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ORIGINAL RESEARCH

Mucosal-Associated Invariant T Cells Are Involved in Acute Ischemic Stroke by Regulating Neuroinflammation

Sho Nakajima, MD; Ryota Tanaka, MD, PhD; Kazuo Yamashiro, MD, PhD; Asako Chiba, MD, PhD; Daisuke Noto, MD, PhD; Toshiki Inaba, PhD; Naohide Kurita, MD, PhD; Nobukazu Miyamoto, MD, PhD; Takuma Kuroki, MD, PhD; Hideki Shimura, MD, PhD; Yuji Ueno, MD, PhD; Takao Urabe, MD, PhD; Sachiko Miyake, MD, PhD; Nobutaka Hattori, MD, PhD

BACKGROUND: Mucosal-associated invariant T (MAIT) cells have been associated with inflammation in several autoimmune diseases. However, their relation to ischemic stroke remains unclear. This study attempted to elucidate the role of MAIT cells in acute ischemic stroke in mice.

METHODS AND RESULTS: We used MR1 knockout C57BL/6 ($MR1^{-/-}$) mice and wild-type littermates ($MR1^{+/+}$). After performing a transient middle cerebral artery occlusion (tMCAO), we evaluated the association with inflammation and prognosis in the acute cerebral ischemia. Furthermore, we analyzed the tMCAO C57BL/6 mice administered with the suppressive MR1 ligand and the vehicle control. We also evaluated the infiltration of MAIT cells into the ischemic brain by flow cytometry. Results showed a reduction of infarct volume and an improvement of neurological impairment in $MR1^{-/-}$ mice (n=8). There was a reduction in the number of infiltrating microglia/macrophages (n=3–5) and in their activation (n=5) in the peri-infarct area of $MR1^{-/-}$ mice. The cytokine levels of interleukin-6 and interleukin-17 at 24 hours after tMCAO (n=3–5), and for interleukin-17 at 72 hours after tMCAO (n=5), were lower in the $MR1^{-/-}$ mice. The administration of the suppressive MR1 ligand reduced the infarct volume and improved functional impairment (n=5). Flow cytometric analysis demonstrated there was a reduction of MAIT cells infiltrating into the ischemic brain at 24 hours after tMCAO (n=17).

CONCLUSIONS: Our results showed that MAIT cells play an important role in neuroinflammation after focal cerebral ischemia and the use of MAIT cell regulation has a potential role as a novel neuroprotectant for the treatment of acute ischemic stroke.

Key Words: cerebral infarction ■ major histocompatibility complex-related protein 1 ■ mucosal-associated invariant T cells ■ neuroinflammation ■ neuroprotection

Ischemic brain injury recruits various immune cells and augments the inflammatory reaction followed by enlargement of the infarct volume and poor prognosis. After cerebral ischemia, endogenous or infiltrating inflammatory cells are activated and release various kinds of inflammatory mediators in the brain.[1,2] These immunological responses exacerbate the cerebral edema and promote cell death, which leads to the expansion of the infarct area.[3]

There is increasing evidence that various types of T cells that infiltrate into the brain parenchyma are involved in the pathophysiology after acute ischemic stroke.[4–7] Recently, innate-like T cells, such as unique T cell subsets, have especially attracted

Correspondence to: Ryota Tanaka, MD, PhD, Stroke Center, Jichi Medical University Hospital, Division of Neurology, Department of Medicine, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke city, Tochigi 329-0498, Japan. E-mail: rtanaka@jichi.ac.jp and Nobutaka Hattori, MD, PhD, Department of Neurology, Juntendo University Faculty of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113–8421, Japan. E-mail: nhattori@juntendo.ac.jp

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CLINICAL PERSPECTIVE

What Is New?

- Knockdown of mucosal-associated invariant T (MAIT) cells ameliorated infarct volume and neurological impairment after transient focal ischemia.
- MAIT cells knockdown was associated with reduced levels of proinflammatory cytokines and microglial activation in the ischemic brain.
- Administration of a suppressive ligand for MAIT cells showed neuroprotective effect by suppressing proinflammatory cytokines and microglial activation.

What Are the Clinical Implications?

- Because MAIT cells are more abundant in human blood in comparison with mice, modulation of MAIT cells could be a potential target for future treatments during the early phase of acute ischemic stroke.

Nonstandard Abbreviations and Acronyms

i6-FP	isobutyryl 6-formylpterin
Iba1	ionized calcium binding adapter molecule-1
MAIT cells	mucosal-associated invariant T cells
mNSSs	modified neurologic severity scores
MR1	major histocompatibility complex-related molecule 1
MR1$^{-/-}$ mice	MR1 knockout C57BL/6 mice
MR1$^{+/+}$ mice	wild-type littermate C57BL/6 mice
TCR	T cell receptor
tMCAO	transient middle cerebral artery occlusion

attention as they share features of both innate and adaptive immunity.[8] These cells have an ability to produce massive amounts of cytokines at the same speed as that seen with innate immunity without mediating clonal proliferation. For example, γδ T cells, which are one of the innate-like T cell subsets, have been shown to play a causal role in devastating ischemic brain injury through the release of interleukin-17 (IL-17) during the subacute phase of ischemic stroke.[7] Another example is the gut dysbiosis alteration of the balance of immunity between the intestinal γδ T and regulatory T cells, which can affect the outcome of experimental stroke because of the difference in the inflammatory responses mediated by cytokine production.[9]

Mucosal-associated invariant T (MAIT) cells are a novel subpopulation of the innate-like T cells. They are defined by the semi-invariant T cell receptor (TCR) α chain Vα7.2-Jα33 in humans and Vα19-Jα33 in mice, which are restricted to the major histocompatibility complex-related molecule 1 (MR1).[10] MAIT cells can be activated by different methods, including after recognition of vitamin B metabolites by TCR, which is dependent on the MR1, or because of activation related to IL-12 and IL-18 cytokine stimulation, which is independent of MR1. Once activated, the MAIT cells rapidly proliferate and then secrete proinflammatory cytokines and other cytotoxic effector molecules.[11] MAIT cells are involved in various cytotoxic or cytoprotective functions in noninfectious autoimmune diseases such as multiple sclerosis,[12,13] rheumatoid arthritis,[14] ulcerative colitis,[15,16] ankylosing spondylitis,[17] and systemic lupus erythematosus.[18,19] However, the role of MAIT cells in the ischemic brain injury remains unknown.

In the current study, we investigated the role of MAIT cells in neuroinflammation during acute and subacute ischemic stroke in an experimental mouse model. In addition, we also investigated whether administration of a suppressive MR1 ligand would be able to affect the outcome after acute ischemic stroke.

METHODS

All data and supporting materials have been provided with the published article.

Mice

C57BL/6 mice were purchased from Oriental Yeast Corporation (Tokyo, Japan) (n=118), and were randomly divided into different experimental groups. MR1$^{-/-}$ (n=41) and MR1$^{+/+}$ (n=45) mice were provided by the Department of Immunology, Juntendo University Faculty of Medicine. MR1$^{-/-}$ mice on a C57BL/6 background, originally provided by Dr. Susan Gilfillan (Washington University School of Medicine, St. Louis, MO), were crossed with C57BL/6 mice in order to obtain the MR1$^{-/-}$ and littermate MR1$^{+/+}$ mice. These mice were genotyped by polymerase chain reaction as previously described.[20] All mice in the study were 9 to 12 weeks of age and weighed 23 to 30 g, and were restricted to male mice to avoid the interactive effects of estrogen. All mice were maintained under specific pathogen-free conditions in accordance with the institutional guidelines of Juntendo University and were housed under controlled lighting and provided with food and water ad libitum.

