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Partial MHC constructs treat thromboembolic ischemic stroke characterized by early immune expansion

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Abstract

Inflammation and thrombosis are tightly linked, with inflammation contributing to thromboembolism and to stroke outcome. Thromboembolism is a frequent cause of ischemic stroke, yet the most used occlusion mouse models of experimental stroke do not effectively replicate thromboembolism. Our group recently described a novel thromboembolic mouse model of stroke that successfully occludes the middle cerebral artery with high reproducibility. In the current study, we characterize the peripheral and local immune outcomes as well as the ischemic response to immune therapy in a clinically relevant mouse model of thromboembolic stroke. Brain and spleen tissues were harvested 24 hours after thromboembolic stroke and cells immunophenotyped by flow cytometry. We observed a significant increase in neutrophils and early activated T cells in the spleen and an increase in neutrophils and activated monocytes/microglia in the ischemic cortex after thromboembolic stroke. Moreover, as was shown previously for transient MCAO models, treatment of thromboembolic stroke with partial MHC constructs significantly reduced ischemic damage indicating an equivalent effect of this immune-based therapy in the thromboembolic model that better mimics the pathophysiology of human stroke.

Keywords

Thromboembolic stroke; RTL1000; Therapy; Immune response; neuroinflammation

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Dr. Offner discloses US patent #8,491,913 B2 for the use of recombinant molecules in treatment of stroke.

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This article does not contain any studies with human participants performed by any of the authors.

Introduction

Ischemic stroke is a leading cause of death and disability in the United States. Eighty-seven [1]percent of all strokes in humans are ischemic and most frequently caused by cerebral thromboembolism [2–4]. Cerebral thrombosis or embolism leads to ischemic stroke with cerebral thrombosis being more common [5, 6]. Cerebral thrombosis is the formation of a blood clot at a congested cerebral vessel due to atherosclerosis of brain arteries, normally caused by an accumulation of fatty deposits inside the blood vessels. An embolus is frequently a fragment of a thrombus, usually from an atherosclerotic plaque that breaks free and travels to the brain.

The immune system plays a pivotal role in the progression of ischemic damage following stroke. Activation of splenocytes after stroke leads to a release of leukocytes into the periphery, followed by infiltration of pathogenic immune cells into the brain and activation of microglial cells, which exacerbate ischemic injury [7–10]. Rodents that are splenectomized prior to middle cerebral artery occlusion (MCAO) exhibit a decrease in infarct volume, associated with reduced inflammatory leukocytes and pro-inflammatory cytokines in the ischemic brain [4, 11]. Additionally, when spleens were irradiated 4 h after MCAO, infarct volume, microglia and infiltrating T cells in the ischemic brain were reduced, presumably due to inhibition of splenocyte efflux [12]. Of the immune cells that contribute to post-ischemic immune responses, neutrophils are early responders that initiate neuroinflammation and are a hallmark of ischemia [13, 14]. T cells that are activated in the periphery and infiltrate the brain can prolong inflammation and promote neuronal damage [15, 16]. Activated macrophages are increased in the periphery and subsequently recruited into the brain 3–7 days after stroke [8, 17].

The mouse embolic stroke model best mimics the most common cause of human stroke and yet remains unreliable. Embolic strokes induced by macrospheres, microspheres or autologous clots injected into the brain continue to be infrequently employed due to the high mortality rate, variability of infarct and unsuccessful induction of stroke [18, 19]. We have recently described a thromboembolic mouse model in C57BL/6 and DR2 male mice that successfully occludes the middle cerebral artery, yields reproducible ischemic lesions, reduces surgery time and better mimics clinical thromboembolic stroke [20].

In the present study, we describe immune responses in the periphery and CNS that contribute to thromboembolic stroke in C57BL/6 and humanized HLA-DR2 expressing mice and further evaluate the therapeutic activity of partial MHC constructs used previously to reduce the severity of MCAO [21]. Our findings indicate that thromboembolic mouse model involves activated monocytes/microglia and neutrophils in the ischemic brain, likely caused by an increased frequency of these cells in the spleen. Additionally, treatment of thromboembolic mice with partial MHC constructs significantly resolved total infarct volume similar to its treatment of MCAO. These results will help to understand the complex role of the immune response and immune based stroke therapies in a model that better mimics human ischemia.

