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# Effect of partial depletion of CD25$^+$ T cells on neurological deficit and tissue damage in acute cerebral ischemia rat models

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ABSTRACT

**Objective**: To evaluate the role of regulatory T cells (Tregs) at late stages of stroke. **Methods**: Anti-CD25 antibody (or PBS as a control) was injected to reduce the pool of Tregs in Wistar rats; then, ischemia was induced transiently by middle cerebral artery occlusion during 60 min and reperfusion was allowed for 7 d. Then, Treg frequency was analyzed in peripheral blood, spleen and lymph nodes. Neurological score (0-6) and infarct volume were also determined. **Results**: Nine days after injection, the CD4$^+$CD25$^+$ T cells were reduced by 70.4%, 44.8% and 57.9% in peripheral blood, spleen and lymph nodes, respectively compared to PBS-treated rats. In contrast, the reduction of CD4$^+$FOXP3$^+$ T cells was lower in the same compartments (38.6%, 12.5%, and 29.5%, respectively). The strongest reduction of CD25$^+$CD4$^+$ T cells was observed in those FOXP3-negative cells in blood, spleen and lymph nodes (77.8%, 52.8%, and 60.7%, respectively), most likely corresponding to activated T cells. Anti-CD25-treated transient middle cerebral artery occlusion rats had a lower neurological deficit and did not develop tissue damage compared with PBS-treated animals. **Conclusions**: These findings suggest that treatment with anti-CD25 in our model preferentially reduce the T cell population with an activated phenotype, rather than the Treg population, leading to neuroprotection by suppressing the pathogenic response of effector T cells.

## 1. Introduction

Stroke remains a global public health problem nowadays[1] It is known that innate and adaptive immune cells[2] promote inflammation and increase the infarct volume[3]. Regulatory T cells (Tregs) characterized by FOXP3 and CD25 expression[4] can promote the stroke progression in animal models by increasing thrombus formation 24 h after transient middle cerebral artery

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occlusion (tMCAO)[5,6] however, Tregs can accumulate in the brain and reduce the infarct size after anti-CD25 antibody or PBS injection[7,8].

Most of the findings on the role of Tregs during ischemia have been performed in mouse models, that have been shown variable results with respect to the size and location of infarcts[9]. Unlike mice, the cerebral vasculature and physiology of rats are similar to that of humans, and also rats are easy to handle and relative homogeneity exists within strains[10]. Thus, we determined the role of an anti-CD25 antibody treatment, and aimed to reduce the Tregs in rats that underwent tMCAO.

## 2. Materials and methods

### 2.1. Animals

Twenty four adult male Wistar rats between eight and nine weeks of age were randomly selected and maintained in pathogen-free environments. All animal procedures were conducted according to the ARRIVE guidelines. All procedures were approved by the ethical committee for animal experimentation from Antioquia University (2003/18084). The protocol was approved in act 70 (June 13-2011).

### 2.2. Study design

Eight rats were randomly assigned to receive an injection with an anti-CD25 antibody or with PBS two days after the animals were submitted to tMCAO. Eight rats were assigned to a sham surgery by using the same anesthesia and surgical procedures with the exception of MCA occlusion, those animals were also pre-treated with anti-CD25 antibody or PBS as tMCAO ones.

### 2.3. Depletion of $CD25^+$ T cells

Two days before the induction of ischemia, the rats were injected intraperitoneally (*i.p.*) with 300 μg of mouse anti-rat CD25 IgG1 monoclonal antibody (NDS61, Raleigh; 300 μg) to decrease the frequency of $CD25^+$ T cells[11]. Since spleen constitutes an important reservoir of cells, the antibody doses were optimized to reduce at least more than 50% of splenic $CD25^+FOXP3^+$ T cells as suggested previously[12]. Additionally, since no reduction of $CD25^+$T cells was observed between animals treated with 300 uL of phosphate buffered saline (PBS) and those treated with IgG control antibody (two rats in each group), thus we use PBS injection as control group in this study.

### 2.4. Middle cerebral artery (MCA) occlusion

Rats were anesthetized and tMCAO was induced by inserting a monofilament for 60 min to occlude the origin of the middle cerebral artery as previously described[13], followed by 7 d of reperfusion.

