TESIS DOCTORAL

ROLE OF REGULATORY T CELLS IN RECOVERY AFTER BRAIN ISCHEMIA

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ABBREVIATIONS

5HPET; 5-hydroxiperoxyeicosatetranoic acid
ACTH; adrenocorticotropin hormone
AIM-2; apoptosis inhibitor of marophagues 2
AMPA; α-amino-2-hydroxy-5methyl acid
AP1; activator protein 1
APC; antigen presenter cell
ATP; adenosin triphosphate
BBB; blood brain barrier
BCL; B cell lymphoma
BrDU; bromodeoxiuridine
^{0}C; Celsius degrees
CBF; cerebral blood flow
CFSE; carboxyfluorescein succinimidyl esterdiacetate
CD28SA; ant- CD28 superantigen monoclonal antibody
$CO_{2;}$ carbón dioxide
COX-2; cyclooxygenase 2
CREB; c-adenosine monophosphate responsable element binding protein
CRF; corticotropin releasing factor
CSF; cerebrospinal fluid
CT; computarized tomography
CTLA-4; cytotoxic T lymphocyte associated protein 4
CX3CR1; CX3C receptor 1
CXCR5; CX chemokine receptor 5
DAMPs; damage-asssociated molecular patterns
DD; death domain
DEREG; depletion of T cells
DNA; deoxiribonucleic acid
DTR; difteria toxin receptor
ELISA; enzyme linked immune sorbent assay
END; early neurological deterioration
EU; European Union
FADD; fas associated death domain

FITC; fluorescence isoTiocyanate
FoxP3; fork head box protein P3
GFAP; glial fibrillary acid protein
GITR; glucoccorticoid induced TNF receptor family related gen
HDACI; histone deacetylase inhibitor
HIV; human immunodeficiency syndrome
HMGB1; chromatin-associated protein termed high mobility group protein B1
H_2O_2; hydrogen peroxide
ICAM; intracelular adhesión molecule
IDO; indoelleamina 2,3 dioxigenase
IL; interleukin
iNOS; inducible nitric oxide synthase
INF: interferon
IPEX; immunodysregulation, polyendocrinopathy, enteropathy X linked syndrome
LACI; lacunar infarct
LAG; lymphocyte activator gene 3
LFA; lymphocyte fynction associated antige
LOX; lipoxygenase
LTA4; leukotriene 4
Mac1; macrophague antigen 1
MadCAM; mucosal vascular addresin cell adhesion molecule
MAPK; mitogen activated protein kinase
MBP; myelin basic protein
MCA; middle cerebral artery
MCAO; middle cerebral artery occlusion
MCP1; monocyte chemoattractant protein 1
MIP1; macrophague inflammatory protein 1
MRI; magetic resonance imaging
MMP; matrix metalloproteinase
MPO; mieloperoxidase
MTPT; 1-methyl-4-phenyl-1,2-3-6 tetrahydroirydine
NADPH; nicotine amide adenine dinucleotid phosphate
NCAM; neuronal cell adhesion molecule

NeuN; neuronal nuclei
NFAT; nuclear factor activated lymphocytes
NFκβ; nuclear factor κβ
nNOS; nitric oxide sinthase
NMDA; N-methyl-D-aspartate
NO; nitric oxidase
NOD; nucleotid binding oligodimmerization domain
NOX; NADPH oxidase
NK; natural killer
OCT; optimum cutting temperature
PACI; partial anterior circulation infarction
PAF; platelet activating factor
PBS; phosphate buffered saline
PD; programmed death
PDL-1; programmed death ligand 1
PE; phycoeritrin
PECAM; platelet endotelial cell adhesión molecule
PGE_2; prostaglandin E2
PGH_2; prostaglandin H2
PLA_2: phospholipase A2
POCI; posterior circulation infarction
PVDF; polyvinylidene difluoride
RIG-1; retinoic acid inducible gen 1
RNA; ribonucleic acid
rNIF; recombinant neutrophil inhibitory factor
ROS; reactive oxygen species
SDS-PAGE; sodium dodecyl sulphate poliacrylamide gel
SEM: standard error of the mean
SPECT; single photon emulssion computed tomography
TACI; total anterior circulation infarction
TBS: Tris Buffered Saline
TCR; T cell receptor
TGFβ; transforming growth factor β
TH; T helper
TIA; transient ischemic atack

TLR; toll-like receptor
TNF; tumor necrosis factor
Treg; regulatory T lymphocytes
TOAST; Trial of Org 10172 in Acute Ischemic Stroke
TWEAK; tumor necrosis factor weak inducer of apoptosis
USA; United States of America
VCAM; vascular adhesion molecule
VEGF; vascular endothelial growth factor
WHO; World Health Organisation
XLAAD; X linked autoinmmunity allergic dysregulation syndrome

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INTRODUCTION

1 ISCHEMIC STROKE

1.1 STROKE DEFINITION

Stroke happens when blood supply to a part of the brain is interrupted or severely reduced, disturbing the normal function of one or more parts of brain. There are different types of stroke, and depending on the nature of the damage we can differentiate between ischemic and hemorrhagic stroke.

Brain ischemia is the endpoint of a reduction in brain blood flow under a level to produce metabolic and biochemical changes, leading to cell death and changes in nervous system function.

The World Health Organization (WHO) definition of stroke is: "rapidly developing clinical signs of focal (or global) disturbance of cerebral function, with symptoms lasting 24 hours or longer or leading to death, with no apparent cause other than vascular origin"[1].

1.2 STROKE EPIDEMIOLOGY

Stroke is the most common neurologic disease and it is an important mortality and disability cause. The WHO, reports an incidence of 200 new cases per 100,000 people worldwide[2], although data vary among countries. Each year 795,000 people experience a new or recurrent stroke. Approximately 610,000 of these are first attacks and 185,000 are recurrent strokes. Of all strokes, 87% are ischemic and 10% are intracerebral hemorrhages, whereas 3% are subarachnoid hemorrhages[3].

Stroke accounted for 1 of every 20 deaths in the United States (USA). When considered separately from other cardiovascular diseases

stroke is the fourth cause of death, after cardiac disease, cancer and chronic respiratory diseases. More than 140,000 people die every year due to cerebrovascular disease in USA.

In Spain, on the basis of the IBERICTUS study[4], 187 new strokes take place per 100,000 people per year. The 80% of these events are ischemic strokes, and only the 20% are hemorrhagic strokes. Incidence rate was slightly higher in men and the hospital mortality rate is 11%. The findings in the CONOCES study showed that in Spain, in-hospital mortality rate is 5.9% and the 1-year mortality rate after stroke is 18.3%. Almost 30% of patients have any disability 1 year after stroke[5, 6].

Stroke is the main cause of disability in adult population. Almost 31% of survivors from stroke need help in their activities of daily living, 20% of survivors need help walking and 16% need nursing care. Almost 39.4 millions disability-adjusted life years were lost because of ischemic stroke in 2010[3].

All these problems cause a high economic cost, estimated of 41 billion dollars per year in USA. In 2011 direct and indirect cost of stroke in USA was 33.6 billion dollars. The mean expense per patient for direct care for any type of service in the US was estimated 4692 dollars[3].

1.3 STROKE CLASSIFICATION

Stroke can be classified in two categories: ischemic and hemorrhagic. Ischemic stroke is caused by the interruption of blood supply, while hemorrhagic stroke results from the rupture of a blood

vessel or an abnormal vascular structure to the brain parenchyma or to the subarachnoid space. Ischemic stroke can be classified in transient ischemic attack and brain infarction. Hemorrhagic stroke can be classified in intracerebral hemorrhage and subarachnoid hemorrhage.

