular, the percentage of CD4+ T lymphocytes significantly decreased until 1 week after stroke compared to that in sham-operated mice (Fig. 5A and 5B). In contrast to the spleen, the percentage of CD44+CD62L- effector T lymphocytes in the blood significantly increased in both CD4+ and CD8+ populations until 1 week after stroke (Fig. 5C and 5D). Activated effector T lymphocytes were probably egressed from the spleen to the blood stream in tMCAO mice.

**Analysis of T lymphocytes in the brain of tMCAO mice**

To investigate the correlation between the thymus, spleen, blood, and brain after stroke, T lymphocytes in the brain were isolated using the Percoll gradient protocol and phenotypes were analyzed. The number of brain CD3+ T cells gradually increased until 2 weeks (Fig. 6A). The number of CD4+ and CD8+ T cells also gradually increased from day 1 up to 2 weeks. In addition, brain CD4-CD8-CD3+ double negative T cells (DNTs) play an inflammatory role in brain injury.[22] The number of DNTs rapidly increased 1 week after stroke (Fig. 6A). Interestingly, when we stained CD4+ T cells in the ipsilateral injured area, they reached a peak at 1 week, which was sustained for up to 6 months after stroke (Fig. 6B). This suggests that CD4+ cells were strongly infiltrated in the injured brain area after 1 week of stroke, and some cells were sustained in the tissue for up to 6 months. In addition, regulatory T cells in the brain may play a role in the regulation of inflammation after stroke [23, 24]. Therefore, we analyzed CD3+CD4+Foxp3+ Tregs in the brain. We observed an increased and sustained regulatory T cell population from 1 d to 2 weeks in the ipsilateral tMCAO brain (Fig. 7A). However, in the ipsilateral cortex injury area, Foxp3+ cells gradually increased up to 6 months. As some regulatory T cells express IL-10 cytokines, we stained both Foxp3 and IL-10 in the infarct area. Interestingly, IL-10 expression strongly increased from 2 weeks to 6 months, and
expressions of co-localized cells with Foxp3 also increased (Fig. 7B). This suggests that the increased levels of IL-10-producing Tregs in the injured area may inhibit inflammation, which may be correlated with increased T lymphocytes and recovery of the NSS and body weight.

Discussion

Here, we aimed to provide a system-based assessment of peripheral responses to cerebral ischemia as well as show dynamic T cell movement and roles beyond the brain from day 1 up to 6 months post-stroke. In our stroke model, the largest infarct size (IS) was observed 1 week post-stroke, and gradually decreased up to 6 months, whereas the EI size did not decrease. This means that the reduction of the infarct area does not necessarily indicate the recovery of neuronal cells from 2 weeks after tMCAO. However, consistent with the IS, the body weight and NSS recovered from 2 weeks post-stroke, which is correlated with the recovery of T lymphocytes. Interestingly, the NSS gradually recovered from 2 weeks to 6 months, which is correlated with increased IL-10+Foxp3 regulatory cells in the brain. The recovery of T lymphocyte numbers and increased IL-10+Foxp3+ regulatory T lymphocytes may be important for the improvement of long-term neurological outcomes.

Lymphocyte number and IFN-γ production decrease up to 2 weeks post-stroke [14], in which the β2-adrenoreceptor antagonist propranolol and a glucocorticoid receptor inhibitor RU486 are involved in the recovery of early lymphocyte activation. However, a detailed analysis of T lymphocytes in relation to brain damage was not reported alongside these findings. Here, we analyzed T lymphocytes in lymphoid organs at the same time in the brain from day 1 up to 6 months to show dynamic T lymphocyte movement. We observed a decreased number of T lymphocytes up to 1 week post-stroke, and the cell numbers recovered from 2 weeks. The percentage of CD44+CD62L- effector T lymphocytes increased in the blood but decreased in the spleen. In addition, we observed increased levels of effector T lymphocytes in tMCAO mice (Supplementary Fig. 2). This suggests that effector T lymphocytes egress from the spleen and enter the injury sites of the brain through the circulating blood stream in the early stage of stroke, whereas naïve T cells likely remain in the spleen. In addition, we observed decreased CD25 expression and cytokine production (such as IFN-γ) up to 1 week post-stroke, when the splenocytes were stimulated with anti-CD3 and anti-CD28 antibodies. It is likely that intrinsic TCR signaling is affected by stroke. Stress hormones, especially catecholamines released by immune cells or the HPA axis, serve as modulators to reduce lymphocyte proliferation and differentiation, and induce cell apoptosis through the β2-adrenergic receptor [25-27]. Here, we also observed a markedly reduced size of the thymus and number of thymocytes in mice following stroke. The cause of thymocyte loss may be stress hormones. The administration of a β2-adrenergic receptor agonist, such as isoproterenol, leads to a decrease in thymocyte number and thymus weight [28]. Moreover, glucocorticoids induce the apoptosis of thymocytes in vivo and in vitro, especially in the DP population [29-31]. As T lymphocytes express catecholamine [32], the dynamic changes in catecholamine levels in immune organs and T lymphocytes post-stroke should be elucidated to understand the relationship between T lymphocytes and neurological disability.
Moreover, we observed the developmental block in the DN stage and the inhibition of thymocyte proliferation at day 1 post-stroke. This means that stroke induced not only thymocyte cell death but also inhibited the development and proliferation of thymocytes in the acute phase. This phenomenon appears to be one of the reasons for T lymphocyte loss in the peripheral immune system. Various selection mechanisms operate in the thymus and, among them, negative selection causes the apoptosis of thymocytes with high TCR gene expression [33-35]. Thymocytes with high avidity TCR/ligand interactions undergo thymic selection due to mutual antagonism between the TCR-mediated signals and glucocorticoids in the thymus [36-38]. It is possible that stroke-induced glucocorticoids increase TCR-β expression and induce the death of immature thymocytes through negative selection in the thymus. As glucocorticoid metabolic enzymes are expressed in thymocyte subsets [37], whether stroke can affect these enzymes requires further investigation.