### 2.5. Neurological score

Neurological deficit was determined after 6 h, 24 h, 48 h, 72 h, and 4 d, 5 d, 6 d and 7 d of reperfusion using a scale as follows: 0, no spontaneous motion; 1, circling spontaneously toward the paretic side; 2, circling toward the paretic side if pulled by the tail; 3, circling toward the paretic side if pulled and lifted by the tail; 4, reduced resistance to lateral push toward the paretic side; 5, consistent flexion of the contralateral forelimb to ischemic injury and 6, normal extension of both forelimbs toward the floor when lifted[13].

### 2.6. 2, 3, 5-triphenyltetrazolium chloride (TTC) staining

The volume of brain infarction was determined 7 d post-MCAO through staining with TTC (Sigma). Stained sections were photographed using a digital camera (Canon) and analyzed using Image J software (National Institute of Health, Bethesda, USA). Edema was corrected by using the formula: infarct size=100 [total contralateral hemisphere area-(total ipsilateral hemisphere area-infarct area)/total contralateral hemisphere area], as described previously[14].

### 2.7. Flow cytometry

Single-cell suspensions from spleen, lymph nodes, and blood were obtained and stained with different antibodies for surface markers: Anti-rat CD3 (1F4; BD Pharmigen), anti-rat CD4 (W3/25; Biolegend), anti-rat CD8a (OX8) and anti-rat CD25 (OX39) from eBioscience. After extracellular staining, the cells were permeabilized (Foxp3 staining kit) followed by staining with anti-rat FOXP3 (FJK-16s) from eBioscience. Isotype controls were used and data were acquired on a FACSCanto ][ and analyzed with FACSDiva software (BD Biosciences).

### 2.8. Statistical analyses

The data are expressed as the mean ± standard deviation. Normal distribution was assessed by Shapiro-Wilk normality test and comparisons between the groups were done using an unpaired one-tailed Student *t*-test or general linear model ANOVA, followed by Dunnett's post hoc test to compare between study groups. Animals were coded by a technician and analysis was done by a different researcher. Statistical analyses were performed with Prism 8.0 (GraphPad Software, La Jolla, CA, USA). A $P<0.05$ was considered statistically significant difference.

## 3. Results

### 3.1. Partial depletion of $CD4^+FOXP3^+$ T cells in rats treated with an anti-CD25 antibody

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First, to ensure that the tMCAO were done on Treg-depleted rats, we evaluated the reduction of Treg percentage two days after anti-CD25 antibody or PBS injection on four naive rats. There was a 60.7% reduction of splenic $CD4^+CD25^+$ T cells, compared with the PBS-treated rats (Figure 1). Then, two days after anti-CD25 antibody injection, rats underwent tMCAO or sham surgery and hereafter all outcomes were determined seven days post-reperfusion. We observed a similar reduction in peripheral blood, splenic and lymphoid $CD4^+CD25^+$ T cells by 70.4% ($P$=0.050), 44.8% ($P$=0.023) and 57.9% ($P$=0.011) compared to PBS-tMCAO rats (Figure 2A). The $CD25^+FOXP3^+$ T cell populations were reduced by 65.3% ($P$<0.01), 67.7 % ($P$=0.006) and 57.8% ($P$<0.01) in blood, spleen and lymph nodes, respectively (Figure 2B). Moreover, there was a 38.6% ($P$=0.030) and a 29.5% ($P$=0.011) reduction of blood and lymphoid $CD4^+FOXP3^+$ T cells respectively, while in spleen the reduction was only of 12.5% ($P$=0.222) (Figure 2C).

The anti-CD25 treatment in our rat model failed to deplete a high proportion of $FOXP3^+$ cells. A total of 52.1% of the peripheral blood $FOXP3^+$ cells, and 44.8% of the splenic and 43.3% lymphoid $FOXP3^+$ cells did not express CD25 as observed in PBS-treated rats (Figure 2D). Interestingly, anti-CD25 treatment particularly reduced the population of $CD4^+CD25^+$ cells that do not express FOXP3, with 77.8% ($P$=0.028), 52.8% ($P$=0.049) and 60.7% ($P$=0.004) decreases in the blood, spleen and lymph nodes, respectively (Figure 2E). Similar reductions in $CD4^+CD25^+$, $CD4^+FOXP3^+$, $CD25^+FOXP3^+$, and $CD25^+$ $FOXP3^-$ T cells were observed sham surgery rats ( Figure 3).