Transient ischemic attack (TIA) is a transient episode of neurologic dysfunction caused by ischemia (loss of blood flow), without acute infarction. In the classic definition, this neurologic dysfunction lasts less than 24 hours. The American TIA Working Group, suggesting temporal criteria for TIA under 1 hour length[7], discussed this definition.

Brain infarction is caused by a qualitative or quantitative interruption of the blood supply to a part of brain, with a neurologic dysfunction lasting more than 24 hours, due to cell death.

The TOAST (Trial of Org 10172 in Acute Stroke Treatment) classification system is based in etiology of ischemic stroke[8]. The TOAST classification denotes five subtypes of stroke: a) large-artery atherosclerosis, b) cardioembolism, c) small-artery occlusion, d) stroke of other determined etiology and e) stroke of undetermined etiology.

a) **Large-artery atherosclerosis:** these group of patients will have clinical and brain imaging findings of either significant (>50%) stenosis or occlusion of a major brain artery or branch cortical artery, presumably due to atherosclerosis. Diagnostic studies should exclude potential sources of cardiogenic origin.

b) **Cardioembolism:** this category includes patients with

arterial occlusions presumably due to an embolus arising in the heart (atrial fibrillation, atrial thrombus, mechanical prosthetic valve, sick sinus syndrome, recent myocardial infarction, left ventricular thrombus, dilated miocardiophaty, akinetic left ventricular segment, atrial myxoma, infective endocarditis). Potential large-artery atherosclerotic sources of thrombosis or embolism should be eliminated.

c) Small-artery occlusion (lacune): this category includes patients with one of the traditional clinical lacunar syndromes (pure motor stroke, pure sensory stroke, ataxic hemiparesis, mixed sensorimotor stroke or dysarthria/clumsy hand) and should not have evidence of cerebral cortical dysfunction. The patient should also have a normal CT (computerized tomography) /MRI (magnetic resonance imaging) examination or a relevant brain stem or subcortical hemispheric lesion with an infarct diameter smaller than 1,5 cm demonstrated. Potential cardiac sources of embolism should be absent, and evaluation of the large extracranial arteries should not demonstrate a stenosis of greater than 50% in an ipsilateral artery.

d) Acute stroke of other determined etiology: this category includes patients with rare causes of stroke, such as no atherosclerotic vasculopathies, hypercoagulable states or hematologic disorders. Patients in this group should have clinical and CT or MRI findings of acute ischemic stroke, regardless of the size or location. Diagnostic studies should reveal one of these unusual causes of stroke. Cardiac sources of embolism and large

artery atherosclerosis should be excluded by other studies.

e) Stroke of undetermined etiology: in several instances, the cause of a stroke cannot be determined with any degree of confidence, some patients will have no likely etiology determined despite an extensive evaluation. This category also includes patients with two or more potential causes of stroke, so that the physician is unable to make a final diagnosis. For example, a patient with an atrial fibrillation and an ipsilateral stenosis of more than 50%.

The OCSP classification (Oxfordshire Community Stroke Project) [9] is a clinical classification, based in stroke localization. It denotes stroke in four subtypes **(Figure 1).**

• **Total anterior circulation infarction (TACI)**, should meet the three criteria;

1.Cortical dysfunction (aphasia, acalculia o visuoespatial disorders)

2. Motor and/or sensitive disturbances in at least two limbs (including face, arm and leg)

3. Homonymous hemianopia

• **Partial anterior circulation infarction (PACI),**

1. Cortical isolated dysfunction (aphasia, acalculia o visuoespatial disorders)

2. When 2 or 3 TACI criteria are met

3. Motor or/and sensitive disturbance more restricted than the LACI

classification

- **Lacunar infarction (LACI),** without cortical dysfunction or hemianopia and meet one of these criteria:

1. Pure motor stroke/hemiparesis in at least 2 limbs (including face, arm and leg)

2. Pure sensory stroke: numbness and mild sensory loss over one side of the body (including face, arm, and leg)

3. Mixed sensorimotor stroke in at least 2 limbs (inluding face, arm and leg)

4. Ataxic hemiparesis: ataxia and weakness in the ipsilateral side of the body

5. Dysarthria/clumsy hand: dysarthria and clumsiness of the hand

- **Posterior circulation infarction (POCI),** one of the following criteria:

1. Ipsilteral cranial nerve dysfunction and contralateral motor and/or sensitive disturbances

2. Bilateral motor or sensitive disturbances

3. Oculomotor palsy

4. Cerebellar dysfunction without motor or sensitive symptoms

5. Isolated homonymous hemianopia

Intracranial hemorrhage represents 20% of all strokes. It is due to a rupture of an artery in the brain resulting in a collection of blood

within the brain tissue. Intracranial hemorrhage can be subdivided in intracerebral hemorrhage and subarachnoid hemorrhage. Intracerebral hemorrhage includes primary intraventricular hemorrhage and intraparenchymal hemorrhage. Intraparenchymal hemorrhage can be located lobar, profound, cerebellar and pontine[10].

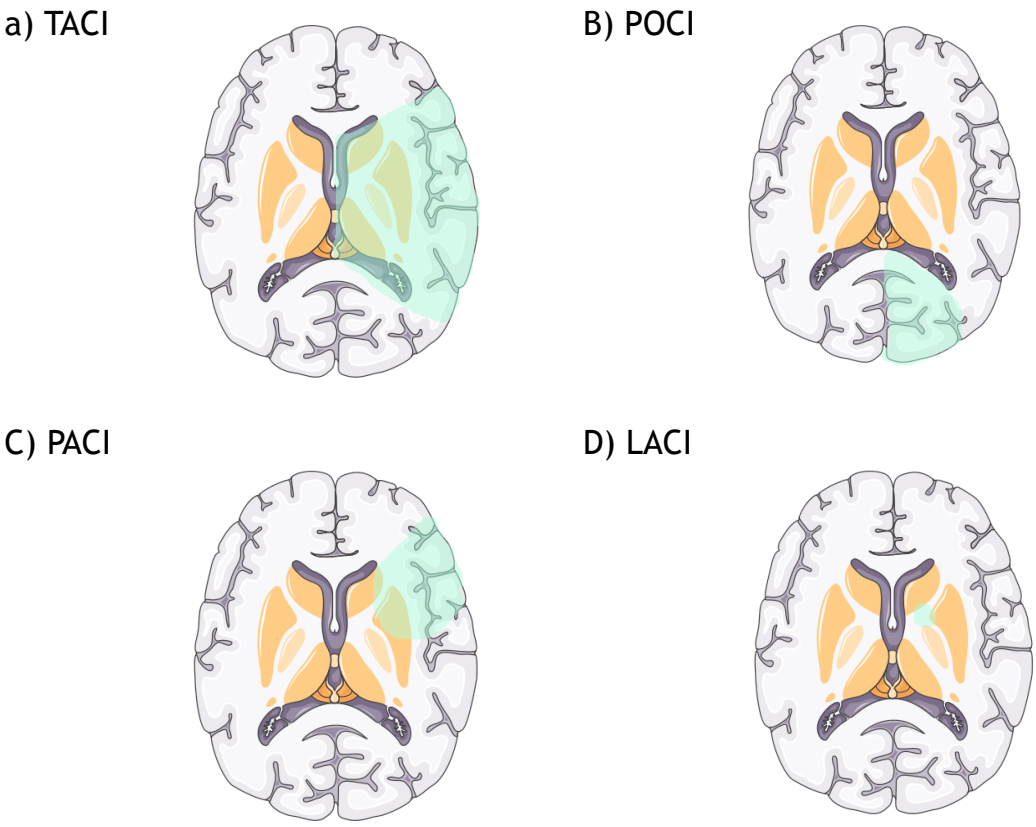

Figure 1: OSCP classification: a) TACI (total anterior circulation infarction), b) POCI (posterior circulation infarction), C) PACI (partial anterior circulation infarction) and d) LACI (lacunar infarction).