Experimental Protocol

We created a 1-hour transient middle cerebral artery occlusion (tMCAO) model using the MR1$^{-/-}$ mice, which are deficient in MAIT cells, and their MR1$^{+/+}$ littermates. Anesthesia was inducted with 4.0% isoflurane (Abbott Japan Co., Ltd., Tokyo, Japan) with animals then maintained on 1.0% to 1.5% isoflurane in 70% N_2O and 30% O_2 using a small animal anesthesia system. All surgical instruments were sterilized before surgery. Before any incision was made, the area was swabbed with ethanol. Transient cerebral focal ischemia was induced by tMCAO for 1 hour using an intraluminal filament technique, as previously described.[21] Briefly, the left common carotid artery and the left external carotid artery were exposed and ligated after a ventral midline neck incision. A silicon-coated nylon monofilament was inserted through the left common carotid artery into the left internal carotid artery to occlude the left middle cerebral artery. After a 1-hour occlusion, the monofilament was withdrawn in order to allow for reperfusion. During the procedure, body temperature was maintained at 37.0±0.5°C using a heating pad. Regional cerebral blood flow was measured in the left temporal window under anesthesia using laser Doppler flowmetry (FLOW-C1; Omegawave Inc., Tokyo, Japan) before and during tMCAO. We excluded mice in which the reduction in regional cerebral blood flow of the laser Doppler signal was below 60%, as compared with the preischemic state. Evaluation of cerebral infarct volume, the degrees of neurological dysfunction, activation of microglia/macrophages collected in the peri-infarct area, and various cytokines in the cerebral infarct hemisphere were compared by using the procedures described subsequently. The 24- or 72-hour survival rates of each group of animals were determined by a Kaplan-Meier analysis. All animal experiments were approved by the Juntendo University Animal Ethics Committee (No. 1286), and efforts were made to minimize the number of animals used and their suffering during all of the experimental procedures.

Measurement of Infarct Volume

For infarct quantification, mice (n=8 each) were anesthetized by an intraperitoneal injection of pentobarbital (50 mg/kg) at 24 and 72 hours after tMCAO and transcardially perfused with 20 mL cold PBS to remove blood from the brain capillaries. Brains of the mice were sliced into 1 mm thick cross sections using Coronal Mouse Brain Matrices (RWD Life Science, Shenzhen, China). Subsequently, 6 consecutive coronal brain sections that were –3.0, –2.0, –1.0, 0, +1.0, and +2.0 mm from the bregma were incubated in 2% 2,3,5-triphenyltetrazolium chloride solution (Sigma-Aldrich, St. Louis, MO) at 37°C for 20 minutes. Photographs of each coronal section were taken immediately and analyzed using the ImageJ program (National Institutes of Health; http://rsb.info.nih.gov/nih-image/). Infarct volumes, which was outlined in unstained white area, were calculated using numerical integration of the respective areas for all the sections and the distance between them.[22]

Neurological Evaluation

Neurological severity was assessed just before each euthanization time point on a scale of 0 (normal) to 14 (maximal deficit) using the modified neurologic severity scores (mNSSs), which is a composite of motor (muscle status, abnormal movement), sensory (visual, tactile, and proprioceptive), and reflex tests. One point is awarded for the inability to perform the tasks or for the lack of a tested reflex.[23] Observers of the behavioral test were blinded to the treatment group.

Immunohistochemical Analysis

At 24 and 72 hours after tMCAO, mice were transcardially perfused with 20 mL cold PBS in order to remove blood from the brain capillaries. After the brains (n=5 from each group for each time point) were perfused with 4% paraformaldehyde in PBS, they were carefully removed and postfixed in 4% paraformaldehyde for at least 2 days at 4°C and then placed in 30% sucrose overnight. Coronal cryostat sections of the brains (20 μm-thick sections) were incubated with rabbit anti-Iba1 (ionized calcium binding adapter molecule-1) antibody (1:500; Wako Pure Chemical Industries, Ltd., Osaka, Japan) overnight at 4°C. Thereafter, sections were incubated with a fluorescein isothiocyanate-conjugated secondary antibody (1:300; Jackson ImmunoResearch, Baltimore, MD) for the identification of Iba1. Subsequently, the slides were covered with Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (Vector Laboratories, Inc., Burlingame, CA). Immunohistochemical images were obtained using a laser scanning microscope (model LSM780, Carl Zeiss, Jena, Germany) and the ZEN 2011 software (Carl Zeiss). To assess the activation of microglia/macrophages, the total number of Iba1-stained cells was calculated in the ischemic boundary area using ImageJ, as previously described.[24] Positively stained cells in 3 adjacent square compartments (0.25 mm^2 each) of the ischemic boundary area were counted in 3 coronal sections at –0.4, 0, and +0.4 mm from the bregma. Immunopositive cells were counted manually by a researcher who was blinded to the experimental conditions. The average cell number for all of the 9 compartments was used as the cell number per mouse. In addition, the degree of activation of the microglia/macrophages was also defined according to the morphological grade, which was classified into 4 groups as previously described[25,26]: (1)

Grade 1, ramified; (2) Grade 2, intermediate; (3) Grade 3, amoeboid; and (4) Grade 4, round phenotypes. The total cell number, which was classified according to the grade for all of the 9 compartments (2.25 mm^2 in total), was used as the cell number per mouse. Iba1-positive cells were counted manually by a researcher who was blinded to the experimental conditions. The 3,3'-diaminobenzidine staining was also performed for the purpose of confirming the validity of the evaluation of microglial activation in fluorescent immunostaining. Briefly, coronal cryostat sections of the brains (20 μm-thick sections) were incubated with anti-ionized calcium-binding adapter molecule antibody (1:500; Wako), treated with biotinylated secondary antibody (1:300; Vector Laboratories), and subsequently processed with an avidin-biotinylated peroxidase complex (Vectastain ABC kit; 1:400; Vector Laboratories). The number of 3,3'-diaminobenzidine-stained cells in 3 predefined ischemic boundary areas (0.25 mm^2) were counted according to the morphological grading scale.

Evaluation of Proinflammatory Cytokine After tMCAO

The cytokine levels assessed by enzyme-linked immunosorbent assay (ELISA) in the ischemic brain were compared between the $MR1^{-/-}$ and $MR1^{+/+}$ mice at 24 hours (tMCAO24) and 72 hours (tMCAO72) after the tMCAO. We also confirmed the difference from the tMCAO model by measuring the cytokine levels in the sham-operated mice without vascular occlusion at 24 hours (sham24) and 72 hours (sham72) after the sham operation (tMCAO24: n=3–5, tMCAO72: n=5, sham24 and sham72: n=3).

The mice were transcardially perfused with 20 mL cold PBS in order to remove the blood from the brain capillaries. We prepared a cerebral hemisphere, which excluded the olfactory bulb, brainstem, and cerebellum for use as an ELISA sample. The cerebral hemisphere was divided into the ipsilateral and contralateral sides with each then homogenized in 1000 μL of PBS using a sonicator (model XL-2000, MISONIX). After performing 2 freeze-thaw cycles at –20°C in order to break the cell membranes, the homogenates were centrifuged at 4°C for 5 minutes at 5000*g*. The supernatant was immediately removed, aliquoted, and stored at –80°C. Before measuring the cytokines by ELISA, all of the frozen aliquot samples were thawed before use. The cytokine IL-17 concentrations in the infarct hemibrain were measured in each animal using the Mouse IL-17 ELISA kit (Abcam, Cambridge, UK; minimum detectable dose 6.1 pg/mL). Similarly, IL-1β (R&D Systems, Minneapolis, MN; minimum detectable dose 2.31 pg/mL), IL-6 (R&D Systems; minimum detectable dose 1.6 pg/mL), and TNF-α (R&D Systems; minimum detectable dose 1.88 pg/mL) were measured using the ELISA kits according to the manufacturer's protocol. Optical density was measured at 450 nm minus those obtained at 570 nm using an ELISA reader (Bio-Rad Laboratories, Inc., Hercules, CA), and the concentration of each cytokine was obtained according to the standard curve. For the cytokine value in each mouse sample, the average of the 2 measured values was used.