No significant changes were observed between anti-CD25 and PBS-treated tMCAO animals in their total cell counts in peripheral blood and spleen, nor the percentages of $CD4^+$ and $CD8^+$ T cells in peripheral blood, spleen and lymph nodes (Figure 4).

### *3.2. Effect of partial depletion of $CD4^+CD25^+$ cells on infarct volume and neurological deficit in rats after tMCAO*

We determined the effect of the partial reduction of $CD4^+CD25^+$ on the infarct volume and neurological deficit after reperfusion in rats that underwent tMCAO or sham surgery. Anti-CD25-treated animals did not exhibit a detectable infarct in TTC-stained coronal slices compared to PBS-treated animals 7 d after reperfusion (Figure 5A), which developed larger infarcts [(71.8 + 27.1) $mm^3$]. Similarly, anti-CD25 injection significantly reduced the neurological deficit in rats 24 h ($P$=0.004), 48 h ($P$=0.080), and 72 h ($P$=0.080) post-reperfusion compared to PBS-treated animals (Figure 5B).

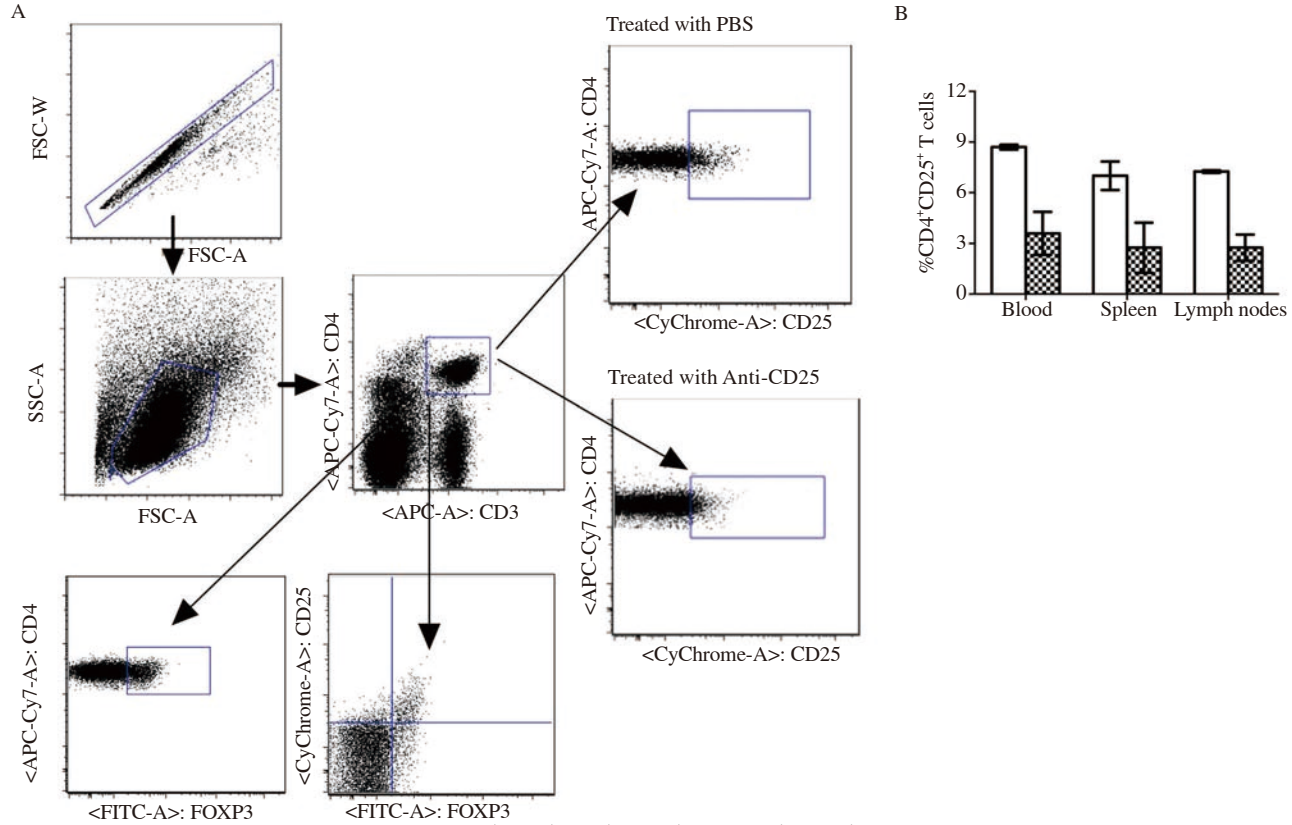


**Figure 1.** Representative gating strategy showing (A) $CD4^+CD25^+$, $CD4^+FOXP3^+$, and $CD25^+FOXP3^+$ T cells obtained from spleen.

The percentage of $CD4^+CD25^+$ T cells was calculated (B) in blood, spleen and lymph nodes from naive rats 2 d after anti-CD25 injection or PBS. Mean ± SD, $n$=2 per group.

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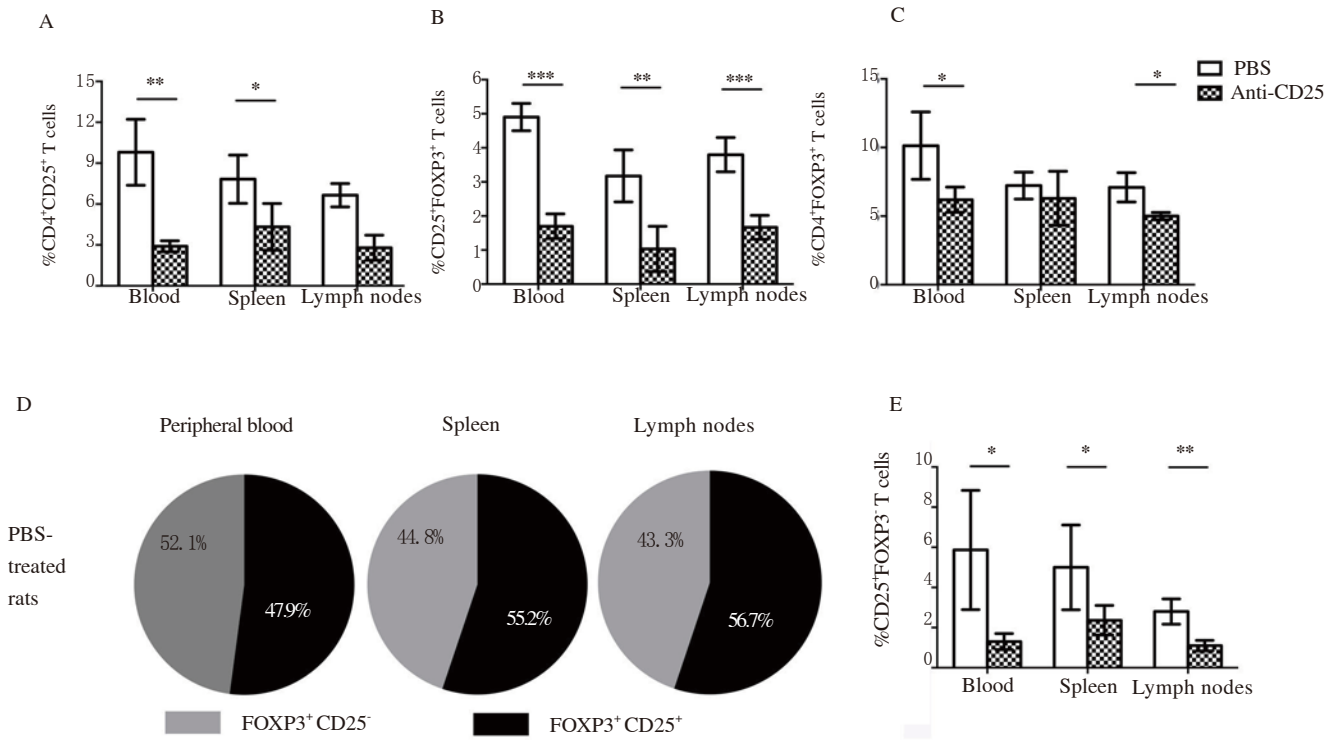


**Figure 2.** Partial depletion of $CD25^+$ T cells in tMCAO rats by anti-CD25 injection.
The percentage of $CD4^+CD25^+$ (A), $CD25^+FOXP3^+$ (B) and $CD4^+FOXP3^+$ cells (C) from tMCAO rats pre-treated with anti-CD25 or PBS. The pie charts show the percentage of $CD25^+$ and $CD25^-$ cells among $CD4^+FOXP3^+$ T cells (D) and the bar graph shows $CD25^+FOXP3^-$ T cells (E). Mean ± SD, $n$=4 per group. $t$-student test. $^*P<0.05$, $^{**}P<0.01$.

