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CD4+FoxP3+ regulatory T-cells in cerebral ischemic stroke

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Abstract

Experimental cerebral ischemic stroke is exacerbated by inflammatory T-cells and is accompanied by systemic increases in $CD4^+CD25^+Foxp3^+$ regulatory T-cells (Treg). To determine their effect on ischemic brain injury, Treg were depleted in $Foxp3^{DTR}$ mice prior to stroke induction. In contrast to a recent Nature Medicine report, our results demonstrate unequivocally that Treg depletion did not affect stroke infarct volume, thus failing to implicate this regulatory pathway in limiting stroke damage.

Keywords

Cerebral ischemia; T lymphocyte; T regulatory cells; middle cerebral artery occlusion; stroke

Introduction

Stroke is a leading cause of death and the most frequent cause of permanent disability worldwide (Sacco *et al*, 2007). Severe brain ischemia causes an initial inflammatory phase in both the CNS and periphery, followed by severe immunosuppression that predisposes to life-threatening infections (Dirnagl *et al*, 2007). Immunoregulatory mechanisms may limit the inflammatory phase as well as mediate immunosuppression. We demonstrated previously that naturally-occurring $CD4^+CD25^+Foxp3^+$ regulatory T-cells (Sakaguchi *et al*, 2006) increase after cerebral ischemia in the face of massive depletion of other immunocytes from spleen and thymus (Offner *et al*, 2006b). A recent report (Liesz *et al*, 2009) indicates that depletion of the $CD25^+$ population with anti-CD25 mAb significantly increased brain infarct volume and worsened functional outcome after brief focal cerebral ischemia. These effects were attributed to $CD4^+CD25^+Foxp3^+$ Treg cells, even though the anti-CD25 mAb only depleted ~50% of this Treg phenotype. CD25, the IL-2 receptor chain-α (IL-2Rα), has a broad expression on early progenitors of the T- and B-cell lineages, as well as on activated mature T-cells and B-cells, thus allowing for the possible contribution of other regulatory

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CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

cell types in the Liesz experiments besides Foxp3^{+} Treg. To target Treg more specifically, we employed the Foxp3^{DTR} mouse, in which the coding sequence of diphtheria toxin receptor (DTR) has been inserted into the *foxp3* allele and is co-expressed on Foxp3+ cells (Kim *et al*, 2007). We tested the hypothesis that Treg are beneficial to the brain stressed with a standard model of middle cerebral ischemia (MCAO).

Methods

Animals Mice used for these experiments were age-matched males and females (7–11 weeks old) that were rested for at least 7 days before treatment. Foxp3^{DTR} mice, which were backcrossed with C57BL/6 (B6) mice, were generously provided by Dr. Alexander Rudensky (Kim *et al*, 2007). Animals were bred and cared for according to institutional guidelines in the animal resource facility at the Veterans Affairs Medical Center, Portland, OR.

Cell isolation Peripheral blood mononuclear cells were prepared by using red cell lysis buffer (eBioscience) following manufacturer's Instructions. Single-cell suspensions from lymph nodes (superficial cervical, mandibular, axillary, lateral axillary, superficial inguinal and mesenteric) and spleens were prepared by mechanical disruption. The cells were then washed twice with RPMI 1640, counted, and resuspended in stimulation medium containing 10% FBS for phenotyping.

Analysis of cell populations by FACS Single-cell suspensions were washed with staining medium (PBS containing 0.1% NaN3 and 2% FCS) and stained with mouse regulatory T cells staining kit (FJK-16s, eBioscience) according to manufacturer's Instructions. After incubation with mAb and washing, cells were analyzed with a FACSCalibur (BD Biosciences). For each experiment, cells were stained with appropriate isotype control antibodies to establish background staining and to set quadrants before calculating the percentage of positive cells. Data were analyzed using Flowjo software (TreeStar).

MCAO The mice were subjected to MCAO as previously published (Offner *et al*, 2006a; Offner *et al*, 2006b) by reversible right MCA occlusion (60min) under isoflurane anesthesia, followed by 96h of reperfusion. Body and head temperatures were controlled at 37±0.5°C. Occlusion and reperfusion were verified in each animal by laser Doppler flowmetry (Moor Instruments).

Neurological Deficit Score Each mouse was scored on emergence from anesthesia and daily after MCAO as previously described (Offner *et al*, 2006b). The graded scoring system ranges from 0 to 2 or 0 to 5 depending on the behavior, with 0 indicating no deficit and positive integers indicating impairment. Consciousness (0–3), interaction (0–2), eye appearance (0–2), breathing (0–2), food/water intake (0–2), ability to clasp (0–2), motor function (0–5) and activity (0–2) were assessed.