Treatments by MR1 Ligand

We induced tMCAO in C57BL/6 mice for 1 hour using the intraluminal filament technique described in the experimental protocol. Subsequently, the mice were administered a ligand that suppressed the MAIT cell activation after tMCAO. We used isobutyryl 6-formylpterin (i6-FP) as the suppressive ligand, which was synthesized by SundiaMediTech Company, Ltd., Shanghai, China. A dose of 15 mg/kg of suppressive MR1 ligand diluted in PBS was injected via the tail vein of the mice 1 time immediately after tMCAO for the 24 hours model or 2 times, with 1 dose given immediately and 1 at 24 hours after tMCAO for the 72 hours model, respectively. Because dimethyl sulfoxide (Thermo Fisher Scientific, Waltham, MA) was also used as a diluent for the i6-FP stock solution, dimethyl sulfoxide diluted with PBS was injected via the mice tail vein as a vehicle control in the same manner. Cerebral infarct volume, neurological severity, immunohistochemistry were evaluated using the brain samples of the ligand-administered mice and the vehicle control at both 24 and 72 hours in the same manner as described (n=5 each).

Cytokine levels assessed by ELISA in the ischemic brain were compared between the ligand-administered mice and the vehicle control at 24 hours (tMCAO24) and 72 hours (tMCAO72) after tMCAO, respectively. We also confirmed the difference from the tMCAO model by measuring the cytokine levels in the sham-operated mice without vascular occlusion at 24 hours (sham24) and 72 hours (sham72) after the sham operation (tMCAO24 and tMCAO72: n=5, sham24 and sham72: n=3).

Evaluation of Cell Death

TUNEL (terminal deoxynucleotidal transferase–mediated biotin–deoxyuridine triphosphate nick-end labeling) staining was performed with an In Situ Cell Death Detection Kit, Fluorescein (Roche, Mannheim, Germany). After incubation in 0.1% sodium citrate in 0.1% PBS containing 0.1% Triton X-100, the sections were incubated with the TUNEL reaction mixture for 60 minutes at 37°C in the dark according to the manufacturer's protocols on 20-μm-thick coronal sections. We compared the number of TUNEL-positive cells in

the ischemic boundary area (0.25 mm^2) of the ligand-administered mice and vehicle control at each time point.

Flow Cytometry

To perform flow cytometry, we used the tMCAO24 model with C57BL/6 mice administered with the suppressive ligand (n=6) and the vehicle control (n=5). Sham-operated mice (n=6) were also evaluated. Briefly, after transcardial perfusion with cold PBS and decapitation, the brain, excluding the olfactory bulb, brainstem, and cerebellum, were removed for the flow cytometry analysis. To isolate immune cells in the brain, the ipsilateral and contralateral sides of the brain were individually homogenized. Homogenates were incubated with Liberase TM Research Grade (Roche, Basel, Switzerland) and DNase I (Roche) and then separated using density gradient centrifugation. Cells were obtained from the interface between the 37% and 70% Percoll (GE Healthcare, Tokyo, Japan) layers. These cells were stained using a Zombie Aqua Fixable Viability Kit and then incubated with CD16/32 to block the interaction with the Fc domain of immunoglobulins. These cells were also stained with combinations of the following monoclonal antibodies: anti-CD3-APC-Cy7 (clone 17A2, dilution 1:50), anti-CD4-PE-CF594 (RM4-5, 1:100), anti-CD8a-BV605 (53-6.7, 1:100), anti-CD11b-APC (M1/70, 1:50), anti-CD45-PE-Cy7 (30-F11, 1:100), and anti-γδTCR-fluorescein isothiocyanate (GL3, 1:100) (all from BioLegend). MR1 tetramers loaded with 5-OP-RU or 6-FP-BV421 (1.2 mg/mL) were used (1:250, National Institutes of Health tetramer core facility located at Emory University, Atlanta, GA). MAIT cells were identified as Zombie $Aqua^{-}CD45^{high}CD3^{+}\gamma\delta TCR^{-}$MR1 tetramer $(5\text{-OP-RU})^{+}$ lymphocytes.

Data were acquired using a FACS LSR Fortessa (BD Biosciences). The absolute number of MAIT cells and the percentage of MAIT cells out of the αβ T lymphocytes that infiltrated into the ischemic brain were analyzed using FlowJo software (TreeStar Inc., San Carlos, CA) as previously described.[19]

Statistical Analysis

Power estimates were calculated based on α=0.05 and β=0.8 in order to obtain group sizes that were appropriate for detecting effect sizes in the range of 30% to 50% for the in vivo models. Between 2 groups of mice, an unpaired Student *t* test was performed to determine the statistical significance, with the exception for the mNSSs and flow cytometry data analysis. For the mNSSs and flow cytometry data analysis, a Wilcoxon rank-sum test or Kruskal-Wallis test followed by post hoc analysis by Dunn's test was used to determine the significant differences. All experiments and measurements were performed in a blinded and randomized manner. The figures for the mNSSs and flow cytometry were constructed with GraphPad Prism 7 software (GraphPad Software Inc., La Jolla, CA). All values except for the mNSSs and flow cytometry data are expressed as mean±SD. Individual values for the mNSSs and flow cytometry data were obtained from individual mice and presented as the median and interquartile range. Significance was defined as $P<0.05$. Statistical analyses were performed using the JMP 13.0.0. software program (SAS Inc., Cary, NC).

RESULTS

MR1 Deficiency Reduces Infarct Volume and Improved Neurological Dysfunction

To investigate whether MAIT cells contribute to the pathological process of acute focal cerebral ischemia, after performing tMCAO in MAIT cell-deficient $MR1^{-/-}$ and $MR1^{+/+}$ mice, we examined the animals at 24 and 72 hours after the procedure, respectively (Figure 1A). The infarct volume in $MR1^{-/-}$ mice tended to be smaller at 24 hours after tMCAO and was significantly smaller at 72 hours after tMCAO versus the $MR1^{+/+}$ mice (Figure 1B and 1C). The mNSSs in $MR1^{-/-}$ mice were lower at 24 hours after tMCAO and significantly lower at 72 hours after tMCAO as compared with that for $MR1^{+/+}$ mice (Figure 1D). The survival rates of these mice at 24 and 72 hours after tMCAO did not differ to a statistically significant extent (Figure S1). These results suggest that the MR1 deficiency reduced the infarct volume and ameliorated the neurological dysfunction after tMCAO.

MR1 Deficiency Attenuates Activation of Microglia/Macrophages After Ischemia Reperfusion

Subsequently, we then evaluated the activation of Iba1-positive microglia/macrophages after tMCAO. Although the number of Iba1-positive cells was significantly reduced in $MR1^{-/-}$ as compared with $MR1^{+/+}$ mice at 24 hours after tMCAO, this difference was lost at 72 hours after tMCAO (Figure 2A). We then evaluated the activation of Iba1-positive cells according to the morphological grading scale, as previously described.[25] The average cell number for Grade 3 and Grade 4, which are the more activated form of the microglia/macrophages, tended to be lower in the $MR1^{-/-}$ mice at 24 hours after tMCAO as compared with the $MR1^{+/+}$ mice (Figure 2B). Moreover, at 72 hours after tMCAO, the average cell number for Grade 4 was significantly lower in the $MR1^{-/-}$ mice versus the $MR1^{+/+}$