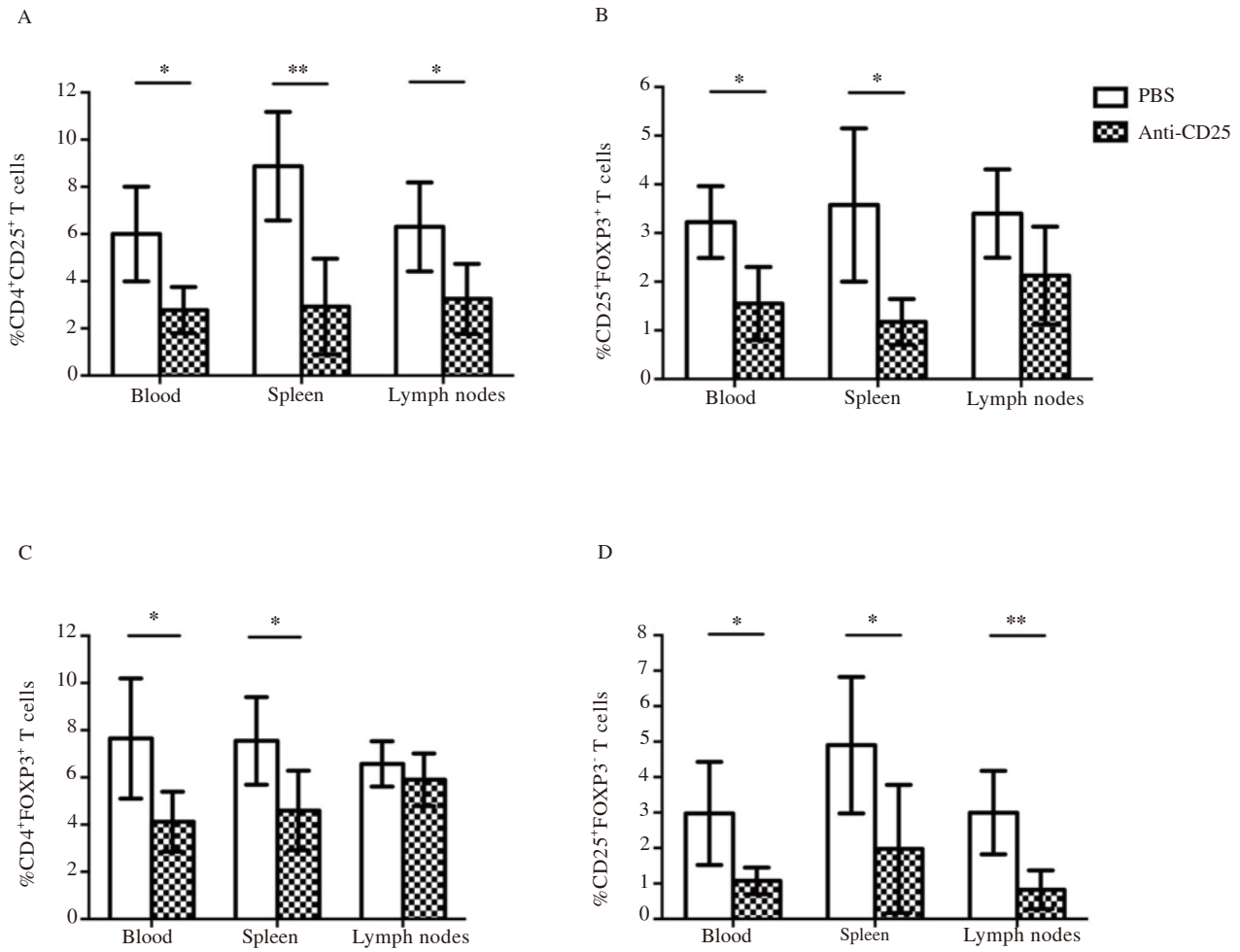


**Figure 3.** The reduction percentage of $CD4^+CD25^+$ (A), $CD25^+FOXP3^+$ (B), $CD4^+FOXP3^+$ (C), and $CD25^+FOXP3^-$ (D) T cells in each compartment was shown in sham surgery rats pretreated with anti-CD25 or PBS. Mean ± SD, $n$=4 per group. $t$-student test. $^*P<0.05$, $^{**}P<0.01$.

