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The role of Interleukin-33 in the modulation of splenic T-cell immune responses after experimental ischemic stroke

Wei Xiao[a,1], Shuang Guo[b,1], Lin Chen[c,d], Yi Luo[b,*]

[a] Department of Nephrology, The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430014, China
[b] Department of Laboratory Medicine, Zhongnan Hospital of Wuhan University, Wuhan 430071, China
[c] School of Physical Education, Jianghan University, Wuhan 430056, China
[d] Wuhan Institutes of Biomedical Sciences, Jianghan University, Wuhan 430056, China

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ABSTRACT

The splenic T-cell immune response to stroke has been identified as an important role in the progression of brain injury following ischemic stroke. Interleukin (IL)-33 as a novel cytokine of IL-1 family has been found to be protective for ischemic brain injury. Here, we determined the contribution of IL-33 to the T-cell immune responses in the spleen after experimental ischemic stroke. Mice were subjected to 30 min of middle cerebral artery occlusion (MCAO) for ischemic stroke induction. Recombinant mouse IL-33 (100 μg/kg) was pre-treated intraperitoneally at 30 min prior to MCAO, then the percentages of T cell subsets, related cytokines and transcription factors in the spleen tissues were measured. Intraperitoneal IL-33 pre-treatment may attenuate neurological deficit scores and infarct volumes after MCAO, which was accompanied by reduced IFN-γ^+ T cells and increased Foxp3$^+$ T cells in the spleen tissues. Meanwhile, IL-33 pre-treatment could decrease the production of IFN-γ and increase the secretion of IL-4, IL-10 and TGF-β from the spleen at 24 h after MCAO. Additionally, the mRNA level of the transcription factor T-bet was downregulated by IL-33, and the levels of GATA-3 and Foxp3 mRNA were upregulated. These results showed that the long-term protective mechanism of IL-33 in ischemic stroke may be partly associated to its modulation role for splenic T-cell immune responses through inhibiting Th1 response and promoting Treg response, suggesting that IL-33 may be a candidate treatment for human stroke via modulating the peripheral immune system following stroke.

1. Introduction

Stroke, a cerebrovascular injury, is the leading cause of disability and death in the world. The most common type of stroke is ischemic stroke, which causes cerebral ischemia with activation of an inflammatory response leading to the exacerbation of primary brain injury. Recent studies have shown that inhibiting the inflammatory response after stroke onset enhances neurosurvival and limits the expansion of infarction (Jin et al., 2010; Lakhan et al., 2009; Nighoghossian et al., 2007). It has been recognized gradually that peripheral immune cell activation is involved in ischemic expansion (An et al., 2014; Stevens et al., 2002). The spleen is a major reservoir of blood cells and the key lymph organ, which can release immune cells into the circulation and central nervous system (CNS). Its important contribution to ischemic brain damage has gained considerable attention (Leonardo and Pennypacker, 2011; Pennypacker and Offner, 2015; Seifert and Offner, 2018). Further investigation into the splenic reaction in stroke patients would provide insight into how the peripheral immune system can be modulated following stroke to improve neurological outcomes. In the animal model of middle cerebral artery occlusion (MCAO), the reduction in spleen size is correlated with the extent of ischemic damage (Ajmo Jr. et al., 2008; Seifert et al., 2012a). The recent clinical studies have also shown similar morphological changes of spleen tissues in stroke patients, which may be associated with the release and infiltration of splenocytes into the CNS (Liu et al., 2015; Sahota et al., 2013). Splenectomy has been shown to reduce neural injury in experimental ischemic stroke (Seifert et al., 2012b; Zhang et al., 2013). However, another study demonstrated that removal of the spleen after MCAO does not reduce the brain infarct (Ran et al., 2018). Additionally, the focal cerebral ischemia could alter the cytokine and immune cell profiles in the spleen (Seifert and Pennypacker, 2014). These issues probably reflect the complexity of splenic functions

* Corresponding author at: The Department of Laboratory Medicine of Zhongnan Hospital of Wuhan University, Donghu Road, No.169, Wuhan, Hubei Province, China.
E-mail address: luoyi929@aliyun.com (Y. Luo).
[1] These authors contributed equally to this work.

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following stroke. T lymphocytes and their subsets play a critical role in ischemia/reperfusion injury (Arumugam et al., 2005; Brait et al., 2012). Splenic leukocytes such as various subsets of T cells may participate in the systemic inflammatory response to stroke and contribute to neurodegeneration (Ajmo Jr. et al., 2008; Leonardo and Pennypacker, 2011; Pennypacker and Offner, 2015). The protective effects of splenectomy on stroke are accompanied by the reductions in the numbers of T cells in the ischemic brain (Seifert et al., 2012b; Zhang et al., 2013). Nevertheless, the recent studies have found that the number of regulatory T (Treg) cells in the spleen were increased after stroke, which may exert the beneficial roles in cerebral ischemia (Offner et al., 2006). Moreover, splenic signaling occurring through Th1-type cytokine interferon gamma (IFN-γ) may be a significant factor in stroke pathology (Seifert et al., 2012b; Yilmaz et al., 2006). Therefore, further understanding splenic immune responses by T cell subsets after stroke may be useful to elucidate the progression of ischemic brain damage and identify the potential target for stroke treatment.

Interleukin (IL)-33, a novel member of the IL-1 cytokine family, has pleiotropic effects on regulating immune responses (Schmitz et al., 2005). As a traditional cytokine, IL-33 can promote the pathogenesis of Th2-related diseases by interacting with its receptor ST2 (Cevikbas and Steinhoff, 2012; Lloyd, 2010; Pushparaj et al., 2009). However, IL-33 has shown the protective effects in several cardiovascular diseases and Th1-mediated inflammatory diseases through modulating the Th1/Th2 balance (Chen et al., 2012; Miller et al., 2008). IL-33 can also function as an intracellular nuclear factor regulating gene transcription or an 'alarmin' to alert tissue damage and stress (Haraldsen et al., 2009). Moreover, the recent studies have found that IL-33 can regulate Th17 response and promote the function of Treg cells in some inflammatory-mediated diseases (Jiang et al., 2012; Schiering et al., 2014). Interestingly, IL-33 is highly expressed in the brain and spinal cord of humans and rodents, indicating its CNS-specific functions (Schmitz et al., 2005; Yasuoka et al., 2011). A study in the model of acute contusion spinal cord injury showed that IL-33 administration can improve the functional recovery after CNS trauma via central and peripheral mechanisms (Pomeshchik et al., 2015). In our previous study, we found that IL-33 may ameliorate ischemic brain injury in experimental stroke through promoting Th2 response and suppressing Th17 response (Luo et al., 2015). Then several other studies also demonstrated the protective immunomodulation of IL-33 in stroke (Korhonen et al., 2015; Luo et al., 2018a; Yang et al., 2017). Except for the neuroprotective effect, the role of IL-33 in the immune modulation of peripheral immune system after stroke may be worth exploring and remains unclear. Based on the significance of spleen in post-stroke inflammation, in this study, we used a mouse MCAO model to determine the effect of intraperitoneal pre-treatment with recombinant mouse IL-33 on the splenic T cell subsets and related cytokines, aiming to further identify the role of IL-33 in the etiology and mechanisms of ischemic stroke.

2. Materials and methods

2.1. Animals

For all experiments, male C57BL/6 mice (8–10 weeks old; body weight 22–26 g) purchased from Wuhan University Center for Animal Experiment (Wuhan, China) were used. All animals were housed in a room with a 12-h light/dark cycle with access to standard laboratory food and filtered clean water ad libitum. The Research Animal Resources and Care Committee of Zhongnan Hospital of Wuhan University approved all animal procedures.

