Modulating monocyte-derived macrophage polarization in cerebral ischemic injury with type 1 diabetes

A-Ra Goh

Department of Medical Science

The Graduate School, Yonsei University

Modulating monocyte-derived macrophage polarization in cerebral ischemic injury with type 1 diabetes

Directed by Professor Jong Eun Lee

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A-Ra Goh

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This certifies that the Master's Thesis of A-Ra Goh is approved.

[Signature]

Thesis Supervisor : Jong Eun Lee

[Signature]

Thesis Committee Member#1 : Bon-Nyeo Koo

[Signature]

Thesis Committee Member#2 : Yong-Ho Lee

The Graduate School
Yonsei University

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ABSTRACT

Modulating monocyte-derived macrophage polarization in cerebral ischemic injury with type 1 diabetes

A-Ra Goh

Department of Medical Science
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Ischemic stroke (IS) which is fatal with high mortality is caused by reduction or blockage of blood flow to the brain. In the case of type 1 diabetes mellitus (T1DM), the risk of ischemic stroke is 6.3 times higher than in normal people. Hyperglycemia in T1DM induces an increase in oxidative stress and Blood-Brain Barrier (BBB) disruption, which increases the influx of blood immune cells into the brain parenchyma. In cerebral ischemia, it has been reported that infiltrating monocytes differentiate into pro-inflammatory or anti-inflammatory macrophages, having a large effect on outcomes of ischemic stroke. In addition, some studies have reported that interleukin-4 (IL-4) and interleukin-13 (IL-13) play a role in the post-ischemia repair process by polarizing infiltrating monocytes to an anti-inflammatory phenotype. In this study, we aimed to figure out the effect of phenotypical polarization of monocyte-derived macrophages on the prognosis in cerebral ischemia with T1DM. We first established a T1DM mice model using streptozotocin (150 mg/kg) and these mice were subjected to transient middle cerebral artery occlusion. We observed that BBB permeability was increased in T1DM mice and T1DM-IS mice exhibited aggravated infarct volume through 2,3,5-triphenyltetrazolium chloride staining, two-photon live imaging, and Evans blue staining. Also, we confirmed increased infiltration of monocyte-derived macrophages and

downregulated anti-inflammatory cells, which are related to tissue remodeling after inflammation, in the infarct area of T1DM-IS mice through immunohistochemistry, western blotting, and flow cytometry. We have observed the phenotypical changes of monocyte-derived macrophages, alleviated infarct volume, and improvement of motor function in T1DM-IS mice administered IL-4 and IL-13. These data suggest that modulating phenotypical changes of macrophages in T1DM mice following ischemic stroke might influence the ischemic recovery.

Key words : type 1 diabetes mellitus, ischemic stroke, monocyte-derived macrophages, phenotypical changes, anti-inflammatory cells

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I. Introduction

Stroke is caused by abnormality in the blood flow to the brain and following brain cell damage. It is accompanied by various neurological defects such as hemiplegia and speech disorders. Also, it has high mortality, aftereffects and complications. Stroke is classified into two categories; first is cerebral infarction, which is caused by blockage or reduction of blood flow to the brain, second is cerebral hemorrhage, which leads to bleeding from cerebral blood vessels. Cerebral infarction accounts for 80% of all strokes[1]. Cerebral ischemia-reperfusion treatment is used to restore blood flow, but it brings about secondary brain damage, causing oxidative stress and neuroinflammation[2].

The relationship between type 1 diabetes mellitus (T1DM) and ischemic stroke has been studied for a long time. T1DM is characterized by loss of insulin secretion function due to the disruption of pancreatic beta cells, accounting for 5-10% of all diabetes cases[3]. Patients with T1DM have a 6.3 times higher risk of ischemic stroke than normal people[4]. Furthermore, their prognosis after stroke is even worse. Several studies have shown that

hyperglycemia induces neuroinflammation and upregulates the expression of pro-inflammatory factors[5,6], which may affect the phenotypical expression of microglia and monocyte-derived macrophages (MDMs) in ischemic brain tissue and aggravate neuroinflammation caused by ischemia. Moreover, many studies have demonstrated that vascular injury has a relationship with abnormal glucose homeostasis. Hyperglycemia increases free radical production and causes Blood-brain barrier (BBB) disorder by damaging the tight junction between brain endothelial cells[7]. BBB disruption may cause more blood-derived monocytes to enter the brain. It is proposed that revealing the role of MDMs in ischemia-induced neuroinflammation is important to understand the mechanisms of ischemic stroke in T1DM and to develop therapeutic strategies for ischemic stroke.

In the brain, resident microglia act as immune cells and are involved in inflammatory reactions. When brain homeostasis changes, microglia are activated and change their morphologies and expression of their surface markers. In ischemic stroke, neuroinflammation serves critical roles in ischemic injury. Reactive oxygen species, cytokines, chemokines, and inflammatory factors are secreted to induce inflammatory reactions. Resident microglia are known to be activated quickly after ischemia[8]. In addition to microglia, MDMs play an important role in neuroinflammation after ischemic stroke. Blood-derived monocytes infiltrate the brain via damaged BBB and are involved in acute inflammatory responses after ischemia. Many studies showed that blood-derived monocytes were recruited to the ischemic parenchyma in the early phase of ischemic stroke[9,10]. Also, there is growing evidence that blood-derived monocytes interact with surrounding microenvironment like activated microglia and differentiate into pro-inflammatory macrophages or anti-inflammatory macrophages, affecting the prognosis of ischemic stroke[11]. Although many studies have been conducted on the effects of microglia and MDMs in the brain after ischemia, their exact role has not yet been determined.

The blood-derived monocytes polarize into pro-inflammatory or anti-inflammatory macrophages in response to their microenvironment. Microglia and macrophages have various phenotypes and functions, but they can be divided into two typical phenotypes. The first is pro-inflammatory phenotype (M1), which causes inflammation. It promotes inflammatory factors such as interleukin-1β (IL-1β), interferon-γ (IFN-γ), tumor necrosis factor α (TNF- α), and inducible nitric oxide synthase (iNOS)[8]. It is characterized by the upregulation of surface marker CD80 and CD86. Another is anti-inflammatory phenotype (M2), which induces tissue recovery and secretion of growth factors. It shows up-regulation of arginase 1, CD206, and Ym-1 and produces interleukin-4 (IL-4), interleukin-13 (IL-13)[8].

In our recent study, we demonstrated that blood-derived monocytes infiltrated damaged tissue in the early stage of stroke and differentiated to M2 phenotype macrophages in the mice brain 3 days after ischemia[12]. Moreover, we showed that IL-4 and IL-13 mediated the phenotypic conversion of the monocyte-derived macrophages to M2 phenotype, and that inhibiting the effect of IL-4 and IL-13 could aggravate infarct volume and prevent beneficial phenotypic conversion of the macrophages. IL-4 and IL-13 are well-known anti-inflammatory cytokines that provide a neuroprotective role in neural injury. The purpose of this study was to confirm the effect of modulating phenotypical changes of monocyte-derived macrophages on ischemic injury in the T1DM conditions and examine whether modulating the phenotypical conversion of blood-derived monocytes by IL-4 and IL-13 affects ischemic injury recovery.

II. Materials and methods

1. Animals

   Male C57BL/6, 8-week-old mice (Central Lab Animal Inc, Seoul, South Korea) were used in this study and all animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Yonsei Laboratory Animal Research Center (YLARC). All animals were under constant temperature with 12 h light/dark cycle and free access to water and food.

2. Establishment of T1DM animal model and experimental groups

   Mice were randomly divided into five groups; Sham, IS, T1DM, T1DM-IS + Veh, T1DM-IS + IL-4, IL-13. T1DM was induced by streptozotocin injection (150 mg/kg). After starving for 6 hours, mice were injected with vehicle (0.1 M citrate buffer, pH 4.5) (Sigma-Aldrich, St. Louis, Missouri, USA) or streptozotocin (Sigma-Aldrich, St. Louis, Missouri, USA) intraperitoneally for 2 consecutive days. We monitored the body weight and blood glucose level of the mice for 2 weeks after streptozotocin injection. Mice with non-fasting blood glucose levels > 300 mg/dL were considered type 1 diabetic mice and underwent a surgery to induce cerebral ischemia. 2 hours after transient middle cerebral artery occlusion surgery, the mice were injected with vehicle (saline) or 2.5 mg IL-4 (Peprotech, London, UK) & 2.5 mg IL-13 (Peprotech, London, UK) intraperitoneally one time.

