



# Endogenous Regulatory T Cells Promote M2 Macrophage Phenotype in Diabetic Stroke as Visualized by Optical Imaging

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## Abstract

Regulatory T cells (Tregs) play an immunosuppressive role in various diseases, yet their function remains controversial in stroke and obscure in diabetic stroke. In the present study, Tregs were found downregulated in the peripheral blood of type 2 diabetes mellitus (T2DM) stroke models and patients compared with controls. In ischemic stroke mice (both T2DM and wild type), endogenous Tregs boosted by CD28SA increased CD206+ M2 macrophage/microglia cells, decreased infarct volumes, and improved neurological recovery. Our results demonstrated the potential of boosting Tregs for treating T2DM stroke. Furthermore, we utilized an optical imaging probe (IRD- $\alpha$ CD206) to target M2 macrophage/microglia cells and demonstrated its effect in visualizing M2 macrophage/microglia cells in vivo in ischemic brain tissue.

**Keywords** Type 2 diabetes mellitus · Ischemic stroke · Regulatory T cells · M2 macrophages/microglia · Optical imaging

## Introduction

Ischemic stroke is a leading cause of death and disability. It causes profound local and systemic inflammatory responses that involve many types of immune cells [1]. Previous studies suggested that neurons in the ischemic penumbra generate interleukin-4 (IL-4), which could induce surrounding microglia/macrophages to an alternative anti-inflammatory M2 state [2]. In addition, ischemia can induce the migration of peripheral immune cells into the brain. Among the infiltrating immune cells, macrophages display remarkable functional plasticity. Compared with peripheral macrophages, invading macrophages possess robust genomic programs that can exert a profound influence over brain injury and repair [3]. Stroke patients often have comorbidities such as hypertension, hyperlipidemia, and diabetes mellitus [4]. Type 2 diabetes mellitus (T2DM), in particular, is a rapidly escalating

medical condition that doubles the risk of ischemic stroke, exacerbates stroke-induced brain damage, and correlates with a worse clinical outcome from stroke [5]. This correlation is partly due to a dysregulated inflammatory response in T2DM patients [6]. Hyperglycemia manifested in T2DM is associated with a state of chronic, systemic low-grade inflammation [7]. Compared with non-diabetic stroke patients, those with diabetes show increased microglial activation and higher levels of pro-inflammation cytokines such as TNF- $\alpha$  and IL-1 $\beta$  [8]. As a subpopulation of T cells that comprise 5–10% of all peripheral CD4<sup>+</sup> T cells, regulatory T cells (Tregs) powerfully influence inflammation processes. Tregs inhibit other immune cells through direct contact or by releasing immunosuppressive cytokines such as TGF- $\beta$  and IL-10 [9]. Ruhnau et al. demonstrated that Tregs derived from ischemic stroke patients' peripheral blood were reduced in number, and that their activation was severely impaired [10]. Endogenous Tregs boosted by CD28 superagonistic monoclonal antibody (CD28SA) have been proven to infiltrate into the peri-infarct area 7 days after stroke [11–13]. Systemic administration of Tregs after ischemic stroke led to a marked reduction in infarct volume and an improvement in neurological function [11]. Thus, CD28SA may be used to increase endogenous Tregs, reduce inflammatory response, and improve stroke outcome [13]. There are, however, concerns that the expansion of Tregs by CD28SA could cause thrombo-inflammatory lesion growth, thus increasing stroke volume and worsening functional recovery [14].

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Clarifying Tregs' role in diabetic stroke requires exploration of specific biomarkers and noninvasive imaging probes that can monitor the effects of Treg activity *in vivo*, which are the aim of this study. In the study, we found that the number of circulating Tregs was decreased in subjects with T2DM than those without after ischemic stroke. In stroke mice models, administration of CD28SA boosted endogenous Tregs and significantly altered macrophage/microglial polarization and improved the outcome of diabetic stroke. To monitor the therapeutic efficacy of CD28SA-boosted endogenous Tregs in real time, we utilized a M2 macrophage-/microglial-targeted probe (IRD- $\alpha$ CD206) to image intracranial M2 microglia/macrophage expression. IRD- $\alpha$ CD206 allowed for accurate and non-invasive profiling of the biological role of Tregs and has potential in therapeutic efficacy evaluation.

## Materials and Methods

### Animal Study

#### Animal Models

The animal experiments were approved by the Institutional Animal Use and Care Committee (IAUCC) of the Medical School of Southeast University (Nanjing, Jiangsu, China; approval ID: SYXK-20130413275). All experiments were performed and reported according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines [15]. A total of 279 C57BL/6J male mice purchased from Yangzhou University (Jiangsu, China) were used. These mice (4 weeks old, 15–17 g) were randomly assigned to the T2DM group or wild-type (WT) group. Animals had free access to fresh food and water at all times. T2DM was induced by feeding the mice a high-fat diet (58 kcal% fat, besfood, Nanjing, China) for 3 weeks and consecutively injecting streptozotocin in sterile citrate buffer (25 mg/kg; Sigma Aldrich, St. Louis, MO) for 5 days [16]. Hyperglycemia was confirmed using blood glucose test strips (OneTouch Ultra®, Tokyo, Japan). Mice were considered diabetic if their glucose level was  $\geq 300$  mg/dL. The WT control group received a normal diet. Cortical ischemic stroke was induced by photothrombosis, as previously described by our group [17]. The animals in sham group were not underwent illumination. Body temperature was closely monitored and maintained at  $37 \pm 0.5$  °C during surgery using a heating pad. T2-weighted images were acquired 3 h post-surgery to confirm stroke lesions. On day 7, post-stroke, mice were sacrificed by inhalation of 1% isoflurane (KeYuan, Shandong, China) in oxygen/air mixture.

