Improvement of ischemic stroke outcome by promoting CD4+CD25+ Treg migration through CCR4 overexpression

Seowoo Lee

Department of Medical Science

The Graduate School, Yonsei University

Improvement of ischemic stroke outcome by promoting CD4+CD25+ Treg migration through CCR4 overexpression

Directed by Professor Jong Eun Lee

The Master's Thesis
submitted to the Department of Medical Science,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Master of Medical Science

Seowoo Lee

December 2023

This certifies that the Master's Thesis of
Seowoo Lee is approved.

[Signature]

Thesis Supervisor : Jong Eun Lee

[Signature]

Thesis Committee Member#1 : Young-Min Hyun

[Signature]

Thesis Committee Member#2 : Je Sung You

The Graduate School
Yonsei University

December 2023

ACKNOWLEDGEMENTS

First, I would like to thank my supervisor Professor Jong Eun Lee and Dr. Jong Youl Kim for always providing the best support and guidance for this current project. Without their guidance I would have never been able to complete my master's research. I would also like to thank my thesis committee members, Professor Young-Min Hyun and Professor Je Sung You for taking their time and providing their insights and advice for this project.

Secondly, I would like to thank my family for providing everything they can to support me all throughout my time here. I thank both of my parents for always being there to help me and give all the advice concerning this project, as well as providing moral support. I cannot express my gratitude and love for them enough, but I hope they know that I always love them.

Next, I would like to thank the lab members. I thank Ji Won Kim for helping me since the very beginning for this project. She taught me everything she knew and could to help me get to this point. She truly is one of the best friend and teacher I have ever had. I also thank Ayoung Shim for providing me insightful advice and help to get me through everything.

Last but not least, I have to thank my colleague, Ara Goh. She is the one who helped me get through all the hard and toiling times. Thank you for always hearing my rants and senseless and silly jokes and complaint. Thank you for being a great friend. I hope we get to still be friends and hang out time to time after all this.

<TABLE OF CONTENTS>

ABSTRACT·····iv

I. INTRODUCTION····· 1
II. MATERIALS AND METHODS····· 4
1. Animal····· 4
2. Primary cell isolation····· 4
3. Cell culture····· 4
4. Microglia oxygen-glucose deprivation (OGD) ····· 5
5. Trans-well migration assay····· 5
6. Mouse chemokine profiling array ····· 6
7. Western blot ····· 6
8. Immunocytochemistry (ICC)····· 7
A. Fluorescence image analysis ·····8
9. CCR4 retrovirus vector design and MSCV packaging ····· 8
10. Treg transduction····· 9
11. Flow cytometry (FACS)····· 9
12. tMCAO surgery ·····10
13. Behavior testing ·····10
A. Bederson score·····11
B. Grip strength test ·····11
C. Elevated body swing test (EBST)····· ····· 11
14. 2,3,5-Triphenyltetrazolium chloride (TTC) test ·····11
15. Statistical analysis····· 12

III. RESULTS ····· 13
1. Both the CD8+ T cells and Tregs showed increased migration into the OGD(+) Direct media·····13

2. OGD(+) Direct media showed an increased level of pro-inflammatory immune cell chemoattractants……15
3. CXCR4 and CCR4 expressed highly on Tregs were selected as the target receptors……18
4. Tregs treated with CCR4 antagonist (sc-221406) showed decreased migration into OGD(+) Direct media……21
5. Tregs showed a mild increase in CCR4 expression after MSCV-CCR4 transduction……23
6. mCcr4 transduced Tregs showed increased migration into the OGD(+) Direct media……26
7. A decrease in infarct size and improved motor function was seen in tMCAO groups injected with Tregs transduced with MSCV-CCR4……28

IV. DISCUSSION ……31
V. CONCLUSION ……35

REFERENCES ……36
ABSTRACT(IN KOREAN) …… 41

LIST OF FIGURES

Figure 1. Trans-well migration assay of CD8+ T cells and Tregs in different primary microglia culture media·····14
Figure 2. Mouse chemokine profiling array of the conditioned primary microglia culture media·····16
Figure 3. Receptor expression confirmation using immunocytochemistry and western blot in CD8+ T cells and Tregs·····19
Figure 4. Observing CCR4 or CXCR4 antagonist treated Treg migration pattern using trans-well migration assay·····22
Figure 5. MSCV-CCR4 retrovirus transduction and CCR4 expression check through immunocytochemistry and flow cytometry·····24
Figure 6. Trans-well migration assay of MSCV-CCR4 retrovirus transduced Tregs into OGD(+) Direct media·····27
Figure 7. Improvement in infarct area and functional testing through TTC and behavior testing in Treg injection groups after tMCAO·····29

LIST OF TABLES

Table 1. Summary of the chemokines of interest and the receptors known to interact with·····17

ABSTRACT

Improvement of ischemic stroke outcome by promoting CD4+CD25+ Treg migration through CCR4 overexpression

Seowoo Lee

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Jong Eun Lee)

The influx of activated pro-inflammatory immune cells post-ischemic stroke significantly heightens the risk of morbidity and developing disability. In response to ischemic stroke (IS), the brain microglia activate and secrete chemokines related to immune responses in the ischemic core. There are numerous studies about neuroinflammation in the initial phase of IS. During the recovery phase of IS, CD4+CD25+Foxp3+ regulatory T cells (Tregs) showed positive effects by ameliorating neuroinflammation, thus reducing the infarct size post-IS. For Tregs to exert neuroprotective effects, trafficking Tregs into the ischemic brain must take place. However, the molecular mechanism of Treg migration into the brain has not yet been defined clearly. Here, we aim to investigate the specific chemokine-receptor interaction regarding the Treg migration during IS, as well as its effect on infarct size and any improvements in motor function. The differences in migratory patterns of the pro-inflammatory CD8+ T cells and Tregs were seen through trans-well migration assay in media collected from the primary microglia culture immediately after oxygen-glucose deprivation (OGD(+) Direct) and after 24-hour reperfusion (OGD(+) Rep). The conditioned microglia culture media were used in murine chemokine profiling array to identify the chemokines released by activated microglia during simulated IS conditions and during the recovery phase. Both the CD8+ T cells and Tregs showed higher migration into the OGD(+) Direct media in response to activated microglia secreting chemokines related to immune cell recruitment, especially T cell

homing. From the above, two candidate chemokine-receptor pairs intimately related to Treg migration were chosen. Then, Tregs were treated with antagonists to block the potential interaction with the above two pairs where the inhibition of CCL22-CCR4 interaction showed significantly diminished migration into the OGD(+) Direct media than that of CXCL12-CXCR4. Next, CCR4 overexpression on Tregs were done using MSCV retrovirus, which were then used to see their effects in vivo after tMCAO in mice. Injection of mCcr4 transduced Tregs showed notable decreased in infarct size and some improvement in motor function in mice after 24 hours post-tMCAO. The results of current study showed the specific factors involved in Treg migration in response to microglia activation, as well as the neuroprotective effects of Tregs in reducing the severity of neuronal damage post-tMCAO in animal models.

Key words : regulatory T cell, ischemic stroke, neuroinflammation, microglia

Improvement of ischemic stroke outcome by promoting CD4+CD25+ Treg migration through CCR4 overexpression

Seowoo Lee

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Jong Eun Lee)

I. INTRODUCTION

Ischemic stroke (IS) is caused by a blockage in the blood vessel leading into the brain, commonly due to thrombosis or embolism formation. The blockage of cerebral blood flow leads to oxidative stress and nutrient depletion. The above conditions together cause neuron cell deaths where the necrotic cells release inflammatory factors like the damage-associated molecular patterns (DAMPs) causing an immune response in the brain (1). Neuroinflammation exacerbates the secondary neuronal damage post-stroke, resulting in negative clinical outcomes in patients (1-6). The ischemic penumbra is exposed to oxidative stress, excitotoxicity, and mitochondria dysregulation, further aggravating neuronal damage. The neuroinflammatory response also leads to the release of various cytotoxic molecules like Matrix Metallopeptidase 9 (MMP-9), disrupting the blood-brain barrier (BBB) (1). The breakdown of BBB augments secondary damage by allowing pro-inflammatory immune cell infiltration into the ischemic brain. Within a few hours, immediate responders enter the brain, known to be neutrophils, monocytes, and cytotoxic CD8+ T cells (2, 3).

Not only the peripheral immune cells, but one of the immediate responders to IS are the brain resident microglia. Microglia undergo functional and morphological changes in

response to IS, into the classical M1 or the alternative M2 phenotype (7, 8). The M1 phenotype exerts a pro-inflammatory response, releasing the reactive oxygen species (ROS), and inflammatory cytokines like interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α). On the other hand, the protective M2 phenotype release transforming growth factor-β (TGF- β) and interleukin-10 (IL-10) and activate the debris clearance process by phagocytosis (7, 9). Not only this, but also the chemoattractants secreted by the activated microglia induce the migration of the inflammatory immune cells like monocytes and inflammatory T cells into the ischemic brain (10). In efforts to subdue the neuroinflammation, many previous studies focus on the inhibition and manipulation of M1 microglia (7, 8, 11).

IS is the most prevalent type of stroke worldwide. The AHA Statistical Update reported a steady overall increase in the number of IS incidents and related deaths (2). Although it is known to be a serious threat to one's health, the recombinant plasminogen activator (rtPA) is the only FDA-approved treatment to this day. Even though rtPA is an accepted treatment for acute IS, there are limitations still. Its use is limited by the time point at which it is applied to the patient, as well as geographically, necessitating the need to find other effective treatments. In search of a novel treatment, studies based on cell therapy, especially about CD4+CD25+Foxp3+ regulatory T cells (Tregs), are ongoing. Studying the role of Tregs in immune homeostasis in autoimmune diseases and cancer was the main focus of Treg research (12). Most studies on Tregs focus on its strong ability to suppress the anti-tumor immune response by the cytotoxic T cells. More recently, more research showed the repair potential of Tregs in various contexts like cardiovascular injury, lung infections, as well as central nervous system (CNS) repair upon spinal cord injury and IS (13-15).

Concerning the influx of the pro-inflammatory immune cells upon the breakdown of BBB, Tregs known for its anti-inflammatory response also migrate into the infarct site

(4, 5). In more recent years, several research proposed that Tregs and its anti-inflammatory functions have neuroprotective effects in the ischemic brain in relation to infarct size reduction and the neuron recovery (1, 7, 16). Tregs express inhibitory molecules like CTLA-4 and programmed cell death-ligand 1 (PD-L1), suppressing the pro-inflammatory immune cells. Tregs interacting with the activated microglia led to the alteration of M1 microglia to M2 by releasing IL-10 and TGF-β (5, 14, 17). Successful suppression of pro-inflammatory IL-17+ γδ T cell and MMP-9 secretion were seen in concert with the increase and accumulation of Tregs in the circulation and the ischemic penumbra (16, 18, 19). Tregs are known to accumulate in the ischemic penumbra region as soon as within 24 hours. Still, its accumulation is more prominent during the recovery phase, which is generally after a week up to 6 months post-stroke. Tregs are proposed to suppress the growth of infarct area and neuroinflammation, ultimately preventing the aggravation of neuronal damage and instead promote tissue repair (3, 19).