1.4 STROKE RISK FACTORS

Stroke risk factors have been classified as modifiable and no modifiable (gender, age, ethnic group). The more prevalent modifiable stroke risk factors are hypertension (52.1%), atrial fibrillation (27.4%), diabetes (20%), hyperlipidemia (16,8%), previous stroke

(16.4%), myocardial infarction (13,3%), transient ischemic attack (12,4%), smoking (11,5%) and peripheral arterial disease (7,8%)[11].

Non-modifiable risk factors:

- **Age**: elderly is one of the main risk factors related to stroke. Stroke incidence rises exponentially with age. The higher incidence rates are over 65 years.

- **Gender**: stroke is more common in men.

- **Ethnic group**: large artery atherosclerosis incidence is higher in caucasic population, and small vessel disease is more prevalent in African and Asian population.

- **Genetic factors:** there is a higher susceptibility to stroke in some families. This relationship can be due to a higher prevalence of other risk factors among these families (hypertension, diabetes and hyperlipidemia), but sometimes the only risk factor in some individuals with stroke is the familiar history of stroke.

Modifiable risks factors

- **Hypertension:** hypertension is the most prevalent modifiable risk factor for stroke with a prevalence of about 30% in the USA[12]. The prevalence of hypertension increases with age, thus as the population ages, hypertension will become an even greater threat to public health, and is likely to increase as life expectancy increases. Hypertension is a risk factor for ischemic and hemorrhagic stroke. Isolated high systolic pressure, isolated high diastolic pressure and systolic and diastolic high pressure increase stroke risk among population. For every 20

mmHg systolic or 10 mm Hg diastolic increase in blood pressure, deaths from ischemic heart disease and stroke are doubled[13].

- **Cardiac disease:** cardiac diseases are related to stroke, mainly atrial fibrillation, valvulopathy, myocardial infarction, left ventricular hypertrophy and cardiomegaly.

- **Diabetes:** The relative risk of stroke in a person with diabetes ranges from 1.8 to 6 and diabetics tend to suffer stroke at a younger age[14]. Pre-diabetes is also a risk factor for the development of diabetes mellitus and, in patients with a history of TIA or stroke, impaired glucose tolerance doubles the risk of stroke[15].

- **Hyperlipidemia:** Several large randomized trials with statins have demonstrated reduction of stroke risk associated with lowering lipid levels. However, observational prospective studies have not found a consistent relation between cholesterol levels and higher incidence of stroke. Some meta-analysis proposed that statin therapy at stroke onset was associated with improved outcome[16]. The beneficial effects of statins over stroke risk reduction may be mediated by additional mechanisms of action such as improvement of endothelial function, antioxidant properties, inhibition of inflammatory responses, immunomodulatory actions, and stabilization of atherosclerotic lesions[17].

- **Smoking:** Smoking is an independent stroke risk factor, increasing the risk of stroke by about 50%[18]. This risk increases proportionally with the number of cigarettes smoked per day and passive smoking also increases the risk of ischemic stroke. Smoking cessation is an

effective measure to reduce stroke risk.

- **Other risk factors:** lack of exercise, obesity, obstructive sleep apnea, fat rich diet, alcohol, and oral contraconceptive pills are also related with a increased risk for stroke.

1.5 PHYSIOPATHOLOGY OF ISCHEMIC STROKE

Cerebral ischemia is caused by the reduction of cerebral blood flow under the normal level for the correct brain function. This process occurs as the result of the balance of some hemodynamic factors and can produce metabolic and biochemical disorders in neurons and glial cells, leading up to cell death[19].

A blood perfusion gradient appears when a blood vessel is blocked, normal cerebral blood flow (CBF) in a brain area decreases under 10 ml/100 g/min, driving to cell death[20].

There are different perfusion thresholds in the brain for the preservation of cellular function, impending cellular death (penumbra) and necrotic cell death (infarct core). The concept of penumbra appears after the observation that restoration of blood flow to a hypoperfused area allows threatened tissue to be saved. Therefore, penumbral tissue was defined as critically hypoperfused tissue destined for infarction, unless normal perfusion could be restored promptly[21, 22].

There are differences in the cause of cellular death between the infarct core (necrosis) and penumbra area (apoptosis). Inside infarct core, necrosis occurs within a few minutes following vessel occlusion. Necrosis is a form of traumatic cell death that results from acute

cellular injury caused by external factors, such as hypoxia in ischemic stroke. However, in the penumbra area, multiple cell death mechanisms are activated: excitotoxicity and ionic imbalance, oxidative stress and apoptotic-like cell death may all occur and progress more slowly than necrosis.

Positron emission tomography studies show differences inside hypoperfused tissue. There is an area with lightly hypoperfusion (CBF over 22 ml/100 g/min) where tissue is threatened (oligemic area) and other area called ischemic penumbra area[23, 24], with a critical hypoperfusion level (CBF < 22 ml/100 g/min) where there is enough oxygen for the tissue survival (misery perfusion) **(Figure 2).**

Penumbral tissue is not always near the necrotic neuronal area, could be a different area with decreased blood flow but with a normal metabolic energetic function[25].

Ischemic penumbra area is the part of brain tissue where cerebral blood flow decreases enough to create electrophisiologic silence, but energetic metabolism and cell membranes preserve a normal function[26]. In ischemic penumbra area there is neuronal functional disorder, but a minimal metabolic function to maintain the structural integrity for a while. Inside this area, autoregulation mechanisms are damaged, CO_2 reactivity, synaptic transmission and ATP levels are normal and glucose level is decreased. These changes can cause neurologic symptoms, but not permanent damage[19]. The most important concept and the basis for ischemic stroke treatment is the fact that neurons inside ischemic penumbral tissue are alive and can function normally when hemodynamic conditions return to previous

levels. There are different factors conditioning cellular necrosis such as hypoxia, hypoglycemia, cerebral blood flow changes and collateral circulation.

1.5.1 Hypoxia

Partial oxygen pressure decrease leads to an increase in cerebral blood flow. Levels under 40 mmHg can cause confusion and less than 20 mmHg can cause coma.

Isolated hypoxia, without acidosis or ischemia does not produce acute brain damage[26]. However hypoxic damaged area is boosted by arterial hypotension as occurring in shock or cardiac arrest. Hypoxia and hypotension lasting more than few minutes, can cause irreversible cerebral damage, especially in grey matter and arterial border areas[27, 28].

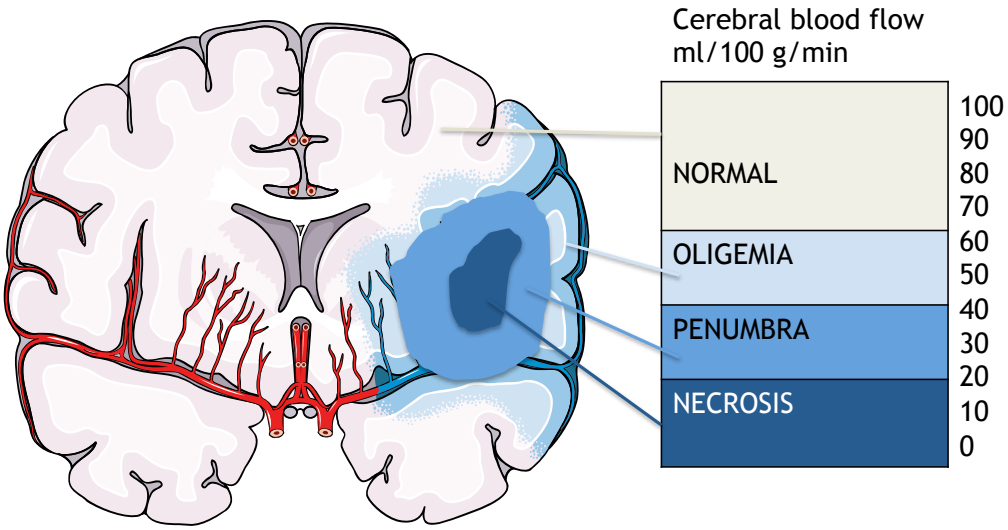

Figure 2. Ischemic penumbra area. There are different perfusion thresholds in the brain for the preservation of cellular function, impending cellular death (penumbra) and necrotic cell death (infarct core). Adapted from[29].