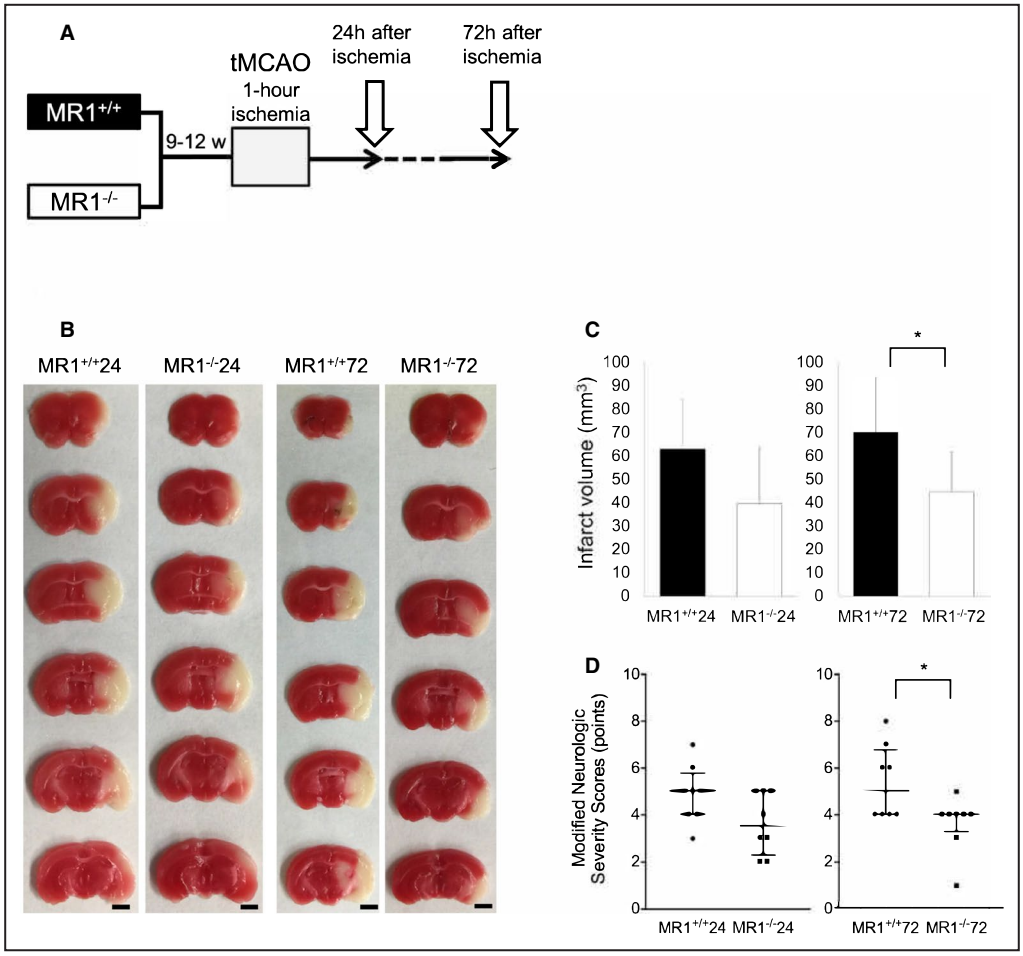


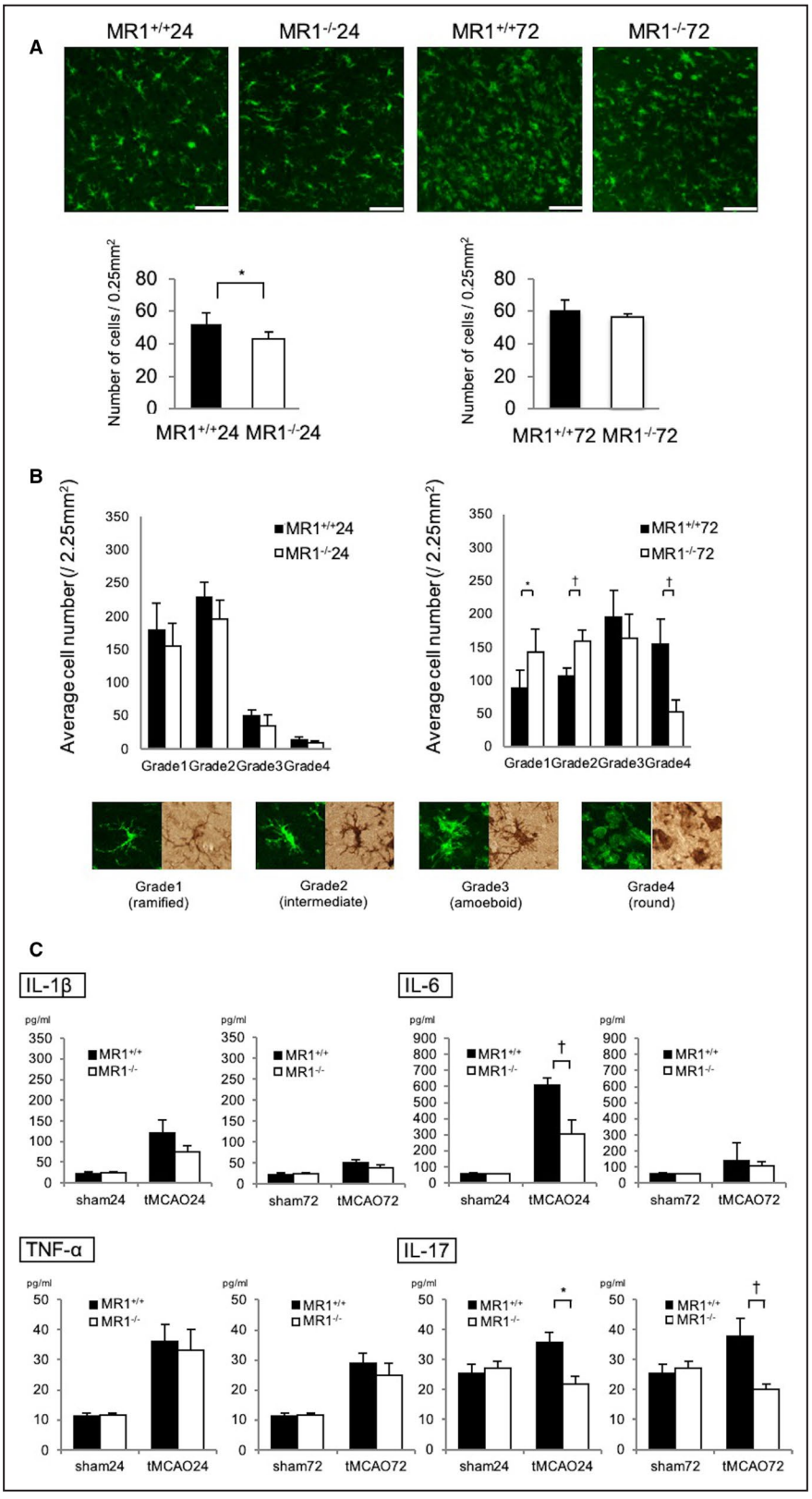
Figure 1. Effect of the major histocompatibility complex-related molecule 1 (MR1) deficiency on the infarct volume and neurological severity.
A, Experimental design using MR1 knockout C57BL/6 ($MR1^{-/-}$) mice and wild-type littermate C57BL/6 ($MR1^{+/+}$) mice. **B**, Representative triphenyltetrazolium chloride-stained sections of each group; $MR1^{+/+}$ mice at 24 hours (24 h) after transient middle cerebral artery occlusion (tMCAO) ($MR1^{+/+}$24), $MR1^{-/-}$ mice at 24 hours after tMCAO ($MR1^{-/-}$24), $MR1^{+/+}$ mice at 72 hours (72 h) after tMCAO ($MR1^{+/+}$72), and $MR1^{-/-}$ mice at 72 hours after tMCAO ($MR1^{-/-}$72). Scale bars=2 mm. **C**, Comparison of cerebral infarct volume between $MR1^{+/+}$ and $MR1^{-/-}$ mice at 24 and 72 hours after tMCAO, respectively. Values are presented as mean±SD and were analyzed by an unpaired Student *t* test. **P*<0.05. **D**, Comparison of the modified neurologic severity scores between $MR1^{+/+}$ and $MR1^{-/-}$ mice at 24 and 72 hours after tMCAO, respectively (n=8 per group). Values are presented as the median and interquartile range and were analyzed by Wilcoxon's rank-sum test. **P*<0.05.

mice (Figure 2B). These results suggest that the MR1 deficiency is involved in not only the number of the microglia/macrophages recruited but also their activation after tMCAO.