Concerning the previous studies on the role of microglia in recruiting peripheral immune cells into the ischemic brain, the molecular mechanism concerning the migration of Tregs into the ischemic brain is still to be elucidated. In addition to the above, there is still more clarification required about the therapeutic role of Tregs in the recovery phase of IS. In this present study, we aim to specify the contributors to Treg migration into the ischemic brain and its effect on infarct size and any improvement in the motor function of the tMCAO animal models.

II. MATERIALS AND METHODS

1. Animal

C57Bl/6 mice (male, 8-week-old) were purchased from Orient Bio Inc. (Gyeonggi-do, Korea). Mice were accommodated in the specific pathogen-free (SPF) environment at the Avison Biomedical Research Center, Yonsei University (Seoul, Korea).

2. Primary cell isolation

Spleen and brain were isolated from C57Bl/6 mouse, then were dissociated using either Spleen Dissociation Kit, mouse or Adult Brain Dissociation Kit, mouse and rat with Miltenyi gentleMACS system (Miltenyi Biotech, Cologne, Germany), respectively, according to the manufacturer's instructions. All cells were isolated using Miltenyi AutoMACS systems and their respective isolation systems. Splenocytes were further processed to isolate CD8+ T cells and Treg cells with CD8α+ T Cell Isolation Kit, mouse (Miltenyi Biotech, Cologne, Germany, #130-095-236) and CD4+CD25+ Regulatory T Cell Isolation Kit, mouse (Miltenyi Biotech, Cologne, Germany, #130-091-041), respectively. Homogenized brain suspension was used to isolate microglia using CD11b (Microglia) MicroBeads (Miltenyi Biotech, Cologne, Germany, #130-093-636). All procedures were done according to the protocols supplied by Miltenyi Biotec (Cologne, Germany).

3. Cell culture

Primary brain CD11b+ microglia cells were cultured in a PDL-coated 6-well plate. The 6-well culture plate was coated with PDL 24 hours before and washed 3 times with 1X PBS for 5 minutes each before seeding. Primary microglia were seeded at a density of $1.0x10^6$/mL in complete RPMI media (10% FBS, 1% Penicillin-streptomycin, 0.5 mM HEPES). Primary Tregs were cultured at a density of $1.5x10^6$/mL in a 24-well culture plate in complete RPMI media with recombinant IL-2 (Sigma-Aldrich, MO, USA) at 2500 U/mL. The isolated Tregs were activated overnight with Gibco™ Dynabeads™

Mouse T-Activator CD3/CD28 for T-Cell Expansion and Activation (Thermo Fisher Scientific, Inc., MA, USA, #11452D). Tregs were seeded at a density of $1.5x10^6$/mL in a 24-well flat-bottom culture plate and were activated at a Treg:Bead ratio of 1:2.

4. Oxygen-glucose deprivation (OGD)

The complete RPMI media from the primary microglia culture was removed and collected (Control), then it was replaced by RPMI-1640 (no FBS or P/S). Above primary microglia were exposed to OGD condition for 40 minutes using Thermo Forma Anaerobic System Model 1025 (Thermo Fisher Scientific, Inc., MA, USA). After exposing microglia in mild OGD condition, media was collected (OGD(+) Direct) and was replaced with complete RPMI-1640. Microglia culture was recovered in standard culture condition for 24 hours, then the media was collected (OGD(+) Rep).

5. Trans-well migration assay

First, 600 μL of Control, OGD(+) Direct, and OGD(+) Rep media were put into each well of the 24-well plate. Then, 24-well SPLInsert™ Hanging (SPL, Gyeonggi-do, Korea, #36024) with 200 μL cell suspension containing 9.0 x 10^5 of primary CD8+ T cells or Tregs were inserted carefully onto each well containing a different medium. They were cultured in standard culture conditions for 48 hours. The number of cells that migrated into each chamber containing different media was then determined with 33342 Hoechst dye (Thermo Fisher Scientific, Inc., MA, USA, #62249). After removing the trans-well inserts before imaging, the plate was briefly centrifuged at 400 g, RT, for 3 minutes. As for the antagonist treatment, CCR4 inhibitor (sc-221406) was obtained from Santa Cruz Biotechnology, Inc. (CA, USA, #864289-85-0) and CXCR4 inhibitor (WZ811) from MedChemExpress (NJ, USA, #HY-15478) were used. The CCR4 antagonist was treated at 20 nM, 40 nM, 60 nM, and 100 nM, and the CXCR4 antagonist was treated at 5 μM, 10 μM, 20 μM, and 50 μM. Tregs were incubated in each inhibitor for 2 hours in standard culture conditions. 0.02 mg/mL DMSO was added

in the antagonist-treated Treg groups as a negative control.

6. Mouse chemokine profiling array

Each collected medium sample was analyzed with the Proteome Profiler Mouse Chemokine Array Kit (R&D Systems Inc., Minneapolis, MN, #ARY020), following the protocols provided by the manufacturer. Each dot's intensity was measured through the ImageJ (Bethesda, MA, USA). In short, each provided cellulose membrane containing 25 capture antibodies was blocked, while 1 mL of each medium collected from control CD11b+ microglia culture and in OGD experiments was mixed with 0.5 mL of Array Buffer 4. 10 μL Detection Antibody Cocktail was added into each sample and was incubated for 1 hour at RT. Then each sample was added onto the cellulose membrane after blocking, then incubated on a shaker overnight at 4°C. Each membrane was washed in 1X Wash Buffer for 10 minutes three times. Membranes were incubated for 30 minutes on a shaker at RT in Streptavidin-HRP solution. Each membrane was incubated in Chemi Reagent Mix for 1 minute at RT, then visualized in LAS 4000 mini (Fujifilm, Tokyo, Japan).

7. Western blot

CD8+ T cell and Tregs were incubated in 1X PMSF Protease Inhibitor in 1X RIPA buffer (T&I, Gangwon-do, Korea) for 1 hour on ice. Each sample was vortexed briefly every 15 minutes. Then they were centrifuged at 14, 000 g for 10 minutes at 4°C.

For protein quantification, BSA standards were serially diluted, then reacted in 49:1 mix of Pierce™ BCA Protein Assay Reagents (Thermo Fisher Scientific, Inc., MA, USA, #23224, #23228), for 20 minutes in standard culture condition. 4–20% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad, CA, USA, #4561096) were used to separate 10 μL of each protein sample at 90 V. The gel was then transferred onto a polyvinylidene difluoride membrane at 20V for 15 hours. Membranes were retrieved and were washed briefly in 0.5% TBST buffer. Membranes were blocked in 5% bovine

serum albumin (BSA) solution for 1 hour, then were incubated in primary antibody solutions diluted in 3% BSA solution overnight at 4°C. Membranes were washed, following incubation in secondary antibody diluted in 3% BSA solution for 2 hours at RT on a shaker. Membranes were washed, then the protein bands were visualized using the Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc., MA, USA, #32109). The images were taken using the chemiluminescent image analyzer LAS 4000 mini (Fujifilm, Tokyo, Japan), then the grayscale values were measured using ImageJ (Bethesda, MA, USA).

Detection of each protein was done using the following antibodies: CXCR4 [UMB2] (monoclonal, Abcam, Cambridge, UK, #ab124824, 1:100), CD4 [9H5A8] (monoclonal, Invitrogen, MA, USA, #MA5-15774, 1:500), CCR4 (polyclonal, Abcam, Cambridge, UK, #ab1669, 1:100), CCR10 (polyclonal, Invitrogen, MA, USA, PA1-21617, 1:100), and β-actin [mAbcam 8226] (monoclonal, Abcam, Cambridge, UK, #ab8226, 1:1000). The secondary antibodies such as goat anti-mouse IgG HRP (Thermo Fisher Scientific, Inc., MA, USA, #sc245915, 1:500), goat anti-rabbit IgG HRP (Thermo Fisher Scientific, Inc., MA, USA, #sa245916, 1:500), and goat anti-rat IgG H&L HRP (Abcam, Cambridge, UK, #ab205720, 1:500) were used.

8. Immunocytochemistry (ICC)

The cells were cultured in cell culture slide 4 well (SPL, Gyeonggi-do, Korea, #30104) coated in PDL. The cells were washed twice in 1X PBS for 5 minutes. The collected cells were fixed in 4% paraformaldehyde (PFA) for 15 minutes at RT. The fixed cells were washed twice in ice-cold 1X PBS for 5 minutes each. The cells were incubated in 0.1% Tween 20 diluted in 1X PBS for 10 minutes, followed by washing three times as indicated above. The cells were blocked in 3% BSA solution for 30 minutes, then were incubated in primary antibody solution overnight at 4°C. The cells were washed three times as indicated above, followed by secondary antibody incubation for 2 hours at RT. DAPI (Invitrogen, Eugene, Oregon, USA) diluted in 1X PBS solution was used for

counterstaining for 5 minutes at RT. Slides were washed, then mounted with mounting solution, covered with microscope cover glass. Confocal microscopy images were taken using Zeiss LSM 710 (Carl Zeiss, NY, USA).

Detection of each receptor were done with the primary antibodies listed: CD8α (monoclonal, Abcam, Cambridge, UK, #ab27344, 1:100), Foxp3 [EPR22102-37] (monoclonal, Abcam, Cambridge, UK, #ab215206, 1:100), CXCR4 [UMB2] (monoclonal, Abcam, Cambridge, UK, #ab124824, 1:100), CD4 [9H5A8] (monoclonal, Invitrogen, MA, USA, #MA5-15774, 1:500), CCR4 (polyclonal, Abcam, Cambridge, UK, #ab1669, 1:100), and CCR10 (polyclonal, Invitrogen, MA, USA, PA1-21617, 1:100). The above was followed by labeling each with fluorescent labeled secondary antibodies such as Goat anti-Rat IgG (H+L) Secondary Antibody, Rhodamine (#31680, 1:1000), Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 594 (polyclonal, Invitrogen, MA, USA, #A-21207, 1:1000), Goat Anti-Mouse IgG Antibody, (H+L) FITC Conjugated (polyclonal, Sigma-Aldrich, MO, USA, #AP124F, 1:100), donkey anti-goat IgG-FITC (polyclonal, Santa Cruz Biotechnology, Inc., TX, USA, #sc-2024, 1:100), and donkey Anti-Rabbit IgG H&L (FITC) (polyclonal, Abcam, Cambridge, UK, #ab6798, 1:1000).

a. Fluorescence image analysis

The corrected total cell fluorescence (CTCF) was measured with ImageJ (Bethesda, MA, USA). Each cell was selected, and the fluorescent intensity was measured for each channel. The intensity values were normalized against DAPI intensity then normalized against FoxP3 or CD8α intensity. The area integrated fluorescent intensity was measured then the CTCF was calculated using the formula here: CTCF= Area integrated fluorescence intensity – (Area of the cell X Background mean fluorescence).