1.5.2 Hypoglycemia

Low glucose levels can cause functional and structural disorders. Glucose level under 20 mg/dl can lead to confusion and under 10 mg/dl to coma. In a first moment, brain consumes glucose and glycogen reserves without causing neurologic symptoms. When reserves are over, brain starts using other substances, producing structural lesions associated to neuronal death with preservation of glial tissue[30].

1.5.3 Cerebral blood flow

Cerebral blood flow depends on blood perfusion pressure and vascular resistance. Vascular resistance depends on blood vessel diameter and blood viscosity. Those parameters can bear on the development of ischemic stroke. Brain perfusion pressure is the result of the relationship between brain blood vessel medium pressure, intracranial pressure and venous pressure[31].

Under physiological conditions, venous pressure and intracranial pressure are low, so brain perfusion pressure is the same than medium diameter arteries pressure. When intracranial pressure rises, causing intracranial hypertension, like in venous thrombosis or mechanical ventilation, brain perfusion pressure decrease, and can lead to the occurrence of ischemic stroke.

Under physiological conditions, brain blood flow autoregulation is independent of big changes in blood pressure thanks to a complex regulation mechanism in brain circulation **(Figure 3),** with the participation of myogenic, biochemical and peptidergic systems[32].

In hypertensive patients, the autoregulation curve changes, so the upper and the lower limits move to higher pressure levels. During brain ischemia a change in brain blood flow occurs. This autoregulation system disappears in the ischemic area and perfusion pressure becomes dependent on arterial pressure (this lack of regulation appears with changes of 40% of cerebral blood flow).

This relationship is stronger with arterial pressure decrease than with arterial pressure increase. That is due to the fact that perfusion pressure in ischemic tissue is under the lower limits of autoregulation in vascular bed. That is why the blood vessels are expanded to the higher point. A decrease in arterial pressure in this scenario cannot be supply with a decrease in vascular resistance[32].

During stroke, autoregulation is also disturbed. Vascular reactivity to CO_2 (carbon dioxide) decreases. Usually, when CO_2 level increases, there is a decrease in blood flow in the ischemic area due to vasodilation in normal arteries. Normal arteries steal flow from ischemic area in response to hipercapnia[24]. Ischemic tissue loses the ability of vasodilation in response to hipercapnia. That situation produces an imbalance in metabolic and hemodynamic control in "luxury perfusion", which leads to a decrease in oxygen extraction and the occurrence of a venous blood network [33].

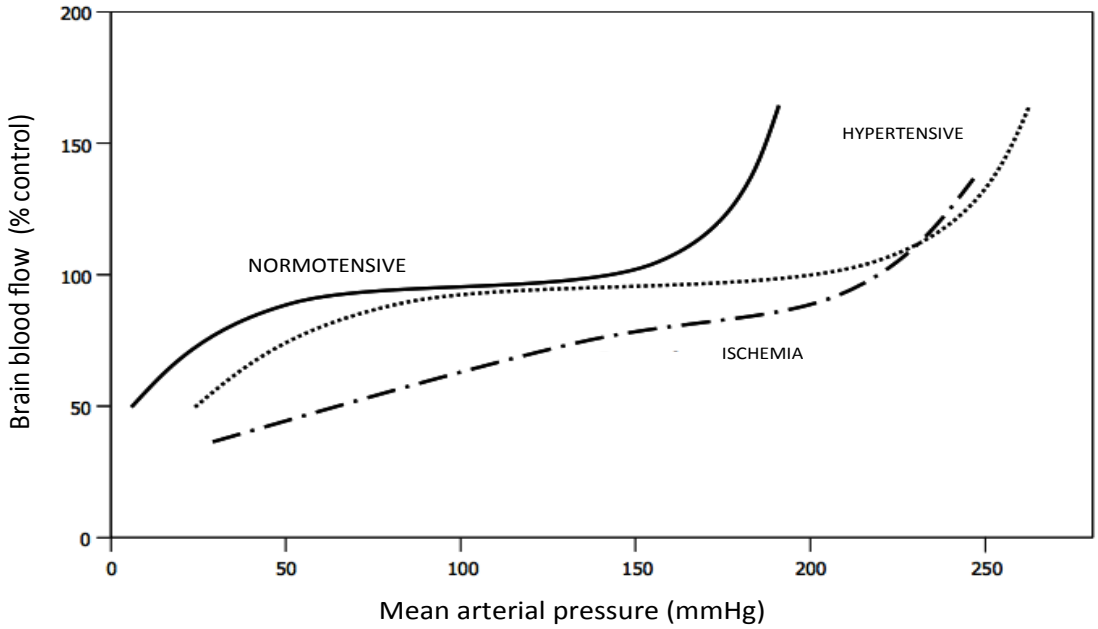

Figure 3. Autoregulation system in brain blood flow in normal situation, hypertensive patients and ischemic tissue.

1.5.4 Collateral circulation

Collateral circulation protects brain from focal ischemia, and establishes an additional security mechanism in brain vascularization.

The main extracranial anastomosis is between both primitive carotid arteries. Intracranial circulation establishes communication with external carotid through ophthalmic artery. Intracranially, communication is made through Willis polygon and leptomeningeal anastomosis.

The degree of collateral circulation to offset impaired blood flow downstream from an arterial occlusion is a principal determinant of ischemia severity in acute stroke. The results form the IMS III trial[34], provide evidence that collateral status is closely related to

revascularization success, defined alternatively as recanalization or reperfusion, and the most importantly, clinical outcome. Collaterals are an influential factor in the angiographic and clinical outcomes across a diverse population of case based on the site of arterial occlusion and particular endovascular strategies. Recanalization success may be associated with more robust collaterals due to hemodynamic factors include increased distribution of thrombolytics to the clot surface. Reperfusion of the downstream territory may also be more complete after opening of an arterial occlusion if such regions are sustained by robust collateral perfusion[34].

Measures of collateral flow on non-invasive modalities and angiography may help to define specific regions of brain at risk of infarction and hemorrhage. Both ischemia and reperfusion, determinants of outcome and hemorrhagic transformation, are influenced by collaterals. Hemorrhage likely results from a combination of severe ischemia followed by reperfusion in regions where collaterals have not offset deleterious changes in downstream resistance. Collaterals and perfusion status may be detailed prior to intervention in acute ischemic stroke with multimodal CT or MRI and angiography. Applications of the diagnostic tools may optimize patient selection and guide therapy in acute ischemic stroke. Imaging-based selection of the patient, which incorporates an understanding of the physiologic changes in brain perfusion and collaterals may help in identification of eligible patients, maximize clinical benefit and improve allocation of resources[35].