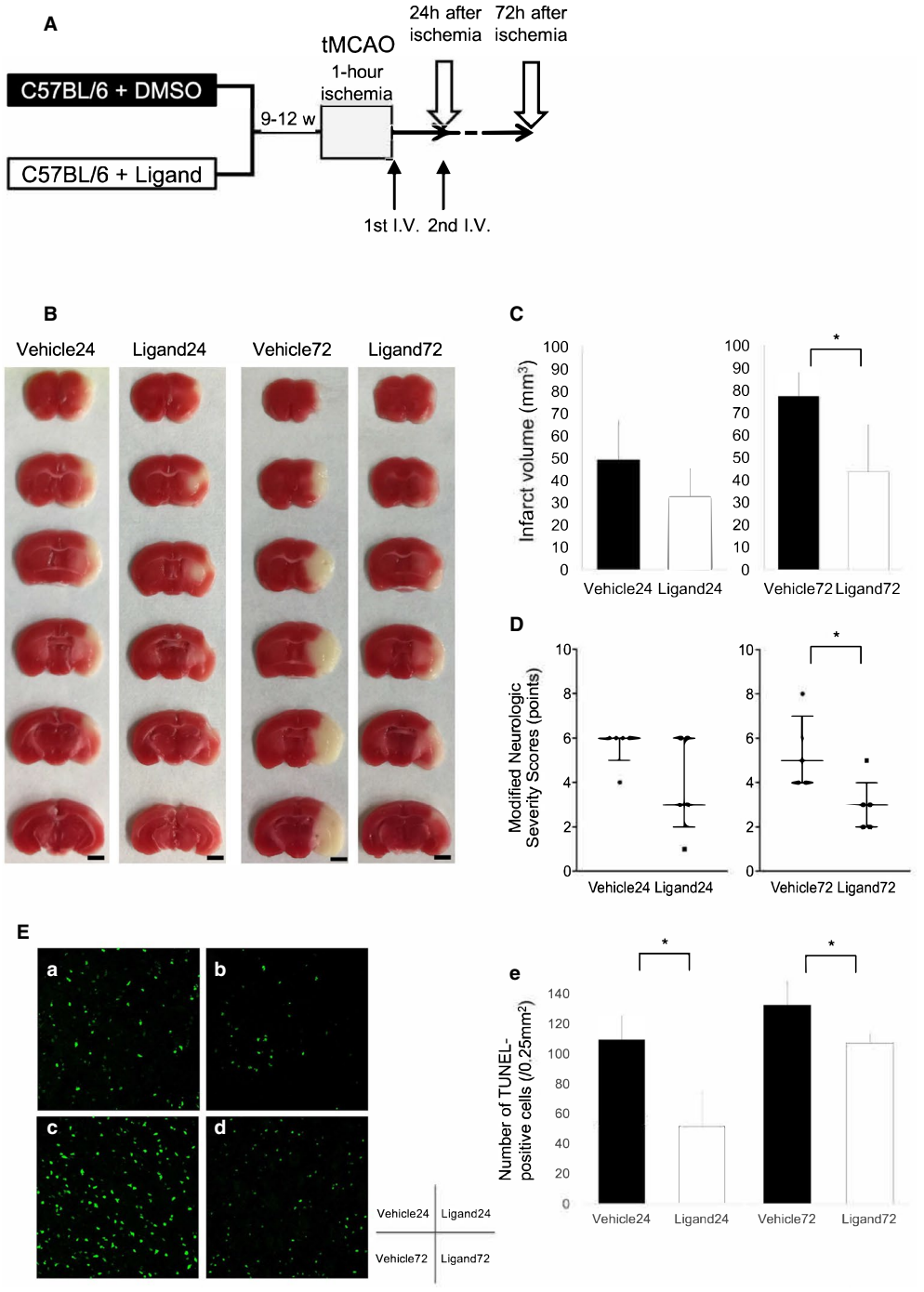
MR1 Deficiency Reduces Proinflammatory Cytokine Production in the Ischemic Brain

Next, we analyzed the proinflammatory cytokines in the ischemic brain using ELISA. The cytokine levels of

Figure 2. Major histocompatibility complex-related molecule 1 (MR1) deficiency and neuroinflammation after transient focal ischemia.
A, Representative images of Iba1 (ionized calcium binding adapter molecule-1) -positive cells and the average cell number of Iba1-positive cells in the ischemic boundary area in $MR1^{+/+}$ and $MR1^{-/-}$ mice at 24 and 72 hours after tMCAO, respectively ($MR1^{+/+}$24 and $MR1^{-/-}$24: n=3–5, $MR1^{+/+}$72, and $MR1^{-/-}$72: n=5 per group). Scale bars=100 μm. **B**, A Comparison of the average cell number of activated Iba1-positive cells between $MR1^{+/+}$ and $MR1^{-/-}$ mice using morphological grading scale at each time point ($MR1^{+/+}$24 and $MR1^{-/-}$24: n=3–5, $MR1^{+/+}$72, and $MR1^{-/-}$72: n=5 per group). **C**, Comparison of the proinflammatory cytokine levels in ischemic hemibrain between $MR1^{+/+}$ and $MR1^{-/-}$ mice at 24 hours (tMCAO24) and 72 hours (tMCAO72) after tMCAO, respectively (tMCAO24: n=3–5, tMCAO72: n=5). Sham-operated mice without vascular occlusion at 24 hours (sham24) and 72 hours (sham72) after sham operation as controls (n=3 each). Values are presented as the mean±SD and were analyzed by an unpaired Student *t* test. **P*<0.05. †*P*<0.01. IL indicates interleukin; tMCAO, transient middle cerebral artery occlusion; and TNF-, tumor necrosis factor alpha.



IL-6 and IL-17 at 24 hours after tMCAO were significantly lower in the MR1$^{-/-}$ versus the MR1$^{+/+}$ mice, with a similar trend seen for IL-1β and TNF-α (Figure 2C). Furthermore, the IL-17 cytokine levels were significantly lower in MR1$^{-/-}$ as compared with the MR1$^{+/+}$ mice at 72 hours after tMCAO (Figure 2C).



Inhibition of MAIT Cell Activation Reduces Infarct Volume and Improves Neurological Dysfunction

Because our data suggested that MAIT cells play a critical role in the pathology of acute focal ischemia, we decided to investigate whether a suppressive MR1 ligand would inhibit MAIT cell activation and potentially reduce infarct volume and improve the neurological dysfunction.

It has been previously reported that 6-formylptrein (6-FP), which is a photodegradation product of folic acid (vitamin B9), is an MR1 ligand that does not cause MAIT cell activation.[27] However, because the inhibitory potency of 6-FP is weak,[27] we used i6-FP as a suppressive ligand in our current study. Moreover, it has also been previously reported that i6-FP was synthesized from 6-FP and confirmed to have an inhibitory effect on MAIT cell activation both in vitro and in vivo.[19] Thus, in accordance

Figure 3. Inhibition of mucosal-associated invariant T (MAIT) cell activation and stroke outcome after transient focal ischemia.
A, Experimental design using C57BL/6 mice administered the suppressive ligand and with dimethyl sulfoxide (DMSO) as a vehicle control. **B**, Representative triphenyltetrazolium chloride-stained sections of each group; DMSO-administered mice at 24 hours after tMCAO (Vehicle24), suppressive MR1 ligand-administered mice at 24 hours after tMCAO (Ligand24), DMSO-administered mice at 72 hours after tMCAO (Vehicle72), and suppressive MR1 ligand-administered mice at 72 hours after tMCAO (Ligand72). Scale bars=2 mm. **C**, Comparison of cerebral infarct volume between the vehicle control and the suppressive MR1 ligand-administered mice at 24 and 72 hours after tMCAO, respectively. Values are presented as the mean±SD and were analyzed by an unpaired Student *t* test. **P*<0.05. **D**, Comparison of the mNSSs between the vehicle control and the suppressive MR1 ligand-administered mice at 24 and 72 hours after tMCAO, respectively (n=5 per group). Values are presented as the median and interquartile range and were analyzed by Wilcoxon's rank-sum test. **P*<0.05. **E**, Representative images of TUNEL-positive cells (**a**, Vehicle24; **b**, Ligand24; **c**, Venicle72; **d**, Ligand72) and the comparison of the number of TUNEL positive cells between the suppressive MR1 ligand-administered mice and the vehicle control at 24 and 72 hours after tMCAO, respectively (**e**). Values are presented as the mean±SD and were analyzed by an unpaired Student *t* test. **P*<0.05. MR1 indicates major histocompatibility complex-related molecule 1; tMCAO, transient middle cerebral artery occlusion; and TUNEL, terminal deoxynucleotidal transferase–mediated biotin–deoxyuridine triphosphate nick-end labeling.