Recently, several studies have reported the various roles of infiltrating T lymphocytes in the brain following stroke [39-41]. Infiltrating DNTs promote microglia-mediated neuroinflammation and increase brain injury in the acute phase following a stroke [22]. However, where the DNTs in brain injury sites following a stroke are primarily derived from is currently unclear. We found that the number of DNTs as well as CD4+ and CD8+ T cells increased gradually from 1 d to 2 weeks in the brain following a stroke. We also observed an increase in the percentage of DNTs in the thymus 1 week post-stroke. Moreover, we observed an increased number of brain CD3+CD4+Foxp3+ Treg cells in the chronic phase up to 6 months post-stroke. Ito et al. found that brain Treg cell numbers increased from 10 d after stroke, which may potentiate neurological recovery during the chronic phase of stroke [23]. They also reported that the brain Treg cells were thymus-derived effector cells. We also observed increased CD4+Foxp3+ nTreg cells in the thymus and gradually increased IL-10+Foxp3+ Treg cells in the brain, which may correlate with the improved NSS. Therefore, the exact role of this population should be investigated in future studies to identify potential therapeutic targets. Overall, T lymphocytes have different infiltration time points to the site of brain injury following a stroke, depending on their pro- or anti-inflammatory functions. Considering the large number of patients with post-stroke disorders, studies regarding the chronic phase are of great importance. Therefore, our study provides fundamental information regarding the T lymphocyte alterations in the brain and periphery from the acute to chronic phase of stroke, which may be helpful for developing potential therapeutic targets for stroke.

**Conclusions**

This study demonstrated that the recovery of T lymphocyte numbers in immune organs and increased IL-10+Foxp3+ regulatory T lymphocytes may be important for the improvement of long-term neurological outcomes. Dynamic changes in T lymphocytes from the acute and chronic phase may play different roles, such as pathological and recovery roles, respectively. This study provides fundamental information regarding the T lymphocyte alterations from the brain to the peripheral immune organs from the acute to the chronic phase of stroke.
Abbreviations
tMCAO: transient middle cerebral artery occlusion; DN: CD4-CD8- double negative; DNT: CD4-CD8-CD3+ double negative T cells; nTreg: natural regulatory T cells; BrdU: 5-Bromo-2’-Deoxyuridine; NSS: neurological severity score

Declarations

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Authors’ contributions

Minha Kim and Myung-Shin Jeon wrote the paper and performed the most experiments. So-Dam Kim performed the tMCAO mouse model. Eun Hae Jeon, Min Gee Kim, Yu-Ree Lim, Enkhmaa Lkhagv-Yondon, Yena Oh, and Kwangmin Na performed the part of experiments. Young Cheul Chung, Byung Kwan Jin, Yun Seon Song, and Myung-Shin Jeon designed the research and provide discussion.

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Availability of data and materials

The datasets generated and/or analyzed in this study are available from the corresponding authors on reasonable request.

Ethics approval and consent to participate

All protocols were approved by the Animal Care and Use Committee of Inha University and conformed to the Guide for the Care and Use of Laboratory Animals by the National Institute of Health, USA.

Consent for publication

Not applicable

Competing interests

The authors declare no conflicts of interest.