9. CCR4 retrovirus vector design and MSCV packaging

First, the vector pMSCV[Exp]-mCcr4[NM_009916.2](ns):P2A:EGFP (Vector ID: VB230314-1872tcs) was designed and created by VectorBuilder, lnc. (Guangzhou,

China). Then the vector was packaged into recombinant MSCV retrovirus by VectorBuilder, lnc. (Guangzhou, China).

10. Treg transduction

The general procedures of Treg transduction followed the paper by Eremenko et al. (20). The Treg culture was briefly centrifuged at 500 g, for 3 minutes at 32°C, then the culture media was collected and stored at 4°C for later use. Transduction media was prepared as follows: RPMI-1640 media with 100 mM HEPES, and 10 μg/mL polybrene. 100 μL of the virus stock was carefully pipetted into each well. The culture was spinoculated at 1200 g for 90 minutes at 32°C. Then it was incubated in the standard culture condition for 4 hours, followed by the exchange of media into the previously collected culture media. Then Tregs were sorted on day 5 or 6 using the BD Aria III (Becton Dickinson Company, Franklin Lakes, NJ, USA), and were cultured in standard conditions until later use.

11. Flow cytometry (FACS)

$1x10^6$ of Treg or mCcr4 transduced Treg (mCcr4-Treg) were washed twice with 1X PBS at 300 g, 4°C, for 5 minutes. Cells were fixed in the cold 2% PFA solution (diluted in PBS) for 30 minutes at RT. Cells were washed three times as indicated above, followed by blocking in 2% FBS solution for 30 minutes on ice. Then cells were centrifuged at 300 g for 5 minutes at 4°C, the supernatant was discarded then extracellular CCR4 staining was done for 30 minutes at 4°C. Cells were washed twice as indicated above. Intracellular staining was done by permeabilizing the cells with 0.7% Tween-20 solution diluted in 1X PBS for 15 minutes on ice. Cells were washed twice, and then Foxp3 was stained in the dark for 30 minutes at 4°C. Cells were washed twice and then were suspended in 500 μL FACS buffer. Flow cytometry analysis was done using BD FACSymphony™ A5 Cell Analyzer (BD Biosciences, Franklin Lakes, NJ, USA). Collected data were further processed using FlowJo™ v10.8 Software (BD Life

Sciences, Franklin Lakes, NJ, USA).

Fluorescent conjugated antibodies used are as follows: Alexa Fluor® 700 anti-mouse FOXP3 Antibody [MF-14] (monoclonal, BioLegend, San Diego, California, USA, #126422, 1:100), and PE anti-mouse CD194 (CCR4) [2G12] (monoclonal, BioLegend, San Diego, California, USA, #131204, 1:100).

12. tMCAO surgery

tMCAO surgery was done in the method described by Koizumi et al. (21). Mice were deeply anesthetized with Zoletil (Virbac, France) at 30 mg/kg mixed with Rompun (Bayer, Germany) at 10 mg/kg via intraperitoneal injection. Their neck fur was shaved, and incision was made. Neck muscles on the right side were separated along the middle until the common carotid artery (CCA) was exposed. A permanent knot was made on the caudal side of the CCA first, then ribbon ties were made just before the bifurcation site, as well as on the external carotid artery (ECA). A small incision was made near the first CCA permanent knot and the silicone rubber-coated monofilament (6-0) (Doccol, MA, USA) was inserted into the middle cerebral artery (MCA). 6-0 fine MCAO suture L56 (Doccol, MA) was purchased from LMS Korea. The occlusion was done for 20 minutes, while the animal was kept under the heating lamp. The ligatures on the caudal side of the CCA and the ribbon ties on the CCA near the bifurcation and ECA remained after removing the MCAO suture. As for the Sham group, all steps until the insertion of the MCAO suture were done. In the experimental groups, animals were left anesthetized for another 20 minutes under the heat lamp. $1.0x10^6$ Treg or CCR4-transduced Treg cells were prepared in 40 uL saline per animal in an insulin syringe. The catheter was inserted into the CCA incision, and cells were slowly injected through the CCA.

13. Behavior testing

After the tMCAO surgery, the animal underwent reperfusion for 24 hours. The next day, the animals were tested for the neurological deficits with the Bederson scoring, and the

grip strength test and the elevated body swing test (EBST) were used for evaluating the degree of motor deficits.

a. Bederson score

The Bederson score was used to measure as indicated by De Meyer et al. (22). The animals were scored from grade 0 to grade 5 according to their behavioral patterns observed 24 hours after tMCAO surgery. Animals were assigned grade 0 if no defects are shown, extending their forelimbs towards the ground when lifted up. Grade 1 was assigned if they show only the forelimb flexion, indicating mild defects. Grade 2 was for animals showing modest defects, where they show reduced resistance to lateral push. Grade 3 was for animals showing severe defects with circling behavior. Grade 4 was for animals showing longitudinal spinning, and Grade 5 was used for animals with no movement.

b. Grip strength test

The Forelimb grip strength test was conducted as described by Takeshita et al. (23). In short, the mouse was held horizontally, parallel to the measuring bar, so that it could grasp the bar. Once forepaws were attached to the bar, the measuring device was tared to 0. The mouse's tail was gently and slowly pulled horizontally until the mouse let go of the bar. The force displayed on the screen in g was recorded. The above was repeated 5 times per mouse.

c. Elevated body swing test (EBST)

EBST was conducted as described by Borlongan et al. (24). Mouse was placed in a new cage, which was left inside for 2 minutes for habituation. Mouse was held up about 10 cm above the ground vertically by its tail, and then the side at which it swung was recorded. The above was repeated 5 times and after each recording, the mouse was placed back into the cage to rest.

14. 2,3,5-Triphenyltetrazolium chloride (TTC) test

Mice were perfused by transcardiac perfusion with 30 mL ice-cold saline. The brain was

isolated and was sliced into 2 mm sections. They were briefly washed in ice-cold saline before incubating in pre-warmed 2% TTC solution in the dark for 30 minutes at 36℃. Then they were incubated in a 2% PFA solution and were photographed. Infarct area analysis was done via ImageJ (Bethesda, MA, USA).

15. Statistical analysis

All statistical analyses were done using Prism software (GraphPad Software, La Jolla, CA, USA), and were only considered significant if $P<0.05$.

III. RESULTS

1. Both the CD8+ T cells and Tregs showed increased migration into the OGD(+) Direct media

To confirm the migration pattern of both the CD8+ T cell and Tregs, a trans-well migration assay was conducted (Fig 1). Isolated primary microglia were exposed to simulated IS through oxygen-glucose deprivation (OGD) conditions in an anaerobic culture chamber. The cell culture media was collected before the microglia were exposed to OGD condition (Control). OGD-exposed culture media was collected immediately after the 40-minute OGD, followed by the collection of the culture media 24 hours post-OGD (OGD(+) Rep) (Fig. 1a). The cell imaging (Fig. 1b) was done with Hoechst 33342 dye where each chamber was counted for the number of CD8+ T cells or Tregs that migrated into the media after 48 hours (Fig. 1c). The confocal imaging results showed 3.1 and 4.1 times more CD8+ T cells and Tregs migrating into the OGD(+) Direct media respectively (Fig. 1b,c) compared to that of Control media. Not only this but the results also showed a noticeable decrease in Treg migration into the OGD(+) Rep media than that of CD8+ T cells (Fig. 1b). Tregs showed a 58.9% drop compared to an 8.6% decrease in CD8+ T cell migration into the OGD(+) Rep media. Together the results showed the possibility of both the CD8+ T cell and Treg migration in response to activated microglia exposed to simulated IS condition during the IS progression rather than during the recovery phase of IS in vitro.

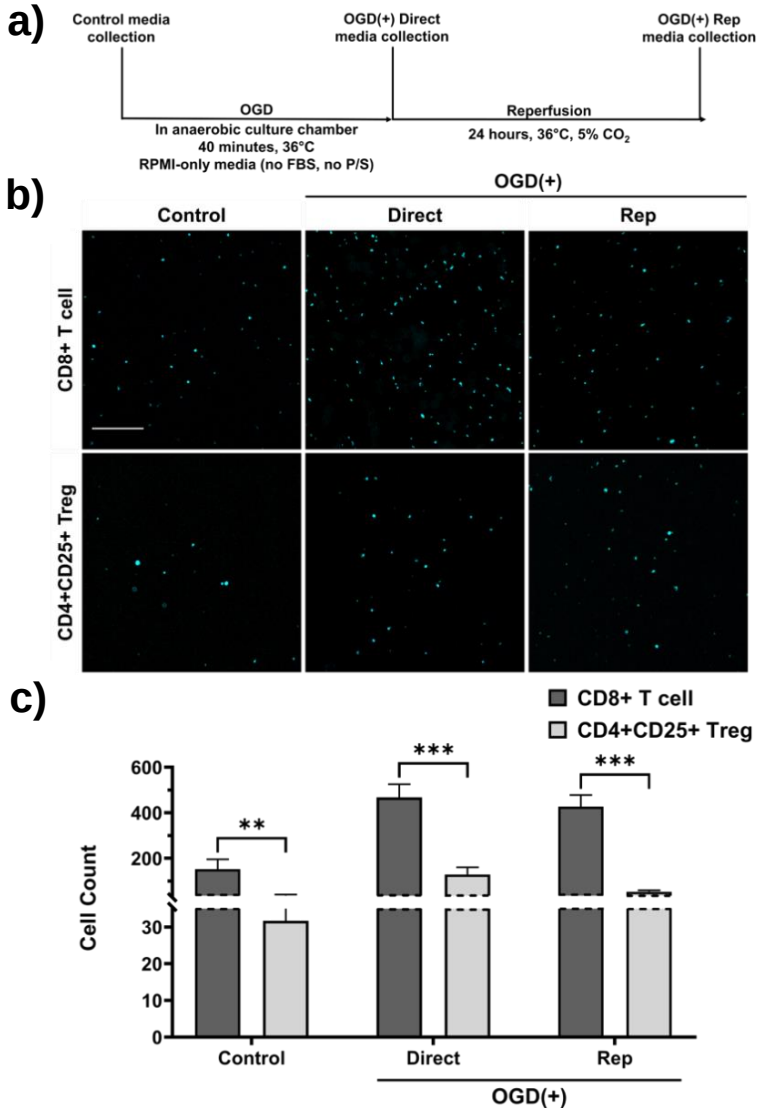

Figure 1. More CD8+ T cell and Tregs migrated into the OGD(+) Direct media compared to the OGD(+) Rep media. (a) The graphical schedule regarding the microglia media collection. (b) Trans-well migration assay confocal imaging results after 48-hours into each of the indicated primary microglia media. Tregs were labeled with 1X Hoechst 33342 dye and imaged at 10x magnification. Scale bar denotes 50 μm. (b) The cell count data of CD8+ T cells and Tregs (** $P \leq 0.01$, *** $P \leq 0.001$, all n=3).