2.2. Recombinant mouse IL-33

Mouse IL-33 was expressed in *Escherichia coli* (BL21 strain) and IL-33 proteins were purified by glutathione affinity chromatography (TransGen Biotech, Beijing, China) as described previously (Luo et al., 2015). Endotoxin was removed by purification with polymyxin B column (GenScript, Piscataway, USA). The purity of IL-33 was > 95% and the endotoxin levels were < 0.1 unit/μg of protein.

2.3. IL-33 treatment

Mice were randomly divided into the IL-33-treated group and PBS-treated group (vehicle group). For dose-response studies, recombinant mouse IL-33 was administered intraperitoneally (i.p.) in doses of 10, 50, 100 and 200 μg/kg (n = 8 in each group) at 30 min before MCAO operation. For the evaluation of time-response, IL-33 groups were administered with IL-33 (100 μg/kg, i.p.) a pre-treatment (at 30 min before MCAO, n = 8) or a post-treatment (just after, and 30 min after reperfusion, n = 8 at each time point). Neurological deficits and the infarct volume were determined at 24 h after MCAO.

2.4. Focal cerebral ischemia mouse model

We used the filament middle cerebral artery occlusion (MCAO) to induce focal ischemia, as previously described (Engel et al., 2011; Luo et al., 2018b; Luo et al., 2015). In brief, animals were anesthetized with 10% chloral hydrate (0.3 mL/100 g) and the right carotid region was exposed. The MCAO was established by inserting a 0.16 mm diameter nylon filament (tip diameter 0.20; Cinontech, Beijing, China) from the right common carotid artery (CCA) to the origin of MCA. After 30 min, reperfusion was established by withdrawal of the filament. Sham control animals were subjected to similar operations without MCA occlusion. During the surgery, the body temperature of mice was kept at 37 ± 0.5 °C with a heating lamp. After the operation, mice were maintained in the same conditions as the preoperative environment until further experiments. The MCAO mice with IL-33 (100 μg/kg) pre-treatment were assigned to four subgroups according to the time of sacrifice (24 h, 48 h, 72 h and 96 h after surgery).

2.5. Behavioral assessment

Neurological impairment was evaluated by using a 5-point neurological deficit score (0, no observable neurological deficits; 1, failed to extend left forepaw; 2, circled to the left; 3, fell to the left; and 4, could not walk spontaneously) with a blinded fashion (Longa et al., 1989; Luo et al., 2015).

2.6. Infarct volume measurement

The infarct volume was measured at 24 h, 48 h, 72 h and 96 h after induction of MCAO. Under brief anesthesia with 10% chloral hydrate, the forebrains of mice were removed and four coronal sections (2 mm-thick) were obtained. Then the coronal slices were stained with 1% 2,3,5-triphenyltetrazolium chloride (TTC, Biosharp, USA) at 37 °C for 20 min followed by immersion in 10% formaldehyde. The stained sections were captured as digital images and analyzed using Image-J software (National Institutes of Health, Bethesda, USA). The infarct volume (%) was quantified using the following formula: (contralateral hemispheric volume – ipsilateral hemispheric non-infarcted volume) /contralateral hemispheric volume (Swanson et al., 1990).

2.7. Flow cytometry analysis (FACS)

Spleens were excised, individually homogenized and then isolated using Ficoll-Hypaque density gradient centrifugation (EZ-Sep Mouse 1× lymphocyte separation medium, Dakota Biotechnology Co., Ltd., Fargo, USA) to enrich leukocytes. Single cell suspensions from the spleen tissue were scrubbed through 40 μm nylon cell strainers. To examine the leukocyte phenotype in the spleen, the cells were stained with the following anti-mouse antibodies (eBioscience, San Diego, USA): anti-CD3e-PE and anti-CD3e-PE-Cy7 (clone 145-2C11), anti-CD4-

FITC (clone GK 1.5), anti-IFN-γ-PE-Cy7 (clone XMG 1.2), anti-IL-4-PE-Cy7 (clone BVD6–24G2), anti-IL-17A-PE (clone eBio17B7) and anti-Foxp3-PE (clone FJK-16 s). For intracellular staining, cells were stimulated for 6 h with 1 μg/ml ionomycin (Sigma-Aldrich, St. Louis, USA) and 50 ng/ml phorbol myristate acetate (PMA, Sigma-Aldrich) in the presence of Brefeldin A (Golgistop, eBioscience). After staining with antibodies against surface markers, cells were permeabilized with Fix/Perm buffer (eBioscience) and finally incubated with antibodies against intracellular cytokines. Cells were analyzed on a FACS LSRIIsystem (BD Biosciences, San Jose, CA). To determine the absolute number of leukocytes in the spleen, a leukocyte gate was first defined for these cells based on forward and side scatter characteristics. The percentage of $CD3^+CD4^+$ cells within this gate was then used to calculate Th cells in the spleen. Intracellular cytokine staining was used to examine Th cell differentiation. IFN-γ has been used as the signature Th1 cytokine, and IL-4 is the most commonly studied Th2 cytokine. IL-17 and Foxp3 staining was typically used to examine Th17 and Treg subtype, respectively. All gates and quadrants were established with the use of appropriate isotype controls.

2.8. RNA isolation and quantitative real-time PCR analysis

Total RNA was extracted from spleen tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA) at 24 h after MCAO, and 5 μg of total RNA from each sample was reverse-transcribed into the first strand cDNA using PrimeScript™ RT reagent kit (Takara, Tokyo, Japan). To determine the expression of T-bet, GATA-3, RORγt and Foxp3 mRNA, relative quantitative real-time polymerase chain reaction (RT-PCR) was carried out using SYBR Green PCR Mix (Thermo Fisher Scientific, Waltham, MA) on the Bio-Rad CFX96 Real-Time PCR System (Bio-Rad, Hercules, CA). The thermal cycling conditions were: 1 cycle at 94 °C for 4 min; 35 cycles at 94 °C for 1 min and 58 °C for 40 s. The relative mRNA expression levels on mouse T-bet, GATA-3, RORγt and Foxp3 were calculated using the $2^{-\Delta\Delta CT}$method afternormalization with the reference GAPDH mRNA level in the same sample. All primers used are listed in Table 1.

2.9. Measurement of cytokines in the supernatants of spleen cell cultures

Splenic mononuclear cells were prepared according to the method described above. Then the cells (1×10^6/L) were cultured in RPMI 1640 medium containing 10% fetal bovine serum with 5 mg/L concanavalin A (ConA, Sigma-Aldrich, St. Louis, USA), and incubated at 37 °C for 48 h. We measured the levels of IFN-γ, IL-4, IL-17, IL-10 and TGF-β in the culture supernatants of spleen cells using commercial ELISA kits following the manufacturers' experimental protocols (eBioscience, San Diego, CA).

Table 1
Sequences of primers for real-time PCR.

