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

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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 occlusion. This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

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

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. 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. 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. 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. Additionally, since no reduction of CD25+ T cells was observed between animals treated with 300 µL 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, 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.

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.

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 CD8(a) (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 II 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
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$^+$CD25$^+$ 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).

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**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.
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.
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

![Figure 4. Number of cells and percentage of CD4+ and CD8+ T cells in blood, spleen and lymph nodes.](image-url)
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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