### Antibody Treatment

CD28SA (200  $\mu$ g; clone 37.51, In Vivo MAb anti-Mcd28; Biocell, Cairo, Egypt) was dissolved in phosphate-buffered saline (PBS) and administered to mice intraperitoneally 3–6 h after ischemia onset. The level of Tregs in peripheral blood was evaluated at day 3 post-ischemia using flow cytometry. IL-2 protein (BioLegend, Cat#:575406) was mixed with anti-mouse IL-2 (BioLegend, Cat #:503705) at 2:1 M ratio (1  $\mu$ g of recombinant murine IL-2 protein and 5  $\mu$ g of anti-IL-2 mAbs) and incubated at 37 °C for 30 min. Mice subjected to ischemia were intraperitoneally injected with 200  $\mu$ l of either IL-2/anti-IL-2 complex or PBS at 2, 24, and 48 h after stroke [18].

### Assessment of Functional Outcomes

On day 7 post-stroke, the modified neurological severity score (mNSS) was applied to identify motor, sensory, reflex, and balance deficits in the mice, and the foot fault test was used to assess locomotor function [19].

### Blood Sample Collection and Flow Cytometry Analysis

Blood samples were collected for flow cytometry analysis of leukocyte subpopulations. We collected peripheral blood from mice and lysed the red blood cells. A single blood sample was around 300–400  $\mu$ l. The total cell count of the PBMCs was about 50,000 per sample. Single-cell suspensions were stained with anti-mouse CD4, CD25, and Foxp3 as well as the appropriate isotype control antibody following the manufacturer's instructions (eBioscience, San Diego, CA). The absolute number of Treg was determined by flow cytometry data from NovoExpress software.

### Bacteriological Analysis

We boosted Tregs on day 7 post-stroke and evaluated bacterial colonies 3 days after CD28SA injection. Blood was collected from eyes. One hundred microliters of blood was diluted and plated onto sheep blood agar plates. The cultures were incubated at 37 °C for 3 days. Bacterial colonies were counted by an observer blinded to experimental group assignments [20].

### MRI and NIRF Studies

Magnetic resonance imaging (MRI) was performed on a 7-T small animal MRI scanner (Bruker PharmaScan, Ettlingen, Germany). T2-weighted images were acquired using a well-defined fast spin echo sequence (repetition time 2000 ms; echo time 36 ms; field of view 2 cm  $\times$  2 cm; slice thickness 1 mm). The regions of interest were manually drawn, and their volumes were calculated as the volume of the contralateral hemisphere minus that of the ipsilateral non-infarct

hemisphere. In vivo near infrared fluorescence (NIRF) imaging was performed using the IVIS-Spectrum system (Perkin Elmer, Santa Clara, CA). The IRD- $\alpha$ CD206 probe (0.5 nmol/mouse, dissolved in 200  $\mu$ L of PBS) was injected via the tail vein and subjected to brain imaging. The probe's excitation and emission wavelengths were 745 nm and 780 nm, respectively. Mice's brain, heart, spleen, liver, kidneys, and lungs were dissected for ex vivo NIRF imaging with the same parameters. To study the microdistribution of  $\alpha$ CD206 in ischemic brain tissue, mice were injected intravenously with FITC- $\alpha$ CD206 (50  $\mu$ g; clone C068C2, Biolegend, San Diego, CA) on day 7 post-stroke. The brains were harvested at 24 h p.i. and immunolabeled with anti-CD206 antibody (Biolegend, San Diego, CA) to determine macrophage/microglial localization.

### Immunostaining

After being sacrificed, mice were transcardially perfused with saline. The brains were removed immediately and fixed in 4% paraformaldehyde for 24 h. Immunostaining was performed on 4- $\mu$ m thick coronal paraffin slides. The slides were stained with antibodies against Arg-1 (sc-18354; Santa Cruz Biotechnology, Texas, USA), CD68 (ab12512; Abcam, Cambridge, MA), and CD206 (Biolegend, San Diego, CA). Next, slides were incubated with Alexa Fluor 488 polyclonal antibody or 546 polyclonal antibody (Life Technologies, Grand Island, NY). Finally, the slides were covered with 4' 6-diamidino-2-phenylindole (DAPI, SouthernBiotech, Alabama, USA) and analyzed using polarized light microscopy (Scope A1; ZEISS, Oberkochen, Germany). To determine the frequency of cells positive for CD68, CD206, and Arg-1, three randomly chosen non-overlapping fields around the infarct lesion were examined. The observers were blinded to group allocation.

### Enzyme-Linked Immunosorbent Assay

Samples were obtained from the right hemispheres of the ischemic brains. Detection of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 were accomplished using commercially available ELISA kits (eBioscience, San Diego, CA) according to the manufacturer's instructions.

### Synthesis of the IRD- $\alpha$ CD206 Molecular Imaging Probe

The anti-CD206 antibody (clone C068C2, IgG2a; Biolegend, San Diego, CA) was mixed with IRDye755-N-hydroxysuccinimide at 1:7 mol ratio in sodium bicarbonate buffer for 12 h. The labeling degree (dye/protein) of IRD- $\alpha$ CD206 was approximately 4.1, as determined by UV/Vis spectroscopy. Sodium dodecyl sulfate polyacrylamide gel

electrophoresis (SDS-PAGE) was used to assess the purity of the IRD- $\alpha$ CD206 probe. The detailed synthesis protocol and in vitro characterization of the IRD- $\alpha$ CD206 probe are provided elsewhere [21]. To evaluate  $\alpha$ CD206 microdistribution, ischemic stroke mice were injected intravenously with 50 mg FITC- $\alpha$ CD206 (clone C068C2; IgG2a; Sungene, Tianjin, China). At 8 h after p.i., brain tissue was harvested, cut on a freezing microtome, and immunolabeled with a CD206 antibody (Biolegend, San Diego, CA).

### Statistical Analysis

SPSS version 18.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Numerical data are presented as the mean  $\pm$  standard error of the mean (SEM). For statistical comparisons, one-way analysis of variance (ANOVA) with the least significant difference (LSD) post-hoc test was applied. Student's *t* test was used for two-group comparisons. *P* values  $< 0.05$  were considered statistically significant.

### Human Study

Human study was approved by the Independent Ethics Committee for Clinical Research of Zhongda Hospital, Southeast University as per the World Medical Association outlined in the Declaration of Helsinki (No. 2013ZDSYLL094.0).