3. Transient middle cerebral artery occlusion (tMCAO)

   To induce focal cerebral ischemia, mice were subjected to transient middle cerebral artery occlusion as described earlier[13]. A mouse was anesthetized with zoletil (100 mg/kg) (Virbac, Westlake, Texas, USA) and Rompun (10 mg/kg) (Bayer, Leverkusen, Germany). The mouse was incised along the midline of the

neck. Glands and connective tissue were gently removed to expose the right common carotid artery (rCCA). After ligating the distal part of the CCA with a permanent tie, a ribbon tie was made around the bifurcation of the CCA using 6-0 silk threads. The right external carotid artery (rECA) was then ligated with a permanent tie and a small hole was made in the rCCA. 6-0 monofilament with a silicon-coated tip (L.M.S. Korea, Seoul, South Korea) was inserted into the rCCA, and advanced to the middle cerebral artery region. After 30 minutes of occlusion, the suture was withdrawn and the incision was closed.

4. Two-photon intravital imaging

Mice were anesthetized with zoletil (100 mg/kg) and Rompun (10 mg/kg), and fixed in a stereotaxic frame (Live Cell Instrument, Seoul, Korea) to make a cranial window. Cranial window surgery was conducted as described previously[12]. A cranial window was established in the right hemisphere, centered 3 mm lateral to the midline and 1.95 mm posterior to bregma, which are correlated with MCA vascular territory[14]. Then, a 5-mm glass coverslip was used to cover the opened skull with cyanoacrylic glue and the periphery of the cranial window was filled with dental resin (B.J.M. laboratory, Or-Yehuda, Israel) with a fixation ring. After the surgery, the mice were treated with enrofloxacin (Baytril, Bayer, Leverkusen, Germany) and meloxicam (Metacam, Boehringer Ingelheim, Ingelheim am Rhein, Germany) to prevent infections or inflammation. 7 to 10 days after the establishment of the cranial window, the mice were anesthetized and injected with FITC-conjugated dextran (Sigma-Aldrich, St. Louis, Missouri, USA) via the retro-orbital sinus. The images were acquired using a two-photon microscope (LSM7MP, Zeiss, Zena, Germany).

5. Brain sample preparation

Mice were anesthetized with zoletil (100 mg/kg) and Rompun (10 mg/kg). Mouse

brains were collected after perfusing with cold saline. For immunohistochemistry, the brains were fixed with 4% paraformaldehyde (PFA) for one day and incubated with 30% sucrose solution until the brains sank into the solution. The brains were stored at -80° C before sectioning. For western blot assay, the ipsilateral brains were stored at -80° C until use.

6. Western blot assay

The ipsilateral brains were treated with lysis buffer and proteins were extracted by using a homogenizer (Dremel, Racine, Wisconsin, USA). After determining the protein concentration using BCA assay kit (Thermo Fisher, Rockford, Illinois, USA), 50 mg of protein was separated on a 10% SDS-PAGE gel at 100 V for 60 min. The proteins were then transferred to the Polyvinylidene difluoride membrane at 25 V overnight. The membrane was blocked with 5% bovine serum albumin (BSA). The membrane was treated with primary antibodies such as CD68 (Invitrogen, Waltham, Massachusetts, USA), Tmem119 (Invitrogen, Waltham, Massachusetts, USA), CD86 (Abcam, Cambridge, Massachusetts, USA), CD206 (Invitrogen, Waltham, Massachusetts, USA), and ß-actin (Abcam, Cambridge, Massachusetts, USA) at 4 ° C overnight. Then the membrane was washed with TBST and reacted with HRP-conjugated IgG antibodies at room temperature for 1 h 30 min. The images were captured by the chemiluminescent image analyzer (LAS 4000, Fujifilm, Tokyo, Japan) and analyzed by ImageJ software.

7. Immunohistochemistry

Frozen brains were sliced into 14-μm thickness and the sections were treated with methanol for 5 min at -20 °C. The sections were permeabilized with 0.25% Triton X-100 in PBS for 20 min and then blocked with 5% BSA at room temperature for 1 h. The sections were incubated with primary antibodies such as CD68 (Bio-Rad, Hercules, CA, USA), Tmem119 (Abcam, Cambridge, Massachusetts, USA),

CD86 (Abcam, Cambridge, Massachusetts, USA), and CD206 (Abcam, Cambridge, Massachusetts, USA) at 4 °C overnight. After washing with PBS, the sections were incubated with secondary antibodies such as Alexa Fluor 488 anti-Rabbit IgG (Invitrogen, Waltham, Massachusetts, USA) and Alexa Fluor 594 anti-Mouse IgG (Invitrogen, Waltham, Massachusetts, USA) at room temperature for 1 h. The samples were washed with PBS 3 times and stained with 1 μg/ml 4',6-diamidino-2-phenylindol (DAPI) (Invitrogen, Waltham, Massachusetts, USA) for 5 min. The images of the peri-infarct zone in ipsilateral brain were acquired by a confocal microscope (LSM710, Carl Zeiss, Jena, Germany).

8. Measurement of infarct volume

Mice who had undergone tMCAO surgery were anesthetized with zoletil (100 mg/kg) and Rompun (10 mg/kg), followed by perfusing with cold saline. The collected brains were cut to 2-mm thickness and dipped in 2% 2,3,5-Triphenyltetrazolium chloride (TTC) (Santa Cruz Biotechnology, Dallas, Texas, USA). After incubating at 37 C° for 20 min, the brains were washed with saline, and infarct size was measured through ImageJ software.

9. Dissociation of microglia/monocyte-derived macrophages

Brains were dissociated into a single-cell suspension. Ipsilateral brains were mechanically dissociated with the GentleMACS program 37 C°_ABDK_01 for 30 min. After filtering with a 70 μm MACS strainer, the cells were centrifuged at 300x g for 10 min and washed with cold DPBS. Debris are removed with Debris Removal Solution (Miltenyi Biotech, Auburn, California, USA) and centrifuged at 3000x g for 10 min. After removing red blood cells with red blood cell removal solution (Miltenyi Biotech, Auburn, California, USA), the cells were washed in cold PB buffer and centrifuged at 1000x g for 10 min. Afterwards, microglia and monocytes/ macrophages were sorted by magnetic cell sorting. The cells were

stained with anti-mouse CD11b for 15 min and centrifuged at 300x g for 10 min.

10. Flow cytometry

Sorted microglia/blood-derived monocytes were blocked with 3% BSA for 20 min and reacted with FITC-conjugated CD11b (Invitrogen, Waltham, Massachusetts, USA), PE-conjugated CD45 (Invitrogen, Waltham, Massachusetts, USA), eFluor450-conjugated F4/80 (Invitrogen, Waltham, Massachusetts, USA), PE-Cy7-conjugated CD206 (Invitrogen, Waltham, Massachusetts, USA), and APC-conjugated CD86 (Invitrogen, Waltham, Massachusetts, USA) for 20 min at 4 C° in the dark. After staining, the cells were washed with FACS staining buffer 3 times and filtered through a cell strainer cap (STEMCELL Technologies, Vancouver, Canada). The cells were analyzed with LSRII flow cytometer (BD Biosciences, Franklin Lakes, New Jersey, USA).

11. Evans blue staining

2% Evans blue (Santa Cruz Biotechnology, Dallas, Texas, USA) were injected intravenously into ischemic mice 2 h before sacrifice. The mice were perfused with cold saline to remove extra solution and brains were collected. The brains were homogenized with formamide (Sigma-Aldrich, St. Louis, Missouri, USA) and centrifuged. The supernatants were collected and analyzed by a spectrophotometer.

12. Behavior test

To evaluate motor function after tMCAO, elevated body swing test and grip strength test were performed. For elevated body swing test, a mouse was lifted 10-15cm above the ground by its tail. The direction in which the mouse turned its head was recorded. Each mouse underwent 20 trials. Grip strength test was performed using an FGE-10XY digital gauge system (Nidec SHIMPO, Kyoto,

Japan). The mouse was lifted by its tail and allowed to hold a bar of the sensor. The mouse was pulled back horizontally until its front paws were separated from the bar. The maximum grip strength was automatically recorded. A total of 3 trials were conducted for each mouse and the measured values were averaged. Both tests were performed 1 day before tMCAO, 1 day, 2 days, and 3 days after tMCAO.

13. Statistical analysis

All statistical analyses were conducted using Prism 9 software (San Diego, California, USA). Student's unpaired t-test and one way ANOVA with Tukey's multiple comparison test were used to determine whether there was a statistical significance in the values of each group. A value of $p < 0.05$ was considered significant.