Gene	Sequences
T-bet	
Forward	5′-CCTCGCACCTGGAGCTGGCTG-3′
Reverse	5′-TTATCAGTTGGGAAAATAGTTA-3′
GATA-3	
Forward	5′-GAATGCCAATGCGGACCCTG-3′
Reverse	5′-CTAACCCATGGCGGTGACCA-3′
RORγt	
Forward	5′-AGTGTAATGTGGCCTACTCCT-3′
Reverse	5′-GCTGCTGTTGCAGTTGTTTCT-3′
Foxp3	
Forward	5′-CAGCTGCCTACAGTGCCCCTAG-3′
Reverse	5′-CATTTGCCAGCAGTGGGTAG-3′
GAPDH	
Forward	5′-TGATGACAGAAGGTGGTGAAG-3′
Reverse	5′ TCCTTGGAGGCCATGTAGGCCAT-3′

2.10. Statistical analysis

Statistical analysis was performed by SPSS 19.0 software (SPSS Inc., Chicago, USA). All data were presented as mean ± SEM. Comparison between two groups was statistically evaluated using the Student's *t*-test. Multiple group comparisons were analyzed by a one-way ANOVA Student-Newman-Keuls test. $P < .05$ was considered significant.

3. Results

3.1. Splenic T-cell responses are altered following transient cerebral ischemia

First we observed changes in the spleen size and weight at 24 h after MCAO to confirm the effect of focal cerebral ischemia on the splenic T-cell immune responses. The spleens were isolated and weighted, and spleen weight index was calculated as organ weight (milligram, mg) per gram (g) of mouse body weight. Compared to the sham group, spleen atrophy occurred and the spleen weight index was lower in the MCAO 24 h mice (Fig. 1A). Then the single cell suspensions from the spleen at MCAO 24 h group and sham group were prepared, stained for cell surface and intracellular markers for FACS. There was no obvious difference in the total percentage of $CD3^+CD4^+$ cells within this gate between sham group and MCAO 24 h group (Fig. 1B). Further we measured the percentages of IFN-γ^+ cells, IL-4^+ cells, IL-17^+ cells and $Foxp3^+$ cells after stimulated with ionomycin, PMA and Brefeldin A, which represented that the splenocytes were stimulated to transform into the amounts of T helper 1 (Th1), Th2, Th17 and Treg cells respectively. Compared with the sham group, the frequency of IFN-γ^+ and IL-17^+ cells in the spleen was increased at24 h after MCAO, while no obvious changes in the frequency of IL-4^+ and $Foxp3^+$ cells could be observed (Fig. 1C). These results showed a transient decrease in spleen size following stroke corresponding to the increased IFN-γ^+ and IL-17^+cells.The stimulated splenocytes in the spleen from MCAO mice may release more IFN-γ^+ and IL-17 as a proinflammatory signal into the circulation.

3.2. Intraperitoneal IL-33 injection also improves the functional outcome and infarct volume after focal ischemic stroke

In our previous study, the protective effect of IL-33 on ischemic stroke was found by intracerebroventricular injection (Luo et al., 2015). Here, we evaluated the optimal treatment dose and therapeutic window using recombinant mouse IL-33 intraperitoneally (i.p.) to determine its effect on the functional outcome and peripheral immune responses. Mice were administered either 0, 10, 50, 100 or 200 μg/kg of IL-33 at 30 min prior to MCAO, then the neurological scores and infarct volumes were tested at 24 h after MCAO. Compared to the vehicle-treated MCAO 24 h mice, all IL-33 doses from 10 to 200 μg/kg were effective in reducing infarct volume. The dose of 100 μg/kg for IL-33 was the most effective, which reduced infarct volume by 40% (Fig. 2A, B). However, the obvious improves in neurological deficit scores were observed only in the 50 and 100 μg/kg doses of IL-33-treated MCAO mice (Fig. 2C). In the following time-response study, IL-33 at 100 μg/kg was administered at 30 min before MCAO, immediately or 30 min after reperfusion. The result showed that the infarct volumes and the neurological deficits were both reduced significantly in MCAO 24 h mice treated with IL-33 at 30 min before MCAO and immediately after reperfusion, with protection at 30 min prior to MCAO being the greatest (Fig. 2D, E). Collectively, these data indicated that intraperitoneal IL-33 (100 μg/kg) pre-treatment may be an effective way to ameliorate ischemic brain injury after MCAO.