### Patients

All patients with acute ischemic stroke admitted between November 2016 and May 2017 were recruited from the Department of Neurology. Signed informed consent for aseptic peripheral venous puncture was obtained from all patients. Inclusion criteria were (1) age of 18 years or older, (2) time of onset within 24 h, and (3) ischemic stroke diagnosed on computed tomography or MRI scan. Exclusion criteria were (1) signs of infection (C-reactive protein levels  $> 50$  mg/L) and (2) confirmed malignancies. T2DM was defined based on the latest criteria published by the American Diabetes Association [21]. Patients who had T2DM for over 1 year were eligible for enrollment in the diabetic stroke group. Characteristics of patients are listed in Supplementary Table 1.

### Blood Sample Collection and Flow Cytometry Assessment

Peripheral blood samples (1–2 mL) were drawn from patients within 24–48 h after stroke onset. Blood samples were incubated with a fluorescence-conjugated CD4, CD25, and Foxp3 monoclonal cocktail (eZFluor™, eBioscience, San Diego, CA). Staining with the appropriate isotype control was performed as per the manufacturer's instructions.

## Statistical Analysis

Statistical analysis was completed with SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). Categorical data, including sex and prevalence of hypertension, smoking, dyslipidemia, and posterior circulation stroke, were analyzed using the chi-squared test. Age, leukocyte subpopulations, neutrophil granulocyte ratio, and lesion volume were presented as mean  $\pm$  standard deviation, while the National Institutes of Health Stroke Scale (NIHSS) score was presented as median and range. These variables were compared between groups (T2DM vs. non-T2DM) using Student's *t* test. *P* values  $<$  0.05 were considered statistically significant.

## Results

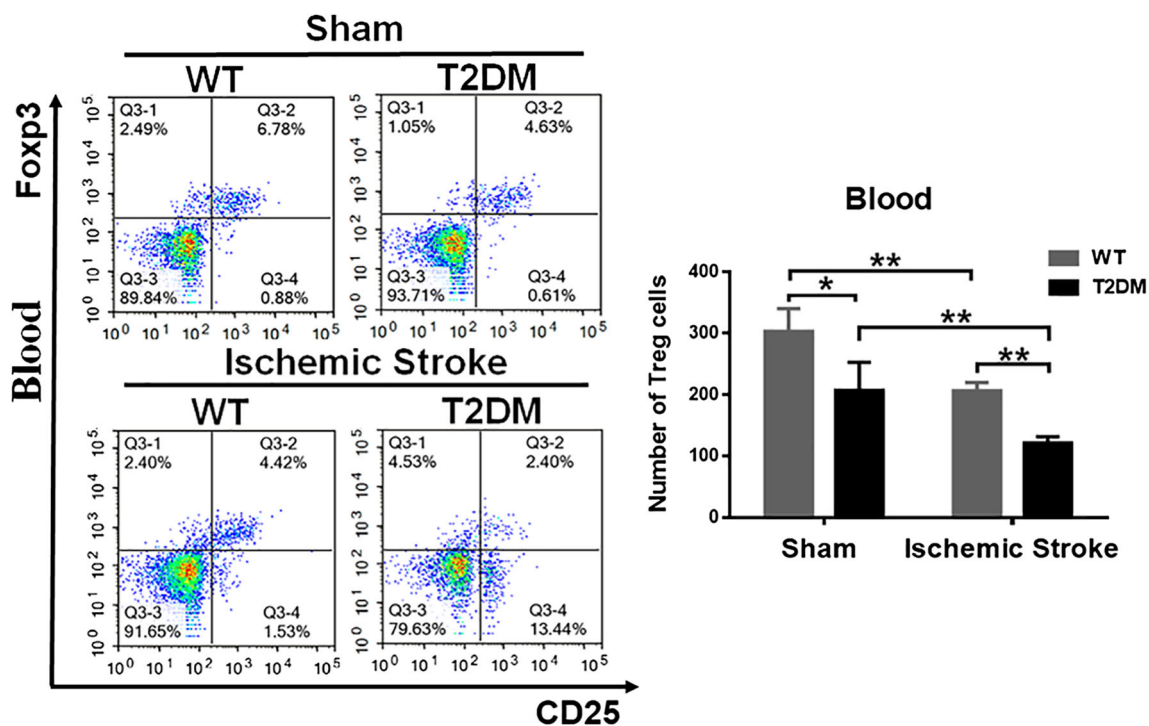
### Decreased Level of Circulating Tregs in T2DM Subjects After Ischemic Stroke

Flow cytometric result showed that ischemic stroke mice had reduced Tregs in peripheral blood compared with the sham group (Fig. 1). In addition, T2DM mice presented significantly lower Tregs numbers than WT mice in peripheral blood (Fig. 1). Consistent with mice model results, T2DM stroke patients had decreased percentages of Tregs

among CD4<sup>+</sup> T cells in peripheral blood lymphocytes (Supplementary Fig. S1).

### Increased Number of Circulating Tregs in T2DM Stroke Mice After CD28SA Treatment

Following our finding that the level of circulating Tregs was reduced in diabetic stroke mice and patients, we sought to test if Treg-boosting therapy could promote recovery after ischemic stroke. CD28SA or vehicle was given intravenously to T2DM and WT mice at 3–6 h after ischemia, before peripheral blood cells were analyzed by flow cytometry. The protocol is shown schematically in Fig. 2a. We analyzed the level of circulating Tregs on day 1, 3, and 7 post-stroke. Kinetic analysis of Tregs in peripheral blood revealed that Treg levels increased and peaked 3 days after CD28SA administration in WT and T2DM mice (Fig. 2b, c), suggesting that CD28SA stimulation successfully boosted the number of Tregs in peripheral blood. To test whether Tregs can cause post-stroke immune suppression, we boosted Tregs at day 7 after stroke by CD28SA and evaluated bacterial loads. Cultures of blood showed no difference of bacterial loads between CD28SA and control groups (Fig. 2d), suggesting that CD28SA treatment does cause immune suppression post-stroke.