2. OGD(+) Direct media showed an increased level of pro-inflammatory immune cell chemoattractants

To search for the potential target molecules participating in Treg migration upon the onset of IS, a mouse chemokine profiling array was done using the primary microglia culture media collected: Control, OGD(+) Direct, and OGD(+) Rep (Fig. 2). The relative mean gray values dramatically increased for chemokines like CXCL12, CCL12, CCL2, CCL6, IL-16, and CCL27 in the OGD(+) Direct media (Fig. 2b,d). Comparing the differences shown in the OGD(+) Direct and OGD(+) Rep, the expression level of CCL12 showed 3.3-fold increase, where CCL2 increased by 2.3. On the other hand, increase of CCL6 levels differed by only 1.7-fold. In the OGD(+) Direct media chemokine array, the biggest increase was seen in the levels of CXCL12 and CCL27, the relative mean gray values for both chemokines showed 3.5-fold change. The OGD(+) Rep media showed an increase in the levels of CCL22 and Hspd1 (Fig. 2d) where the chemokines increased by 4.8- and 2.5-fold compared to their expression levels in the OGD(+) Direct media. The receptors that are known to interact with the above chemokines were investigated and listed (Table 1). Though it will further be discussed, the general trend of an increase in pro-inflammatory chemokines is observed in the OGD(+) Direct media, whereas the OGD(+) Rep media showed a decrease in the above pro-inflammatory chemokines, and instead showed an increase in chemokines related to Treg functions. The above results narrowed down the possible target receptor that can be focused on relating to the migratory potential of Tregs into the ischemic brain in response to the chemokines secreted by activated microglia.

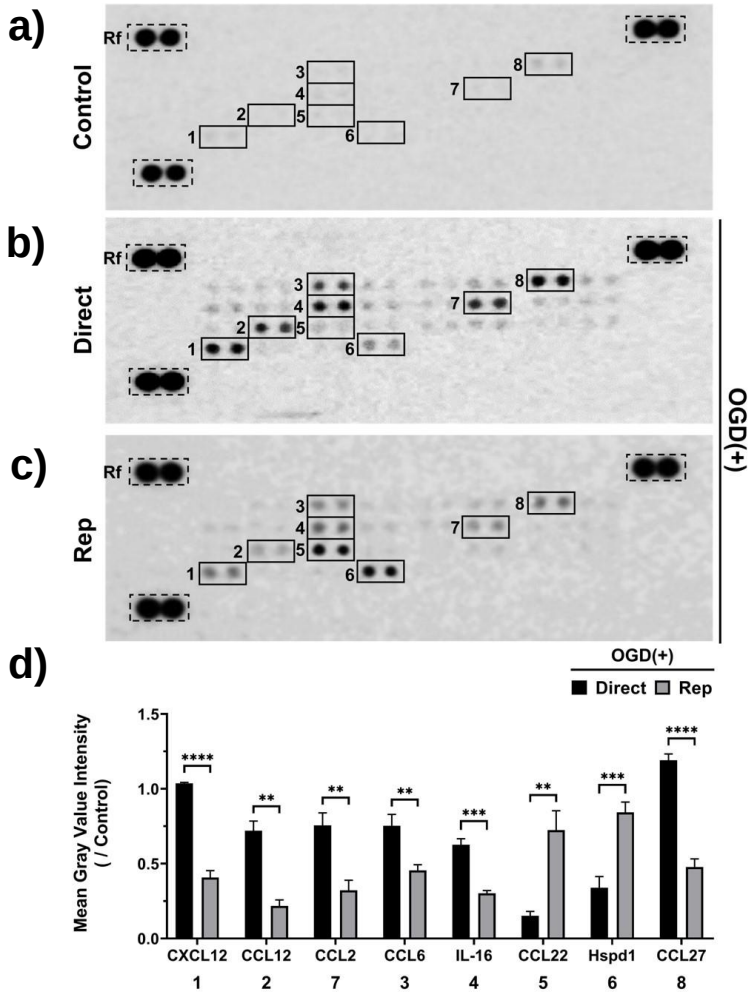

Figure 2. The differential expression of chemokines secreted by the primary microglia during the simulated IS and during the recovery phase *in vitro*. Murine chemokine profiling array chemiluminescence data shown for each collected media: Control (a), OGD(+) Direct (b), and OGD(+) Rep (c). (d) Normalized mean gray intensity value for the dot blot incubated in each of the corresponding microglia media: OGD(+) Direct and OGD(+) Rep. The numbers below correspond to the numbers in (a-c). Asterisks denote the significance of data from P-values obtained by performing the t-test (** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, all n=3). All gray values for each dot blot in each media was measured relatively to the reference dot blot (Rf, dotted boxes).

Table 1. Summary of the chemokines observed in murine chemokine array in conditioned primary microglia culture media and the receptors known to interact with

#	Chemokine	Receptor	Roles	Ref.
1	CXCL12	CXCR4	T cell, B cell, monocyte, and Treg recruitment	(28)
2	CCL12	CCR2	Activation of microglia and complement activation in the brain	(29)
7	CCL2			
3	CCL6	CCR6	Migration of macrophage, neutrophil, astrocyte, and microglia	(30)
4	IL-16	CD4	Skin inflammation through over sensitization, CD4+ T cell expansion	(31)
5	CCL22	CCR4	Suppression of T cell response through Treg activity	(35-37)
6	Hspd1	TLR9/TLR2	Anti-inflammatory response through inhibiting T cell migration indirectly	(32-34)
8	CCL27	CCR10	Lymphocyte recruitment, inflammation, and allergic reaction in skin Inflammatory immune response in the brain	(27)

The numbers on the left (#) represent the chemokines shown in Fig. 2 (a-c).

3. CXCR4 and CCR4 expressed highly on Tregs were selected as the target receptors

In order to further narrow down the target receptor to be studied in relation to Treg migration, the expression patterns of the known specific markers and selected receptors on the primary CD8+ T cells and Tregs were observed. The primary CD8+ T cells and Tregs were fluorescently labeled for CD8α and FoxP3 respectively, as well as CXCR4, CD4, CCR4, and CCR10 (Fig. 3a-c). The presence and the degree of expression were also quantified using western blot, detecting each listed receptor (Fig. 3d,e). The immunocytochemistry (ICC) results showed both cell populations expressing CXCR4, whereas the western blot results showed a lower expression level in CD8+ T cells than in Tregs where the expression level was 2.5 times higher. The CD4 and CCR4 expression in the CD8+ T cells were notably lower in both ICC and in western blot compared to that of Tregs, 2.7 and 2.3 times less in CD8+ T cells respectively. On the other hand, the CCR10 expression on the CD8+ T cells was significantly higher, with about 5.0-fold difference, compared to Tregs in both ICC and western blot. When the ICC data were analyzed by ImageJ to measure the differences in fluorescence intensity shown as the corrected total cell fluorescence (CTCF), it showed a similar trend as the western blot expression intensity data (Fig. 3c). In summary, the expression of potential target receptors was seen in both the CD8+ T cell and the Treg populations, where the focus was set to the receptors CXCR4 and CCR4 expressed highly on Tregs compared to CD8+ T cells.

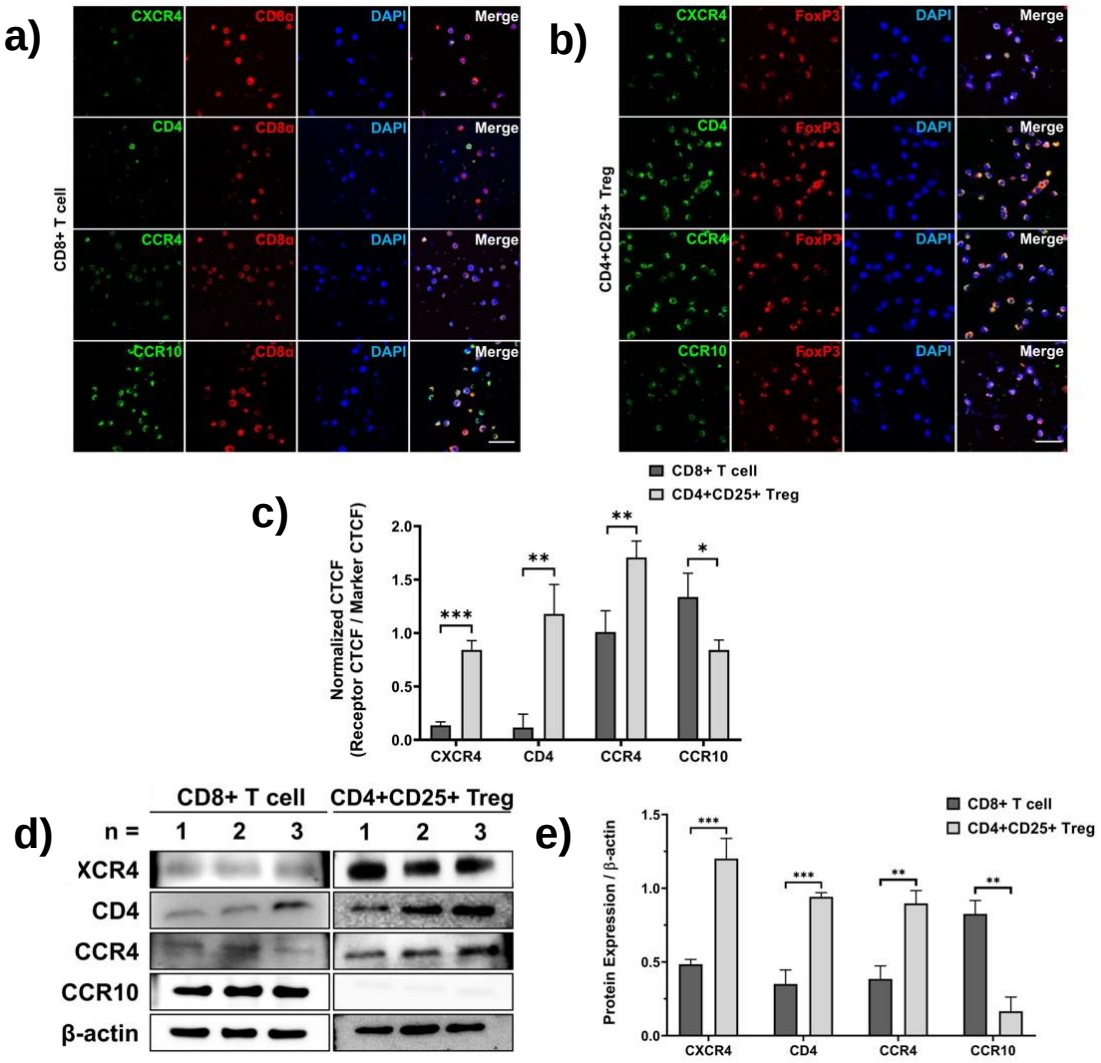

Figure 3. Confirmation of the selected potential target receptors were checked on CD8+ T cells and Tregs through immunocytochemistry (ICC) and western blot. (a, b) ICC confocal imaging results showing the expression of the screened receptors: CXCR4, CD4, CCR4, CCR10 on CD8+ T cells (a) and Tregs (b), as well as its defining markers, CD8α and FoxP3 respectively. Imaging was done at 80x magnification. The scale bar indicates 25 μm. (c) The corrected total cell fluorescence (CTCF) was measured with ImageJ and was normalized against either the CD8α or FoxP3 respectively. (d) Western

blot chemiluminescence results of the receptors listed above, as well as β-actin as a reference in CD8+ T cells and Tregs. (e) The above western blot band intensity for each receptor was measured using ImageJ and normalized against the β-actin bands in CD8+ T cells and Tregs. Asterisks denote the significance from P-values obtained by performing the t-test (** $P \leq 0.01$, *** $P \leq 0.001$, all n=3).