3.3. IL-33 modulates T-cell immune responses in the spleen after MCAO

We tested the percentages of splenic IFN-γ^+ cells, IL-4^+ cells, IL-

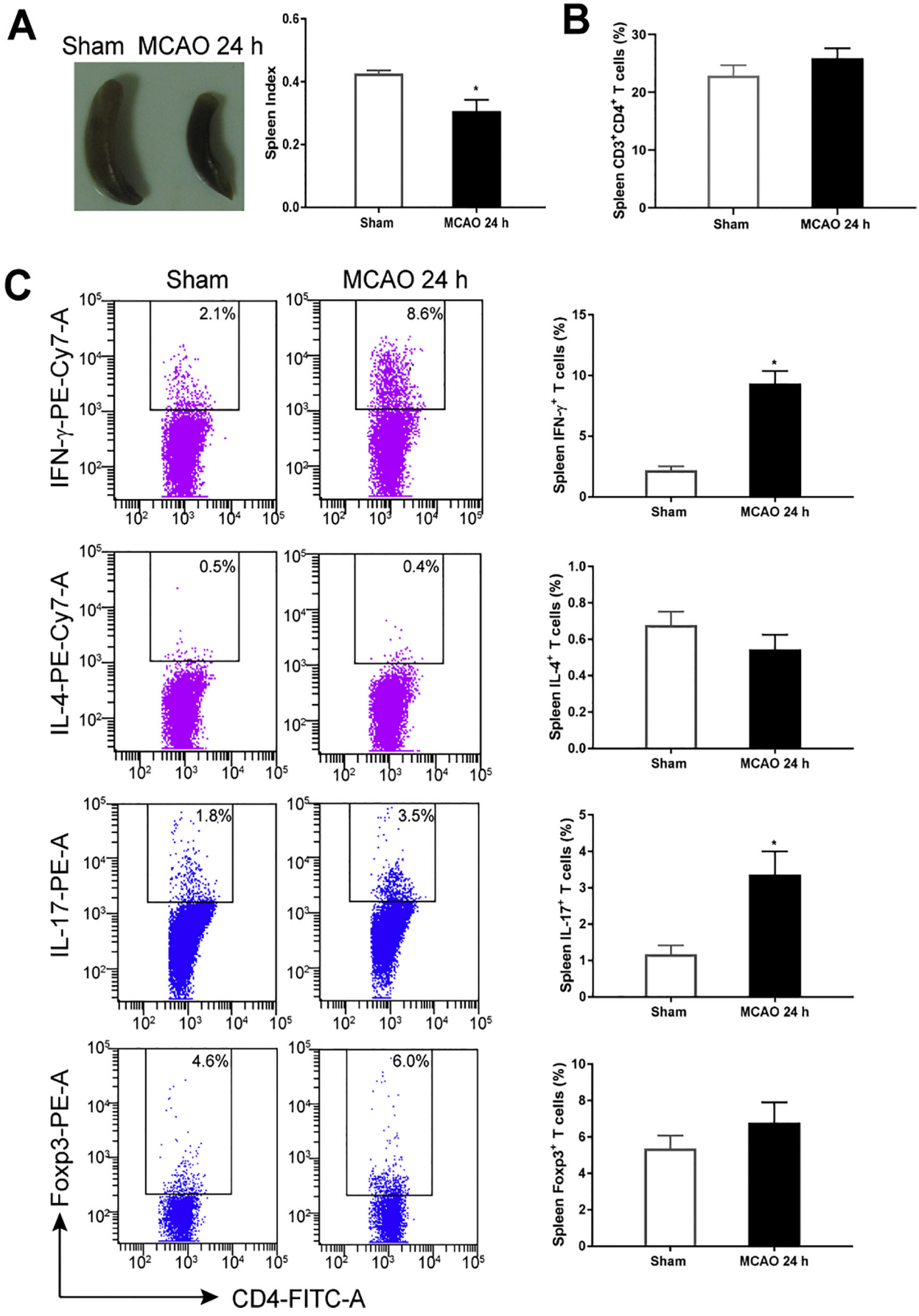

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Fig. 1. Focal cerebral ischemia affects the levels of splenic T cell subsets. (A) Representative image and quantification of spleen weight index show the spleen size from sham control mice and mice subjected to 30 min of middle cerebral artery occlusion (MCAO) followed by 24 h reperfusion (n = 6 mice per group). The spleen weight index was calculated as organ weight (milligram, mg) per gram (g) of mouse body weight. (B) Statistical analysis for the percentage of $CD3^+CD4^+$ cells in the spleen within this gate from sham group and MCAO 24 h group (n = 6 mice per group). (C) Representative FACS plots and statistical analysis for IFN-γ^+ T cells, IL-4^+ T cells, IL-17^+ T cells and $Foxp3^+$ T cells in the spleen from sham group and MCAO 24 h group (n = 6 mice per group). For intracellular staining, cells were stimulated with ionomycin, PMA and Brefeldin A. Data are shown as means ± SEM. *p < .05 compared with the sham group (paired Student's t-test).

17^+ cells and $Foxp3^+$ cells from MCAO 24 h mice pre-treated with IL-33 (100 μg/kg) at 30 min before occlusion to further determine the effect of IL-33 on the splenic T-cell immune responses. No obvious spleen atrophy occurred in the IL-33-treated MCAO 24 h mice, and the splenic index was larger than that in PBS-treated MCAO 24 h group (Fig. 3A). Compared to PBS-treated MCAO 24 h group, no significant difference in the percentage of $CD3^+CD4^+$ cells within this gate was found in IL-33-treated MCAO 24 h group (Fig. 3B). However, there was a significant decrease in the frequency of splenic IFN-γ^+ cells and an increase in the percentage of splenic $Foxp3^+$ cells from MCAO 24 h mice pre-treated with IL-33 (Fig. 3C). Nevertheless, there was no significant change in the amounts of splenic IL-4^+ and IL-17^+ cells between the IL-33-treated and PBS-treated MCAO 24 h mice (Fig. 3C). Thus, the stimulated splenocytes from MCAO 24 h mice pre-treated with IL-33 were capable of producing more $Foxp3^+$ cells, but the production of IFN-γ^+ cells was inhibited.

Also, we examined the cytokines secreted from spleen in MCAO 24 h mice pre-treated with IL-33. The levels of IFN-γ, IL-4, IL-17, IL-10 and TGF-β in the culture supernatants of spleen cells were analyzed by ELISA. Consistent with the cellular phenotype profiles observed in the spleen tissues from MCAO 24 h mice, the levels of IFN-γand IL-17 in the culture supernatants of spleen cells were significantly increased (Fig. 4). IL-33 pre-treatment may decrease the secretion of IFN-γand promote the secretion of IL-4, IL-10 and TGF-β from spleen cells at 24 h after MCAO (Fig. 4). However, no obvious difference in the level of IL-17 was observed between the IL-33-treated and PBS-treated MCAO 24 h mice (Fig. 4). Together, these data may suggest that IL-33 pre-treatment can down-regulate Th1 cell immune response and up-regulate Treg cell immune response in the spleen at 24 h after MCAO.

3.4. IL-33 regulates T cell-related transcription factors in the spleen after MCAO

The differentiation of T cells is determined by the induction of several key transcription factors: T-bet is important for the generation of Th1 cells, GATA-3 is an indispensable factor for Th2 cells, RORγt plays a critical role in the differentiation of Th17 cells, and Foxp3 plays an important role in Treg generation and function (Yu et al., 2015). To further understand the mechanism of IL-33 regulating splenic T-cell immune responses after MCAO, we examined the mRNA levels of the