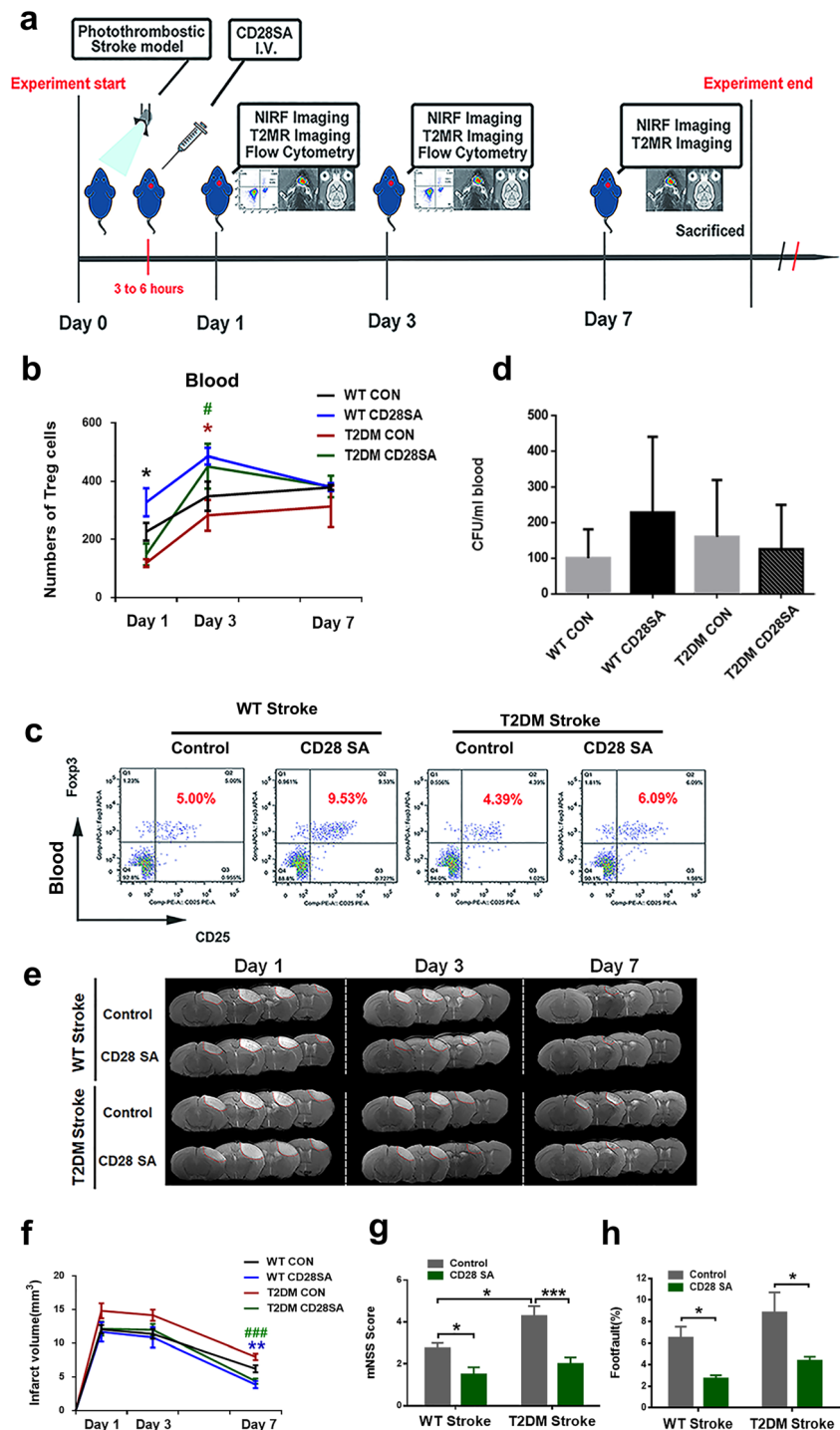


**Fig. 1** T2DM mice showed decreased Tregs in peripheral blood with and without ischemic stroke. Flow cytometry analysis of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells in WT and T2DM mice with and without ischemic stroke. Peripheral blood of the ischemic stroke group was collected 24 h post-

ischemia. T2DM mice had significantly less Tregs in both ischemic stroke and non-ischemic stroke groups compared with WT mice. In addition, Tregs were significantly decreased after stroke in WT and T2DM mice.  $n \geq 4$  per group, \**P* < 0.05, \*\**P* < 0.01, mean  $\pm$  SEM



**Fig. 2** CD28SA treatment increased the percentage of endogenous Tregs in peripheral blood. In T2DM mice, CD28SA treatment significantly improved stroke outcomes compared with the control group. **a** Schematic diagram of the study design. **b** Flow cytometric analysis of Treg cells in peripheral blood on day 1, 3, and 7 post-stroke. Kinetic analysis revealed that Tregs increased and peaked 3 days after CD28SA administration in WT and T2DM mice. ( $n = 4-7$  mice/group.  $*P < 0.05$  in black, WT control vs. T2DM control;  $\#P < 0.05$  in green, WT control vs. WT CD28SA;  $*P < 0.05$  in red, T2DM control vs. T2DM CD28SA.). **c** Representative flow cytometry plots showing expression of circulating Tregs on day 3 post-CD28SA administration. **d** Blood homogenates were collected and then cultured on blood agar plates. The bacterial loads demonstrated no difference between control and CD28SA-treated mice in both diabetic and non-diabetic stroke mice. ( $n = 3-6$ /group). **e** Representative T2-weighted images of T2DM and WT stroke mice in various groups on day 1, 3, and 7 post-stroke. **f** Quantitative calculation of infarct volume using T2-weighted images. ( $n \geq 5$ /group,  $*P < 0.01$  in blue, WT control vs. WT CD28SA;  $###P < 0.001$  in green, T2DM control vs. T2DM CD28SA). **g** Modified neurological severity test and **h** foot fault test were performed on day 7 post-stroke ( $n = 6-8$ ,  $*P < 0.05$ ,  $**P < 0.01$ ). All data are presented as the mean  $\pm$  SEM