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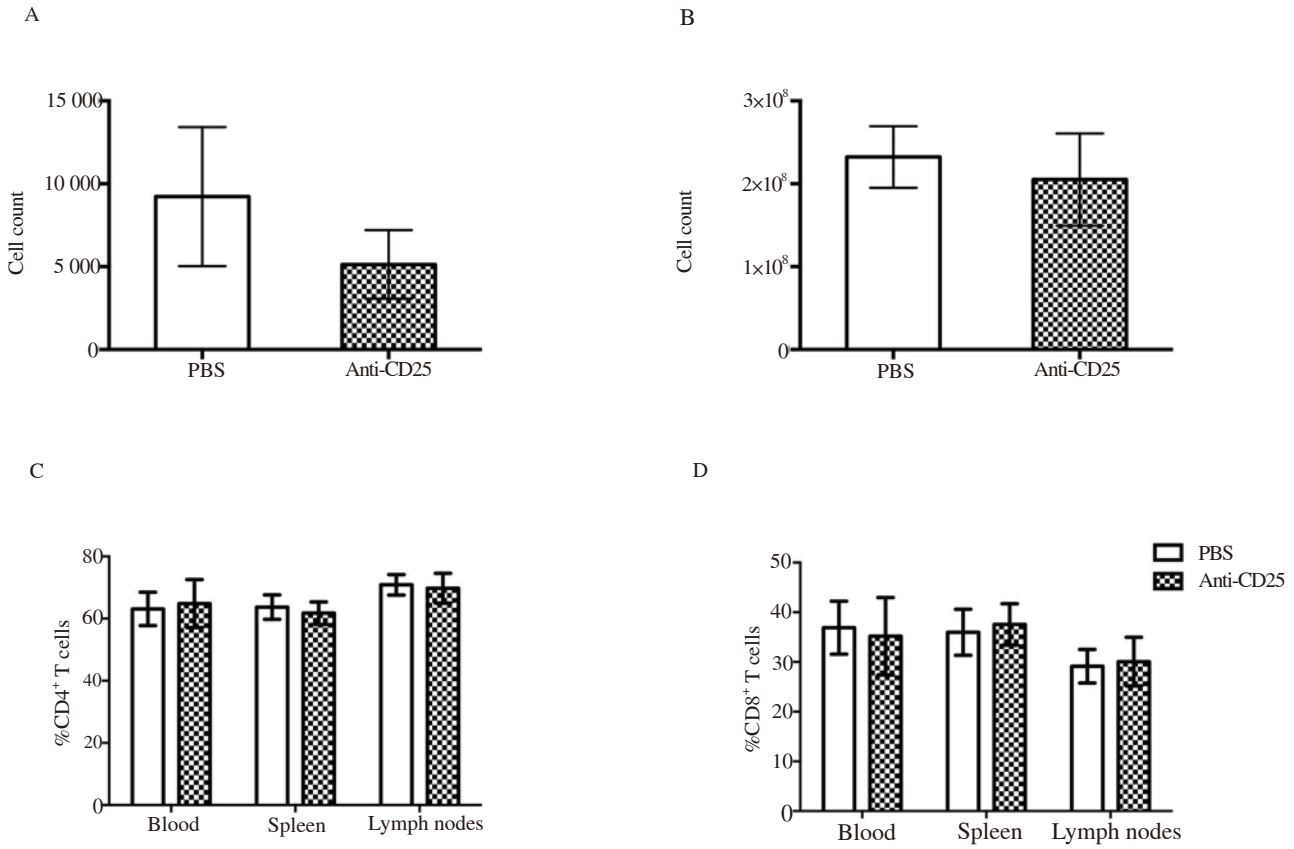


**Figure 4.** Number of cells and percentage of $CD4^+$ and $CD8^+$ T cells in blood, spleen and lymph nodes.

The total number of cells in blood (A) and spleen (B) from tMCAO rats 9 d after anti-CD25 or PBS injection. The percentages of $CD4^+$ (C) and $CD8^+$ T cells (D) are showed as Mean ± SD, $n$=4 per group. $t$-student test.