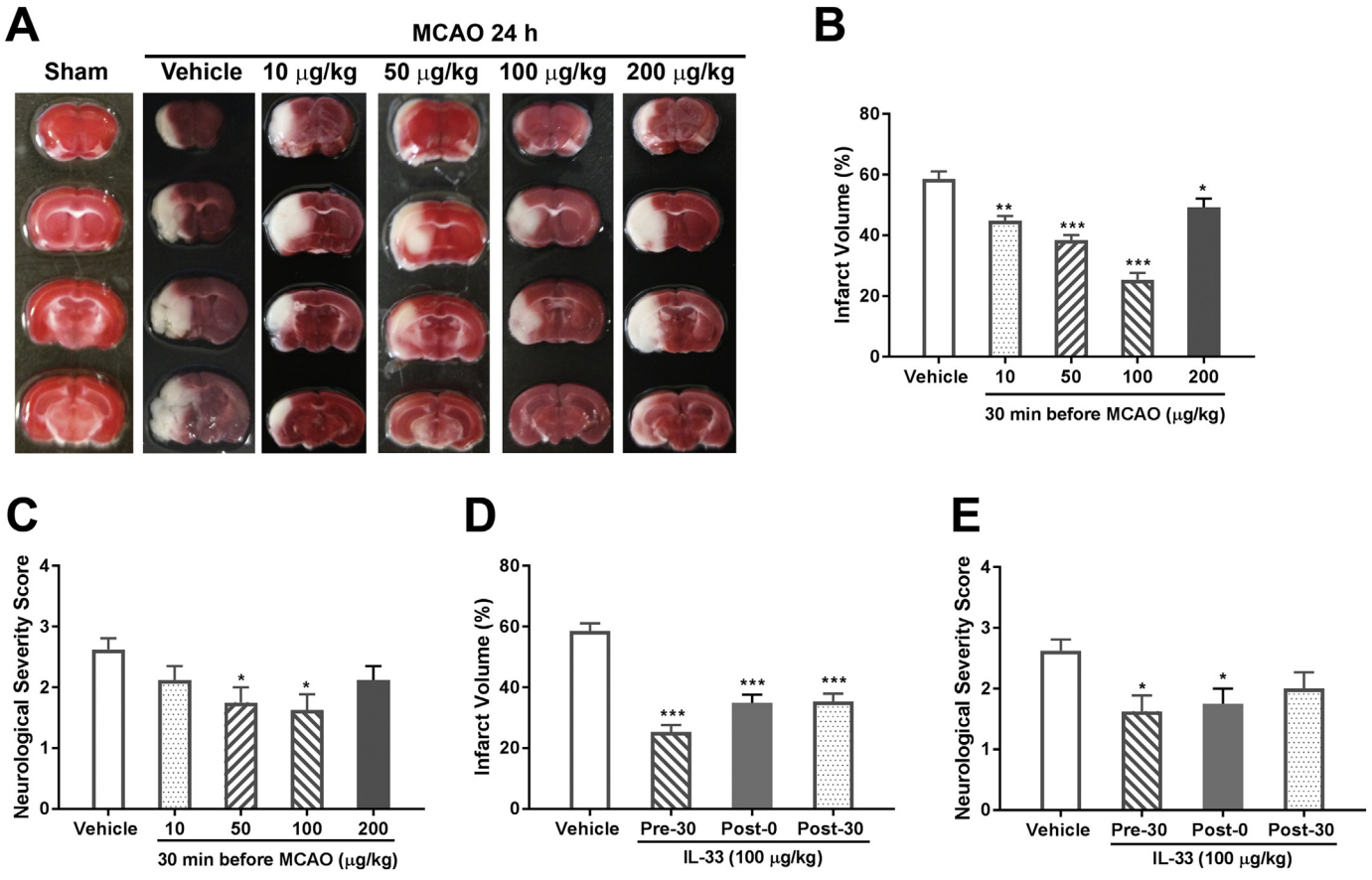

Fig. 2. Determination of the dose range and treatment window of efficacy for IL-33. (A) Examples of triphenyltetrazolium chloride (TTC) stained serial brain sections from sham control mice and MCAO 24 h mice treated with the indicated doses of recombinant mouse IL-33 (vehicle 0, 10, 50, 100 and 200 μg/kg), administered 30 min before MCAO (n = 8 mice per group). (B, C) Quantification of brain infarct volumes and neurological deficit scores in MCAO 24 h groups pre-treated with the different doses of IL-33. (D, E) Quantification of infarct volumes and neurological deficit scores in MCAO 24 h mice treated with IL-33 for 100 μg/kg at the indicated time points (n = 8 mice per group), including 30 min before MCAO (Pre-30), just after reperfusion (Post-0) and 30 min after reperfusion (Post-30). Data are shown as means ± SEM. *p < .05, **p < .01, ***p < .001 compared to the value for the corresponding vehicle-treated MCAO 24 h mice (paired Student's t-test).

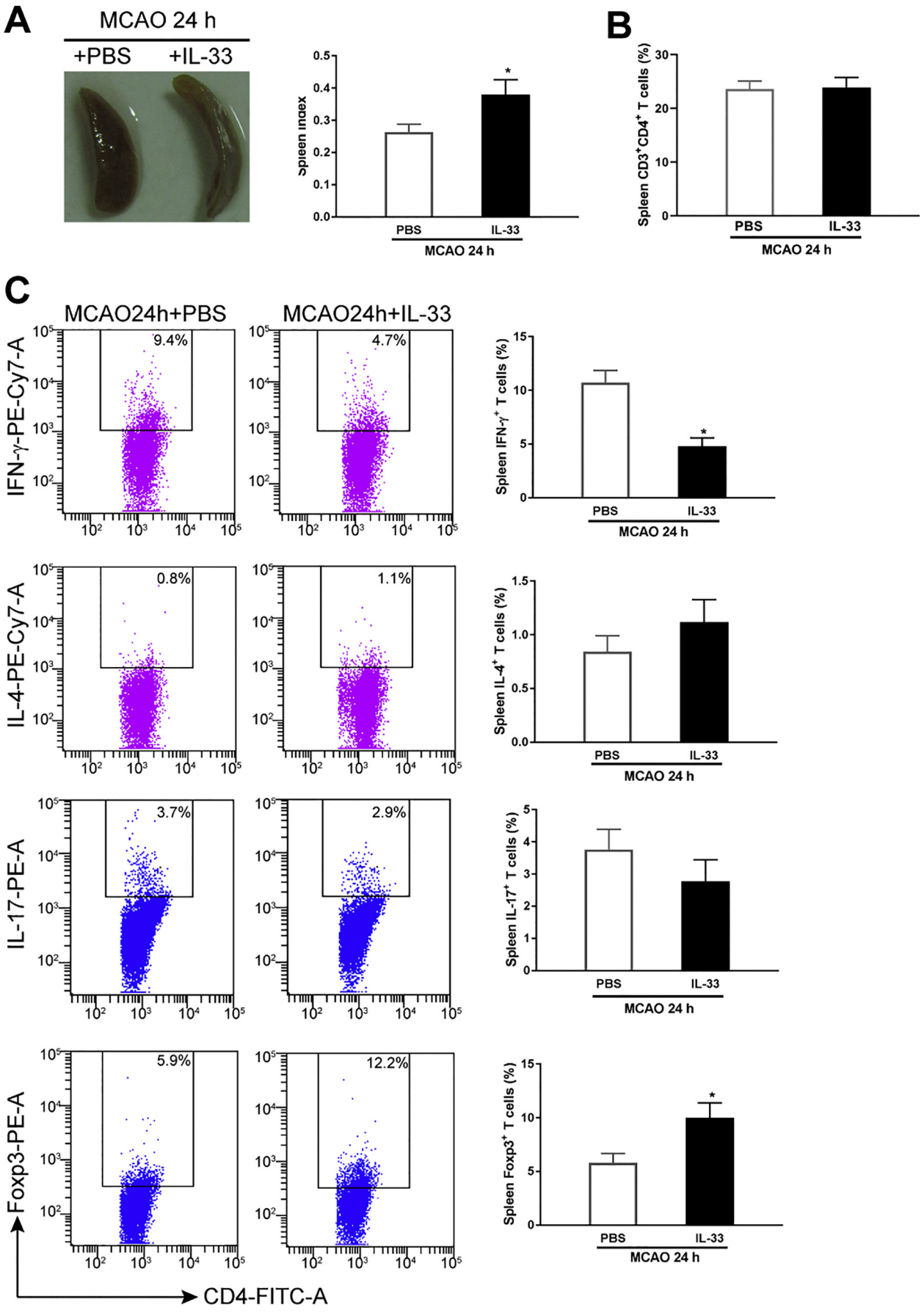

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Fig. 3. Recombinant mouse IL-33 pre-treatment reduces IFN-γ^+ T cells and increases Foxp3$^+$ T cells in the spleen at 24 h after MCAO. (A) Representative image and quantification of spleen weight index show the spleen size from PBS-treated MCAO 24 h mice and IL-33-treated MCAO 24 h mice (n = 8 mice per group). Recombinant mouse IL-33 (100 μg/kg) or PBS was administered 30 min before MCAO. (B) Statistical analysis for the percentage of CD3$^+$CD4$^+$ cells in the spleen within this gate from PBS-treated MCAO 24 h group and IL-33-treated MCAO 24 h group (n = 6 mice per group). (C) Representative FACS plots and statistical analysis for IFN-γ^+ T cells, IL-4$^+$ T cells, IL-17$^+$ T cells and Foxp3$^+$ T cells in the spleen from PBS-treated MCAO 24 h group and IL-33-treated MCAO 24 h group (n = 8 mice per group). Cells were stimulated with ionomycin, PMA and Brefeldin A for intracellular staining. Data are shown as means ± SEM. *$p < .05$ compared with the PBS-treated MCAO 24 h group (paired Student's *t*-test).