III. Results

1. A T1DM mice model exhibited a significantly increased level of blood glucose, hemoglobin A1c, microalbumin urea creatinine

Body weight, the level of blood glucose, hemoglobin A1c, microalbumin, and urea creatinine were measured to evaluate a streptozotocin-induced T1DM mice model. (Figure 1A-1E) T1DM mice showed slightly decreased body weight compared with normal mice, but there was no notable difference. The blood glucose levels of T1DM mice were over 300 mg/dL, which was significantly higher than that of normal mice. (Normal vs T1DM *** $p< 0.001$) Additionally, the levels of hemoglobin A1c were significantly increased in T1DM mice compared with normal mice. (Normal vs T1DM ** $p= 0.0013$) The levels of microalbumin and urea creatinine were measured to evaluate kidney damage caused by diabetes, which is well known as a complication of diabetes[15]. T1DM induced a significant increase in microalbumin (Normal vs T1DM ** $p= 0.0039$) and urea creatinine levels. (Normal vs T1DM *** $p< 0.001$) These results indicate the establishment of the T1DM mice model.

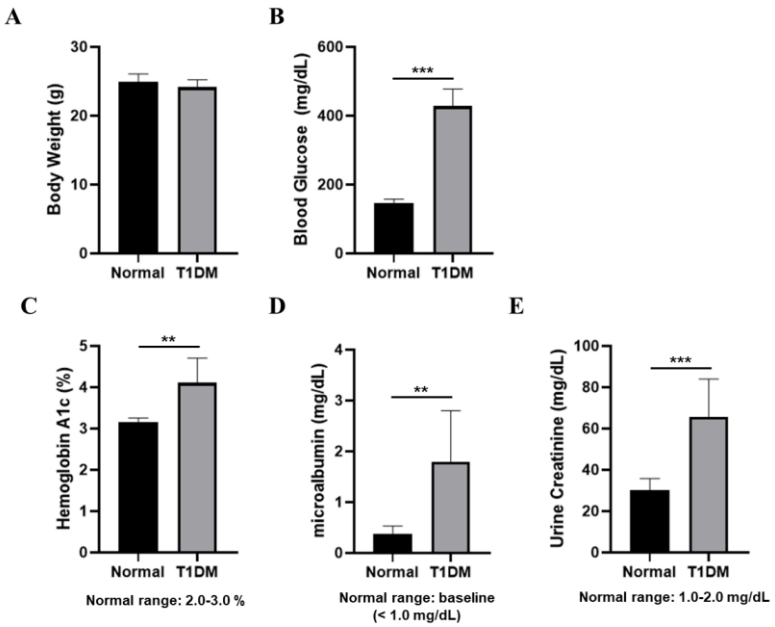


Figure 1. Confirmative examination of a T1DM mice model. (A) Estimation of body weight. (B) Estimation of blood glucose levels; *** $p < 0.001$. (C) The levels of hemoglobin A1c; ** $p < 0.01$. (D) The levels of microalbumin; ** $p < 0.01$. (E) Urea creatinine level; *** $p < 0.001$. All values are expressed as mean ± SEM and unpaired t-test. Normal group n= 5-7, T1DM group n= 6-15.

2. Increased infarct volume and Blood-brain barrier disruption were observed in T1DM mice

Infarct volume was measured by TTC staining in IS and T1DM-IS mice. TTC staining results showed that T1DM-IS mice exhibited considerably increased infarct volume compared with IS mice 1 and 3 days after ischemia. (Figure 2A, 2B) (IS vs T1DM-IS * p= 0.0450, IS vs T1DM-IS ** p= 0.0020) To investigate BBB integrity in T1DM mice, intravital imaging and Evans blue staining were performed. For intravital imaging, a cranial window was made in MCA vascular territory of the right hemisphere and the mice were imaged through the cranial window after intravenous injection of FITC-conjugated dextran. Measuring GFP intensity leaking out from the vessels can predict BBB breakdown. In Figure 2C and 2D, GFP intensity was increased in T1DM mice compared with normal mice. (Normal vs T1DM *** p< 0.001) Afterwards, Evans blue staining was conducted to estimate BBB disruption after tMCAO in T1DM mice. Evans blue dye can penetrate into the brain parenchyma from blood vessels under damaged BBB conditions. Dye leakage was slightly increased in T1DM mice compared with normal mice. Also, tMCAO surgery increased dye leakage at the ipsilateral hemisphere in IS and T1DM-IS group, with significantly increased dye leakage in T1DM-IS mice compared with IS mice. (Figure 2E, 2F) (IS vs T1DM-IS *** p< 0.001) Those data suggest that T1DM increases infarct volume and induces severe damage to BBB integrity after ischemia.

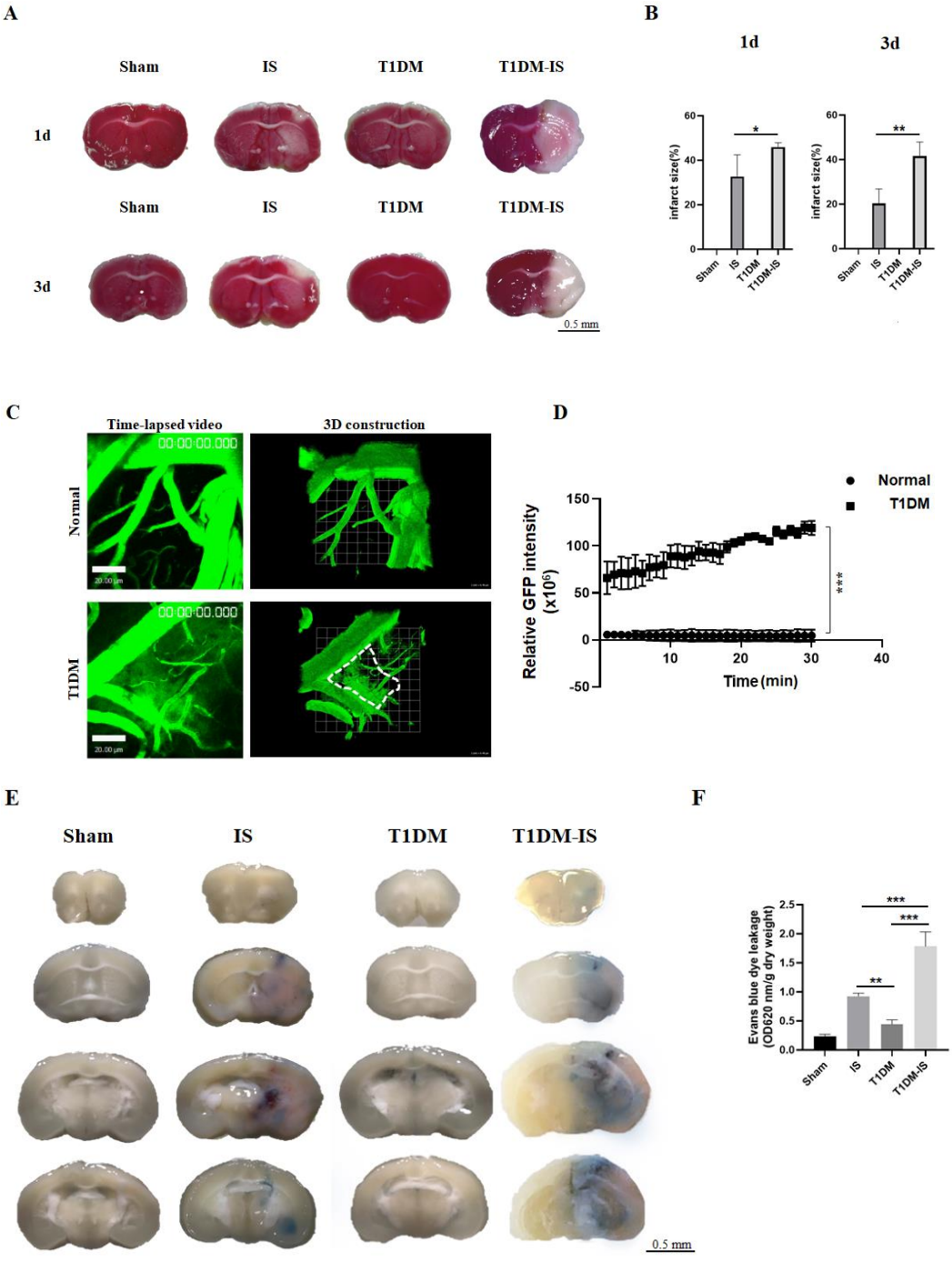
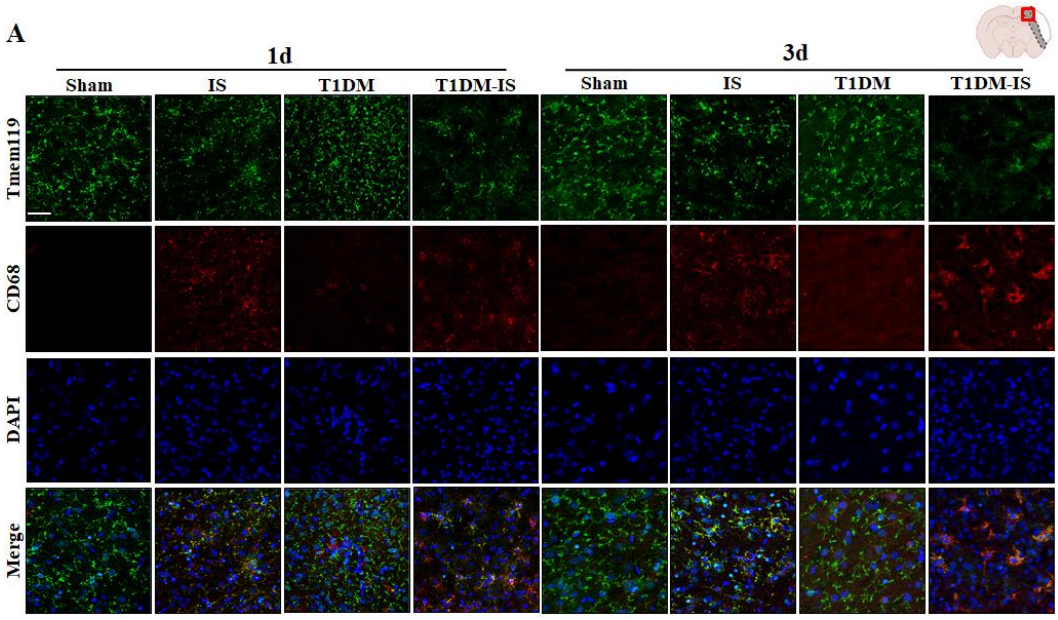
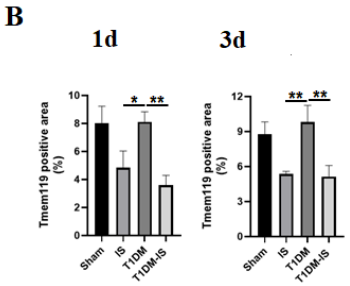