References
1. Murray CJ, Vos T, Lozano R, Naghavi M, Flaxman AD, Michaud C, Ezzati M, Shibuya K, Salomon JA, Abdalla S, et al: Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* 2012, 380:2197-2223.

2. Lozano R, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, Abraham J, Adair T, Aggarwal R, Ahn SY, et al: Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* 2012, 380:2095-2128.

3. Emsley HC, Hopkins SJ: Acute ischaemic stroke and infection: recent and emerging concepts. *Lancet Neurol* 2008, 7:341-353.

4. McColl BW, Allan SM, Rothwell NJ: Systemic infection, inflammation and acute ischemic stroke. *Neuroscience* 2009, 158:1049-1061.

5. McColl BW, Rothwell NJ, Allan SM: Systemic inflammatory stimulus potentiates the acute phase and CXC chemokine responses to experimental stroke and exacerbates brain damage via interleukin-1- and neutrophil-dependent mechanisms. *J Neurosci* 2007, 27:4403-4412.

6. 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, 13:220-227.

7. Offner H, Subramanian S, Parker SM, Wang CH, Afentoulis ME, Lewis A, Vandenbark AA, Hurn PD: Splenic atrophy in experimental stroke is accompanied by increased regulatory T cells and circulating macrophages. *Journal of Immunology* 2006, 176:6523-6531.

8. Chiu NL, Kaiser B, Nguyen YV, Welbourne S, Lall C, Cramer SC: The Volume of the Spleen and Its Correlates after Acute Stroke. *Journal of Stroke & Cerebrovascular Diseases* 2016, 25:2958-2961.

9. Ajmo CT, Jr., Vernon DO, Collier L, Hall AA, Garbuzova-Davis S, Willing A, Pennypacker KR: The spleen contributes to stroke-induced neurodegeneration. *J Neurosci Res* 2008, 86:2227-2234.

10. Chauhan A, Al Mamun A, Spiegel G, Harris N, Zhu L, McCullough LD: Splenectomy protects aged mice from injury after experimental stroke. *Neurobiol Aging* 2018, 61:102-111.

11. Hurn PD, Subramanian S, Parker SM, Afentoulis ME, Kaler LJ, Vandenbark AA, Offner H: T- and B-cell-deficient mice with experimental stroke have reduced lesion size and inflammation. *J Cereb Blood Flow Metab* 2007, 27:1798-1805.

12. Yilmaz G, Arumugam TV, Stokes KY, Granger DN: Role of T lymphocytes and interferon-gamma in ischemic stroke. *Circulation* 2006, 113:2105-2112.

13. Ran YY, Liu ZJ, Huang S, Shen JM, Li FW, Zhang WX, Chen C, Geng XK, Ji ZL, Du HS, Hu XM: Splenectomy Fails to Provide Long-Term Protection Against Ischemic Stroke. *Aging and Disease* 2018, 9:467-479.

14. Prass K, Meisel C, Hoflich C, Braun J, Halle E, Wolf T, Ruscher K, Victorov IV, Priller J, Dirmagl U, et al: Stroke-induced immunodeficiency promotes spontaneous bacterial infections and is mediated by sympathetic activation reversal by poststroke T helper cell type 1-like immunostimulation. *J Exp Med* 2003, 198:725-736.
15. Cao Y, Sun N, Yang JW, Zheng Y, Zhu W, Zhang ZH, Wang XR, Shi GX, Liu CZ: Does acupuncture ameliorate motor impairment after stroke? An assessment using the CatWalk gait system. *Neurochem Int* 2017, 107:198-203.

16. Combs DJ, D'Alecy LG: Motor performance in rats exposed to severe forebrain ischemia: effect of fasting and 1,3-butanediol. *Stroke* 1987, 18:503-511.

17. Yang J, Ahn HN, Chang M, Narasimhan P, Chan PH, Song YS: Complement component 3 inhibition by an antioxidant is neuroprotective after cerebral ischemia and reperfusion in mice. *J Neurochem* 2013, 124:523-535.

18. Pino PA, Cardona AE: Isolation of brain and spinal cord mononuclear cells using percoll gradients. *J Vis Exp* 2011.

19. Kim E, Woo MS, Qin L, Ma T, Beltran CD, Bao Y, Bailey JA, Corbett D, Ratan RR, Lahiri DK, Cho S: Daidzein Augments Cholesterol Homeostasis via ApoE to Promote Functional Recovery in Chronic Stroke. *J Neurosci* 2015, 35:15113-15126.