Materials and Methods

Experimental Animals

All animal procedures were conducted in accordance with the National Institutes of Health guidelines for the use of animals in research, and protocols were approved by the Animal Care and Use Committees at Oregon Health & Science University and the Portland Veteran Affairs Medical Center. Male HLA-DRB1*1502 transgenic mice (DR2-Tg, 10–14 weeks of age and weighing 23–28 g) were produced at the Portland VA Medical Center using foundation breeders provided by Dr. Chella David [22]. The mice were housed in temperature-controlled rooms on a 12 hour light and 12-hour dark cycle with water and food ad libitum. Mice were randomized to one of the following experimental groups: sham surgery, stroke surgery.

Thromboembolic Stroke Model

Methods for stroke surgery were previously described [20]. Briefly, mice were anesthetized with isoflurane (5% induction, 1%–1.5% maintenance) and temperature was maintained with warm water pads and heating lamp. A modified PE-8 catheter, filled with 2 NIH U α -thrombin (10 μ L of 0.2 NIH U/ μ L) was gently introduced into the external carotid artery (ECA) lumen through a small puncture. A sample of 3 μ L blood was withdrawn from ECA into this catheter, which was then exposed to air for 15 min to accelerate clot formation at the tip of the catheter. 8-mm length of catheter was reintroduced into the ECA and gently advanced 7–8 mm into ICA. The blood clots and 50% of the thrombin were briskly injected to occlude the vessel, which was confirmed by the sudden drop in laser-Doppler perfusion (LDP; < 30% of baseline) over the MCA territory. The rest of thrombin was then very gently injected over 1 min making sure LDP remained low. At 10 min after injection, the catheter was withdrawn and the right ECA was ligated. For sham surgeries, the modified PE-8 catheter was placed in the ECA, but not advanced into ICA. No blood was withdrawn and no thrombin was injected in sham animals.

Monitoring Laser-Doppler Perfusion (LDP)

During the procedure, cerebrocortical perfusion was monitored by laser-Doppler (Model DRT4, Moor Instruments Inc., Wilmington, DE, USA) using a metal probe affixed to the surface of right side of the skull at the mid-point between the right ear and right eye (core of MCA territory). LDP was measured before CCA ligation (baseline), and measurements continued throughout procedure and 5 min after release of CCA ligature. Animals were excluded if LDP did not drop below 30% of baseline after all thrombin was infused [22].

Treatment with partial MHC constructs (RTL1000)

Mice were randomized to receive 0.1mL (100 μ g) RTL1000 or 0.1mL vehicle (5% dextrose in Tris-HCl, pH8.5) by subcutaneous injection one hour after stroke surgery. The dose of RTL was empirically determined by our lab [21, 23]. Both RTL1000 and vehicle treated mice were euthanized at the 24h time-point after stroke onset.

Measurement of Ischemic Lesion Volume

Mice were euthanized and brains collected at 24 hours after occlusion for measurement of infarct size using 2, 3, 5-triphenyltetrazolium chloride (TTC; Sigma, St. Louis, MO, USA) histology and digital image analysis, as previously described [24]. Four slices of 2-mm thick coronal sections were incubated in 1.2 % TTC for 15 min at 37 °C, then fixed in 10% formalin overnight. Images were analyzed using Sigma Scan Pro 5.0 Software (Systat Software, Inc., Point Richmond, CA, USA). To control for edema, infarct size in each region (cortical, striatal and total hemispheric infarct) was determined by subtracting the ipsilateral non-infarcted tissue volume from the volume of the contralateral region, then dividing the difference by the contralateral region volume. The ratio is multiplied by 100 to yield regional infarct volume as a percent of the contralateral region.

Leukocyte isolation from spleen and brain

Spleens from individual mice were removed and a single-cell suspension was prepared by passing the tissue through a 100 µm nylon mesh (BD Falcon, Bedford, MA). The cells were washed using RPMI 1640 and the red blood cells lysed using 1× red blood cell lysis buffer (eBioscience, Inc., San Diego, CA) and incubated for 1 min at room temperature. The cells were then washed with RPMI 1640, counted on a Cellometer Auto T4 cell counter (Nexcelom, Lawrence, MA), and resuspended in staining medium (PBS containing 0.1% NaN₃ and 1% bovine serum albumin (Sigma, Illinois)) for flow cytometry. The brain was divided into the ischemic (right) and nonischemic (left) hemispheres and the cortex was separated for analysis using a dissection microscope. The brain tissue was digested for 60 min with 1 mg/ml Type IV collagenase (Sigma Aldrich, St. Louis, MO) and DNase I (50 mg/ml, Roche Diagnostics, Indianapolis, IN) at 37°C with intermittent shaking. Samples were mixed with a 1 ml pipette every 15 min. The suspension was washed 1× in RPMI, resuspended in 80% Percoll overlaid with 40% Percoll and centrifuged for 30 min at 1600 RPM. The cells were then washed twice with RPMI 1640 and resuspended in staining medium for flow cytometry.