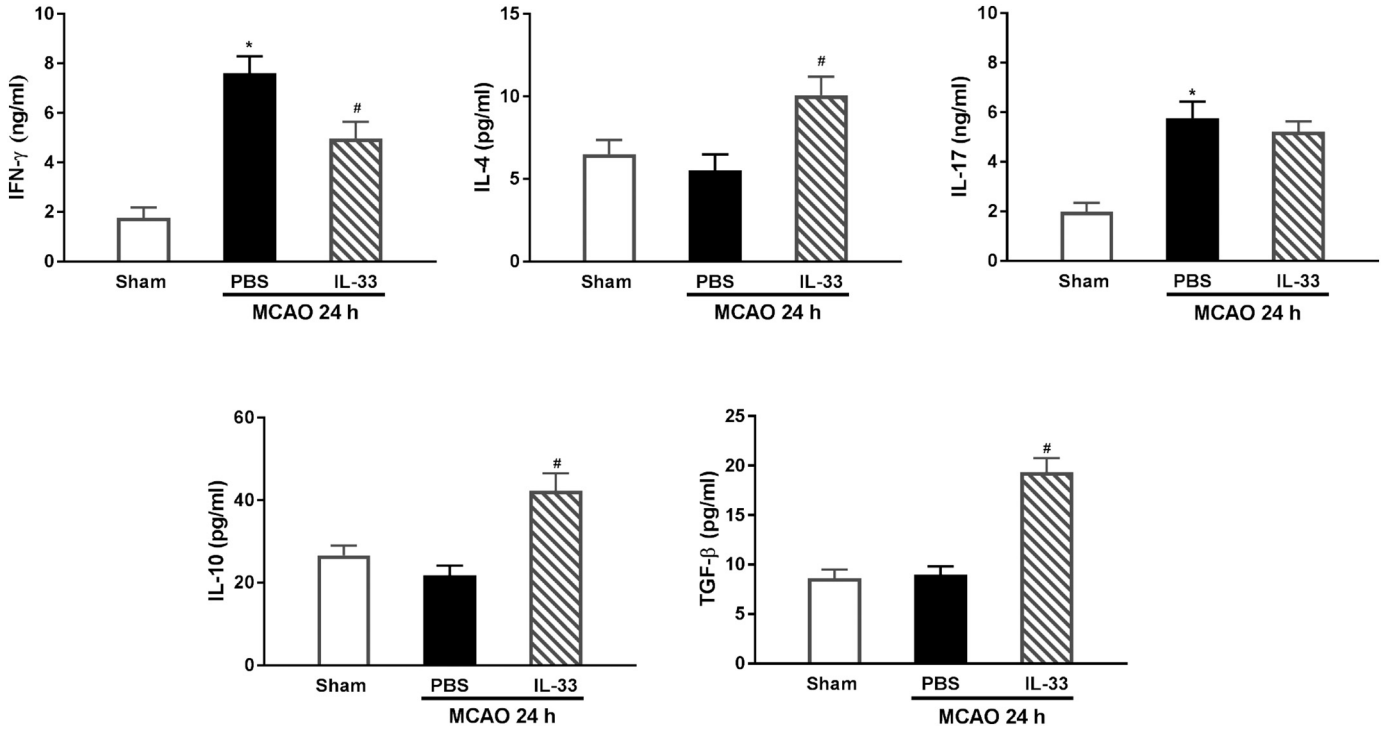


Fig. 4. Cytokine concentrations in the culture supernatant of spleen cells from MCAO 24 h mice pre-treated with IL-33. Spleens were collected from sham group, PBS and IL-33-treated MCAO 24 h group (n = 8 mice per group). Recombinant mouse IL-33 (100 μg/kg) or PBS was administered 30 min before MCAO. The concentrations of cytokines in the supernatant of spleen cells including IFN-γ, IL-4, IL-17, IL-10 and TGF-β were determined by ELISA separately. Data are shown as means ± SEM. *$p < .05$ compared with sham group, $^{\#}p < .05$ compared with the PBS-treated MCAO 24 h group (repeated measures ANOVA).

above transcription factors in the spleen from MCAO 24 h mice pre-treated with IL-33 (100 μg/kg) at 30 min prior to MCAO. The mRNA expression of T-bet and RORγt was increased at 24 h after MCAO, revealing that acute cerebral ischemia may induce immune-shift of Th cells to Th1 and Th17 cell subsets in the spleen (Fig. 5). Compared to the PBS-treated MCAO 24 h group, IL-33 pre-treatment could reverse the elevated T-bet mRNA level and increase the mRNA levels of GATA-3 and Foxp3 in the spleen at 24 h after MCAO (Fig. 5). These results showed that IL-33 pre-treatment may suppress the differentiation of Th1 cells and promote the generation of Treg cells in the spleen after MCAO via regulating the related transcription factors T-bet, GATA-3 and Foxp3.

3.5. The time course effect of IL-33 on infarct volume, neurological deficit score, and splenic T cell subsets after MCAO

To investigate the effect of IL-33 pre-treatment on ischemic brain injury at the different time points after MCAO, we further tested the infarct volumes, neurological deficit scores and the percentages of splenic IFN-γ^+ and Foxp3$^+$ cells at 48 h, 72 h and 96 h after MCAO pre-treated with IL-33 (100 μg/kg) at 30 min prior to MCAO. Pre-treatment with IL-33 also significantly reduced the brain infarct volume and neurological severity score at 48 h, 72 h and 96 h after MCAO (Fig. 6A). Compared to the corresponding PBS-treated MCAO subgroup, IL-33 pre-treatment may decrease the number of IFN-γ^+ cells and increase the amount of Foxp3$^+$ cells in the spleen at 48 h, 72 h and 96 h after MCAO. The percentage of Foxp3$^+$ cells was found to reach its peak in the MCAO 48 h mice pre-treated with IL-33 (Fig. 6B, C). We also observed changes in the spleen mass from 24 h to 96 h after MCAO, and found that no obvious spleen atrophy occurring in the IL-33-treated MCAO mice from 24 h to 96 h (Fig. 6D). These data showed that IL-33 pre-treatment may play a long-term protective role in the development of acute cerebral ischemia via suppressing Th1 response and promoting Treg response peripherally.

4. Discussion

A salient finding of our study is that intraperitoneal IL-33 pre-treatment may attenuate ischemic brain injury via regulate splenic Th1 cell immune responses and Treg cell immune responses. Recently, an increasing number of studies have shown that peripheral immune response originating from the spleen appears to be a potential target for the treatment of stroke (Ajmo Jr. et al., 2008; Pennypacker, 2014; Seifert and Offner, 2018). The spleen contains vast quantities of peripheral immune cells, and spleen-derived T lymphocytes have emerged as a principal target for dampening the pro-inflammatory peripheral immune responses (Kim et al., 2014; Pennypacker and Offner, 2015). Ischemic spleens have been found a reduction in CD8$^+$ T cells that was accompanied by an increased CD4/CD8 ratio after experimental stroke, which may reflect the loss of CD8$^+$ T cells and indicate several possible CD4$^+$ T cell responses: proinflammatory Th1 response or anti-inflammatory Treg response (Gendron et al., 2002; Leonardo and