### Endogenous Tregs Boosted by CD28SA Reduced Infarct Volume and Improved Neurological Recovery in Ischemic Stroke Mice

T2-weighted imaging was performed on days 1, 3, and 7 post-stroke to assess infarct volume (Fig. 2e). No significant differences between CD28SA-treated and control mice were noted on day 1 and day 3. On day 7 post stroke, CD28SA-treated

mice showed smaller infarct volumes than control mice (Fig. 2f). To examine the effect of CD28SA on neurological deficits, the mNSS test (Fig. 2g) and foot fault test (Fig. 2h) were conducted on day 7 post-stroke. T2DM mice showed significantly more severe neurological function deficits than WT mice. However, both CD28SA-treated T2DM and WT mice showed significant improvement in functional tests. These findings suggest that CD28SA significantly decreased

ischemic stroke–induced loss of ipsilesional activation in T2DM mice. In addition, we experimented with the IL-2/IL-2mAb complex, which has also been shown to expand endogenous Tregs. The level of Tregs was increased in IL-2/IL-2mAb complex-treated T2DM mice on day 7 post-stroke (Supplementary Fig. S2), and T2-weighted images and stroke volume quantification showed that IL-2/IL-2mAb complex–treated mice presented significantly smaller infarction lesions than control mice (Supplementary Fig. S3).

### CD28SA Treatment Upregulated M2–Dominant Macrophage/Microglial Expression in T2DM Stroke Mice

To investigate the effect of CD28SA on macrophage/microglia polarization, we analyzed ischemic brain tissue immunohistochemically on day 7 post-stroke (Fig. 3a). Microscopic examination revealed that the number of CD206+ (M2 marker) and CD68+ (macrophage/microglial marker) cells in the peri-infarct area was much higher in CD28SA-treated mice than in control mice, in both the T2DM and WT groups (Fig. 3b). Furthermore, CD28SA treatment significantly increased the number of Arg-1+ (M2 marker) and CD68+ (macrophage/microglial marker) cells in T2DM mice (Fig. 3c). Thus, CD28SA treatment expanded the population of M2 macrophages/microglia in diabetic stroke mice.

### CD28SA Treatment Reduces the Levels of Cytokines in the Ischemic Brain

Macrophages/microglia play a key role in the recovery process after cerebral ischemia, and their functions are partly mediated by the release of inflammatory cytokines. We performed enzyme-linked immunosorbent assays to quantify the expression of various immune factors on day 7 post-stroke. TNF- $\alpha$  levels were significantly lower in CD28SA-treated groups than in the corresponding control groups (Fig. 3d). In addition, IL-6 and IL-1 $\beta$  levels were lower in CD28SA-treated mice than in control mice, but the difference was not statistically significant (Fig. 3e, f).

### Characterization of the IRD- $\alpha$ CD206 Probe

Anti-CD206 monoclonal antibody was conjugated with a near-infrared dye (IRD), DyLight 755, to synthesize the IRD- $\alpha$ CD206 probe (Fig. 4a). An isotype-matched control probe (IRD-IgG) was synthesized by conjugating anti-rat IgG antibody with DyLight 755 using the same protocol. Immunofluorescence staining of the brain tissue sections showed that CD206 (red) merged well with FITC- $\alpha$ CD206 (green), indicating the ability of the IRD- $\alpha$ CD206 probe to target CD206-positive (M2 macrophages/microglia) cells in

ischemic brain tissue (Fig. 4b). In vivo imaging of ischemic stroke mice showed that the uptake of IRD- $\alpha$ CD206 in ischemic tissue was significantly higher than that of the control IRD-IgG. (Fig. 4c, d). This result demonstrates the potential ability of IRD-CD206 in in vivo imaging of ischemic brain tissue.

### In Vivo Imaging and Ex Vivo Bio-distribution of IRD- $\alpha$ CD206 Probe

A series of in vivo images were acquired from the ischemic brain from 20 min to 48 h after probe injection. Peak signal intensity of IRD- $\alpha$ CD206 occurred at 8 h after probe injection (Fig. 4e, f). The bio-distribution of IRD- $\alpha$ CD206 in the heart, liver, lung, spleen, intestine, kidney, and brain was evaluated through ex vivo imaging, which showed strong fluorescence signals in the liver, kidney, and ischemia brain (Fig. 4g–h).