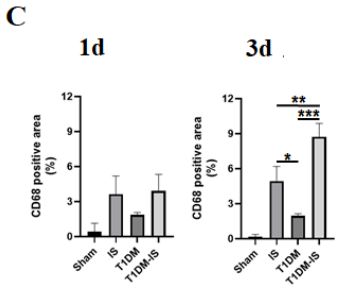
Figure 2. Estimation of BBB integrity and infarct volume in T1DM mice. (A) TTC staining image representing infarct volume in IS and T1DM-IS mice. (B) Quantification of infarct size in IS and T1DM-IS mice; * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$. (C) Captured image of blood vessels in the MCA territory by two-photon microscope in normal and T1DM mice. (D) Quantification of relative GFP intensity in normal and T1DM mice; *** $p < 0.001$. (E) Representative brain images of Evans blue staining. (F) Quantitative analysis of Evans blue dye leakage; ** $p< 0.01$, *** $p < 0.001$. All values are expressed as mean ± SEM, unpaired t test, and one-way ANOVA followed by Tukey's multiple comparison tests. Each group n= 3.

3. Monocyte infiltration was increased in T1DM-IS mice

Immunohistochemistry and western blot assay were performed to confirm monocyte infiltration to the ischemic parenchyma in T1DM-IS mice brain, using microglia-specific marker Tmem119 and microglia & macrophage marker CD68. Tmem119 is a stable marker for microglia that can distinguish microglia from other macrophage populations[16]. CD68 expression is observed in macrophages such as monocytes[17] and upregulated in activated microglia[18]. Immunohistochemistry data noted that Tmem119 and CD68 double-positive cells were decreased after tMCAO in the penumbra of IS and T1DM-IS group at 1 and 3 days after ischemia. (Figure 3A-3C) Tmem119-negative and CD68-positive cells were invisible in the sham group, and significantly increased in T1DM-IS group compared with IS group 3 days after ischemia. (IS vs T1DM-IS ** p= 0.0026) There is no significant change in Tmem119 expression between Sham and T1DM group, but CD68-positive cells were slightly increased in T1DM group compared with sham group. Similarly, in western blot assay, Tmem119 expression level was decreased in both IS and T1DM-IS group 1 and 3 days after tMCAO. (Figure 3D, 3E) tMCAO surgery induced an increase of CD68 expression in both IS and T1DM-IS group, showing a significant increase in T1DM-IS group compared to IS group 3 days after ischemia. (Figure 3F) (IS vs T1DM-IS * p= 0.0496) Flow cytometry analysis was also conducted to investigate infiltrating monocytes at a single-cell level in a time-dependent manner. (Figure 3G, 3H) The results showed that the number of CD11b-positive and $CD45^{high}$ monocytes/macrophages was increased over time after stroke, which was further increased in T1DM-IS group compared to IS group at 3 days after ischemia. (IS vs T1DM-IS *** p< 0.001) These data indicate that monocyte infiltration was increased in T1DM-IS mice.







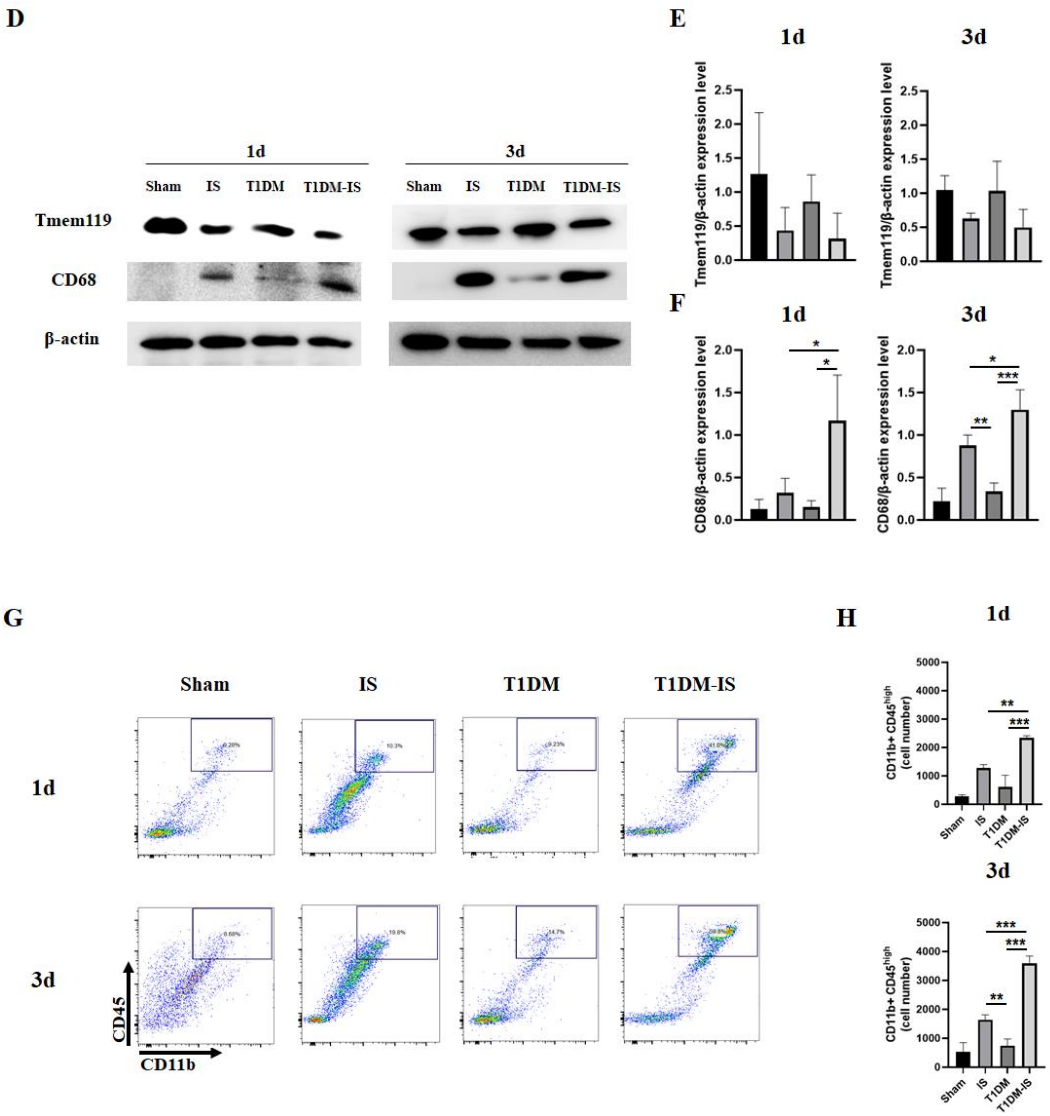
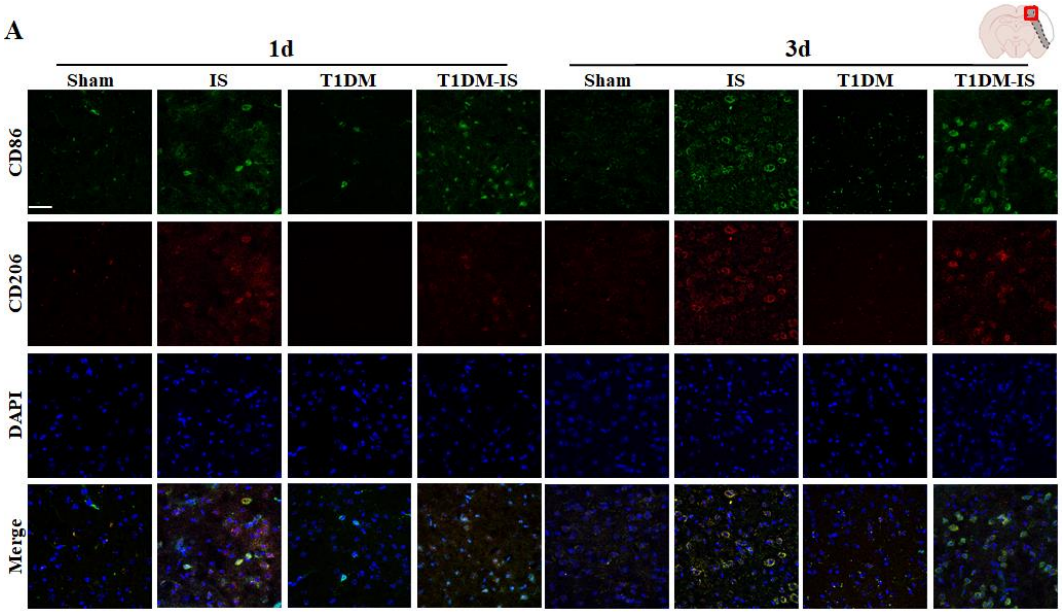