with our protocol, the suppressive MR1 ligand was injected via the tail vein of the mice 1 time immediately after tMCAO for the 24 hours model or 2 times in the 72 hours model, with 1 dose given immediately and 1 at 24 hours after tMCAO, respectively (Figure 3A). The infarct volume in the ligand-administered mice tended to be smaller at 24 hours after tMCAO and was significantly reduced at 72 hours after tMCAO as compared with that for the vehicle control (Figure 3B and 3C). The mNSSs in the ligand-administered mice was lower at 24 hours after tMCAO and significantly lower at 72 hours after tMCAO as compared with that for the vehicle control (Figure 3D). The number of TUNEL-positive cells at 24 and 72 hours after tMCAO was significantly lower in ligand-treated mice in comparison with vehicle-treated control mice (Figure 3E). Ligand injection did not alter the survival rate at 24 and 72 hours after tMCAO (Figure S1).

Inhibition of MAIT Cell Activation Attenuates the Activation of Microglia/Macrophages After Ischemia-Reperfusion

Although the number of Iba1-positive microglia/macrophages was significantly reduced in ligand-administered mice as compared with vehicle control at 24 hours after tMCAO, this difference was lost at 72 hours after tMCAO (Figure 4A). At 24 hours after tMCAO, the average cell number of the morphological Grade 3 in the ligand-administered mice tended to be lower, whereas that for Grade 4 was significantly lower than that observed in the vehicle control (Figure 4B). However, at 72 hours after tMCAO, the average cell numbers for Grade 3 and Grade 4 were significantly lower in the ligand-administered mice as compared with the vehicle control (Figure 4B). Morphological analysis of 3,3'-diaminobenzidine staining was also performed to confirm the activation of microglia/macrophages, but the results were almost the same in those of fluorescent immunostaining.
These results demonstrated that the inhibition of MAIT cells by the suppressive MR1 ligand was similar to the effect seen for the MR1 deficiency.

Inhibition of MAIT Cell Activation Reduces Proinflammatory Cytokine Production in the Ischemic Brain

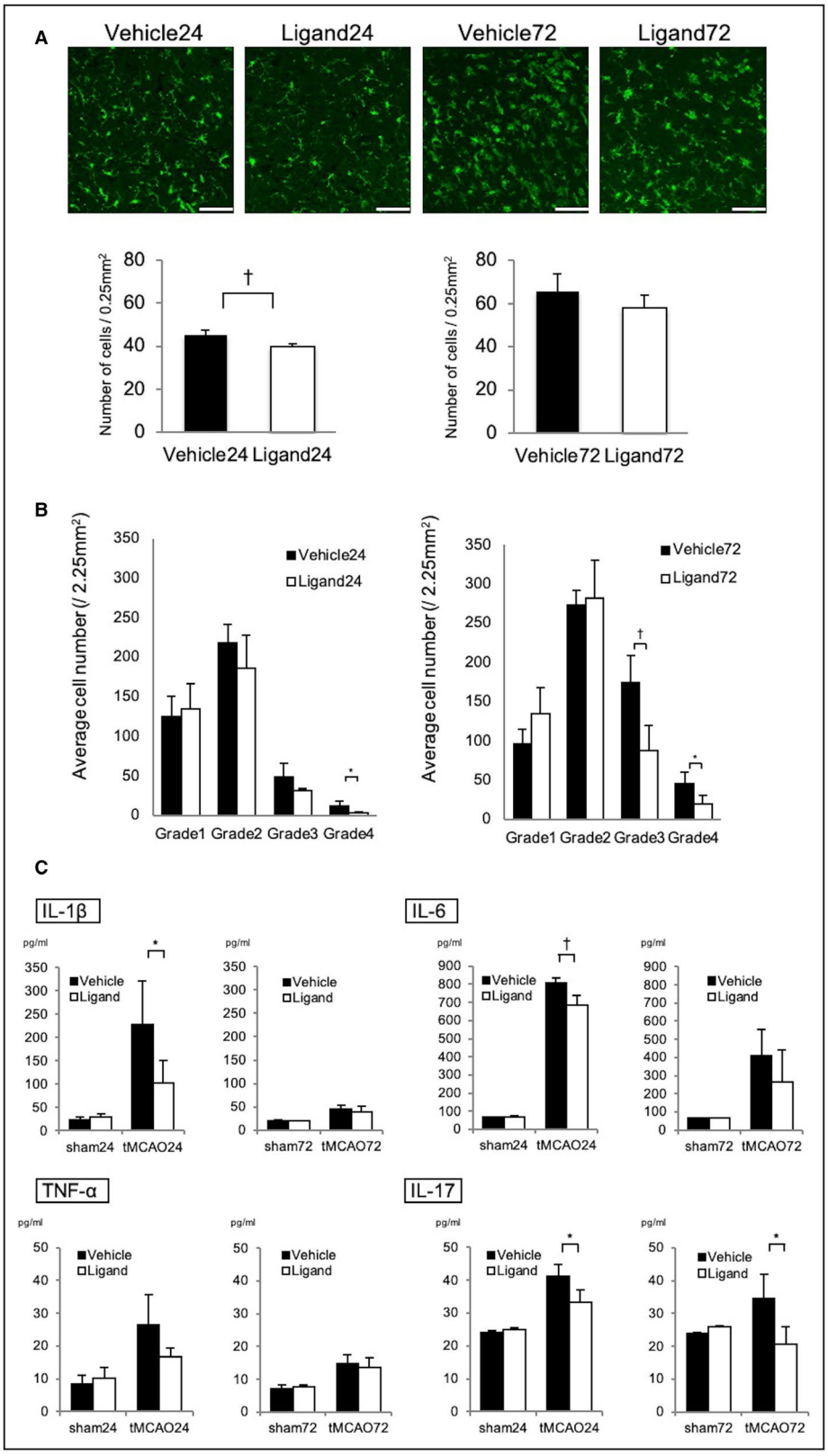
The cytokine levels of IL-1β, IL-6, and IL-17 in the ischemic brain at 24 hours after tMCAO were significantly lower in the ligand-administered mice versus the vehicle control (Figure 4C). In addition, IL-17 cytokine levels were significantly lower in the ligand-administered mice as compared with the vehicle control at 72 hours after tMCAO (Figure 4C).

Infiltration of MAIT Cells in the Ischemic Brain

Because results similar to the knockout and wild-type mice models were obtained when using the ligand-administered and the vehicle control mice model, we attempted to use flow cytometry to directly confirm whether or not the infiltration of MAIT cells into the cerebral infarction was reduced when using the ligand-administered mice and the vehicle control. We found a certain number of MAIT cells (Zombie Aqua$^-$ CD45^{high} CD3$^+$ γδTCR$^-$ MR1 tetramer$^+$) in the sham-operated normal cerebral hemisphere (Figure 5A). The number of MAIT cells was significantly greater in the control mice than in the sham-operated mice (70.4 [35.5–111.9] per hemisphere versus 10.7 [7.2–28.6] per hemisphere, *P*<0.05), but this was not observed in the ligand-administered group (31.7 [14.0–45.7] per hemisphere) (Figure 5A). A similar trend was also observed in the proportion of MAIT cells; however, there were no statistically significant differences among 3 groups (Figure 5B).