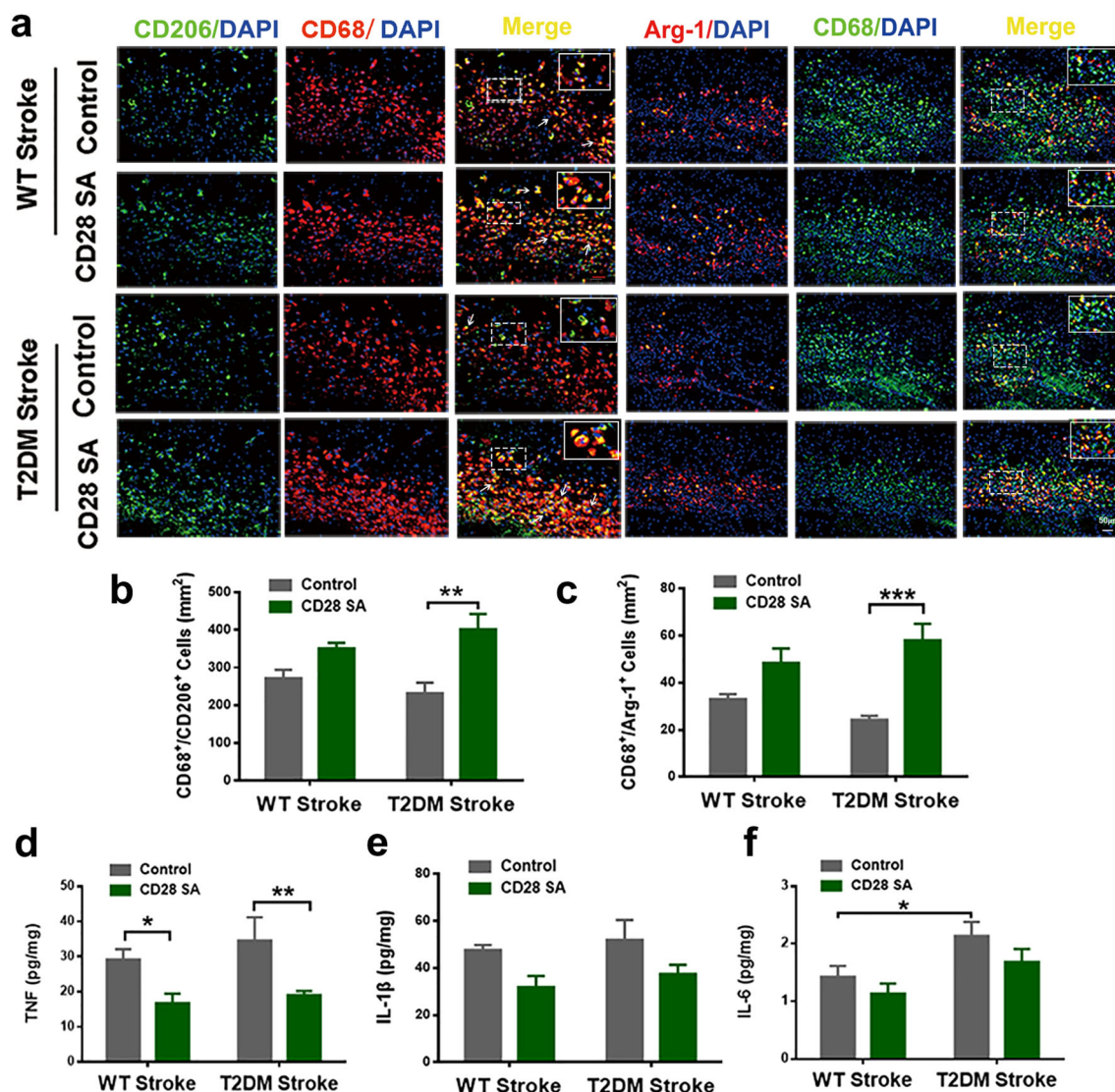
### Evaluating the Therapeutic Efficacy of CD28SA Using IRD- $\alpha$ CD206 Probe

To evaluate the therapeutic efficacy of CD28SA, we acquired in vivo NIRF images on days 1, 3, and 7 post-stroke (Fig. 5a). Target-to-background ratio (TBR) did not differ significantly between CD28SA-treated and control mice on day 1 and 3 post-stroke (Fig. 5b, c). On day 7, TBR values in both T2DM and WT mice treated with CD28SA were significantly increased (Fig. 5d).

## Discussion

Immunity and inflammation are key elements of the pathobiology of ischemic stroke, and inflammatory signaling is involved in all stages of the ischemic cascade [22]. Brain-resident and circulating pro-inflammatory cells and cytokines accumulate in the ischemic region, leading to inflammatory injury [23]. Down-regulation of neuro-inflammatory signaling and systemic inflammation is necessary for brain tissue repair and remodeling to occur [24, 25].

T2DM is a chronic, systemic, low-grade inflammatory disease associated with altered immune functions and levels of circulating inflammatory factors [6]. It is a well-known, independent risk factor for stroke that predicts poor prognosis in ischemic stroke patients [26]. The levels of pro-inflammatory agents, including IL-1 $\beta$ , IL-6, and monocyte chemoattractant protein-1, are elevated in diabetic patients compared with non-diabetic controls [7]. After ischemic stroke, the expression of pro-inflammatory cytokines in the brain is significantly higher in diabetic mice than in WT mice [7]. In addition, diabetic mice exhibit dysregulation of inflammatory responses and impaired ability to curtail inflammation [6].



**Fig. 3** Expression of M2 phenotype microglia/macrophages was increased in CD28SA-treated mice. Inflammatory cytokines were reduced by CD28SA administration. **a** Representative triple staining of CD206, CD68, and DAPI (4'6-diamidino-2-phenylindole) and triple staining of Arg-1, CD68, and DAPI of the ischemic brain on day 7 post-stroke. **b** Quantitative analysis of the number of CD206<sup>+</sup>/CD68<sup>+</sup> positive cells in

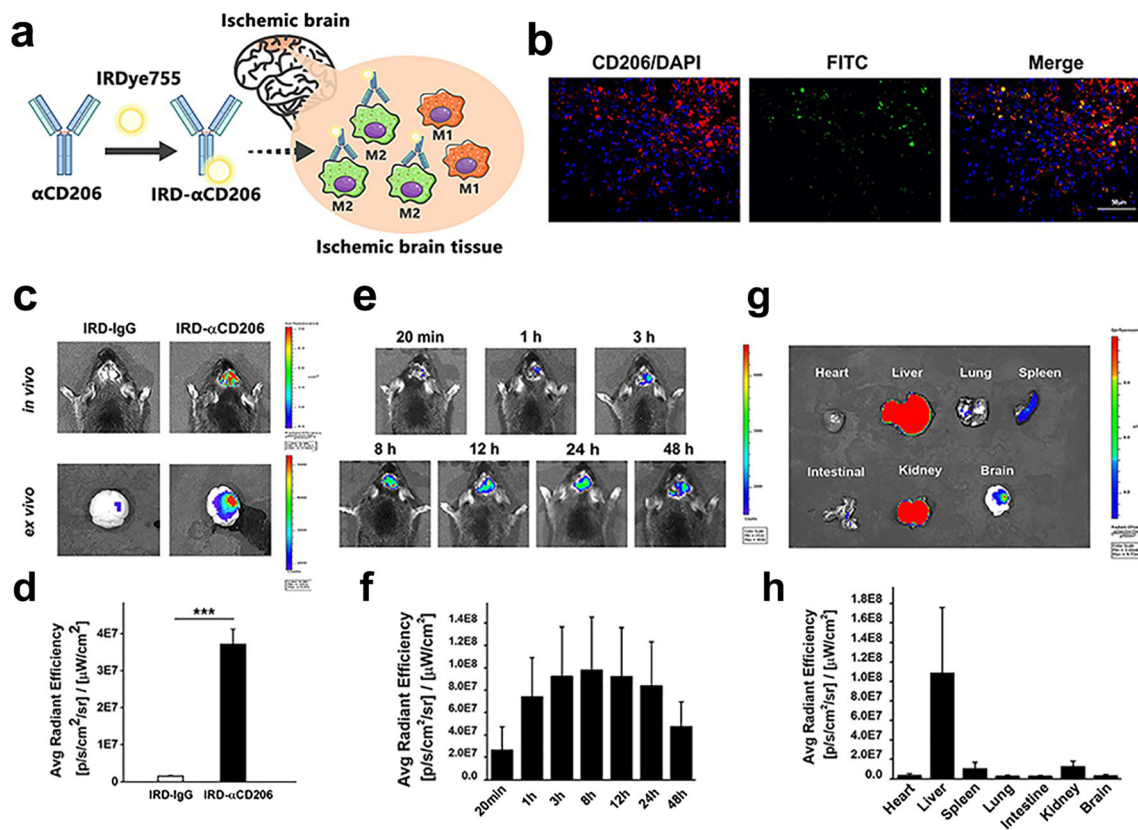
sections from each group ( $n = 3-4$ ,  $**P < 0.01$ ). **c** Quantitative analysis of the number of Arg-1<sup>+</sup>/CD68<sup>+</sup> positive cells in sections from each group ( $n = 4-5$ ,  $***P < 0.001$ ). **d-f** Enzyme-linked immunosorbent assays of the expressions of inflammatory factors: TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in the ischemic lesion ( $n = 4-6$ ,  $*P < 0.05$ ,  $**P < 0.01$ ). All data are presented as the mean  $\pm$  SEM