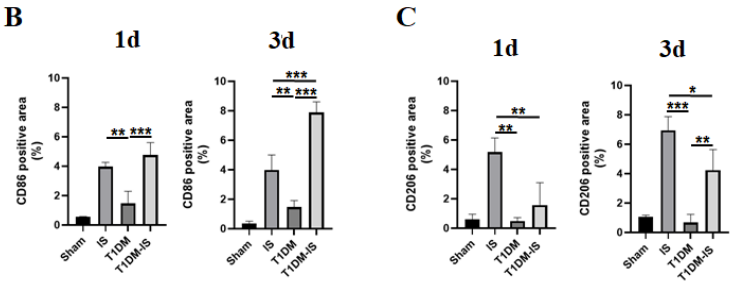
Figure 3. Increased monocyte infiltration in T1DM-IS mice. (A) Immunohistochemistry data showing Tmem119 and CD68 positive cells from the peri-infarct zone in the ipsilateral brain at 40X magnification. Scale bar= 50 μm. (B) Tmem119 positive area. * p< 0.05, ** p< 0.01. (C) CD68 positive area. * p< 0.05, ** p< 0.01, *** p< 0.001. (D) Western blot analysis of Tmem119 & CD68. (E) Quantitative analysis of Tmem119

expression level. (F) Quantification of CD68 expression level; * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$. (G) Flow cytometric analysis gated by CD11b+ and $CD45^{high}$. (H) Quantitative analysis of CD11b+ $CD45^{high}$ cell number; ** $p< 0.05$, *** $p< 0.001$. All values are expressed as mean ± SEM and one-way ANOVA followed by Tukey's multiple comparison tests. Each group n= 3.

4. Alternatively activated monocyte-derived macrophages were decreased in T1DM-IS mice

To ascertain the phenotype of monocyte-derived macrophages after ischemia, immunohistochemistry and western blot assay using CD86 (pro-inflammatory phenotype marker) and CD206 (anti-inflammatory phenotype marker) were conducted. In T1DM-IS group, CD86 expression level was increased compared with IS group as shown in Fig. 4A, 4B, and 4C. In contrast, T1DM-IS group showed decreased CD206 expression level compared to IS group 3 days after ischemia. (IS vs T1DM-IS * $p= 0.0228$) A little increase in CD86 expression and a decrease in CD206 expression was observed in T1DM group compared to sham group, but there was no significant difference. In line with immunohistochemistry data, western blot data also represented increased CD86 expression level and significantly decreased CD206 expression level at 3 days after ischemia, shown in Fig. 4D, 4E, and 4F. (IS vs T1DM-IS * $p= 0.0303$) To determine MDM phenotype at a single cell level, flow cytometry analysis was performed. MDM was defined as CD11b-positive, $CD45^{high}$, and F4/80-positive populations. (Figure 4G) The number of CD86-positive and CD206-negative MDMs was significantly increased in T1DM-IS group at 3 days after ischemia compared with IS group. (IS vs T1DM-IS *** $p< 0.001$) (Figure 4H) However, the number of CD86-negative and CD206-positive MDMs was considerably decreased in T1DM-IS group at 1 and 3 days after ischemia. (IS vs T1DM-IS *** $p< 0.001$) (Figure 4I) There was a slight increase in CD86 expression and a decrease in CD206 expression in T1DM group compared to sham group without a significant difference. These data indicate that T1DM-IS condition induced an increase in pro-inflammatory MDMs and decrease in anti-inflammatory MDMs.