4. Tregs treated with CCR4 antagonist (sc-221406) showed decreased migration into OGD(+) Direct media

To further study the role of CCR4 and CXCR4 in relation to Treg migration, a trans-well migration assay was performed (Fig. 4). The CCR4 and CXCR4 antagonists, sc-221406 and WZ811 respectively, were treated on Tregs to block their interactions with CCL22 and CXCL12 secreted by activated microglia. Tregs were incubated in sc-221406 or WZ811 in the indicated concentrations for 2 hours before seeding on the trans-well sleeves above the OGD(+) Direct media. To test for the most effective concentration of each antagonist, sc-221406 or WZ811 was treated at indicated concentration (Fig. 4a). The confocal imaging showed a gradual decrease in Treg migration into the OGD(+) Direct media when Tregs were treated with sc-221406 or WZ811. In comparison to DMSO control, CCR4 blockage with sc-221406 showed about 19.4% less migration into the OGD(+) Direct media compared to that of WZ811 treatment (Fig. 4b). The above results show how CCR4 might be the receptor more closely related to Treg trafficking into the ischemic condition in response to chemokines released by the activated microglia, making CCR4 the receptor of focus for this study.

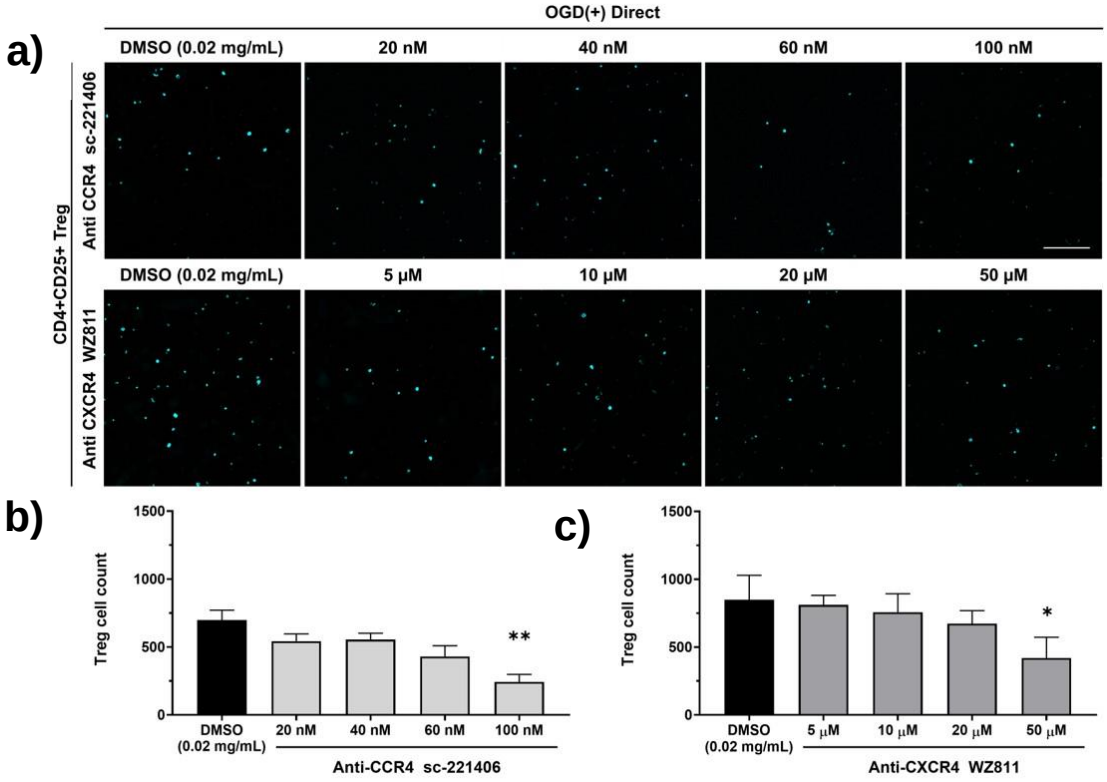

Figure 4. The migration pattern check for CCR4 antagonist (sc-221406) or CXCR4 antagonist (WZ811) treated Tregs using trans-well migration assay using the OGD(+) Direct media. (a) Confocal imaging of antagonist treated Tregs labeled with 1X Hoechst 33342 under 10x magnification. Tregs were incubated with either the CCR4 antagonist (sc-221406) or CXCR4 antagonist (WZ811) at indicated concentration for 2 hours before the trans-well assay was conducted. (b,c) Number of Tregs treated with either sc-221406 (b) and WZ811 (c) that migrated into the OGD(+) Direct media after 48 hours. Asterisks denote the significance from P-values obtained by performing the t-test (* $P \leq 0.05$, ** $P \leq 0.01$, all n=4).

5. Tregs showed a mild increase in CCR4 expression after MSCV-CCR4 transduction

To potentially increase the migration rate of Tregs into the ischemic brain, retrovirus transduction was conducted (Fig. 5). The mouse CCR4 sequence was incorporated into the murine stem cell virus (MSCV) retroviral vector by VectorBuilder. The promoter MSCV ψ+ controls the expression of the virus vector, and the mCcr4 sequence is linked with the reporter gene EGFP with P2A linker (Fig. 5a). Treg transduction and sorting were done following the shown schedule (Fig. 5b). To confirm the successful transduction and efficiency, ICC was performed to check the presence of EGFP in the MSCV-CCR4 transduced Treg (TD) group (Fig. 5c), whereas the flow cytometry analysis (FACS) was done to quantify the increase in the CCR4 receptor expression level (Fig. 5e). After the MSCV-CCR4 transduction, TD Tregs showed clear EGFP reporter expression in ICC (Fig. 5c). The CTCF for EGFP expression was measured, where it showed about a 6.9-fold difference when UT and TD Tregs were compared. The above was followed by quantification of CCR4 expression on UT and TD Tregs (Fig. 5d). Both TD and UT Tregs were first selected with high levels of FoxP3-Alexa Fluor 700 expression, 9.28% and 24.0% respectively. Then, within the FoxP3+ population, EGFP+ groups and CCR4-PE high populations were gated (Fig. 5e). The UT Tregs showed 0.62% EGFP+ population, whereas the TD Tregs showed a 7.19% of EGFP+ population. Among the FoxP3+ population, CCR4-PE expression was then observed where the UT Tregs showed 63.1% and TD Tregs at 85.3%. The FACS result showed a mild, approximately 22%, increase in the expression levels of CCR4 in the TD group (Fig. 5f). These results together indicate the successful transduction of Tregs using the MSCV-CCR4, which resulted in an increase in the CCR4 expression on Tregs.

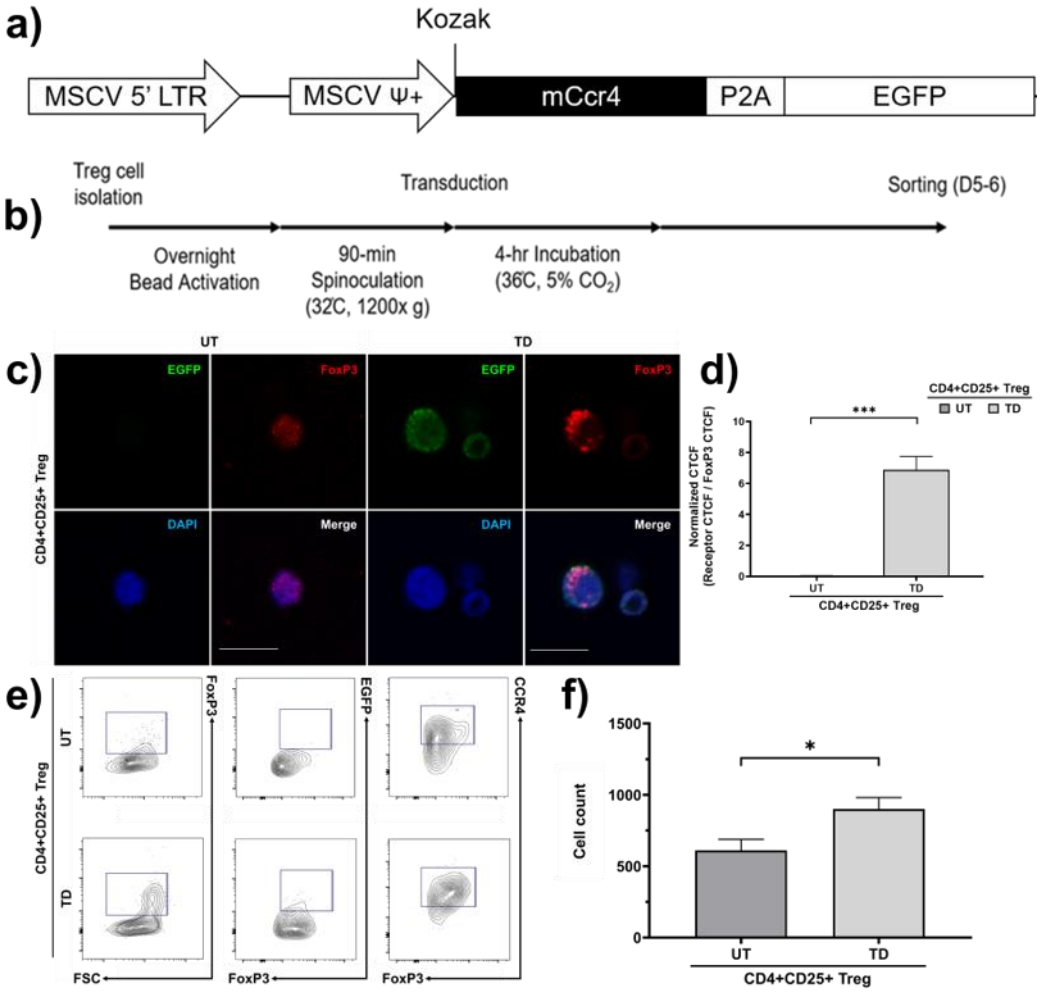

Figure 5. The MSCV-CCR4 retrovirus efficiency check via immunocytochemistry (ICC) and flow cytometry analysis (FACS). (a) The MSCV retrovirus vector construct designed using VectorBuilder. The mCcr4 sequence was linked with EGFP reporter gene with P2A link, where the expression was controlled by the MSCV ψ+ promoter. (b) Treg transduction was performed following the schedule shown here. (c) ICC imaging of the UT Tregs and TD Tregs. (d) The CTCF was measured with ImageJ program and were normalized against either the CD8α or FoxP3 fluorescence measurement. All imaging was done under 40x magnification at 8.1X zoom. (e) The detailed quantification of

changes in the CCR4 expression was observed using FACS. (f) Graph of FoxP3+ Treg population that are expressing CCR4 highly. FoxP3 was labeled with Alexa Fluor 700 and CCR4 was labeled with PE. EGFP expression was from the mCcr4:P2A:EGFP vector construct. Asterisks denote the significance from P-values obtained by performing the t-test (* $P \leq 0.05$, *** $P \leq 0.001$, all n=3).