Tregs are essential for maintaining peripheral tolerance and limiting chronic inflammation. They can suppress acute inflammatory responses by inhibiting cytokines and cytotoxicity [27] and are therapeutic targets for ischemic stroke via multiple treatment approaches (e.g., adoptive cell transfer and CD28SA administration) [28]. It was recently revealed that in metabolic disorders, Tregs accumulate in adipose tissues to protect against obesity-related inflammation and insulin resistance [7]. In this study, we determined the numbers of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs in peripheral blood of stroke mice with and without T2DM. Additionally, we estimated the level of circulating Tregs in T2DM and non-diabetic stroke patients. In both experiments, the level of Tregs was significantly lower

in T2DM than in non-diabetic subjects at 24 h post-stroke, highlighting the relevance of Tregs and T2DM in ischemic stroke.

Recent studies have reported that macrophages/microglia are important in tissue and vascular remodeling after ischemic stroke [29]. Activated macrophages/microglia are classified into classic (M1-like, pro-inflammatory) and alternative (M2-like, anti-inflammatory) phenotypes. M2 macrophage/microglial polarization is associated with decreased neuro-inflammation and may promote axonal outgrowth and angiogenesis following ischemic stroke [11, 30]. After focal cerebral ischemia, the infiltrating macrophages/microglia assume the M2 phenotype and downregulate the expression of inflammatory factors,





**Fig. 4** Characterization of the IRD- $\alpha$ CD206 probe. **a** The chemical structure of IRD- $\alpha$ CD206. **b** Immunofluorescence images of CD206 (red) overlapping with FITC- $\alpha$ CD206 probe (green), suggesting that probe was targeting CD206 positive cells. **c** In vivo NIRF images and **d** signal quantitation of IRD-IgG and IRD- $\alpha$ CD206 at 8 h after probe injection ( $n = 3$ ,  $***P < 0.001$ ). **e** Representative NIRF images and **f** quantitative

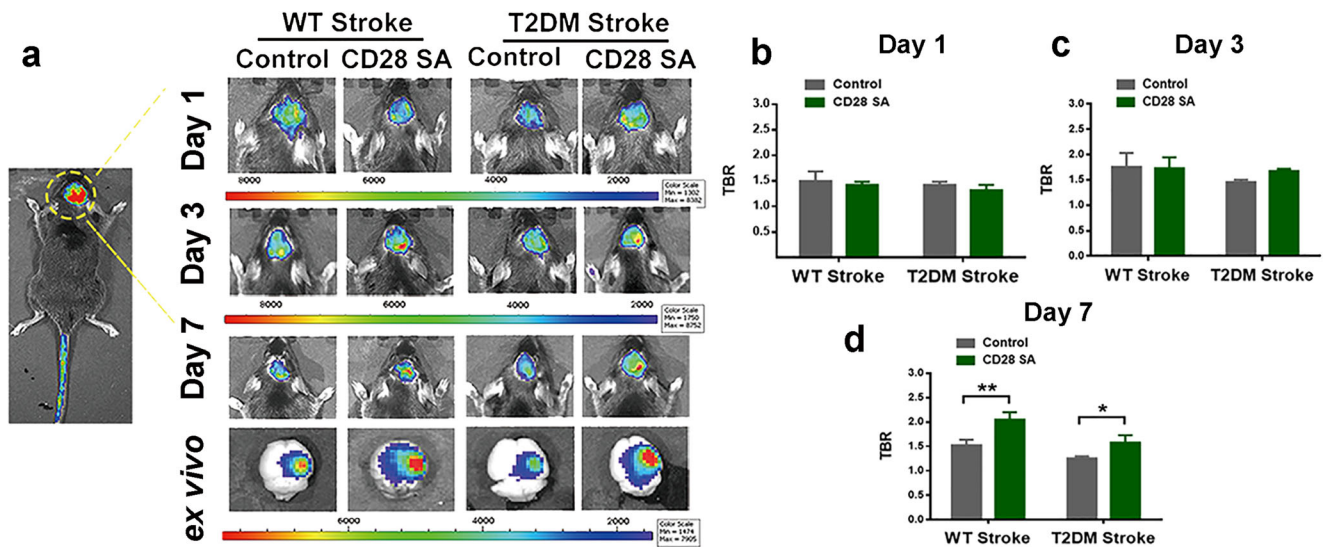
analysis of average radiant efficiency at 20 min, 1 h, 3 h, 8 h, 12 h, 24 h, and 48 h after IRD- $\alpha$ CD206 injection. **g** Bio-distribution of IRD- $\alpha$ CD206 in ex vivo organs dissected from mice sacrificed 8 h after probe injection on day 7 post-stroke. **h** Quantitative analysis of the average radiant efficiency of different organs on NIRF images.  $n = 3$ –5 per group. All data are presented as the mean  $\pm$  SD