Analysis of cell populations by flow cytometry

All antibodies were purchased (BD Biosciences, San Jose, CA or eBioscience, Inc., San Diego, CA) as published. Four-color (FITC, PE, APC and PerCP) fluorescence flow cytometry analyses were performed to determine the phenotypes of splenocytes and brain cells. One million splenocytes or total separated brain leukocytes were washed with staining medium, blocked with Anti-mouse CD16/CD32 Mouse BD Fc Block™ (BD Biosciences, San Jose) for 15 minutes at 4°C and then incubated with combinations of the following monoclonal antibodies: CD11b (MAC-1), CD45 (Ly-5), CD3 (145-2C11), CD11c (HL-3), CD19 (1D3), CD4 (GK1.5), CD8 (53–6.7), Ly6C (AL-21), Ly6G (RB6-8C5), CD122 (TM-β1), CD44 (IM7), CCR5 (HM-CCR5), CD25 (7D4) and NK1.1 (PK136) for 20 min at 4°C. 7-AAD was added to examine cell death. Data were collected with BD Accuri™ C6 software on a BD Accuri™ C6 (BD Biosciences, San Jose, CA).

Statistical Analysis

Data are presented as mean $\pm$ SEM. Differences between sham and ischemic group were evaluated by Student's t-test. Statistical significance was set at $p < 0.05$. Statistical analyses were performed using Prism (GraphPad, La Jolla, CA).

Results

Increased peripheral neutrophils at 24 hours after thromboembolic stroke

Our previous study demonstrated that thromboembolic stroke induces sizable ischemic damage in DR2-Tg mice [20]. To examine the response of peripheral immune subsets to brain ischemic injury 24 hours following thromboembolic MCAO, we identified splenocyte phenotypes compared to sham surgery. We found no significant difference in splenocyte number or splenocyte death with between the thromboembolic stroke mice or sham mice (Fig. 1A). Differences in immune subsets were illustrated by an increase in total CD11b+ myeloid cells with thromboembolic stroke mice (Fig. 1B). To examine the specific subtype of CD11b+ that was elevated after stroke, we co-stained with Ly6G and Ly6C, markers for neutrophil and monocyte cells, respectively. CD11b+Ly6G+ neutrophils, not Ly6C+ monocytes, were found to be the contributing factor in CD11b+ increase in the spleen following stroke, increasing (Figure 1B, Table 1). We did not see any significant difference in total T cells or CD4+/CD8+ T cell subsets between thromboembolic stroke or sham mice (Figure 1C, Table 1). There is, however, a significant decrease in the frequency of B cells in the spleen following thromboembolic stroke (Figure 1C, Table 1). B cells have been shown to have a therapeutic effect on stroke [25], therefore a decrease in peripheral B cells could also contribute to infarction.

Thromboembolic stroke induces stages of early activation in splenic T cells

Because we did not see a difference in frequency of T cells in the spleen, we examined their activation status after 24 hours of thromboembolic stroke. We found a significant increase in splenic T cells expressing CD25 and CD122 (Fig. 2A,B) indicating early activation in spleen-derived T cells 24 hours after thromboembolic stroke. Thus, T cells may contribute to the immune response later in the peripheral reaction to thromboembolic stroke.

T cells do not exhibit an effector phenotype 24 hours after thromboembolic MCAO

In addition to examining early T cell activation in the spleen 24 hours after thromboembolic MCAO, we determined if there were differences in the effector phenotype or migration capabilities of those T cells. We looked at the expression of CD44, expressed on effector memory T cells, on CD3+ T cells. We found no significant change in CD44 expression on T cells between stroke or sham mice (Fig. 3A) indicating that 24 hours following thromboembolic stroke, T cells have not yet transitioned to an effector phenotype. Additionally, we examined if T cells were expressing CCR5, a chemokine receptor that allows cell homing into tissue. Again, there was no significant change in CCR5 expression on T cells between stroke or sham mice (Fig. 3B). These data illustrate that T cells do not contribute to the immune system's effects 24 hours after thromboembolic stroke.