6. mCcr4 transduced Tregs showed increased migration into the OGD(+) Direct media

After confirming the successful transduction of Tregs with MSCV retrovirus containing mCcr4:P2A:EGFP vector, sorted cells were used to perform trans-well migration assay to see the changes in their migration patterns (Fig. 6). The trans-well migration assay was done using the OGD(+) Direct media as indicated in the previous sections. The number of live-Tregs that migrated into the OGD(+) Direct media over the 48-hour period was imaged using the Hoechst33342 dye (Fig. 6a) and counted (Fig. 6b). The TD Tregs showed increased migration into the OGD(+) Direct media by 1.5-fold compared to that of UT Tregs. This result suggests that the overexpression of CCR4 on Tregs increase the migration ability in response to the microglia derived CCL22 during the OGD.

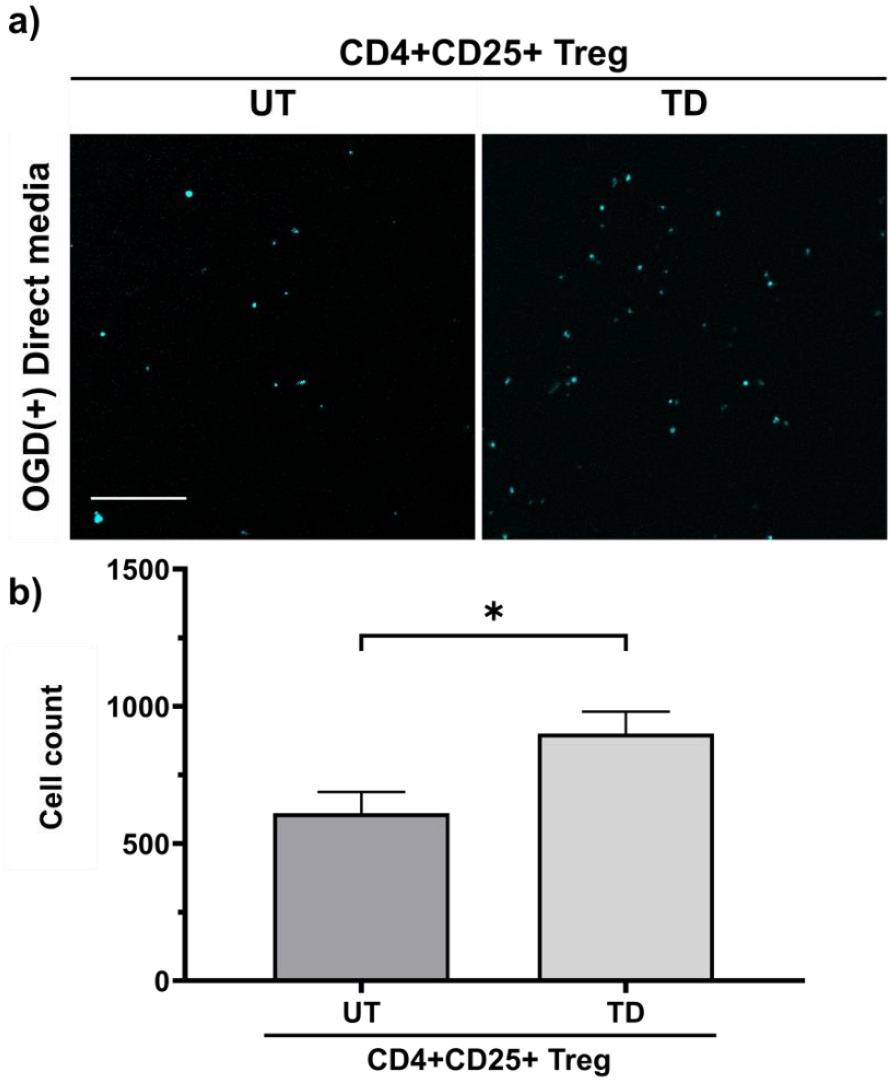

Figure 6. Increased Treg cell migration rate of TD Tregs compared to that of UT Tregs into the OGD(+) Direct media. (a) Confocal images of Tregs that migrated into the chamber containing OGD(+) Direct media. Tregs were labeled with 1X Hoechst 33342 dye and images were taken under 20x magnification (zoom 2.0). (b) The number of Tregs that migrated into the OGD(+) Direct media after 48 hours. Asterisks denote the significance from P-values obtained by performing the t-test (* $P \leq 0.05$, all n=3).

7. A decrease in infarct size and improved motor function was seen in tMCAO groups injected with Tregs transduced with MSCV-CCR4

To confirm the effects of Tregs during IS, the infarct size was observed using TTC staining assay and functional improvement by behavior testing (Fig. 7). The above experiments were done following the graphical schedule (Fig. 7a). TTC staining assay indicated a dramatic decrease in the infarct size measured after 24 hours post-tMCAO upon the UT or TD Treg injection compared to the saline control (tMCAO) group (Fig. 7b). Quantitatively comparing the Treg injection groups with the tMCAO control group, the injection of UT Tregs showed a 25.7% reduction in infarct size where the TD injection group showed a significant 50.7% infarct size reduction. Furthermore, comparing the infarct size between the UT Treg and the TD Treg injection group, TD Treg injection showed about 33% more reduction (Fig. 7c). Then, the animals were tested for any functional improvement by performing the Bederson scoring, the forelimb grip strength test (GT), and the elevated body swing test (EBST) (Fig. 7c-e). The Bederson score was assigned to each animal by observing their behavior before performing other tests. The UT and TD groups showed a slight decrease in the severity of neurological deficits compared to the tMCAO control, the score value differences being 0.7 and 1.0 respectively (Fig. 7d). The GT results showed further improvement in the TD Treg injected group (Fig. 7e). The strength values measured in the UT Treg group showed a 1.3-fold increase, whereas the TD Treg group showed a 2.7-fold increase compared to the tMCAO group. Next, the EBST results showed a dramatic decrease in the left-swing bias in both the UT Treg and TD Treg injection groups (Fig. 7f). The TD Treg injection group showed 60% less infarct size compared to the tMCAO control but showed only 9.8% differences when compared to UT Treg injection group. Taken together, the results showed a notable decrease in the infarct size when TD Treg was injected into the animal after tMCAO surgery, showing some improvement in the neurological and motor functions in animals.

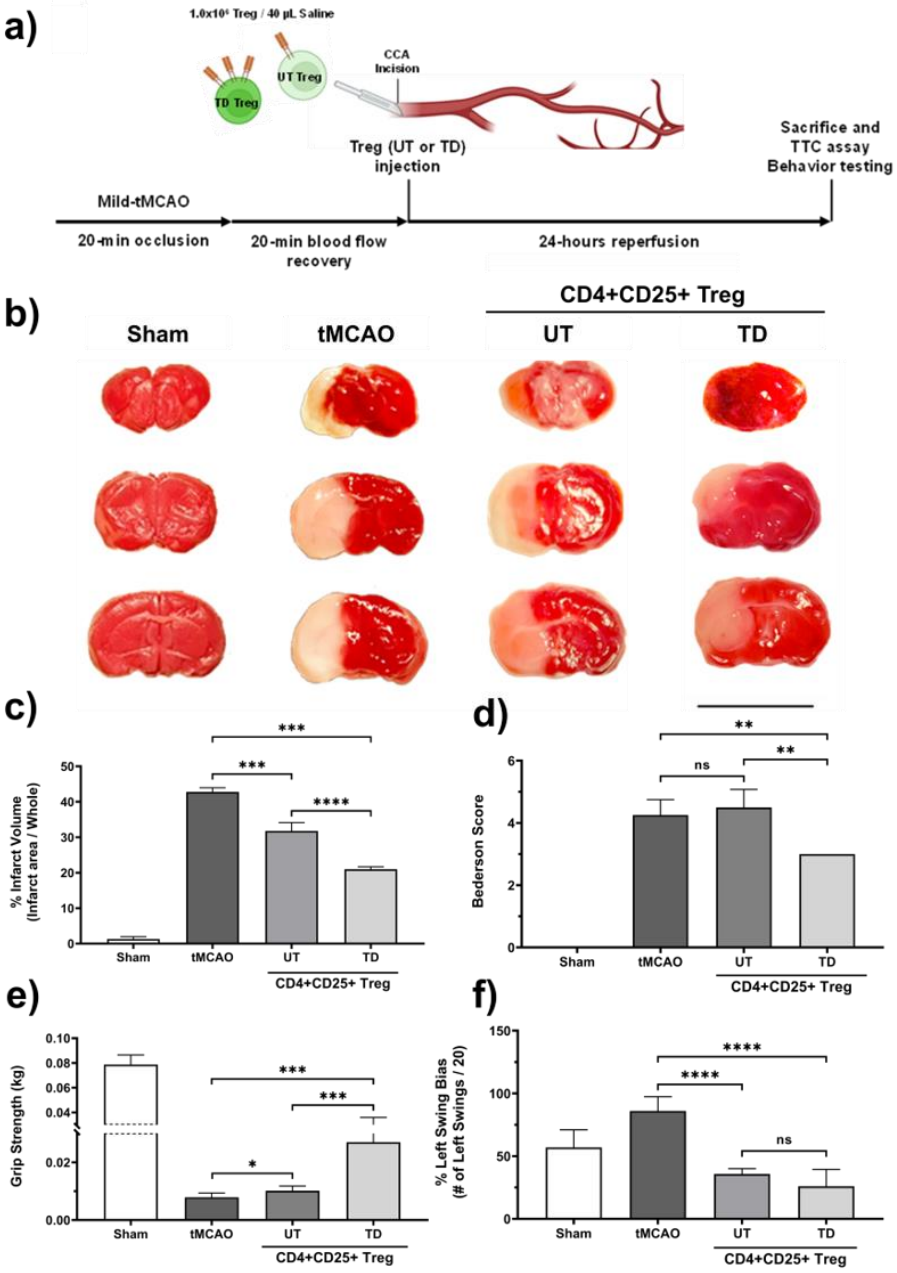

Figure 7. Summarized data of infarct size differences observed by TTC assay and behavior testing in tMCAO animal models injected with either the UT Tregs or TD Tregs. (a) All experiments followed the graphical schedule shown here. $1.0x10^6$ UT or

TD Tregs in 40 μL Saline were injected through catheter into the CCA incision made during tMCAO surgery. (b) TTC assay results for the indicated groups. The scale bar indicates 1 cm in length. (c) The infarct size was measured using ImageJ. (d) Bederson scoring was done to measure the overall deficits in neurological functions of animals 24-hours after tMCAO surgery. (e, f) Motor function of the animals post-tMCAO modeling was done using grip strength test (GT) (n=7) and the elevated body swing test (EBST). Asterisks denote the significance of data from P-values obtained by performing the t-test (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, all n=5).