1.5.5 Biochemistry of cerebral ischemia

Brain ischemia triggers a sequence of molecular phenomena in the short and the long term, initiated with an energetic failure related to the interruption of oxidative phosphorylation processes and the deficient production of adenosine tri-phosphate (ATP) in neurons **(Figure 4).** The cease of transmembrane ionic gradients due to the failure of sodium-potassium-ATPase pump and other ATP-dependent ionic pumps is the key step of the physiopathologic mechanism in stroke, especially of cell death in the ischemic core, when the vascular occlusion lasts a few minutes[36]. Neurons and glial cells suffer an extreme depolarization because of the entrance of sodium, chloride, calcium and water into the cytoplasm[37]. In addition, potassium leaves the cell, inducing a sudden increase in extracellular potassium[38].

The energetic failure and the associated ionic changes originate an increment in glutamate, an hyperexcitability of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and NMDA (N-methyl-D aspartate) receptors, which induces an even higher increase of intracellular calcium[39-41].

The increase of intracellular calcium does not exclusively depend on the activation of glutamate receptors, but in the stimulation of calcium voltage-dependent channels. Hyperexcitability causes a depolarization phenomenon in the periphery of the infarct which increases the energetic cost while the membrane tries to re-polarize itself[42-44].

Calcium increase, together with acidosis and peri-infarct depolarization, contributes to initiate the damage; later on, inflammation and activation of apoptotic phenomena contribute to increase the lesion[41, 45]. During ischemia, and particularly during reperfusion, free radicals are generated. Such molecules are highly reactive and are produced at both the initial and late stages of brain ischemia via different physiopathological mechanisms. In first place, oxygen reactive species are produced by the metabolism of arachidonic acid and the neuronal NO (Nitric Oxide) synthase (nNOS). During intermediate stages, free oxygen radicals are provided by the infiltration of neutrophils in the ischemic area and at late stages are produced via the synthesis and activation of inducible NO synthase (iNOS) enzymes and cycloxygenase-2 (COX-2) [46]. Ischemic stroke triggers a series of complex molecular events, among which activation and expression of genes are included. Some of those events arise from the immediate reaction of neurons to damage[47], and others are associated to cellular processes that determine the fate of the affected neurons[48] or coordinate the repair mechanisms[49, 50].

Ischemic cellular death can take place in two different ways. The most common one, described in the classical treatises, is necrosis[51]. This type of cellular death is the result of the acute energetic failure, with loss of morphology and, in the end, cellular lysis, triggering inflammatory processes. On the other hand, apoptotic or programmed cell death can be observed, when energy-dependent intracellular mechanisms are activated, leading to regulated cell degradation, being later eliminated by phagocytic cells without inflammatory reaction[45, 52].

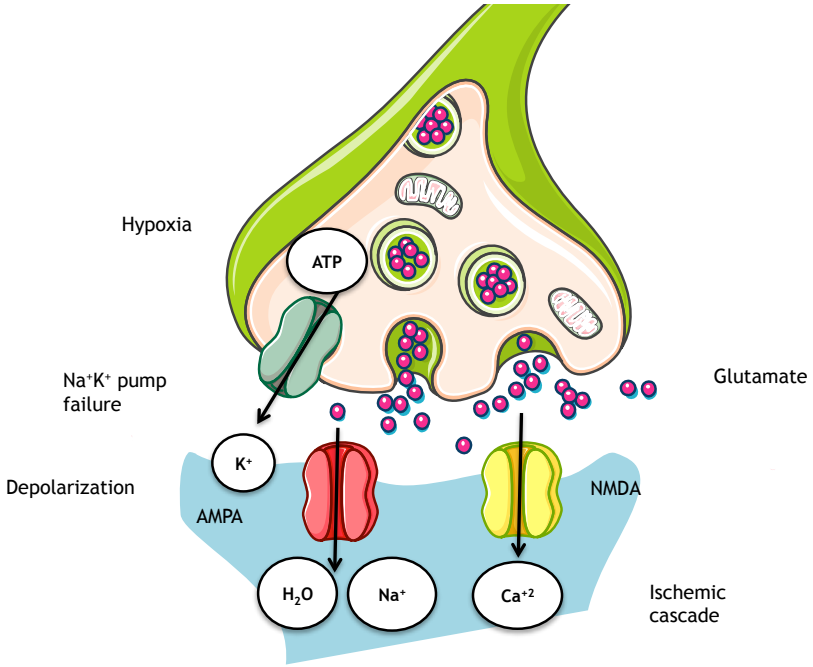

Figure 4: Pathophysiology of ischemia in neurons: Hypoxia induces energetic failure, leading to a membrane depolarization, increasing glutamate levels. Glutamate acts on AMPA and NMDA receptors.

In physiological conditions, astrocytes have a fundamental action in controlling glutamate action, and they are the responsibles for glutamate reuptake in synaptic cleft. The glutamate transporters are membrane sodium gradient-dependent. Once inside the astrocytes, glutamate is transformed for new neutrotransmitters production.

After ischemia, the first change is astrocyte edema, due to the energetic failure. This edema decreases glutamate reuptake inside the cell. Free radical and lactic acid released from anaerobic glucose metabolism in ischemia also decrease glutamate reuptake. That increases glutamate levels in extracellular space and induces even higher cell damage. Other glial cells, such as oligodendrocytes and microglia, also play a role in brain ischemia. In oligondendrocytes,

energy decrease causes a change in ion gradient, altering sodium and calcium transporters function and increasing these molecules inside cells. Microglia also contributes to tissue injury through inflammatory molecules and free radical release.

1.6 INFLAMMATION AND STROKE

Although different mechanisms are involved in stroke pathogenesis, there is increasing evidence showing that inflammation accounts for its progression[53, 54].

An important inflammatory reaction follows ischemic stroke **(Figure 5)**. After ischemia onset, inflammatory cells such as blood-derived leukocytes and microglia are activated and accumulated within brain tissue, subsequently leading to inflammatory injury. Increasing evidence shows an important relationship between central nervous system and immune system[55]. Cytokine levels in cerebrospinal fluid (CSF) and blood increase in stroke patients, and these changes have a clinical correlation with infection, functional outcome or mortality[56, 57].

Decrease in cerebral blood flow leads to energy depletion and necrotic neuron death, triggering immune response leading to inflammatory cell activation and infiltration[54].

Reperfusion of the occluded vessel, either due to compensation by the collateral circulation, spontaneous or therapeutic recanalization, leads to the generation of reactive oxygen species (ROS) by reperfusion with oxygenated blood or production within brain and immune cells. ROS can stimulate ischemic cells, even ischemic neurons,

to secrete inflammatory cytokines and chemokines that cause, among other things, adhesion molecules upregulation in the cerebral vasculature and peripheral leukocyte recruitment, respectively.