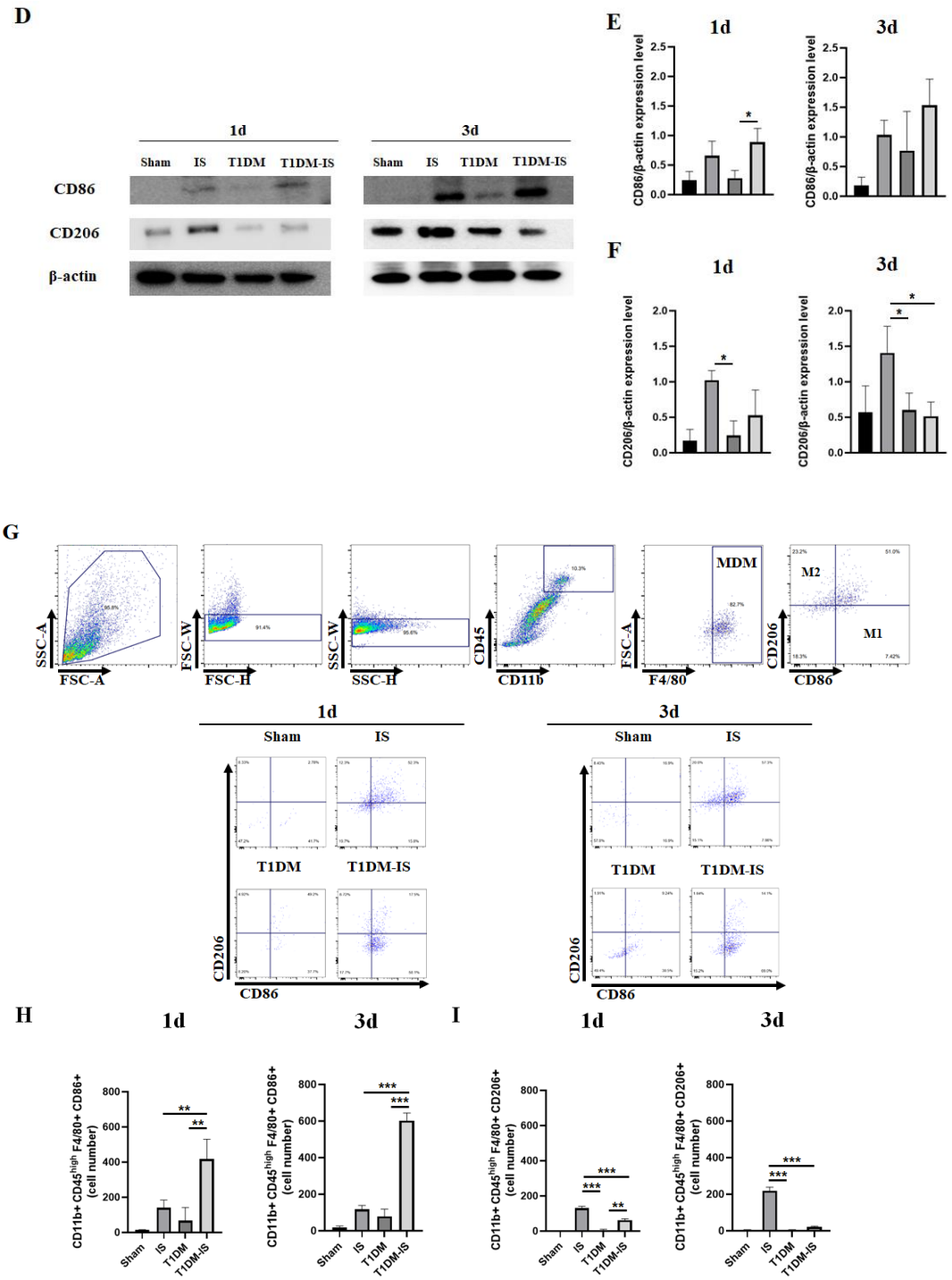


Figure 4. The decline of anti-inflammatory monocyte-derived macrophages in T1DM-IS mice. (A) Immunohistochemistry data showing CD86 and CD206 positive cells in the peri-infarct zone of the ipsilateral brain at 40X magnification. Scale bar= 50 μm. (B) CD86 positive area; ** $p< 0.01$, *** $p< 0.001$. (C) CD206 positive area; * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$. (D) Western blot assay showing CD86 and CD206 expression. (E) Quantitative analysis of CD86 expression level; * $p < 0.05$. (F) Quantification analysis of CD206 expression level; * $p< 0.05$. (G) Flow cytometric analysis of CD86 and CD206 in monocyte-derived macrophages gated by CD11b+ $CD45^{high}$ F4/80+ 1 and 3 days after tMCAO. (H) Quantification of flow cytometry analysis for the number of CD86 expressing MDMs; ** $p< 0.01$, *** $p< 0.001$. (I) Quantification of flow cytometry analysis for the number of MDMs expressing CD206; ** $p< 0.01$, *** $p< 0.001$. All values are expressed as mean ± SEM and one-way ANOVA followed by Tukey's multiple comparison tests. Each group n= 3.

5. IL-4 and IL-13 treatment resulted in decreased infarct volume and recovery of motor function in T1DM-IS mice

To evaluate the effect of IL-4 and IL-13 in T1DM-IS, TTC staining and behavior tests were performed after IL-4 & IL-13 treatment in T1DM-IS mice 3 days after tMCAO. Elevated body swing test and grip strength test are widely used to assess motion deficit after ischemia in an animal model. TTC staining data showed decreased infarct volume in IL-4 and IL-13-treated group compared with vehicle-treated T1DM-IS group 3 days after tMCAO. (T1DM-IS + Veh vs T1DM-IS + IL-4 & IL-13 * p= 0.0379) (Figure 5A, 5B) In elevated body swing test, swing bias was not observed in sham and T1DM group. (Figure 5C) Swing bias in vehicle-treated T1DM-IS group was higher than IS group, but IL-4 & IL-13 treatment reduced swing bias in T1DM-IS mice at 3 days after ischemia. (T1DM-IS + Veh vs T1DM-IS + IL-4 & IL-13 * p= 0.0414) In grip strength test, the difference in grip strength between sham and T1DM group was not found. T1DM-IS group showed a significantly low grip strength score compared with IS group. (IS vs T1DM-IS + Veh *** p< 0.001) Also, grip strength was higher in IL-4 and IL-13 treated group 3 days after tMCAO compared with vehicle-treated T1DM-IS group. (T1DM-IS + Veh vs T1DM-IS + IL-4 & IL-13 *p= 0.0145) (Figure 5D) These data indicate the reparative effects of motor function and infarct damage by IL-4 and IL-13 treatment in T1DM-IS.

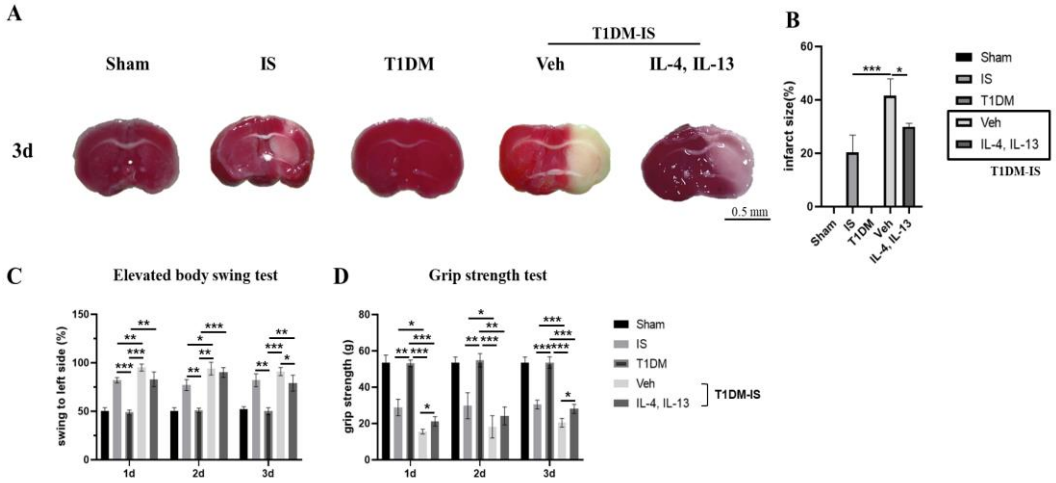
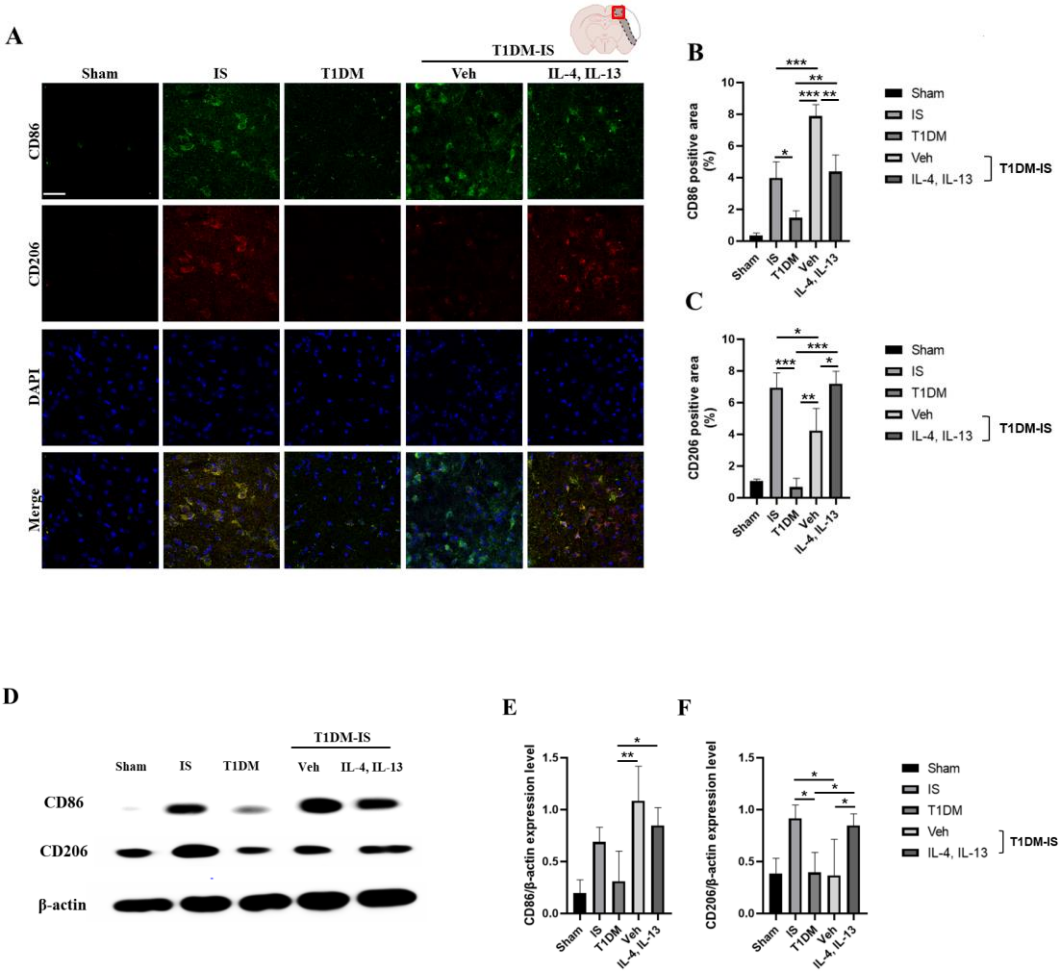