Thromboembolic MCAO induces infiltration of activated microglia/monocytes and neutrophils

We also examined the effect of thromboembolic stroke on immune composition in the brain. Cortices from the ischemic hemisphere were harvested 24 hours following thromboembolic MCAO and examined for infiltrating immune cells. Due to the large amount of cell death, the striatum was manually excluded from the analysis. Thromboembolic MCAO significantly increased the percent of activated microglia/monocytes in the ischemic hemisphere versus sham surgery (Fig. 4A,B). Infiltrating neutrophils were also significantly elevated in the ischemic hemisphere over the sham surgery (Fig. 4C,D). The increase of neutrophils in the ischemic tissue correlates with the increase in neutrophils observed in the periphery 24 hours post thromboembolic MCAO. There was no change in the ischemic hemisphere between thromboembolic MCAO and sham surgery in percentage of infiltrating T cells, B cells, dendritic cells or NK cells (Table 2). These findings support our previous infarct data indicating that activated microglia/macrophages and neutrophils are the main immunological responders at 24 hours after thromboembolic stroke. Other immune cell types may not be likely to appear in the ischemic hemisphere until a later time point after stroke.

Thromboembolic MCAO induces significant infarction in brain tissue that can be resolved with RTL1000 therapy

DR2 mice were subject to thromboembolic MCAO and infarct was measured by TTC staining 24 hours later. Recombinant T-cell receptor ligand (RTL) molecules consist of the $\alpha 1$ and $\beta 1$ domains of MHC class II molecules expressed as a single polypeptide with antigenic amino terminal extensions [23, 26]. We have previously demonstrated that treatment with RTL1000 can significantly reduce infarct volume and neuroinflammation in a transient MCAO model of stroke [27]; therefore, we tested the therapy in the new thromboembolic model. Cerebral blood flow was measured by laser Doppler flowmetry to ensure success of surgeries and indicated no difference between the two treatments in the severity of ischemia (Fig. 5A). As illustrated in the representative TTC-stained cerebral sections in Figure 5B and quantified in 5C, infarct size in the cortex and striatum 24 hours following thromboembolic MCAO averaged $46.7 \pm 3.6\%$ and $71.6 \pm 7.5\%$, respectively. Total infarct was $49.3 \pm 5.1\%$. RTL1000 therapy significantly reduced infarct size in the cortex and hemisphere (Fig 5B,C). RTL1000 therapy also significantly reduced neurological deficit score 24 hours after thromboembolic stroke (Table 3). Of the 45 total DR2 mice that underwent thromboembolic MCAO surgeries for RTL and vehicle treated studies, 3 mice resulted in a failure to advance the catheter or failed to receive the thrombin clot and 3 mice underwent successful surgery but did not survive 24 hours post-surgery. Therefore, the success rate of the thromboembolic MCAO was 42/45 (93.3%) with a mortality rate of 2/21 (9.5%) in RTL vehicle-treated group and 1/21 (4.8%) in RTL-treated group.

Discussion

Ischemic stroke accounts for approximately 87% of all acute strokes and are a result of cerebral thrombosis or embolism [1]. Several stroke models have been developed in rodents to mimic ischemic stroke in humans and have helped to understand the outcomes of cerebral

ischemia. However, no model has accurately reproduced the natural progression and pathophysiology of cerebral thrombosis [28]. Our group recently developed a reproducible thromboembolic mouse model with low mortality and variability, which requires a short surgery time and a single anesthesia exposure [20]. In the present manuscript, we characterized the peripheral and ischemic brain immune response to thromboembolic stroke and evaluated treatment effects on the model with an emerging stroke therapy.