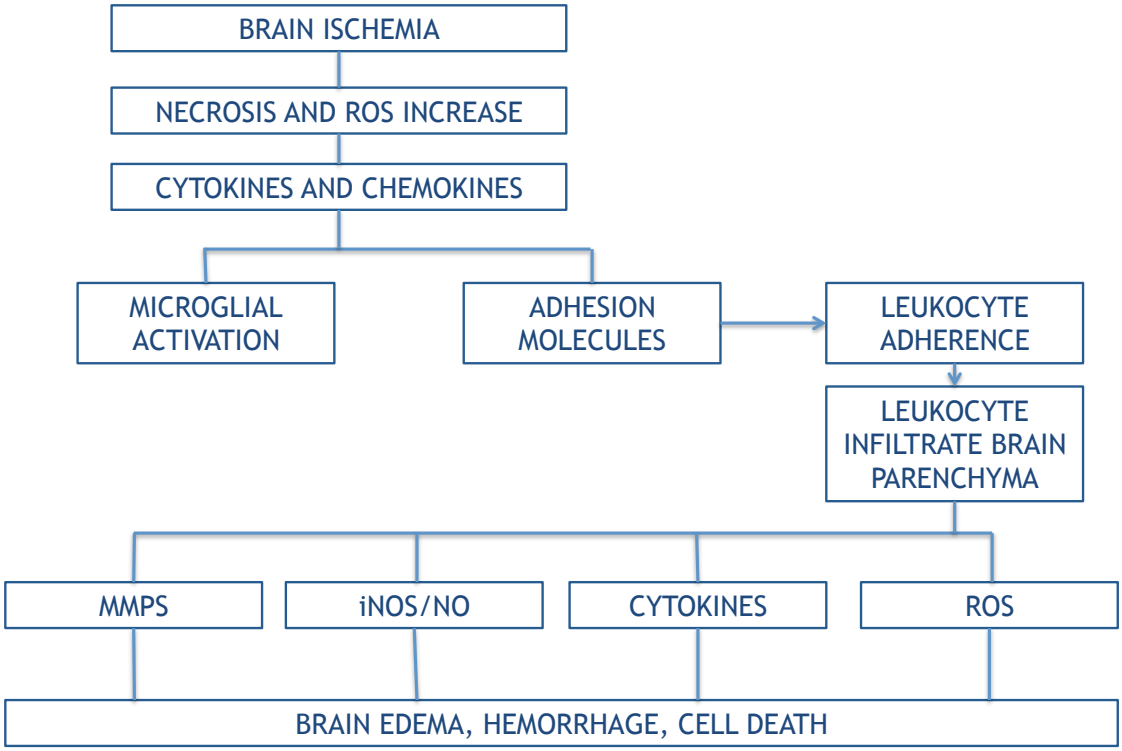

Figure 5. Inflammation and stroke: brain ischemia triggers inflammatory responses due to the presence of necrotic cells and infiltration into the brain parenchyma. Adapted from[54].

Once activated, inflammatory cells can release a variety of cytotoxic agents including more cytokines, matrix metalloproteinase (MMPs), nitric oxide (NO) and more ROS . These substances may induce more cell damage as well as disruption of the blood-brain barrier (BBB) and extracellular matrix[58, 59]. BBB disruption can further potentiate brain tissue injury and contribute to a secondary ischemic brain damage by permitting serum elements and blood to enter the brain. Secondary damage is developed as a consequence of brain edema, post-ischemic microvascular stasis and vasomotor/hemodynamic deficits leading to

hypoperfusion and postischemic inflammation, thus involving activation of microglia and brain infiltration of peripheral inflammatory cells.

Immune response following ischemic stroke is produced firstly by an innate system response and further by a response of acquired immune system.

The **innate immune system** is the responsible of an initial and early response of the organism against cell damage. Is compounded of chemical and physical barriers, inflammatory cells (macrophages, neutrophils and natural killer lymphocytes) and blood proteins (complement system, inflammatory mediators…).

Following ischemic damage, neuron destruction leads to DAMPs (damage-associated molecular patterns) release to extracellular environment, triggering innate and adaptive immunity and stimulating ischemic cascade[60].

DAMPs include HMGB1 (chromatin-associated protein termed high mobility group protein B1), uric acid, heat shock proteins, ATP, S100 protein, heparan sulphate, DNA (deoxyribonucleic acid) and RNA (ribonucleic acid). DAMPs release can have both positive and deleterious effect. HMGB1 inhibition had a protective effect in some experimental studies and in some of them improve functional outcome[61]. Uric acid administration appears to be neuroprotective following transient focal cerebral ischemia and higher uric acid levels are related to a higher reperfusion level in patients after intravenous thrombolysis[62].

Receptors involved in antigen recognition are also part of innate inflammatory response, such as Toll-like receptors, RIG-1 (retinoic acid inducible gen I) receptors, NOD (nucleotid binding oligodimerization domain) receptors, C-lecitin type receptors and AIM2 (apoptosis inhibitor of macrophagues 2) type receptors [63] and receptors that activate signalling pathways through nuclear factor κβ (NFκβ) or mitogen activated protein kinase or interferon 1 (INF1) signalling pathways, turning up proinflammatory mediators, chemokines, ROS and costimulator signals. Receptor activation leads to an upgrade of reactive T cells to specific antigens.

Monocytes are part of innate immunity, playing an important role in immune regulation and tissue repair. Monocytes levels increase in blood after ischemic stroke and show a decrease in antigen presenting cell molecules and a decrease in TNF (tumor necrosis factor) production, related to infection risk after stroke[64].

Complement system is activated following ischemic stroke. Lecitin complement pathway is activated through MBL (mannose binding lecithin) and associated protease[46].

After innate immunity, **adaptive immune response** provides a more specific system against cell damage. Adaptive response is slower but more specific than innate immunity. Adaptive immune response can "remember" a previous exposition to a pathogen and offers an earlier response if there is a new exposition to the antigen. T and B lymphocytes, cytokines and antibodies are the main compounds of adaptive response. Response starts after recognition of antigen by lymphocytes, followed by a proliferation and differentiation of effector

cells, causing cytotoxic or protector effects.

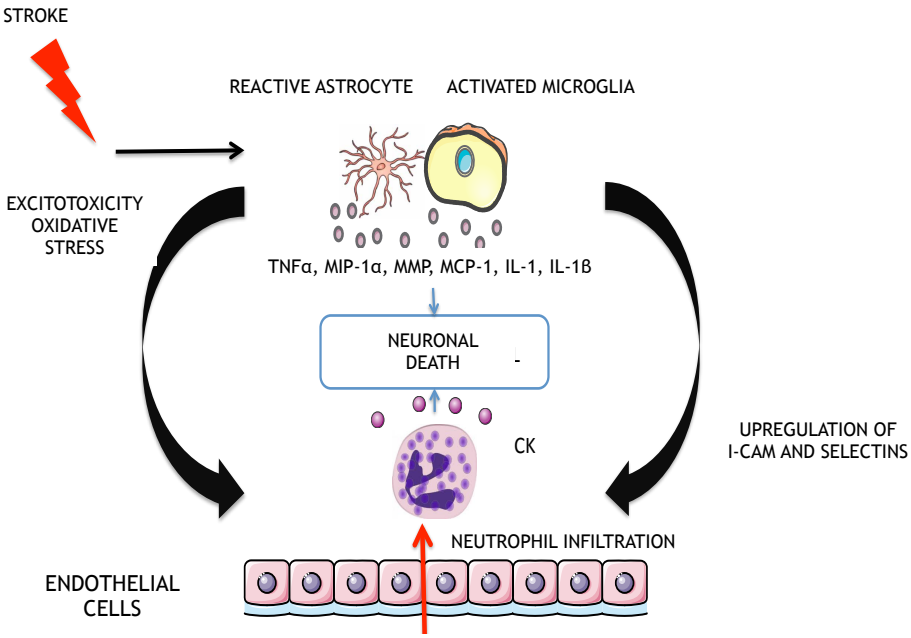

Figure 6. Inflammatory response after stroke. Modified from[66].

Within seconds to minutes after the loss of blood flow to a region of the brain, the ischemic cascade is rapidly initiated [65]. Ischemic cascade comprises a serial of subsequent biochemical events that eventually lead to disintegration of cell membranes and neuronal death at the core of infarct. Ischemic stroke begins with severe focal hypoperfusion, which leads to excitotoxicity and oxidative damage that in turn cause microvascular injury, blood-brain barrier dysfunction and initiate post-ischemic inflammation. These events all exacerbate the initial injury and can lead to permanent cerebral damage (**Figure 6**). The amount of permanent damage depends on several factors, mainly the degree and the duration of ischemia and the capability of the brain to recover and repair itself.

Different elements are implied in inflammatory response following stroke: cellular response to ischemic stroke, adhesion molecules,

inflammatory mediators and regulatory transcription factors.