IV. DISCUSSION

Although some studies were done on the effects of Tregs during IS, the heterogeneity of results still does not explain the therapeutic potential of Tregs in IS. This study investigated the specific molecular factors in Treg migration upon microglia activation in the brain under ischemic stress and the effects of Tregs in the context of neurological and motor functions. The trans-well migration assay results showed an increased migration into OGD(+) Direct media for CD8+ T cells and Tregs. The murine chemokine profiling array showed an increase in chemokines like CCL27, CCL2, and CXCL12 in the OGD(+) Direct media, whereas the OGD(+) Rep media showed higher expression of Hspd1 and CCL22. ICC and western blot results showed the receptors known to interact with the listed chemokines. These results suggest that the activated microglia affect pro-inflammatory immune cells and Treg trafficking. To further screen the specific factors for Treg trafficking, a trans-well migration assay with antagonist-treated Tregs was conducted. Blocking CCR4 caused a significant decrease in migration into the OGD(+) Direct media, indicating that CCL22-CCR4 interaction can be an important factor in Treg migration. When the mCcr4:P2A:EGFP MSCV retrovirus vector construct was transduced into Tregs (TD Tregs), an increase in the CCR4 expression level was seen. Injection of TD Tregs post-tMCAO showed further reduction in the infarct size. In agreement with the reduced infarct size, some functional improvement was observed. The animals showed better grip strength and a lower left-swing bias in EBST. The results of this study indicate that increased expression of CCR4, hence the Treg migration into the infarct site responding to the chemokines secreted by activated microglia, has noteworthy effects on reducing the severity of IS.

This study is the first to investigate the relationship of activated microglia and Treg migration during IS in the molecular context. Although there are many studies regarding the role of Tregs in the recovery phase of IS (25), conclusive evidence on the neuroprotective effects of Tregs is still absent. Previous studies showed the accumulation

of Tregs in the bloodstream and ischemic penumbra (4). Tregs are known to reduce neuroinflammation through the expression of PD-L1 to inhibit the activity of MMP-9 released by neutrophils as well as the activity of cytotoxic astrocyte via the amphiregulin (AREG)/epidermal growth factor receptor (EGFR) axis (16). Furthermore, Tregs provide neuroprotection indirectly through IL-10 and TGF-β secretion, shifting microglia into the M2 phenotype that promotes brain repair (17). Recently, studies showed how Treg injection enhanced white matter integrity and repair and showed better functional recovery when Treg proliferation was promoted in animal models (26, 27).

The specific interactions involved in the Treg migration into the ischemic brain due to microglia-derived factors are still not studied thoroughly. The accumulation of Tregs around the brain infarct region and the cerebral vasculature suggests that Tregs react and migrate in response to microglia-derived factors during ischemia. The chemokines profiled in the OGD(+) Direct media showed an increase in chemokines involved in various physiological pathways like nociception in pathological conditions, causing adverse effects in cancer patients (28, 29). The levels of CCL27 and CXCL12 showed the largest increase in the OGD(+) Direct media. CCL27 interacts with CCR10 and is most studied concerning skin lymphocyte recruitment, inflammation, and allergic reaction in the skin, but alternatively spliced CCL27 is also found in the brain tissue (30). The alternatively spliced CCL27 is involved in neuroimmune responses, suggesting a pro-inflammatory role in the brain. In addition, CXCL12-CXCR4 interaction is responsible for recruiting T cells, B cells, and monocytes. Not only this, but CXCL12 also induces anti-inflammatory Treg recruitment (31). Also, the chemokines like CCL12, CCL2, CCL6, and IL-16 increased in the OGD(+) Direct media. CCL12 and CCL2 interact with CCR2 and contribute to expressing Iba1 and activating the brain complement system through C1qa (32). CCL6-CCR6 interaction regulates the migration of macrophages, neutrophils, astrocytes, and microglia in various inflammatory disorders (33). Like CCL27, the IL-16-CD4 axis is related to increased sensitization, resulting in skin inflammation, and is also

involved in CD4+ T cell expansion (34).

On the other hand, OGD(+) Rep media showed high expression of chemokines like Hspd1 and CCL22. The chemokine Hspd1, or Hsp60, is a chaperone protein secreted under cellular stress or during immune responses (35). Previous reports suggest that Hsp60 is involved in an autoimmune response in the CNS and reduced inflammation in inflammatory disorders (36). Though the Hsp60-TLR9/TLR2 interaction is an appealing target for the study, previous studies showed that Hsp60 is not the direct factor resulting in the chemotaxis of T cells (35). CCL22, being one of the selective CCR4 ligands, is known to be secreted by monocytes, agreeing with the chemokine profiling array results shown in this study. Like CXCL12, CCL22 is an effective chemoattractant for Tregs (37). Notably, few studies showed a significant reduction of CCL22 during the acute phase of IS in circulation and in the infarct core (38). Previous reports showed a decrease in CCL22 after IS results in a higher National Institute of Health Stroke Scale (NIHSS), meaning the severity of stroke is high. Recently, Rapp et al. (39) also reported that CCL22-CCR4 is a crucial factor in regulating the suppression of T cell activity through the intervention by Treg. Together, this suggests that activated microglia release chemokines for pro-inflammatory immune cell trafficking and the anti-inflammatory immune cell chemoattractant to balance the detrimental neuroinflammation. The above observation was seen in the OGD(+) Direct media, but the level significantly increased in the OGD(+) Rep media in this study. Yet in the context where the reduction of CCL22 during the initial phase of IS exacerbated the damage, the significance of CCL22-CCR4 inducing Treg migration to alleviate IS progression cannot be overlooked.

CXCR4 and CCR4 were chosen as the two potential target receptors. However, CCR4 blockage further inhibited Treg migration into the OGD(+) Direct media. Therefore, manipulation of CCR4 has become the main target of the study. Consistent with the previous reports, the current study showed a notable reduction in the infarct size 24 hours

post-tMCAO in the animals injected with either the UT Tregs or TD Tregs. Among the two groups, TD Treg injection groups showed further infarct size reduction. This may be because of the potential increase in the mobility of Tregs in response to the activation of microglia and micro-derived chemokine release. The above was supported in the trans-well migration assay of the TD Tregs, showing a notable cell count increase in the OGD(+) Direct media. The above is consistent with previous studies reporting the importance of CCL22-CCR4 interaction in Treg migration (28, 37, 39), proposing the therapeutic potential of Tregs to reduce ischemic damage and promote recovery in the brain post-IS. Taken together, the importance of CCL22-CCR4 interaction in reducing the IS severity via Treg recruitment can be a potent cell therapy candidate.

Most previous studies showed positive effects of adoptive transfer of Tregs into IS patients where they found reduced infarct volume and better prognosis post-IS (16, 17). Still, there are controversies about the efficacy and safety of Treg-based therapy for IS. Some studies reported that Tregs did not reduce infarct size, but others reported thrombosis formation through CD40 and ICAM-1 pathways during Treg recruitment worsening the damage. Despite the controversial effects of Tregs in IS concerning the infarct size and the functional outcome, our study showed a clear reduction of infarct size in the Treg injection group in the TTC assay and improvement in the functional evaluation. Relating to the previous studies regarding the recruitment of Tregs by CCL22-CCR4 interaction and the ameliorating effects of Tregs in infarct size and motor function, the potential neuroprotective Treg cell therapy can be further developed.

V. CONCLUSION

Tregs are proposed to have neuroprotective effects exerting anti-inflammatory responses to reduce neuroinflammation and promote brain tissue repair. This study showed the specific interaction of chemokines secreted by activated microglia in response to IS and Treg, supporting the neuroprotective role of Treg in IS. The notable findings of the current study are:

- CD8+ T cells and Treg showed more migration into the OGD(+) Direct media mimicking the onset of IS where microglia activation happens.
- During the onset of ischemic stroke, activated microglia release chemokines resulting in pro-inflammatory immune cell migration.
- Overexpression of CCR4 in Tregs resulted in increased migration into OGD(+) Direct media.
- Successful increase of CCR4 expression on Tregs using the MSCV retrovirus and the injection of it after tMCAO showed an infarct size reduction and a slight improvement in motor function.

In conclusion, the results of this study propose the novel idea of enhancing Treg migration into the infarct site by CCR4 overexpression in response to the chemokines secreted by activated microglia in IS, reducing the severity of ischemic stroke *in vivo*.

REFERENCES

1. Iadecola C, Buckwalter MS, Anrather J. Immune responses to stroke: mechanisms, modulation, and therapeutic potential. J Clin Invest. 2020;130(6):2777-88.

2. Virani SS, Alonso A, Aparicio HJ, Benjamin EJ, Bittencourt MS, Callaway CW, et al. Heart Disease and Stroke Statistics-2021 Update: A Report From the American Heart Association. Circulation. 2021;143(8):e254-e743.

3. Lei TY, Ye YZ, Zhu XQ, Smerin D, Gu LJ, Xiong XX, et al. The immune response of T cells and therapeutic targets related to regulating the levels of T helper cells after ischaemic stroke. J Neuroinflammation. 2021;18(1):25.

4. Santamaria-Cadavid M, Rodriguez-Castro E, Rodriguez-Yanez M, Arias-Rivas S, Lopez-Dequidt I, Perez-Mato M, et al. Regulatory T cells participate in the recovery of ischemic stroke patients. BMC Neurol. 2020;20(1):68.

5. Wang S, Zhang H, Xu Y. Crosstalk between microglia and T cells contributes to brain damage and recovery after ischemic stroke. Neurol Res. 2016;38(6):495-503.

6. Faura J, Bustamante A, Miro-Mur F, Montaner J. Stroke-induced immunosuppression: implications for the prevention and prediction of post-stroke infections. J Neuroinflammation. 2021;18(1):127.

7. Dong R, Huang R, Wang J, Liu H, Xu Z. Effects of Microglial Activation and Polarization on Brain Injury After Stroke. Front Neurol. 2021;12:620948.

8. Zhang Y, Lian L, Fu R, Liu J, Shan X, Jin Y, et al. Microglia: The Hub of Intercellular Communication in Ischemic Stroke. Front Cell Neurosci. 2022;16:889442.

9. Hu X, Leak RK, Shi Y, Suenaga J, Gao Y, Zheng P, et al. Microglial and macrophage polarization-new prospects for brain repair. Nat Rev Neurol. 2015;11(1):56-64.

10. McCulloch L, Alfieri A, McColl BW. Experimental Stroke Differentially Affects Discrete Subpopulations of Splenic Macrophages. Front Immunol. 2018;9:1108.