Figure 5. The reparative effects of IL-4 and IL-13 in T1DM-IS. (A) TTC staining image showing infarct volume in T1DM-IS + Veh and T1DM-IS + IL-4, IL-13 group. (B) Quantification of infarct size; * $p< 0.05$, *** $p< 0.001$. (C) Elevated body swing test data, * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$. (D) Grip strength test data; * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$. All values are expressed as mean ± SEM, one-way ANOVA followed by Tukey's multiple comparison tests, and two-way ANOVA followed by Tukey's multiple comparison tests. For TTC staining, each group n= 3. For behavior test, each group n= 5.

6. IL-4 and IL-13 treatment induced M2 phenotype monocyte-derived macrophages in T1DM-IS mice

To determine the phenotypical changes in MDMs after IL-4 and IL-13 treatment in T1DM-IS condition 3 days after ischemia, immunohistochemistry, western blot assay, and flow cytometry analysis were conducted. Immunohistochemistry data noted that CD206-positive cells were increased in the ischemic penumbra of T1DM-IS + IL-4 & IL-13 group compared with T1DM-IS + Veh group. (Figure 6A-6C) (T1DM-IS + Veh vs T1DM-IS + IL-4 & IL-13 * $p= 0.0121$) Western blot assay results showed that CD206 expression level was significantly increased in T1DM-IS + IL-4, & IL-13 group compared to T1DM-IS + Veh group. (T1DM-IS + Veh vs T1DM-IS + IL-4 & IL-13 * $p= 0.0310$) (Figure 6D-6F) In flow cytometry analysis, the expression pattern of CD86 and CD206 in CD11b-positive, $CD45^{high}$, and F4/80-positive MDMs was analyzed. (Figure 6G) IL-4 and IL-13 administration significantly reduced CD86-positive and CD206-negative MDMs (T1DM-IS + Veh vs T1DM-IS + IL-4 & IL-13 *** $p< 0.001$) and increased CD86-negative and CD206-positive MDMs. (T1DM-IS + Veh vs T1DM-IS + IL-4 & IL-13 *** $p< 0.001$) (Figure 6I, 6J) In addition, CD86 and CD206 double-positive cells were also increased in T1DM-IS + IL-4 & IL-13 group compared with T1DM-IS + Veh group. (T1DM-IS + Veh vs T1DM-IS + IL-4 & IL-13 *** $p< 0.001$) (Figure 6K) These data suggest that IL-4 and IL-13 treatment induced MDMs in the brain to an anti-inflammatory phenotype in T1DM-IS.



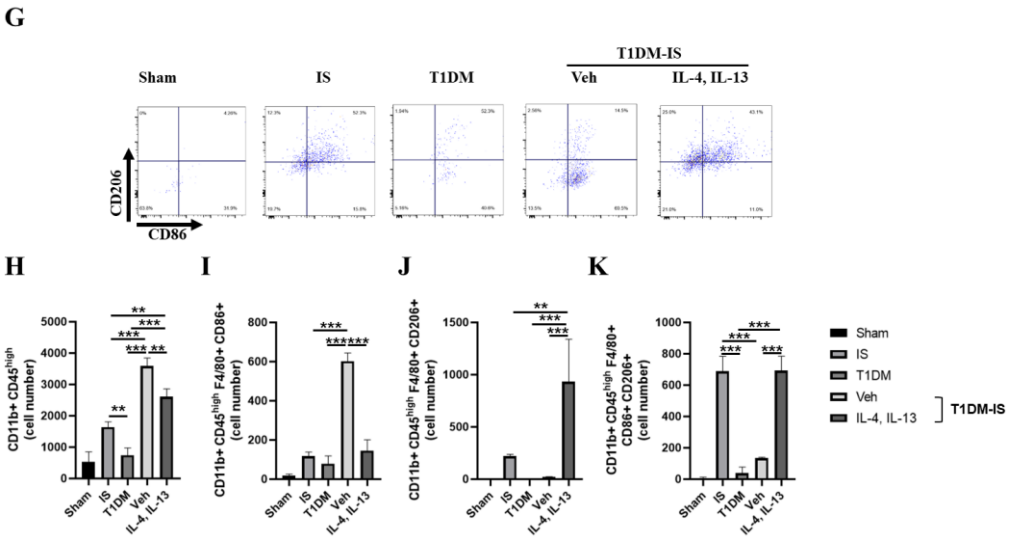


Figure 6. Transition of monocyte-derived macrophage into anti-inflammatory phenotype in IL-4 and IL-13-treated T1DM-IS mice. (A) Immunostaining data showing CD86 and CD206 positive cells from the ischemic penumbra in the ipsilateral brain at 40X magnification. Scale bar= 50 μm. (B) CD86 positive area; * p< 0.05, ** p< 0.01, *** p< 0.001. (C) CD206 positive area; * p< 0.05, ** p< 0.01, *** p< 0.001. (D) Western blot assay presenting CD86 and CD206 expression. (E) Quantitative analysis of CD86 expression level; * p< 0.05, ** p< 0.01. (F) Quantification analysis of CD206 expression level; * p< 0.05. (G) Flow cytometric analysis of CD86 and CD206 in monocyte-derived macrophages gated by CD11b+ $CD45^{high}$ F4/80+ 3 days after tMCAO. (H) Quantitative analysis of flow cytometry analysis for the number of CD11b+ $CD45^{high}$ monocytes/macrophages; ** p< 0.01, *** p< 0.001. (I) Quantification of flow cytometry analysis for the number of CD86-expressing MDMs; *** p< 0.001. (J) Quantification of flow cytometry analysis for the number of CD206-expressing MDMs; ** p< 0.01, *** p< 0.001. (K) Quantification of flow cytometry analysis for the number of CD86 & CD206 double expressing MDMs; *** p< 0.001. All values are expressed as mean ± SEM and one-way ANOVA followed by Tukey's multiple comparison tests. For immunohistochemistry and flow cytometry, n= 3. For western blot assay, n= 4.

IV. Discussion

In this study, it showed that (1) T1DM induces BBB disruption and infiltration of blood-derived monocytes/macrophages to the brain parenchyma after cerebral ischemia, (2) Blood-derived monocytes/macrophages exhibited mainly pro-inflammatory phenotype rather than anti-inflammatory phenotype, (3) IL-4 and IL-13 treatment in T1DM-IS could switch the monocyte/macrophage polarization into anti-inflammatory phenotype, relieve infarct damage and motor function deficit after ischemia.

This investigation has shown that T1DM increased BBB permeability. Hyperglycemia activates toll-like receptor (TLR)-2 and -4, produces reactive oxygen species[19], and upregulates phosphorylation of extracellular signal-regulated kinase (ERK) which promotes pro-inflammatory cytokines like TNF-α and IL-1β via NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway[20,21,22]. In hyperglycemic mice, BBB components such as tight junctions, basement membrane proteins, and astrocytic endfeet were impaired[23]. Many studies have demonstrated that T1DM induces BBB disruption and therefore increases the risk of various diseases. T1DM increased BBB permeability in the epileptic seizure animal model[7]. Talyor SL et al. found damaged BBB integrity and cognitive impairment in a T1DM mice model[24]. T1DM-IS resulted in significantly increased BBB leakage and brain hemorrhage[25]. Thus, there is a need to find ways to alleviate inflammation and reduce damage in ischemic stroke with T1DM conditions.

Also, some studies described that hyperglycemia induces an inflammatory micro-environment in the brain. Gliem M et al. found skewed pro-inflammatory phenotypes in monocytes/macrophages of a streptozotocin-induced hyperglycemic mice brain[26], which is in line with this study. Non-inflammatory myeloid cells were significantly reduced in an animal model administered glucose before MCAO surgery[27].