which improves patient recovery and outcome. Tiemessen et al. found that CD4+CD25+ Tregs inhibit the pro-inflammatory responses of human monocytes/macrophages to lipopolysaccharides and induce characteristics typical of alternative activation in monocytes/macrophages [31]. In our study, the levels of pro-inflammatory cytokines released by the microglial fraction (IL-1 $\beta$ , TNF- $\alpha$ , and IL-6) were reduced, while the expression of M2 markers (CD206 and Arg-1) was significantly increased in CD28SA-treated T2DM mice. In addition, CD28SA treatment was associated with an increased number of circulating Tregs in both T2DM and WT mice on day 3 post-stroke. CD28SA-treated T2DM mice also showed smaller infarct volumes and improved functional recovery following ischemic brain injury. Taken together, these results suggest that boosting endogenous Tregs with CD28SA can promote M2 macrophage/microglia polarization post-stroke, alleviate inflammatory response, and promote recovery in diabetic ischemic stroke.

Multiple studies have demonstrated that CD28SA application effectively expands peripheral and brain Tregs in healthy mice and disease models [32–36]. Na et al. [13], for example, found that CD28SA treatment increased the number of Tregs in

the spleen after permanent middle cerebral artery occlusion. Moreover, the augmented accumulation of Tregs in the brain was confirmed by flow cytometry [13]. However, the effect of Treg-boosting therapy in stroke remains uncertain. While most studies, as the present one, reported improvements, a few noted increased infarct volumes and poor recovery [14]. Therefore, there is an urgent need for biomarkers and imaging methods that can dynamically monitor the effect of Tregs after stroke. A variety of imaging techniques have been explored to non-invasively assess the molecular events that follow ischemic stroke. Positron emission tomography, MRI, and NIRF have been used in animal studies. Among these techniques, NIRF imaging is the preferred choice for small animal models due to its low cost, simple operation, high sensitivity, and easy data analysis. Given Tregs' ability to modulate M2 macrophages/microglia and the latter's role in the repair and regeneration of ischemic brain tissue, we utilized a CD206-targeted imaging probe to monitor M2 macrophages/microglia as prognostic biomarkers and dynamically monitor the effect of Tregs in vivo. CD206 (mannose receptor) is a cell surface marker for M2 microglia/macrophages [37]. Our probe, which is comprised of monoclonal anti-CD206 antibody conjugated to a near-





**Fig. 5** Evaluating the therapeutic efficacy of CD28SA using the IRD- $\alpha$ CD206 probe. **a** Representative NIRF images of ischemic brain tissue of T2DM and WT mice acquired on day 1, 3, and 7 post-stroke, 8 h after IRD- $\alpha$ CD206 injection. **b–d** Representative quantification of target-to-

background ratio (TBR) values of ischemic brain tissue on day 1, 3, 7 post-stroke.  $n = 3–5$  per group, \* $P < 0.05$ , \*\* $P < 0.01$ . All data are presented as the mean  $\pm$  SEM

infrared phthalocyanine dye, enabled us to visualize the dynamic changes in M2 microglia/macrophage expression in vivo. Mice treated with CD28SA showed significantly increased IRD- $\alpha$ CD206-related signal intensity on in vivo NIRF imaging. To the best of our knowledge, this is the first study to report a decreased level of circulating Tregs following T2DM ischemic stroke and to dynamically monitor the efficacy of Treg-boosting therapy in vivo. Our results show that the IRD- $\alpha$ CD206 probe can be used to non-invasively monitor CD206+ M2 macrophage/microglia polarization, which represents a promising strategy to evaluate the therapeutic efficacy of endogenous Treg-boosting treatment.

There are several limitations to this study. CD206+ M2 microglia/macrophages were not depleted prior to CD28SA treatment to ensure the relationship between boosted Tregs and increased CD206+ M2 microglia/macrophages. In future studies, we hope to deplete CD206+ microphage/macrophages in vivo and then study the effect of CD28SA treatment. Second, because of its high molecular weight (~150 KDa), the IRD- $\alpha$ CD206 probe has a long circulating half-life leading to a high background accumulation in vivo, which might limit its use. Third, the sample size of ischemic stroke patients recruited in this study is small; larger human peripheral blood samples should be collected to confirm our results.

In conclusion, our results show that the number of Tregs in peripheral blood of mice and humans was lower in diabetic stroke compared with non-diabetic stroke. Using CD28SA to boost, the endogenous Treg population post-stroke promoted CD206+ M2 macrophage/microglia polarization and improved the prognosis. In addition, the present study provided

a novel NIRF-imaging probe for non-invasive and dynamic visualization of CD206+ M2 macrophage/microglia polarization in vivo after ischemic stroke. Using this probe, we successfully demonstrated that endogenous Treg boosting was capable of modulating CD206+ M2 macrophage/microglia polarization after ischemic stroke. Taken together, our study revealed a novel regulatory mechanism of T2DM stroke brain damage and provided an in vivo monitoring technology with potential in therapeutic efficacy evaluation.

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### Compliance with Ethical Standards

The animal experiments were approved by the Institutional Animal Use and Care Committee (IAUCC) of the Medical School of Southeast University (Nanjing, Jiangsu, China; approval ID: SYXK-20130413275). All experiments were performed and reported according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines [15]. Human study was approved by the Independent Ethics Committee for Clinical Research of Zhongda Hospital, Southeast University as per the World Medical Association outlined in the Declaration of Helsinki (No. 2013ZDSYLL094.0).

**Conflict of Interest** The authors declare that they have no competing interests.

**Ethical Approval** We followed all institutional and national guidelines for the care and use of laboratory animals and the study of human subjects.

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