11. Laffer B, Bauer D, Wasmuth S, Busch M, Jalilvand TV, Thanos S, et al. Loss of IL-10 Promotes Differentiation of Microglia to a M1 Phenotype. Front Cell Neurosci. 2019;13:430.

12. Lu DR, Wu H, Driver I, Ingersoll S, Sohn S, Wang S, et al. Dynamic changes in the regulatory T-cell heterogeneity and function by murine IL-2 mutein. Life Sci Alliance. 2020;3(5).

13. Zhuang R, Feinberg MW. Regulatory T cells in ischemic cardiovascular injury and repair. J Mol Cell Cardiol. 2020;147:1-11.

14. Pluijmert NJ, Atsma DE, Quax PHA. Post-ischemic Myocardial Inflammatory Response: A Complex and Dynamic Process Susceptible to Immunomodulatory Therapies. Front Cardiovasc Med. 2021;8:647785.

15. He N, Mao XJ, Ding YM, Zuo T, Chen YY, Wang LL. New insights into the biological roles of immune cells in neural stem cells in post-traumatic injury of the central nervous system. Neural Regen Res. 2023;18(9):1908-16.

16. Wang H, Wang Z, Wu Q, Yuan Y, Cao W, Zhang X. Regulatory T cells in ischemic stroke. CNS Neurosci Ther. 2021;27(6):643-51.

17. Zhang Y, Liesz A, Li P. Coming to the Rescue: Regulatory T Cells for Promoting Recovery After Ischemic Stroke. Stroke. 2021;52(12):e837-e41.

18. Brea D, Agulla J, Rodriguez-Yanez M, Barral D, Ramos-Cabrer P, Campos F, et al. Regulatory T cells modulate inflammation and reduce infarct volume in experimental brain ischaemia. J Cell Mol Med. 2014;18(8):1571-9.

19. Stubbe T, Ebner F, Richter D, Engel O, Klehmet J, Royl G, et al. Regulatory T cells accumulate and proliferate in the ischemic hemisphere for up to 30 days after MCAO. J Cereb Blood Flow Metab. 2013;33(1):37-47.

20. Eremenko E, Taylor ZV, Khand B, Zaccai S, Porgador A, Monsonego A. An optimized protocol for the retroviral transduction of mouse CD4 T cells. STAR Protoc. 2021;2(3):100719.

21. Koizumi J-I, Yoshida Y, Nakazawa T, Ooneda G. Experimental studies of ischemic brain edema. Nosotchu. 1986;8(1):1-8.

22. De Meyer SF, Schwarz T, Schatzberg D, Wagner DD. Platelet glycoprotein Ibalpha is an important mediator of ischemic stroke in mice. Exp Transl Stroke Med. 2011;3:9.

23. Takeshita H, Yamamoto K, Nozato S, Inagaki T, Tsuchimochi H, Shirai M, et al. Modified forelimb grip strength test detects aging-associated physiological decline in skeletal muscle function in male mice. Sci Rep. 2017;7:42323.

24. Borlongan CV, Sanberg PR. Elevated body swing test: a new behavioral parameter for rats with 6-hydroxydopamine-induced hemiparkinsonism. J Neurosci. 1995;15(7 Pt 2):5372-8.

25. Wang YR, Cui WQ, Wu HY, Xu XD, Xu XQ. The role of T cells in acute ischemic stroke. Brain Res Bull. 2023;196:20-33.

26. Yuan C, Shi L, Sun Z, Xu F, Wang C, Shan J, et al. Regulatory T cell expansion promotes white matter repair after stroke. Neurobiol Dis. 2023;179:106063.

27. Shi X, Luo L, Wang J, Shen H, Li Y, Mamtilahun M, et al. Stroke subtype-dependent synapse elimination by reactive gliosis in mice. Nat Commun. 2021;12(1):6943.

28. Bogacka J, Pawlik K, Ciapala K, Ciechanowska A, Mika J. CC Chemokine Receptor

4 (CCR4) as a Possible New Target for Therapy. Int J Mol Sci. 2022;23(24).

29. Farris BY, Monaghan KL, Zheng W, Amend CD, Hu H, Ammer AG, et al. Ischemic stroke alters immune cell niche and chemokine profile in mice independent of spontaneous bacterial infection. Immun Inflamm Dis. 2019;7(4):326-41.

30. Gunsolly C, Nicholson JD, Listwak SJ, Ledee D, Zelenka P, Verthelyi D, et al. Expression and regulation in the brain of the chemokine CCL27 gene locus. J Neuroimmunol. 2010;225(1-2):82-90.

31. Zhang B, Wu C, Zhang Z, Yan K, Li C, Li Y, et al. CXCL12 is associated with FoxP3(+) tumor-infiltrating lymphocytes and affects the survival of patients with oral squamous cell carcinoma. Oncol Lett. 2019;18(2):1099-106.

32. Popiolek-Barczyk K, Ciechanowska A, Ciapala K, Pawlik K, Oggioni M, Mercurio D, et al. The CCL2/CCL7/CCL12/CCR2 pathway is substantially and persistently upregulated in mice after traumatic brain injury, and CCL2 modulates the complement system in microglia. Mol Cell Probes. 2020;54:101671.

33. Nakagawa S, Izumi Y, Takada-Takatori Y, Akaike A, Kume T. Increased CCL6 expression in astrocytes and neuronal protection from neuron-astrocyte interactions. Biochem Biophys Res Commun. 2019;519(4):777-82.

34. Richmond JM, Strassner JP, Essien KI, Harris JE. T-cell positioning by chemokines in autoimmune skin diseases. Immunol Rev. 2019;289(1):186-204.

35. Zanin-Zhorov A, Tal G, Shivtiel S, Cohen M, Lapidot T, Nussbaum G, et al. Heat shock protein 60 activates cytokine-associated negative regulator suppressor of cytokine signaling 3 in T cells: effects on signaling, chemotaxis, and inflammation. J Immunol. 2005;175(1):276-85.

36. Marino Gammazza A, Colangeli R, Orban G, Pierucci M, Di Gennaro G, Lo Bello M, et al. Hsp60 response in experimental and human temporal lobe epilepsy. Sci Rep. 2015;5:9434.

37. Korobova ZR, Arsentieva NA, Totolian AA. Macrophage-Derived Chemokine MDC/CCL22: An Ambiguous Finding in COVID-19. Int J Mol Sci. 2023;24(17).

38. García-Berrocoso T, Giralt D, Llombart V, Bustamante A, Penalba A, Flores A, et al. Chemokines after human ischemic stroke: From neurovascular unit to blood using protein arrays. Translational Proteomics. 2014;3:1-9.

39. Rapp M, Wintergerst MWM, Kunz WG, Vetter VK, Knott MML, Lisowski D, et al. CCL22 controls immunity by promoting regulatory T cell communication with dendritic cells in lymph nodes. J Exp Med. 2019;216(5):1170-81.

ABSTRACT(IN KOREAN)

CCR4 과발현을 통한 CD4+CD25+ 조절 T 세포 이동 촉진으로 허혈성 뇌졸중 결과의 개선

<지도교수 이종은>

연세대학교 대학원 의과학과

이서우

뇌 내의 면역세포인 미세아교세포는 허혈성 뇌졸중으로 인해 활성화한다고 알려져 있다. 미세아교세포는 허혈성 뇌졸중에서 괴사하는 뉴런과 함께 면역 반응에 관련된 인자들을 분비한다. 혈액-뇌 장벽이 파괴되면서 활성화된 전염증성 면역 세포 유입으로 인해 일어난 이차 조직 손상은 사망률과 장애 수준을 극적으로 증가시킨다. 허혈성 뇌졸중의 급성기에서 신경염증의 중요성은 집중적으로 연구되어 왔지만 급성기의 CD4+ CD25+ Foxp3+ 조절 T (Treg) 세포의 긍정적인 효과에 대해서는 아직 확실한 결과가 나오지 않았다. 하지만, 회복 단계 동안 Treg 세포의 긍정적인 효과는 신경염증을 개선하고 허혈성 뇌졸중 이후 경색 크기를 줄이는 것과 관련된다고 보고된 바 있다. Treg 세포가 이러한 신경 보호 효과를 발휘하려면 허혈성 뇌로 이동해야 하지만 Treg 이동에 관여하는 미세아교세포 유래의 인자는 아직 명확하게 정의되지 않았다. 따라서 우리는 허혈성 뇌졸중 동안 Treg 세포 이동과 관련된 특정 케모카인과 수용체 간의 상호 작용과 Treg 세포가 경색 크기 및 운동 기능에 미치는 영향을 조사하는 것을 목표로 했다. 염증성 CD8+ T 세포와 Treg세포의 이동 패턴의 차이는 산소-포도당 결핍(OGD(+) Direct)에 노출된 1차 미세아교세포 배지와

24시간 재관류 후 수집한 배지 (OGD(+) Rep)를 사용한 trans-well 이동 분석을 통해 확인되었다. 위 조건에서 수집된 배양 배지는 활성화된 미세아교세포 유래 케모카인을 식별하기 위해 케모카인 프로파일링에 사용되었다. OGD(+) Direct 배지에서 높게 나타난 T 세포 homing 관련 케모카인에 반응하여 CD8+ T 세포와 Treg 세포 모두 OGD(+) Direct 배지로 더 많은 이동을 보여주었다. In vitro 실험을 통해 Treg 세포의 이동과 밀접하게 관련된 두 개의 후보 케모카인-수용체가 선택되었으며, Tregs를 길항제로 처리하여 trans-well 실험을 실행하였을 때, CCL22-CCR4 상호 작용의 억제가 CXCL12-CXCR4보다 OGD(+) Direct 미디어로의 이동을 크게 감소시켰다. 다음으로, Treg 세포에서 CCR4 과발현은 MSCV 레트로바이러스 (MSCV-CCR4)를 사용하여 수행되었다. MSCV-CCR4 형질도입을 하지 않은 untreated Treg 세포 (UT Treg) 과 형질도입을 한 Treg 세포 (TD Treg)를 이용해 OGD(+) Direct 배지로의 trans-well 이동 분석과 마우스에서 transient middle cerebral artery occlusion (tMCAO) 후 생체 내 효과를 확인했다. UT Treg과 비교하여 TD Treg에서 EGFP와 CCR4의 발현 및 OGD(+) Direct 배지로의 이동이 증가했다. tMCAO 수술 이후 TD Treg을 주입하였을 때, 경색 크기가 눈에 띄게 감소하고 운동 기능이 일부 개선된 것으로 나타났다. 결론적으로 우리는 Treg 세포의 이동에 관련된 미세아교세포 유래의 케모카인과 수용체를 발굴하였고 CCR4 발현을 늘린 Treg 세포를 주입한 tMCAO 동물 모델에서 신경 손상의 심각성을 줄임으로써 허혈성 뇌졸중에 대한 Treg세포의 신경 보호 효과를 보여주었다.

핵심되는 말 : 조절 T 세포, 허혈성 뇌졸중, 신경염증, 미세아교세포