20. Savino W: The thymus is a common target organ in infectious diseases. *PLoS Pathog* 2006, 2:e62.

21. Starr TK, Jameson SC, Hogquist KA: Positive and negative selection of T cells. *Annu Rev Immunol* 2003, 21:139-176.

22. Meng H, Zhao H, Cao X, Hao J, Zhang H, Liu Y, Zhu MS, Fan L, Weng L, Qian L, et al: Double-negative T cells remarkably promote neuroinflammation after ischemic stroke. *Proc Natl Acad Sci U S A* 2019, 116:5558-5563.

23. Ito M, Komai K, Mise-Omata S, Iizuka-Koga M, Noguchi Y, Kondo T, Sakai R, Matsuo K, Nakayama T, Yoshie O, et al: Brain regulatory T cells suppress astrogliosis and potentiate neurological recovery. *Nature* 2019, 565:246-250.

24. Liesz A, Suri-Payer E, Veltkamp C, Doerr H, Sommer C, Rivest S, Giese T, Veltkamp R: Regulatory T cells are key cerebroprotective immunomodulators in acute experimental stroke. *Nat Med* 2009, 15:192-199.

25. Bergquist J, Tarkowski A, Ekman R, Ewing A: Discovery of endogenous catecholamines in lymphocytes and evidence for catecholamine regulation of lymphocyte function via an autocrine loop. *Proc Natl Acad Sci U S A* 1994, 91:12912-12916.

26. Meisel C, Schwab JM, Prass K, Meisel A, Dirnagl U: Central nervous system injury-induced immune deficiency syndrome. *Nat Rev Neurosci* 2005, 6:775-786.

27. Schulze J, Vogelgesang A, Dressel A: Catecholamines, steroids and immune alterations in ischemic stroke and other acute diseases. *Aging Dis* 2014, 5:327-339.

28. Durant S: In vivo effects of catecholamines and glucocorticoids on mouse thymic cAMP content and thymolysis. *Cell Immunol* 1986, 102:136-143.

29. Brewer JA, Kanagawa O, Sleckman BP, Muglia LJ: Thymocyte apoptosis induced by T cell activation is mediated by glucocorticoids in vivo. *J Immunol* 2002, 169:1837-1843.
30. Herold MJ, McPherson KG, Reichardt HM: Glucocorticoids in T cell apoptosis and function. *Cell Mol Life Sci* 2006, 63:60-72.

31. Wang D, Muller N, McPherson KG, Reichardt HM: Glucocorticoids engage different signal transduction pathways to induce apoptosis in thymocytes and mature T cells. *J Immunol* 2006, 176:1695-1702.

32. Laukova M, Vargovic P, Vlcek M, Lejavova K, Hudecova S, Krizanova O, Kvetnansky R: Catecholamine production is differently regulated in splenic T- and B-cells following stress exposure. *Immunobiology* 2013, 218:780-789.

33. Baldwin KK, Trenchak BP, Altman JD, Davis MM: Negative selection of T cells occurs throughout thymic development. *J Immunol* 1999, 163:689-698.

34. Kurd N, Robey EA: T-cell selection in the thymus: a spatial and temporal perspective. *Immunol Rev* 2016, 271:114-126.

35. Van De Wiele CJ, Marino JH, Murray BW, Vo SS, Whetsell ME, Teague TK: Thymocytes between the beta-selection and positive selection checkpoints are nonresponsive to IL-7 as assessed by STAT-5 phosphorylation. *J Immunol* 2004, 172:4235-4244.

36. Vacchio MS, Papadopoulos V, Ashwell JD: Steroid production in the thymus: implications for thymocyte selection. *J Exp Med* 1994, 179:1835-1846.

37. Rocamora-Reverte L, Reichardt HM, Villunger A, Wiegers GJ: T-cell autonomous death induced by regeneration of inert glucocorticoid metabolites. *Cell Death & Disease* 2017, 8.

38. Mittelstadt PR, Taves MD, Ashwell JD: Cutting Edge: De Novo Glucocorticoid Synthesis by Thymic Epithelial Cells Regulates Antigen-Specific Thymocyte Selection. *Journal of Immunology* 2018, 200:1988-1994.

39. Brait VH, Arumugam TV, Drummond GR, Sobey CG: Importance of T lymphocytes in brain injury, immunodeficiency, and recovery after cerebral ischemia. *J Cereb Blood Flow Metab* 2012, 32:598-611.

40. Jian Z, Liu R, Zhu X, Smerin D, Zhong Y, Gu L, Fang W, Xiong X: The Involvement and Therapy Target of Immune Cells After Ischemic Stroke. *Front Immunol* 2019, 10:2167.

41. Selvaraj UM, Stowe AM: Long-term T cell responses in the brain after an ischemic stroke. *Discov Med* 2017, 24:323-333.