DISCUSSION

Neuroinflammation play a critical role in the pathogenesis of ischemic brain and is related to the neurological severity, with recent data suggesting that infiltrating T lymphocytes are associated with the progression of tissue damage.[28] Early experimental studies showed that T cells were recruited into the ischemic brain lesion during the delayed stages.[29] However, recent studies



using more sophisticated methods have shown that some innate-like T and T cell subsets were rapidly recruited much earlier than 24 hours after the induction of tMCAO.[4,30] Thus, the role of the T lymphocyte during the acute stage of ischemic stroke is believed to be involved in the deterioration of brain tissue. Mice

Figure 4. Inhibition of MAIT cell activation and neuroinflammation after transient focal ischemia.
A, Representative images of Iba1 (ionized calcium binding adapter molecule-1)-positive cells and the average cell number of Iba1-positive cells in the ischemic boundary area in tMCAO C57BL/6 mice administered with the suppressive MR1 ligand and the vehicle control at 24 and 72 hours after tMCAO, respectively (n=5 each). Scale bars=100 μm. **B**, A comparison of the average cell number of activated Iba1-positive cells between the vehicle control and ligand-administered mice using a morphological grading scale at each time point (n=5 each). **C**, A comparison of the proinflammatory cytokine levels in the ischemic hemibrain between the vehicle control and the ligand-administered mice at 24 hours (tMCAO24) and 72 hours (tMCAO72) after tMCAO, respectively (n=5 each). Sham-operated mice without vascular occlusion at 24 hours (sham24) and 72 hours (sham72) after the first injection of the ligand or DMSO in the experimental protocol as controls (n=3 each). Values are presented as the mean±SD and were analyzed by an unpaired Student *t* test. **P*<0.05. †*P*<0.01. IL indicates interleukin; MAIT, mucosal-associated invariant T; tMCAO, transient middle cerebral artery occlusion; and TNF-, tumor necrosis factor alpha.

with deficient T or B lymphocytes have been shown to exhibit a significant reduction of infarct volume and have milder neurological impairment after focal cerebral ischemia.[31,32] Furthermore, attenuation of lymphocytes by sphingosine 1-phosphate receptors resulted in a significant neuroprotective effect not only in animal models of ischemic stroke[33,34] but also in human patients with strokes.[35,36] Thus, the modulation of T lymphocytes is attractive as both a neuroprotectant and enhancer of neurological improvement in acute ischemic stroke.

Our results additionally demonstrated that the MR1 deficiency was related to the attenuation of the inflammatory reaction followed by a reduction in infarct volume and subsequent neurological improvement. Data from many previous research studies have indicated that MAIT cells were related to several immune-mediated diseases. For example, although the frequency of MAIT cells is reduced in the peripheral blood of patients with inflammatory bowel diseases,[15,37] IL-17 production by MAIT cells was shown to be increased in patients with ulcerative colitis with the CD69 expression levels on these cells demonstrated to be correlated with the disease severity.[16] In inflammatory arthritis animal models, MAIT cells have been shown to enhance the arthritic inflammation. For example, MR1 deficiency reduced the severity of collagen-induced arthritis, whereas reconstitution with MAIT cells in MR1-deficient mice induced severe arthritis.[14] In contrast, MAIT cells have been shown to

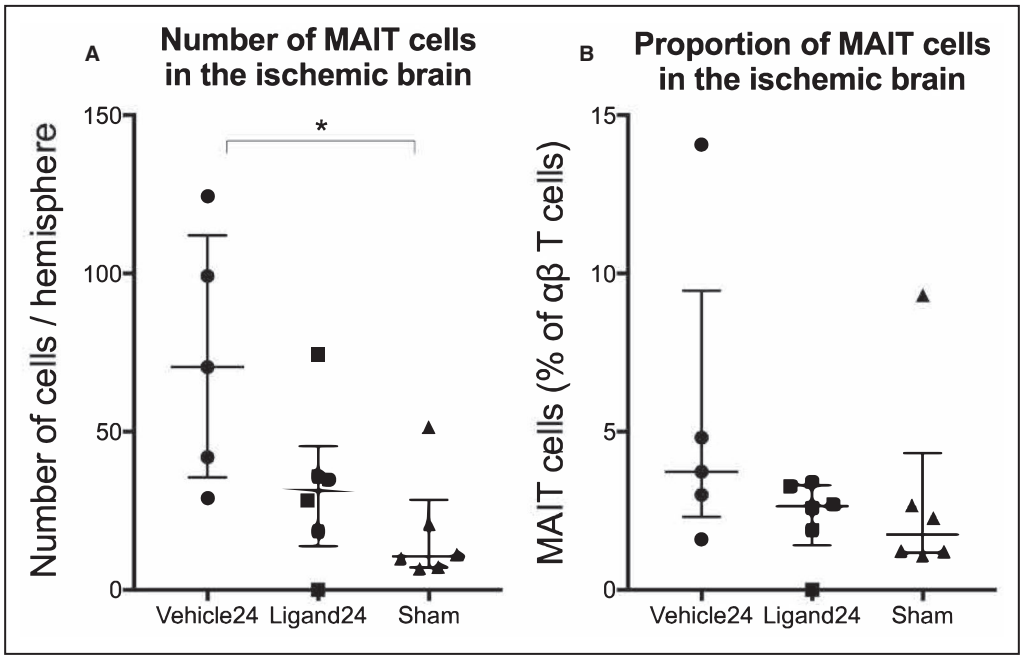


Figure 5. Administration of the suppressive MR1 ligand (isobutyryl 6-formylpterin) and infiltrating MAIT cells in the ischemic brain.
A, Absolute MAIT cell numbers per cerebral hemisphere. **B**, MAIT cell percentages of the αβ T cells among tMCAO C57BL/6 mice administered with the suppressive MR1 ligand (n=6), the vehicle control mice (n=5), and the sham-operated C57BL/6 mice (n=6). Each symbol represents data from an individual mouse. Values are presented as the median and interquartile range and were analyzed by the Kruskal-Wallis test followed by a post hoc analysis with Dunn's test. **P*<0.05. MAIT indicates mucosal-associated invariant T; MR1, major histocompatibility complex-related molecule 1; and tMCAO, transient middle cerebral artery occlusion.

have a protective role in experimental autoimmune encephalomyelitis, which is the mouse model of multiple sclerosis. The disease development and progression were attenuated in transgenic mice of the invariant Vα TCR in the T cells that augment MAIT cells, with the MR1-deficient mice developing more severe experimental autoimmune encephalomyelitis as compared with that observed in the control mice.[12] These studies suggest that the MAIT cells exert different roles depending upon the particular pathological condition.

In our current study, we found that MAIT cell deficiency suppressed both the number of Iba1-positive microglia/macrophages and their activation, as well as attenuating cytokine production, such as IL-1β, IL-6, and IL-17, after tMCAO. MAIT cells produce several cytokines such as IL-17, TNF-α, and IFN-γ, and these promote inflammation in different inflamed tissues.[38] In addition, IL-17 is secreted by MAIT cells and is involved in several autoimmune disorders and ischemic stroke.[39] A number of IL-17-expressing cells are elevated in both the brain and peripheral blood cells as early as 1 hour after focal ischemia in rats.[40] IL-17A deficient (*Il17ra*$^{-/-}$) mice have been shown to have a significant reduction of infarct size and a milder disability after tMCAO. Moreover, neutralization of IL-17A by anti-IL-17A also resulted in a similar effect.[41] Thus, these results suggest that IL-17 may play an important role in neuroinflammation after cerebral ischemia. Shichita et al demonstrated that IL-17 producing γδ T cells played a pivotal role during the subacute phase (day 4) of ischemic brain infarction.[7] In addition, although another group demonstrated that the T and B cell deficient (RAG$^{-/-}$) mice exhibited a significant reduction of cerebral infarction, other mice lacking γδ T cells did not exhibit any protective effect when evaluated at 24 hours after ischemia.[5] Our current data showed that MR1-deficient mice exhibited a reduction of the infarct volume along with significantly less IL-17 production and Iba1-positive microglia/macrophage inactivation as early as 24 hours after tMCAO. These data suggest that MAIT cells might be involved in the neuroinflammation earlier than other innate-like T lymphocytes. Thus, the modulation of MAIT cells during the acute stages of ischemic stroke might be an attractive new strategy for treatments. The mechanism underlying the correlation between MAIT cells and microglial activation in the ischemic brain is unclear. However, IL-17 plays a critical role in microglial activation, and IL-17 knockdown or anti-IL-17 antibody treatment reduce microglial activation after acute focal cerebral ischemia.[26] Such data suggest that there may be a synergistic correlation between MAIT cells and microglial activation under the pathological condition of brain ischemia.