1.6.1 Cellular response to ischemic stroke

After ischemia onset, inflammatory cells such as blood-derived leukocytes and microglia are activated and accumulated within the brain tissue leading to inflammatory injury. Cells implied in inflammatory process are: leukocytes, macrophages and microglia, macroglia, astrocytes, oligodendrocytes, epithelial and endothelial cells.

1.6.1.1 LEUKOCYTES

Four-six hours after ischemia onset, circulating leukocytes adhere to vessel walls, leading to migration and accumulation into ischemic brain and releasing proinflammatory mediators. Brain SPECT (single-photon emission computed tomography) studies showed leukocyte deposit inside ischemic areas[67]. Microscopic studies in brain microcirculation after brain ischemia demonstrated leukocyte accumulation in postcapilar veins followed by platelet aggregation, with a proinflammatory and prothrombotic effect.

Neutrophils are generally the first leukocyte subtype recruited into the ischemic brain and into the penumbra area around infarct tissue[67]. Within six and twelve hours following ischemic stroke there is an accumulation of neutrophils in ischemic tissue. Neutrophil levels stay high during 6 or 9 days, when neutrophil level decreases and return to normal values[68]. There is also a correlation between neutrophils accumulation in stroke area and higher stroke volume and worse functional outcome.

Under physiological conditions, neutrophils circulate and have no interaction with endothelial cells, whereas lymphocytes and monocytes pass through capillary walls constantly. When endothelial cells are damaged, neutrophils cleave to these cells through a rolling mechanism, boosted by extravascular stimuli, such as bacterial derived products or other endogenous inflammatory mediators. In a first place, the rolling process is conducted by P-selectin (inside endothelial cells) to L-selectin (in leukocytes) union. Later, the relationship is conducted by E-selectin (endothelial cells) to L-selectin union. Once leukocytes join together the endothelial cells through selectins, leukocyte integrin receptors are activated thanks to platelet activating factor (PAF) in the endothelial membrane and to the chemokines secreted by inflammatory cells.

Lymphocyte levels increase in peripheral blood after neutrophils following permanent middle cerebral artery occlusion in rats[69]. Studies show that lymphocytes have strong proinflammatory and tissue-damaging properties. The upregulation of circulating lymphocytes is correlated to an increased risk of stroke recurrence and death[70].

T lymphocytes are the most relevant cells in inflammatory response following ischemic stroke. Inactive naive T cells do not go through the blood brain barrier, but that fact changes if there is a response of T lymphocyte against antigens started in lymphoid organs.

Hours following stroke, immunologic agents are presented by APC (antigen presenter cells) to effector cells and T lymphocytes are reestimulated. There are different types of cells in the nervous system that could act as antigen presenter cells (microglia, macrophages…) [71]

and produce proinflammatory cytokines such as TNFα, IL-1β (Interleukin 1β) or IL-6 (Interleukin 6). These proinflammatory cytokines stimulate adhesion molecules production and enhance T lymphocyte recruitment. T cells can join the vein endothelium, penetrate into the tissue and produce cellular necrosis.

In brain ischemia there is short time between damage and T cells infiltration. That is why the immune response is though to be non-specific, because there is not enough time for an antigen specific response.

T lymphocytes act by different mechanisms in tissue damage after ischemic stroke **(Figure 7).** They are the most significant effector cells in late stage of brain ischemia. T lymphocyte levels in ischemic brain increase 24 hours following ischemia/reperfusion and reach the highest levels 3 days following stroke[72]. T lymphocytes are distinctively located in frontier of ischemic area, near blood vessel. Lymphocyte subgroups that have an important role in brain ischemia are: Tγδ lymphocytes (20-30%), CD8+cytotoxic T lymphocytes (20-30%) and CD4+ T lymphocytes (30-40%).

Cytotoxic CD8+ T lymphocytes recognize their antigen in the context of major histocompatibility complex (MHC) class I antigens on the surface of target cells. In normal human brain tissue, class I antigens are constitutively expressed on all cerebral endothelial cells and on some microglia cells. Under inflammatory conditions, these MHC molecules may appear on any nervous system cell, and their expression is regulated by the intensity of the proinflammatory stimulus (IFNϒ and TNFα). All nervous system cells may become

targets for cytotoxic T-lymphocytes. Cytotoxic T-cells may kill their target cells through cytotoxic granules, containing perforin and granzymes, or through TNF family receptor-mediated cytotoxicity, by the interaction between Fas-receptor with Fas ligand[72]. For these reasons, direct CD8+ T-cell mediated cytotoxicity could play a major role mediating inflammatory damage in the CNS following brain ischemia/reperfusion.

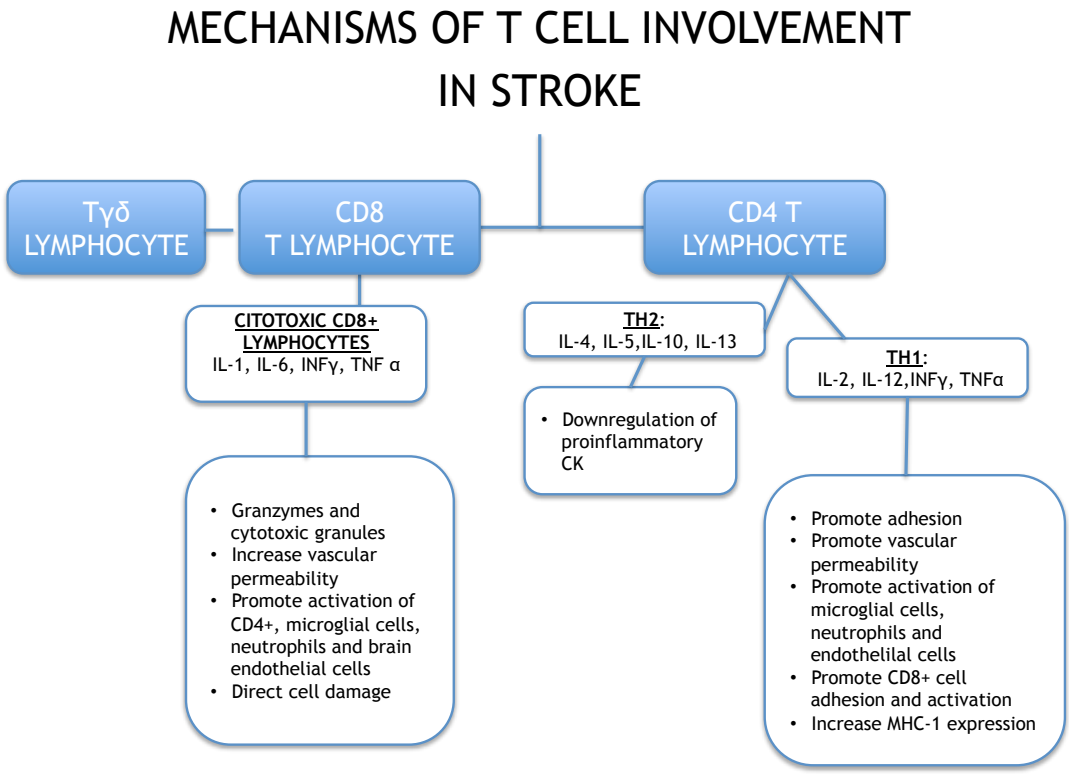

Figure 7. T cell mechanisms involved in stroke. Lymphocyte subgroups that have an important role in brain ischemia are Tγδ lymphocyte (20-30%), CD8+cytotoxic T lymphocyte (20-30%) and CD4+ T lymphocyte (30-40%). Adapted from[72].