The peripheral immune response to stroke plays an important role in the progression of cerebral infarction. Previous work published by our laboratory illustrates a shift in immune subset frequencies in the spleen after MCAO [8] and it was thus of interest to carry out a similar evaluation of the immune composition of the spleen after thromboembolic stroke. Our data demonstrate an increase in neutrophils in the spleen 24 hours after thromboembolic stroke, consistent with previous studies documenting these cells as the initial activated immune subset following stroke. As well, previous reports characterized an increase in T cell populations 96 hours after transient MCAO [8]. Although our current findings do not indicate a shift in the frequency of T cell populations or effector T cells in the spleen 24 hours after thromboembolic stroke, there was an increase in early T cell activation markers, CD25 and CD122. CD25 and CD122 are two of the three possible proteins that comprise the IL-2 receptor in response to early T cell activation. However, they are also markers of CD4 and CD8 regulatory T cells, respectively, which are a hallmark of ischemic stroke [29, 8, 30–32]. It is possible that in time, a portion of the early activated T cells will transition to a regulatory phenotype, contributing to an increase in regulatory T cells that is observed by 96 hours after experimental stroke [8]. The decrease we see in B cell frequency in the spleen has also been observed at later time points in transient MCAO [8]. B cell subsets have been found to be protective in experimental stroke [25, 33, 34] and their decrease in the periphery could also exacerbate ischemic brain damage. Another hallmark of stroke is splenic atrophy [9]. Although a trend was observed, there was not a significant decrease in splenocyte numbers 24 hours after thromboembolic stroke. However, it is possible that the spleen size would continue to decrease over time as previously observed [8] until a significant and biologically relevant difference was apparent.

As anticipated, thromboembolic stroke also increased inflammatory cells in the ischemic brain hemisphere. Mice that were subjected to thromboembolic stroke exhibited a sizable and significant increase in infiltrating and activated monocytes/microglia 24 hours after stroke onset. Likewise, infiltrating neutrophils were significantly elevated. After ischemic injury, rapid activation of resident microglia was immediately followed by infiltration of circulating neutrophils [17]. While T cells can have early effects in stroke, the recruitment of peripheral lymphocytes into the brain typically occurs 3–7 days post stroke [17, 35], explaining the lack of significant T cell elevation in the ischemic hemisphere 24 hours after thromboembolic stroke. There is a possibility that the early activated T cells observed in the spleen could infiltrate into the ischemic tissue. Therefore, examining early activated T cell infiltration at a later time point in future studies would be of interest. It is also noteworthy that an elevated neutrophil to lymphocyte ratio was reported in human stroke subjects 24 hours after symptom onset that correlated with a worse stroke outcome [36].

Having characterized the immune response to thromboembolic stroke, we then tested an emerging stroke therapy, RTL1000, in the thromboembolic model. RTLs are partial agonists that render autoreactive T cells nonpathogenic [37, 38]. Our lab has previously shown that RTLs could treat both experimental autoimmune encephalomyelitis (EAE) and experimental stroke induced by transient MCAO in mice [23, 39, 37, 40, 41, 21, 42, 43]. RTL1000 is comprised of an HLA-DR2 $\alpha 1\beta 1$ moiety linked to human MOG-35-55 peptide [44] and has been shown to reduce infarct size in young, middle aged and older humanized DR2 mice treated with RTL following experimental stroke [21, 45, 22, 27, 46]. RTL1000, delivered one hour after stroke onset significantly reduced neurological scores and infarct volumes in the cortex and hemisphere after 24 hours.

The current study demonstrates that the mouse model of thromboembolic stroke is characterized by early expansion of peripheral and brain infiltrating neutrophils and early stages of T cell activation, comparable to what is observed in humans and other animal models of stroke. Additionally, RTL1000 therapy was able to reduce infarct damage induced by thromboembolism. Together, these data help to understand the complex role of the immune response and immune based therapy (RTL1000) in an animal model that better mimics the pathophysiology of human stroke.

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Fig. 1.

Increased peripheral neutrophils in spleen at 24 hours after thrombotic stroke. Spleens were harvested 24 hours after thromboembolic stroke and immunophenotyped by flow cytometry. Total splenocytes were enumerated; n=10 sham, n=13 stroke (A). Splenocyte death was determined by 7-AAD staining (B). The frequency of total CD3+ T cells, CD19+ B cells and CD11b+ myeloid cells were determined (C,D,E). Neutrophils were CD11b+Ly6G+ (F). Values represent mean numbers ($\pm$ SEM) of 7 mice in the sham group and 9 in the stroke group. * indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$ by t-test

Fig. 2.

Thromboembolic stroke induces stages of early activation in splenic T cells. Spleens were harvested 24 hours after thromboembolic stroke and immunophenotyped by flow cytometry. The frequency of CD25+CD4+ T cells was determined by gating on CD4+ cells and measuring expression of CD25(A). The frequency of CD122+CD8+ T cells was determined by gating on CD8+ cells and measuring expression of CD122(B). Values represent mean numbers ($\pm$ SEM) of 5 mice in the sham group and 9 in the stroke group

Fig. 3.