Quantification of Infarct As previously published (Offner *et al*, 2006a; Offner *et al*, 2006b), brains were collected at 96h for standard 2, 3, 5-triphenyltetrazolium chloride (TTC) histology and digital image analysis of infarct volume. To control for edema, volume is expressed as a percentage of contralateral structure, i.e. cortex, striatum or total hemisphere.

Results

We accomplished near complete deletion of Foxp3 expression in the peripheral blood, lymph nodes and spleens (≤0.3%) by treating the mice with two daily i.p. injections of 50μg diphtheria toxin (DT) per kg body weight (Fig. 1A) prior to induction of middle cerebral artery occlusion (MCAO) for 60min on Day 3, followed by a final DT treatment on Day 4

that maintained deletion of $Foxp3^+$ Treg as assessed on Day 7 (Fig. 1B). In comparison, normal levels of $Foxp3^+$ Treg cells were observed in vehicle-treated $Foxp3^{DTR}$ mice undergoing MCAO (Fig. 1).

Depletion of $Foxp3^+$ Treg did not affect infarct volume in cortex or striatum in male, female or pooled male and female mice 96h after MCAO and reperfusion (outline in Fig. 2A) compared to vehicle treated mice (Figs. 2B, 2C & 2D). Moreover, there were no significant effects of Treg depletion on behavioral evaluations (data not shown).

Discussion

These data show that Treg did not limit brain damage or improve functional outcomes in mice undergoing MCAO. We believe that there are at least two plausible explanations that account for the different outcomes in our study vs. the single previous evaluation of Treg cells in cerebral ischemia. First, we selectively depleted essentially all Foxp3-expressing cells using a genetic approach. In Liesz et al., anti-CD25 mAb was used to deplete $CD25^+$ cells that included precursor and mature T- and B-cells, but only ~50% of Foxp3-expressing Treg. Moreover, reconstitution experiments in this report involved only $CD25^+$ cells, not $Foxp3^+$ cells. Thus, increased histological damage and improved functional outcomes observed in their study cannot be attributed solely to the $CD4^+CD25^+Foxp3^+$ Treg subpopulation, although other regulatory cell types expressing CD25 would still be implicated. Second, we utilized a standard experimental stroke model of moderate duration that reproducibly yields infarction, analyzed as a percentage of contralateral structure to account for tissue edema. While the effect of $CD25^+$ cell depletion were detectable in mice with small infarcts (~$15mm^3$, after 30 min vascular occlusion), the effect could not be demonstrated in mice with larger damage (>$100mm^3$ after 90min occlusion) (Leisz et al., 2009). In contrast, lesion volumes in our $Foxp3^{DTR}$ mice were intermediate (~$50mm^3$ after 60min MCAO). These comparisons may indicate that putative protective effects of $CD25^+$ regulatory cells are relevant only under conditions of mild cerebral ischemic damage.

Our failure to implicate $CD4^+Foxp3^+$ T-cells in limiting brain lesion volume after MCAO is of general importance to the field because of the increased interest in increasing $CD4^+Foxp3^+$ Treg as a possible therapeutic approach in stroke. Our results do not support this rationale, but do highlight the need to identify other regulatory pathways that can limit stroke-induced inflammatory damage to the CNS.

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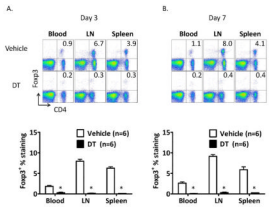

Figure 1.

Characterization of Foxp3^{+} Treg cell ablation in Foxp3^{DTR} mice. (A) On day 3 (after 2 DT or Vehicle treatments) and (B) on day 7 (after 3 DT or Vehicle treatments), mice were euthanized and lymphoid organs removed. Flow cytometric analysis of blood, LN-derived cells and splenocytes shows efficient depletion of Foxp3^{+} cells in DT-treated Foxp3^{DTR} mice compared with Vehicle-treated control mice (upper right quadrant). The percentage of cells in each quadrant is indicated. *Vehicle vs. DT treatment, $p<0.001$.

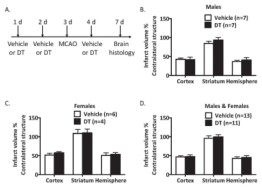

Figure 2.

Depletion of Treg cells does not change ischemic lesion volume. (A) Scheme for experimental design. (B) Infarct volume at 96h reperfusion after 60min MCAO in male mice, (C) female mice and (D) combined data from both genders.