## 4. Discussion

In recent years there has been a controversy concerning the effect of Treg on ischemic stroke; however, several pieces of evidence point towards a neuroprotective effect by reducing infarct volume and inflammation around of infarcted tissue. Here, we found that the injection of an anti-CD25 antibody on Wistar rats partially eliminated the Treg population with no detectable infarct with TTC staining compared to PBS-treated counterparts.

Previous reports have shown a partial depletion of $CD4^+FOXP3^+$ T cells after anti-CD25 treatment, ranging from 40% to 70%[15-17] along with a lower efficiency of Treg elimination in lymph nodes. Moreover, resistance to elimination of these cells in lymph nodes could be due to their ability to capture IL-2 in these niches more efficiently compared to conventional T cells[18]. We anticipate that higher doses could increase the efficiency of elimination in these compartments, but a single injection reduces the $CD25^+$ cell population for over one week[19,20]. In addition, clone PC61, that is the most used antibody to deplete $CD25^+$ T cells, induces a partial reduction of Tregs, similar to that obtained using the NDS61 clone[11,21,22]. In addition, it is known that the use of the same antibody clone for both the cell depletion and detection leads to an overestimation of the reduction of $CD25^+$ T cells[17].

Similar to previous report in healthy rats[23], we found an important population of $FOXP3^+$ cells that do not express CD25, thus those cells would not be targeted by the anti-CD25 antibody. Other authors have reported similar proportions of $CD4^+FOXP3^+$ T cells that do not express CD25[17,24,25], which could constitute a Treg reservoir that after activation could express CD25[26] along with their suppressive function[17,27]. However, a defect in their functionality cannot be ruled out, since the lack of expression of CD25 controls the expression of suppressive molecules, such as CTLA-4, CD39/CD73[28,29] and FOXP3[30]. Interestingly, the major target of anti-CD25 was $CD4^+CD25^+$ cells that do not express FOXP3, probably, this population represents activated T cells[31,32]. Futures studies are necessary to confirm the identity of those depleted cells. Taken together, these results suggest that the treatment with anti-CD25 partially reduces $CD25^+FOXP3^+$ cells in rats along with a higher reduction of activated $CD25^+FOXP3^-$ T cells.

We found that the partial reduction of $CD4^+CD25^+$ cells was accompanied by non-detectable infarct by TTC staining on anti-CD25-treated tMCAO rats compared to PBS-treated rats, which developed large infarcts. We do not rule out that a subjacent lesion could have occurred since TTC staining cannot efficiently detect the infarcted region at late stages of ischemia[33]. Other methodologies, such as magnetic resonance imaging, would allow detecting cerebral damage at late stages of cerebral ischemia, allowing to characterize the spatiotemporal evolution of stroke[34]. Similarly, anti-CD25 injection also reduced the neurological deficit in rats 24 h and 48 h post-reperfusion compared with PBS-treated animals. Our