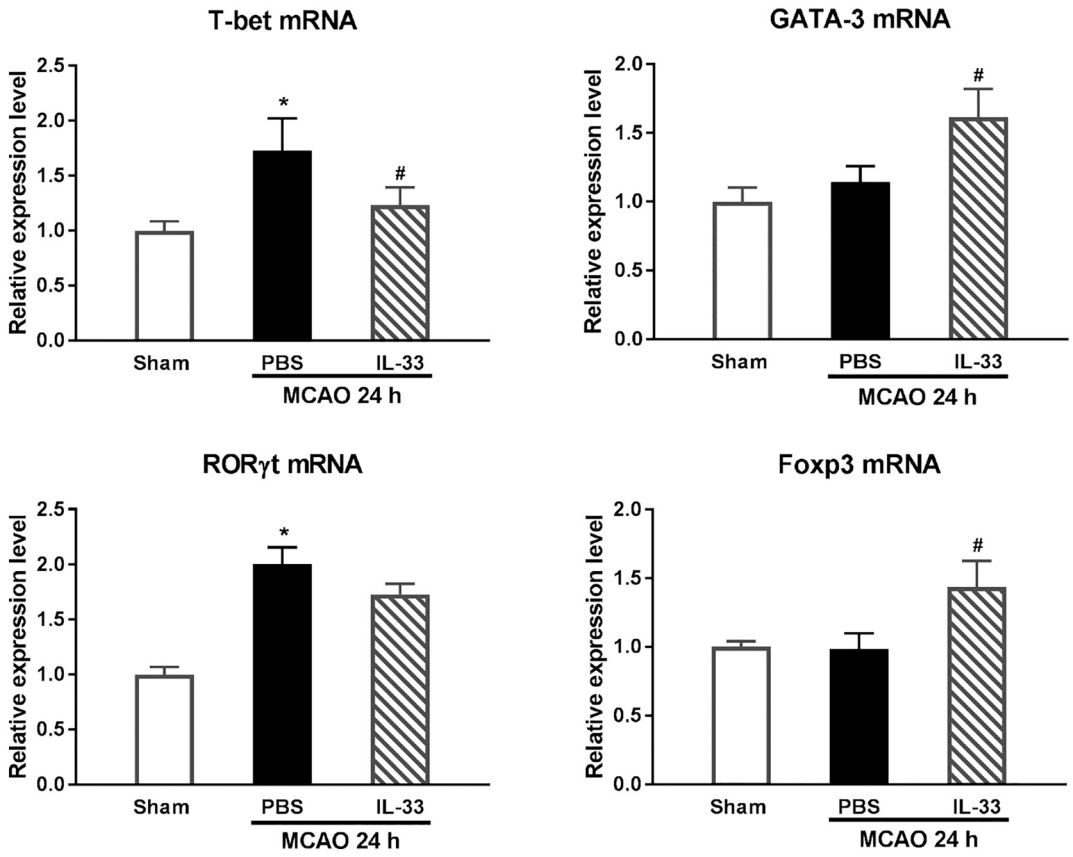
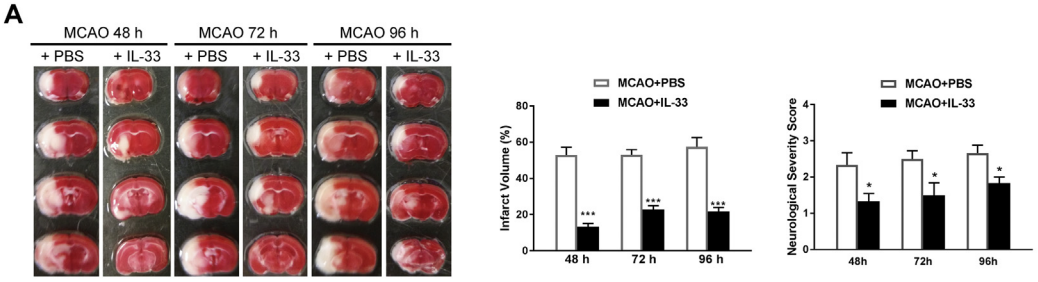


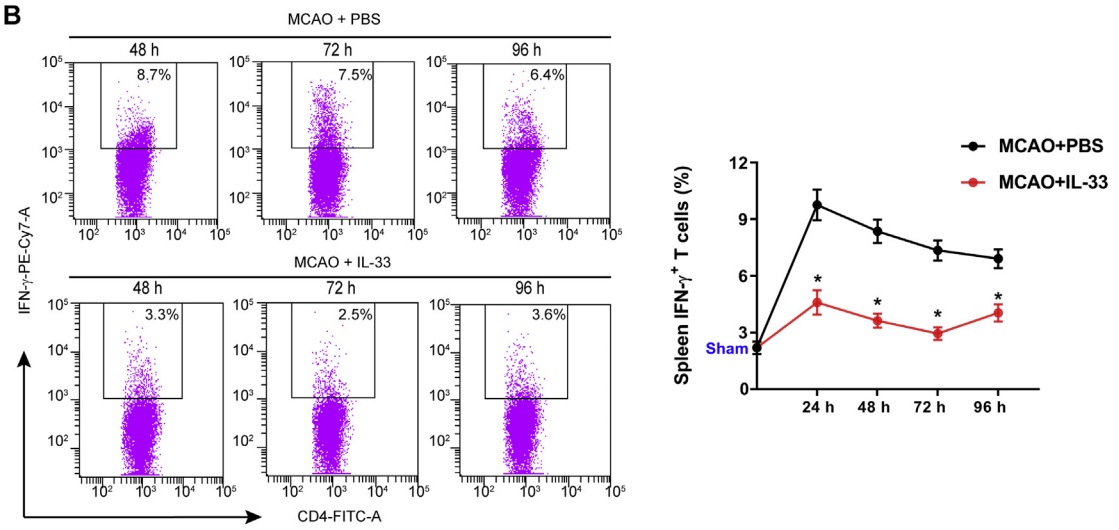
Fig. 5. Quantitative PCR analysis of the mRNA levels of transcription factors T-bet, GATA-3, RORγt and Foxp3 in spleen tissues from MCAO 24 h mice pre-treated with IL-33.Total RNA in spleen tissues from sham group, PBS and IL-33-treated MCAO 24 h group was extracted (n = 6 mice per group), and the mRNA levels of transcription factors were determined by quantitative real-time PCR analysis. Recombinant mouse IL-33 (100 μg/kg) or PBS was administered 30 min before MCAO. Normalized gene expression was derived from the ratio of the mRNA expression of T-bet, GATA-3, RORγt and Foxp3 to the GAPDH mRNA expression. Data are shown as means ± SEM. $^{*}p < .05$ compared with sham group, $^{\#}p < .05$ compared with the PBS-treated MCAO 24 h group (repeated measures ANOVA).

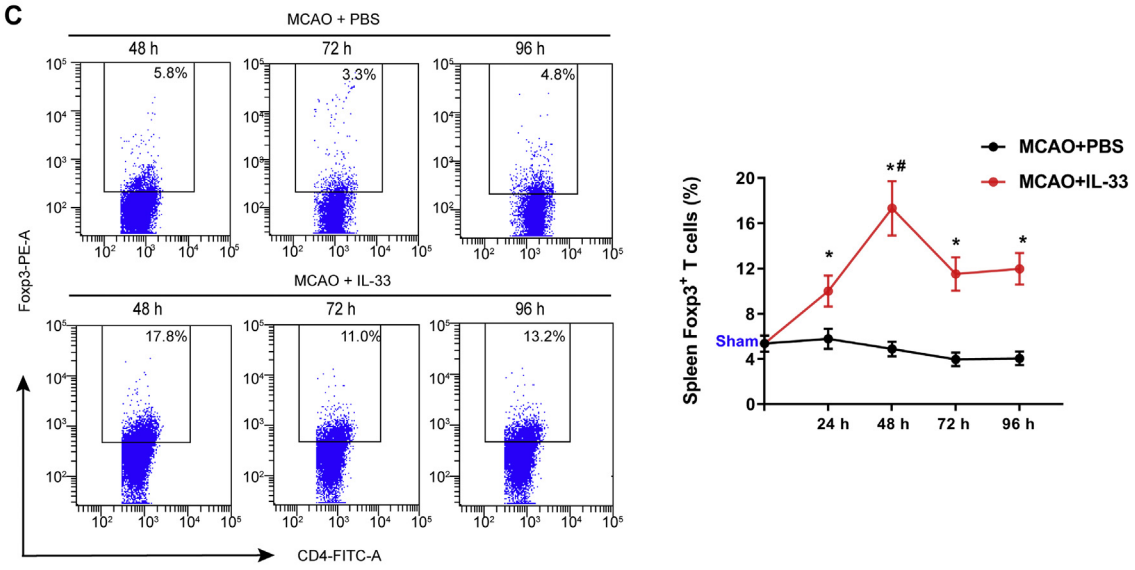
Pennypacker, 2011). Thus, $CD4^+$ T cell immune responses in the spleen may be important for stroke development. In our present study, we found that the spleen size decreased after MCAO in mice, which was consistent with the previous reported studies (Ajmo Jr. et al., 2008; Seifert et al., 2012a). Additionally, the spleen size changes were shown to accompany with the increased percentages of splenic Th1 and Th17 cells at 24 h after MCAO. The related cytokines IFN-γ and IL-17 were produced more from spleen at 24 h after MCAO, which may migrate into the brain and aggravate brain damage and inflammation. Previous study reported that the splenic atrophy was accompanied by increased Treg cells at 96 h after MCAO (Offner et al., 2006). However, the amounts of Treg cells were just presented a rising trend at 24 h after MCAO in our present study, and no statistical difference between control and MCAO group was shown. We also found that the percentage of $IL\text{-}4^+$ T cells was very low in the spleen. Maybe down-regulation of Th1 and Th17 immune responses and up-regulation of Treg responses in the spleen would be the effective strategy to improve stroke.