In our current study, we used i6-FP as the suppressive ligand, as it has been shown to inhibit the MAIT cells activation both in vitro and in vivo.[19] Inhibition of MAIT cells by i6-FP exhibited a similar effect to that which was confirmed in an ischemic model using MR1-deficient mice. The FACS data also support that the attenuation of intracranial infiltration of MAIT cells was associated with a reduced infarct volume. Furthermore, intracranial microglia/macrophages in i6-FP treated group were reduced in comparison with the control group. Thus, the attenuation of neuroinflammation by the regulation of MAIT cells might result in the amelioration of ischemic brain damage. MAIT cells are preferentially located in mucosal tissue but are also abundant in peripheral blood.[42] Because T cells usually infiltrate the ischemic brain tissue as early as 3 hours after the onset of focal ischemia,[30] we believe that the main sources of infiltrated MAIT cell are derived from a circulating one. Thus, these results suggest that regulation of MAIT cells immediately after cerebral ischemia may ameliorate the infarct volume and the neurological severities that occur after ischemic stroke.

Study Limitations

There were several limitations for our present study. First, although our study used a tMCAO model that is highly reproducible and widely used in basic stroke research, this model is not comparable with naturally occurring clinical strokes. Thus, studies that examine other stroke models, such as the permanent MCAO, the thromboembolic clot model, or the use of aged or female animals, will need to be undertaken in the future. The second limitation of our study is that we initially administered i6-FP to the mice immediately after tMCAO, which was then followed by a second administration at 24 hours after tMCAO. Thus, we will need to determine the appropriate dose and timing of i6-FP injections in future experiments. Third, the mechanisms of MAIT cell infiltration into the ischemic brain unclear. Several chemokines are involved in the mobilization of MAIT cells.[43] Activated human MAIT cells upregulate the integrin very late antigen-4, an important integrin for T cell migration through the blood brain barrier.[44] Further studies are needed to clarify this issue in acute ischemic stroke.

CONCLUSIONS

In conclusion, our study demonstrated that MAIT cells are involved in neuroinflammation and can exacerbate ischemic brain injury. Modulation of MAIT cells could be a potential target for future treatments during the early phase of acute ischemic stroke.

ARTICLE INFORMATION

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Affiliations

From the Department of Neurology, Juntendo University Faculty of Medicine, Tokyo, Japan (S.N., R.T., T.I., N.M., T.K., Y.U., N.H.); Stroke Center and Division of Neurology, Department of Medicine, Jichi Medical University, Tochigi, Japan (R.T.); Department of Neurology, Juntendo University Urayasu Hospital, Chiba, Japan (K.Y., N.K., H.S., T.U.); and Department of Immunology, Juntendo University Faculty of Medicine, Tokyo, Japan (A.C., D.N., S.M.).

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Disclosures

None.

Supplementary Material

Figure S1

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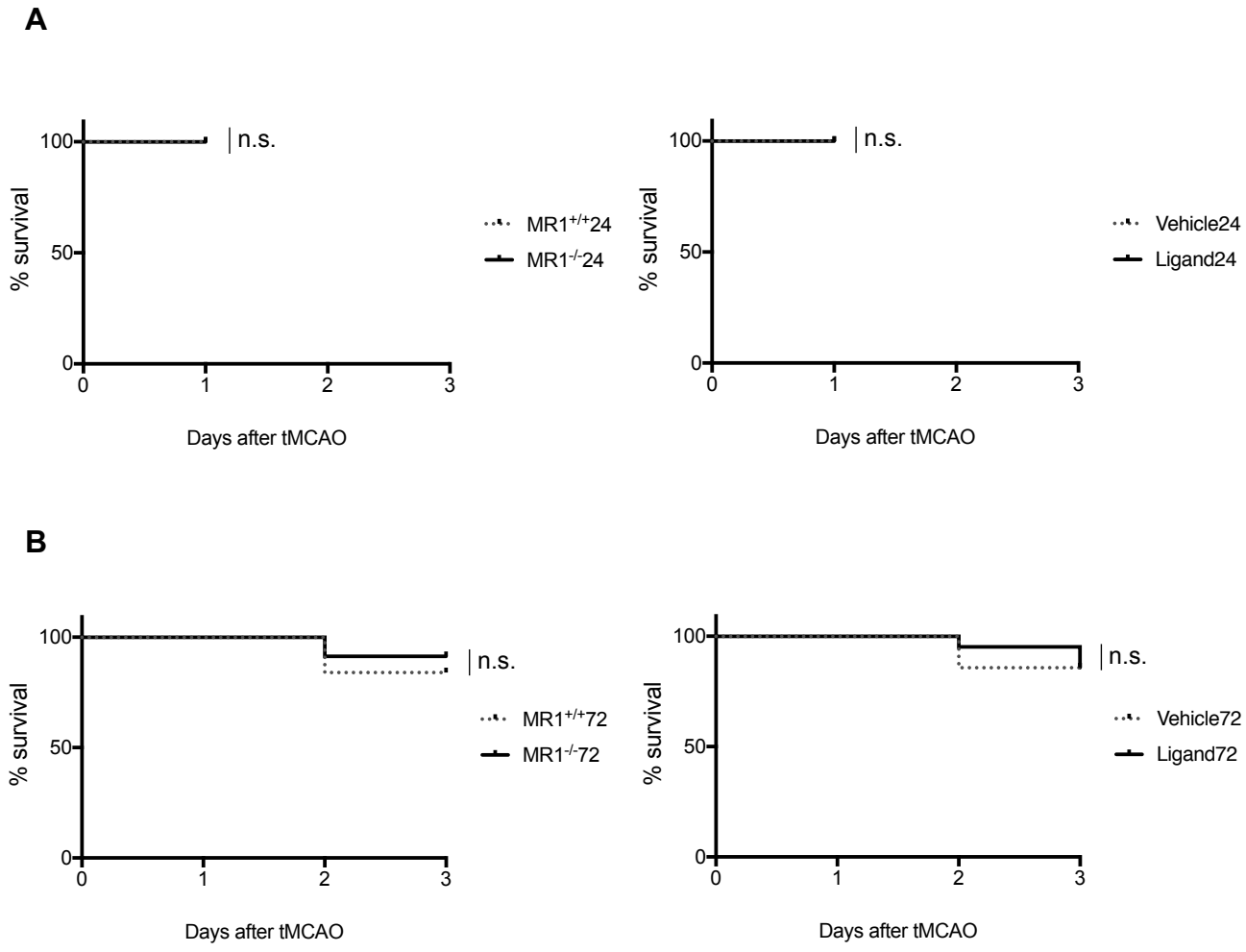
Supplemental Material

Figure S1. Survival rate at each time point after tMCAO.



A, Survival rate at 24 hours after tMCAO in each group (n=5-8 per group). **B,** Survival rate at 72 hours after tMCAO in each group (n=6-9 per group). Kaplan-Meier curve followed by the log rank test.