T cell activation and migration do not contribute to peripheral activity following thromboembolic MCAO. Spleens were harvested 24 hours after thromboembolic stroke and immunophenotyped by flow cytometry. The frequency of activated T cells was determined by gating on CD3+ cells and measuring expression of CD44 (A). The frequency of migrating T cells was determined by gating on CD3+ cells and measuring expression of CCR5 (B). Values represent mean numbers ($\pm$ SEM) of 7 mice in the sham group and 9 in the stroke group

Fig. 4.

Thromboembolic stroke increases the percentage of activated microglia/monocytes and neutrophils in ischemic cortex. Brain cortices were harvested 24 hours after thromboembolic stroke, processed and immunophenotyped by flow cytometry. Infiltrating and activated monocytes/microglia in the ischemic hemisphere were determined by gating on CD11b⁺ cells and examining CD45 high cells (A, B). Infiltrating neutrophils in the ischemic hemisphere were CD11b⁺Ly6G⁺ (C, D). Values represent mean numbers ($\pm$ SEM) of 7 mice from each group for monocyte/microglia stain; n=3 mice from each group for neutrophil stain. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

Fig. 5.

Assessment of infarct volume and cerebral blood flow after initiation of thromboembolic MCAO. Male DR2 mice were subject to thromboembolic stroke with vehicle or RTL1000 treatment. Cerebral blood flow (CBF) during thromboembolic stroke was measured by laser-Doppler (A). Brains were harvested 24 h after MCAO and brain slices were stained with 2,3,5-triphenyltetrazolium chloride (TTC) (B). Infarct volumes were quantified and compared (C). Values represent mean numbers ($\pm$ SEM) of 9 mice from the vehicle group and 10 mice for the RTL group. * indicates $p < 0.05$

Table 1

Immune subsets in the spleen 24 hours after thromboembolic stroke

Splenocyte frequency	Sham thromboembolic stroke	Thromboembolic stroke
Splenocyte # ($\times 10^7$)	4.34 $\pm$ 0.41	3.53 $\pm$ 0.37
CD3+ (%)	23.39 $\pm$ 1.5	23.14 $\pm$ 1.6
CD4+ (%)	15.65 $\pm$ 0.76	15.2 $\pm$ 0.75
CD8+ (%)	8.97 $\pm$ 0.66	9.24 $\pm$ 0.64
CD19+ (%)	66.94 $\pm$ 1.02	55.84 $\pm$ 2.12***
CD11c+ (%)	1.89 $\pm$ 0.11	1.40 $\pm$ 0.20
total CD11b+ (%)	5.08 $\pm$ 0.44	11.88 $\pm$ 1.82**
CD11b+Gr-1+ (%)	3.44 $\pm$ 0.52	8.67 $\pm$ 1.61*
CD11b+Ly6C+ (%)	1.24 $\pm$ 0.11	1.56 $\pm$ 0.33
7AAD (%)	7.27 $\pm$ 1.44	7.82 $\pm$ 1.56

* indicates $p < 0.05$,** indicates $p < 0.01$ and*** indicates $p < 0.001$ by t-test.

Table 2

Immune subsets in the brain 24 hours after thromboembolic stroke

Ischemic hemisphere	Sham thromboembolic stroke	Thromboembolic stroke
CD11b+CD45 ^{hi} (%)	5.06±0.60	30.13±5.10 ***
CD3+ (%)	2.91±0.57	3.25±0.50
CD19+ (%)	0.34±0.10	0.13±0.07
CD11c+ (%)	0.65±0.03	2.43±1.37
NK1.1+ (%)	0.17±0.03	0.24±0.08
CD11b+Ly6G+ (%)	1.77±0.77	9.97±2.16 *

* indicates p<0.05,

*** indicates p<0.001 by t-test.

Distribution of neurological deficit scores in vehicle and RTL1000 treated DR2-Tg mice 2h and 24 h after thromboembolic MCAO

Table 3

NDS	2 h post stroke					24 h post stroke				
	0	1	2	3	4	0	1	2	3	4
Vehicle (n=9)	0	0	3	5	1	0	0	3	6	0
RTL (n=10)	0	0	3	5	2	0	1	8	1	0
<i>P</i> value	0.724					0.029 *				

* $p < 0.05$ compared with vehicle group determined by Mann-Whitney Rank Sum Test.