Other investigators have found that the decrease in spleen size following transient MCAO is due to apoptosis of the cells and a loss of functional centers within the spleen (Offner et al., 2006; Bao et al., 2010). The splenic contraction is associated with an increase in circulating immune cells, at least a part of which are from the spleen tissue (Bakovic et al., 2005). While there are still differences among the different findings this could be due the different species used or the type of stroke surgery performed in the different studies. A previous study found that blocking the α1-adrenergic receptors with carvedilol prevents the decrease in spleen size following MCAO while carvedilol is also neuroprotective (Savitz et al., 2000; Ajmo Jr. et al., 2009). As a new member of IL-1 cytokine family, IL-33 has shown pleiotropic effects on regulating T cell immune responses under the different disease conditions, such as atherosclerosis, multiple sclerosis and spinal cord injury (Jiang et al., 2012; Miller et al., 2008; Schmitz et al., 2005; Pomeshchik et al., 2015). In our previous study, we have reported that intracerebroventricular IL-33 treatment may ameliorate ischemic brain injury in experimental stroke through promoting Th2 response and suppressing Th17 response in the brain (Luo et al., 2015). In this study, we found that the neuroprotective role of IL-33 may be partly associated to its modulation to splenic T-cell immune responses. And we speculated that blocking the decrease in the spleen mass with IL-33 may be due to inhibiting Th1 cells and related proinflammatory cytokines released into the circulation. Moreover, a research has reported that IL-33/ST2 signaling can provide neuroprotection through inhibiting apoptosis in a mouse model of traumatic brain injury (Gao et al., 2018). Maybe IL-33 could block the decrease in the size of the spleen, at least in part, by a mechanism involving suppressing apoptosis of the cells and related functional deficit within the spleen, which deserves further investigation.

Meanwhile, we found that IL-33 pre-treatment could decrease the amount of Th1 cells and increase the percentage of Treg cells in the spleen at 24 after MCAO. Correspondingly, IL-33 pre-treatment inhibited the secretion of the proinflammatory cytokine IFN-γ from the spleen and promoted the production of anti-inflammatory cytokines IL-4, IL-10 and TGF-β. Secretion of proinflammatory cytokines from splenic T cells can activate microglia/macrophage at the site of injury, which would be deleterious to the survival of neural cells following stroke (Seifert et al., 2012a). Our study may contribute to further understand the regulation of T cell infiltration after acute CNS injuries. Moreover, the prominent expression of the IL-33 receptor ST2 on







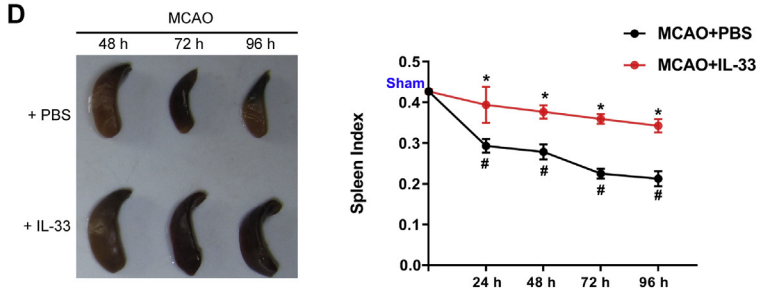


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Fig. 6. The time course effects of IL-33 pre-treatment on infarct volume, neurological deficit score, and splenic T cell subsets after MCAO. (A) Representative triphenyltetrazolium chloride staining and quantification of brain infarct volume and neurological severity scores in MCAO 48 h, 72 h and 96 h mice pre-treated with IL-33 (100 μg/kg) or PBS at 30 min before MCAO (n = 8 mice per group). (B, C) FACS plots and statistical analysis for IFN-γ^+ T cells and Foxp3$^+$ T cells in the spleen at sham group (representative FACS plots shown in Fig. 1C), MCAO 24 h (representative FACS plots shown in Fig. 3C), 48 h, 72 h and 96 h pre-treated with IL-33 (100 μg/kg) or PBS at 30 min before MCAO (n = 8 mice per group). For intracellular staining, cells were stimulated with ionomycin, PMA and Brefeldin A. (D) Representative image and quantification of spleen weight index show the spleen size from sham group (representative image shown in Fig. 1A), MCAO 24 h (representative image shown in Fig. 3A), 48 h, 72 h and 96 h pre-treated with IL-33 (100 μg/kg) or PBS at 30 min before MCAO (n = 8 mice per group). Data are shown as means ± SEM. $^*p < .05$, $^{***}p < .001$ compared with the corresponding PBS-treated MCAO subgroup, $^{\#}p < .05$ compared with sham group and other PBS-treated MCAO subgroups in spleen Foxp3$^+$ T cells (repeated measures ANOVA).

GATA3$^+$ Th2 cells demonstrated that IL-33 could have a direct impact on T cells (Schmitz et al., 2005). Recent observations reveal that T-bet$^+$ Th1 cells and Foxp3$^+$ Treg cells can also express the ST2 receptor (Alvarez et al., 2019). Another study also found that Th17 cells could express ST2 and be controlled by the alarmin IL-33 (Pascual-Requant et al., 2017). As such, IL-33 can have an important effect on the dynamics of T cell populations. Our results showed that IL-33 may regulate gene transcription of T-bet, GATA-3 and Foxp3 in the splenocytes as a nuclear factor to influence the differentiation of Th cells, which may be the possible molecular mechanism for IL-33 regulating T cell phenotypes.

T cells have been determined to play a role as mediators in the delayed phase of brain ischemia (Yilmaz et al., 2006), so the long-term effects of IL-33 pre-treatment on splenic Th1 response and Treg response is also interesting. Our results showed that intraperitoneal IL-33 pre-treatment was also neuroprotective for cerebral ischemia, and may sustain the amount of splenic Th1 cells in a low level. The percentage of Treg cells in the spleen increased from 24 h and reached the peak at 48 h after MCAO pre-treated with IL-33. Recent study has reported that Treg cells in the spleen tissue could be expanded by IL-33 (Pomeshchik et al., 2015; Delacher et al., 2017). We speculated that IL-33 pre-treatment may have a long-term protective role on stroke outcome, which was mainly associated to the increased splenic Treg cells in the delayed stage.

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Conflict of interest

The authors declare no conflict of interest.

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