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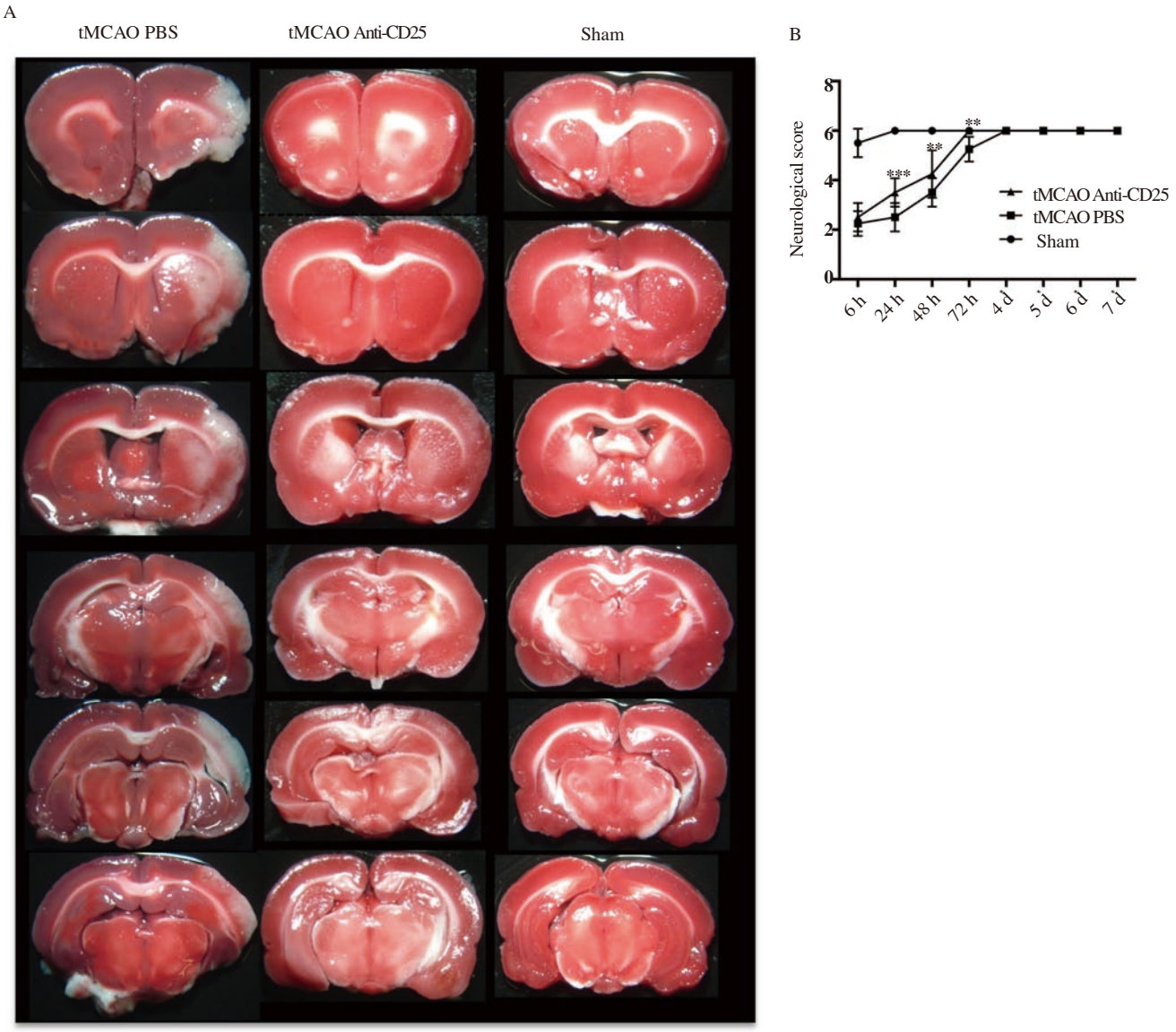


**Figure 5.** Effect of anti-CD25 treatment on infarct volume and neurological deficit.

Representative coronal brain slices representative of 3 animals stained with TTC (A). Neurological function (B) was determined at different time points after reperfusion in groups of study. Mean ± SD, $n$=4 per group. General linear model ANOVA, Dunnett's *post-hoc* test. $^{**}P<0.01$. $^{***}P<0.001$.

results contrast with those reported by Liesz *et al*, probably due to the anatomical differences between mice and rats, the ischemia induction techniques and the variations in the efficiency of $CD25^+$ T cells elimination[8]. Altogether, these results suggest that the use of an anti-CD25 antibody preferentially reduces a population of activated $CD25^+FOXP3^-$ T cells, being more evident in lymph nodes, which could be associated with the reduction of infarct volume and neurological deficit. However, we cannot rule out that Treg cells that escape elimination are contributing to the protection observed. Although a protecting role of Tregs has been demonstrated in other pathological conditions, the role of Tregs during cerebral ischemia remains controversial[5,8,35]. Understanding the Treg-mediated immunomodulatory mechanisms underlying cerebral ischemia is essential for the development of novel therapies that limit the inflammatory response observed in cerebral ischemia.

## Conflict of interest statement

The authors report no conflict of interest.

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