Persisted pro-inflammatory phenotype of monocytes/macrophages and reduced growing factors are observed in the diabetic wound mice model[28]. In this study, it was suggested that T1DM suppressed the polarization of MDMs into the M2 phenotype and significantly increased the number of MDMs polarized into a pro-inflammatory phenotype. And it was reported that decreased CD86-positive populations and increased CD206-positive populations from immunostaining and flow cytometry results. Biased polarization of MDMs into M1 phenotype might aggravate inflammation and have negative effects on the outcomes of cerebral ischemia. Interestingly, not only CD86-negative and CD206-positive cells but also CD86 and CD206 double-positive populations increased in IL-4 and IL-13-treated T1DM-IS group compared with T1DM-IS group in flow cytometry data. Several studies have suggested that macrophages expressing CD206 and CD86 are in the transition stage of differentiation to either side[29,30]. Therefore, it could be inferred that CD86 and CD206 double positive MDMs in these results are in a state of polarization from M1 to M2.

During Inflammation in ischemic stroke, various inflammatory mediators including cytokines are produced to play a role in the activation of immune cells and regulate inflammation in ischemic stroke. IL-4 and IL-13 are well-known cytokines that enhance anti-inflammatory responses and suppress pro-inflammatory cytokines. IL-4 increases TGF-β expression, which enhances Ym1 and M2 polarization of microglia[31]. IL-13 is an anti-inflammatory cytokine that shares properties with IL-4. IL-4-deficient mice exhibited more pro-inflammatory microglia/macrophages, higher infarct volume, and poor outcomes in motion and cognitive function, whereas IL-4 recombinant treatment in these mice after cerebral ischemia enhanced functional recovery[32]. In addition, IL-13 administration in a permanent MCAO mice model induced M2a polarization of microglia/macrophages and functional recovery[33]. In our previous study, we demonstrated that IL-4 and IL-13 could switch blood-derived macrophages into anti-inflammatory phenotype after cerebral ischemia and exert a reparative role in stroke injury[12]. In this

study, IL-4 and IL-13 treatment in T1DM-IS mice efficiently resulted in alleviated infarct volume and recovery of motor function. Besides, IL-4 and IL-13 treatment reduced the number of infiltrating monocyte-derived macrophages, which is significantly increased in T1DM-IS mice. IL-13 treatment in a cerebral ischemic mice model significantly lessened CD45-positive leukocyte infiltration in the ischemic core[33]. In another similar study, it has been reported increased infiltration of CD45-positive leukocytes in myocardial infarction of IL-13-deficient mice[34]. Taken together, these data suggest the therapeutic effects of IL-4 and IL-13 that decrease infiltration of blood-derived immune cells and induce M2 polarization of monocytes/macrophages in in ischemic stroke with T1DM.

Although it has been determined the early response of IL-4 and IL-13 treatment after ischemic stroke with T1DM, further studies that examine the effects of IL-4 and IL-13 treatment in the late phase of stroke are needed. In cerebral ischemia, microglia and infiltrating myeloid cells are known to show various temporal expression patterns and phenotypes depending on the ischemic stage. It was reported that monocyte-derived macrophages adopted an anti-inflammatory phenotype in the early stage of stroke and then changed into pro-inflammatory phenotypes[35]. Hence, it is important to elucidate how microglia and blood-derived myeloid cells behave in each ischemic stage.

In addition, the effect of microglia in T1DM-IS was not identified in this study. Extensive research has been conducted to investigate the role of microglia in ischemic stroke[36,37]. After ischemia, the proliferation of microglia/macrophages peaks at 48-72h, and their expression persists for several weeks[36,37]. In addition, there are many studies about the polarization states of microglia in ischemic stroke[38,39]. Some studies showed that microglia are polarized into M1 phenotype by hyperglycemia in ischemic stroke[38], while another study reported that M2 phenotype microglia were observed at 12h after ischemia[39]. Monocyte/macrophage depletion aggravated infarct damage and increased the expression of pro-inflammatory markers in CD11b+ microglial/macrophages of an

ischemic animal model[36]. Regarding that microglia are the first cells to respond to ischemic stroke and highly plastic cells that can rapidly change into various states in response to the microenvironmental stimulus, further studies on the interaction between microglia and MDM are needed.

In this study, the infiltration of blood-derived monocytes/macrophages into the brain parenchyma was increased in T1DM, and the blood-derived monocytes/macrophages mainly displayed a pro-inflammatory phenotype. However, it showed that IL-4 and IL-13 treatment can be converted the phenotype of blood-derived monocytes/macrophages to an anti-inflammatory phenotype, and that reduced infarct size and alleviated motor function deficits against cerebral ischemic injury in T1DM.

V. Conclusion

It was proposed that T1DM resulted in increased monocyte infiltration and polarization of monocyte-derived macrophages into pro-inflammatory phenotype. Also, modulating phenotypical changes in T1DM-IS by IL-4 and IL-13 might result in recovery of ischemic damage. This study provides a potential mechanism for modulating infiltrating monocyte-derived macrophage polarization to improve stroke outcomes in T1DM conditions. In this study, the following results could be achieved;

- T1DM induced BBB disruption and severe infarct damage.
- T1DM increased infiltration of blood-derived monocytes/macrophages to the brain parenchyma after cerebral ischemia.
- The infiltrating blood-derived monocytes/macrophages in T1DM-IS exhibited a pro-inflammatory phenotype rather than an anti-inflammatory phenotype.
- IL-4 and IL-13 treatment could switch the monocytes/macrophages polarization into an anti-inflammatory phenotype, relieve infarct damage, and improve motor function in T1DM following ischemia.

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Abstact in Korean

제1형 당뇨를 수반한 허혈성 뇌졸중 손상에서 단핵구 유래 대식세포의 표현형 조절

<지도교수 이종은>

연세대학교 대학원 의과학과

고아라

높은 사망률로 치명적인 허혈성 뇌졸중 (Ischemic stroke, IS)은 뇌로 가는 혈류의 감소나 막힘에 의해 발생한다. 제1형 당뇨병 (Type 1 diabetes mellitus, T1DM) 환자의 경우 허혈성 뇌졸중 위험이 정상인보다 6.3배 높다. T1DM의 특징인 고혈당증은 산화 스트레스의 증가와 혈액-뇌 장벽 붕괴를 유도하여 단핵구를 포함한 혈액 면역 세포의 뇌 실질조직으로의 유입을 증가시킬 수 있다. 대뇌 허혈에 따른 염증 반응에서, 단핵구는 뇌 실질조직으로 침투해 전염증성 또는 항염증성 대식세포로 분화되어 허혈성 뇌졸중의 결과에 큰 영향을 미친다고 보고되었다. 또한, interleukin-4 (IL-4)와 interleukin-13 (IL-13)이 허혈 후 복구 과정에서 뇌 실질조직에 침투한 단핵구를 항염증성 표현형으로 분극화하는 역할을 한다는 연구가 존재한다. 본 연구에서는 단핵구 유래 대식세포의 표현형 분극화가 제1형 당뇨병으로 인한 뇌 허혈의 예후에 미치는 영향을 알아보고자 하였다. 우리는 먼저 스트렙토조토신 (150 mg/kg)을 사용하여 T1DM 마우스 모델을 확립하였고, 이 마우스들은 일시적인 중뇌동맥 폐색 수술을 받았다. 우리는 2,3,5-triphenyltetrazolium chloride 염색과 two-photon live imaging, Evans blue staining을 통해 T1DM 마우스와 T1DM-IS 마우스에서 혈액-뇌 장벽의 손상과 경색 부피가 증가하는 것을 관찰하

였다. 또한 T1DM-IS 마우스에서 단핵구의 침투가 증가하며 항염증성 단핵구 유래 대식세포가 감소해 있음을 면역조직화학법, 웨스턴 블랏, 유세포 분석을 통해 확인하였다. 다음으로, 우리는 IL-4와 IL-13을 투여한 T1DM-IS 마우스에서 단핵구 유래 대식세포의 항염증성 표현형으로의 분극이 증가하였으며 경색 부피가 완화되고 운동기능이 회복됨을 관찰했다. 이러한 결과는 당뇨와 같이 대사성 질환을 가지고 있는 동물에서 허혈성 뇌졸중 발생 시에, 단핵구 유래 대식세포의 표현형 조절이 허혈성 뇌졸중으로 인한 손상을 회복시킬 수 있음을 시사한다.

핵심되는 말: 허혈성 뇌졸중, 제1형 당뇨, 단핵구 유래 대식세포, 표현형 분극, 항염증성 세포