Tγδ lymphocytes differentiation (20-30%) is conducted by IL-23, a cytokine produced by macrophages infiltrating the ischemic tissue on the first day following stroke. This lymphocyte subgroup produces IL-17, a proinflammatory cytokine. IL-23 and IL-17 deficiencies, reduce

infarct size one to four days following ischemic stroke[73]. IL-17 receptor is universally expressed in brain cells and modifies inflammatory response in CNS.

IL-17 receptor promotes proinflammatory cytokine expression and macrophage chemokine production[74]. It also regulates endothelial barrier function, promoting matrix metalloproteinase and ICAM-1 expression[75, 76]. IL-1β, IL-23, IL-6 and TGFβ, are needed for IL-17 production by Tγδ lymphocytes. Clinical studies using neutralizing antibodies against IL-23 and IL-17 have been used for different diseases treatment (asthma e.g.).

Although central nervous system is isolated form peripheral blood thanks to blood brain barrier, ***CD4+ T lymphocytes or helper lymphocytes***, can infiltrate CNS following ischemia. Once CD4+ lymphocytes go through blood brain barrier, microglia stimulates them to turn into different lymphocyte subgroups, mainly T helper type 1 (TH1) and T helper type 2 (TH2).

CD4+ lymphocytes include, TH1, TH2 and other subgroups of lymphocytes such as follicular T helper, TH17, TH9 and regulatory T lymphocytes[77].

The exact mechanism of CD4+ lymphocytes involvement in tissue damage is unknown. CD4+ can participate in cellular apoptosis through proinflammatory cytokine production (IL-2, INFϒ, TNFα or FAS dependent). Inside CNS there are not as many inflammatory mediators or costimulatory molecules as in other lymphoid tissues, so inflammatory response cannot be regulated. Activated T lymphocytes

can contribute to stroke pathogenesis thanks to their accumulation inside microcirculation, disturbing perfusion and increasing hypoxia.

TH1 lymphocytes produce proinflammatory cytokines: IL-2, IL-12, INFϒ and TNFα. INFϒ has a neurotoxic effect, acting directly over neurons, inducing cell death[78]. However, clinical studies do not show neuroprotective effect of INFϒ deficit. IL-12, produced by macrophages, dendritic cells and neutrophils has a main role in differentiation of T helper lymphocytes.

TH2 lymphocytes secrete anti-inflammatory cytokines: IL-4, IL-5, and IL-10. IL-4 deficiency increases ischemic tissue damage and neurologic symptoms[79].

Follicular TH lymphocytes can express high CXCR5 (C-X-C chemokine receptor type 5) levels and secrete anti-inflammatory cytokines such as IL-10 and IL-21.

TH17 lymphocytes produce IL-1A and play a main role in the induction and the propagation of autoimmunity disorders in diseases such as multiple sclerosis, rheumatoid arthritis or allergy.

The last subpopulation of CD4+ cells is regulatory T lymphocytes (Treg). These subgroup of lymphocytes will be analyzed later in this text.

1.6.1.2 MICROGLIA AND MACROPHAGES

Macrophages levels increase between 12 and 24 hours following ischemia and reperfusion and reach a peak the third day after ischemia[80]. Macrophages produce proinflammatory cytokines such as IL-1β, TNFα and IL-23, promoting inflammation. Macrophages and

microglia (resident macrophages of the brain), play a critical role as immunocompetent and phagocytic cells in the central nervous system[81] and serve as scavenger cells in the event of infection, trauma, ischemia and neurodegeneration. Once activated, microglia can undergo morphologic transformation into phagocytes, making them virtually indistinguishable from circulating macrophages.

This activation may be induced by cerebral ischemia, causing a release of cytotoxic and/or cytoprotective substances. Via CD4, microglia are activated, followed by stimulation of Toll-like receptor 4 (TLR-4). How microglia are activated following ischemia is not completely clear, but some receptors have been documented in monocytes and activated microglia in brain of stroke patients.

Whether microglia and macrophages are necessarily deleterious in brain ischemia remains unclear, but a few lines of evidence suggest that activated microglia may contribute to injury. *Edaravone*, a novel free radical scavenger, significantly reduced the infarct volume and improved the neurological deficit scores in brain ischemic models mice by reducing microglial activation[82]. In spontaneously hypertensive rats with permanent MCAO, repetitive hyperbaric oxygen treatment obviously reduced the infarct volume by suppressing microglia activation[83]. Minocycline, a tetracycline antibiotic, was shown to provide significant protection against brain ischemia by inhibition of microglial activation and proliferation[84].

However, some studies indicate that microglia and macrophages or their secreted factors may actually protect cells after ischemic stroke[85]. No effect on infarct size was seen after depleting peripheral

macrophages using liposome-encapsulated clodronate[80]. However, this latter study did not deplete brain macrophages and may suggest that brain macrophages, rather than circulating macrophages, are important in brain ischemia pathogenesis.

1.6.1.3 MACROGLIA: ASTROCYTES AND OLIGODENDROCYTES

Following ischemia, astrocytes are activated, resulting in increased expression of GFAP (glial fibrilary acid protein) and leading to a reactive gliosis, characterized by specific structural and functional changes[86]. Astrocytes also participate in brain inflammation by expressing major histocompatibility complex (MHC) and costimulatory molecules, developing TH2 immune response (anti-inflammatory response) and suppressing IL-12 expression. Astrocytes are also capable of secrete inflammatory factors such as cytokines, chemokines and inducible nitric oxide synthase (iNOS) [87]. Furthermore, inducible nitric oxide synthase (iNOS) in astrocytes has been show to potentiate ischemia like injury to neurons.

A member of the tumor necrosis factor superfamily, the tumor necrosis factor-like weak inducer of apoptosis (TWEAK), is though to be produced by neurons, astrocytes and endothelial cells. Expression of TWEAK can stimulate proinflammatory molecules.

These data suggest that, while astrocytes normally play important role in neuron maintenance and function, activated astrocytes have the potential to produce harm to ischemic brain[87].

1.6.1.4 ENDOTHELIAL CELLS

The main function of endothelial cells is the maintenance of vessel

integrity. Under normal conditions, endothelium plays an anticoagulant and antithrombotic role, improving blood flow. Following injury, such as produced in brain ischemia, endothelial dysfunction happens, disturbing homeostatic properties of endothelium, leading to an increase of adhesion and permeability to leukocytes and platelets[88]. Damaged endothelium changes from an anticoagulant role to a procoagulant role. As a response against endothelial damage, lipids and monocytes are accumulated within ischemic areas. Due to inflammatory response, acute phase reactants are released from liver, such as C reactive protein, fibrinogen, prothrombin… C reactive protein stimulates mononuclear cells that release tisular factor, which activates coagulation, activates complement pathway and neutralizes platelet activator factor[89]. Fibrinogen plays an important role in hemostasia, because it is transformed into fibrin thanks to thrombin. All these mechanisms lead to a prothrombotic status, hindering blood circulation.

1.6.2 Adhesion molecules

Adhesion molecules play a pivotal role in the infiltration of leukocytes into the brain parenchyma after stroke and may represent important therapeutic targets. Three major steps, rolling, adhesion and transendothelial migration of leukocytes, are involved in the access of leukocytes to the brain through the endothelial wall.

Some studies showed that blocking adhesion molecules and inhibiting leukocyte adhesion reduces neurologic damage following stroke[90].

Interaction between leukocytes and vascular endothelium is mediated by three main groups of cell adhesion molecules: selectins (P-selectin, E-selectin and L-selectin), the immunoglobulin superfamily and integrins.

1.6.2.1 SELECTINS

Selectins mediate cell to cell adhesion and rolling of leukocytes on the postcapillary venules endothelium. There have been identified three kinds of selectins: E-selectin, P-selectin and L-selectin. They are expressed on the outer cell membrane immediately upon cell activation by stimulants such as thrombin or histamine[91]. While E and P-selectin are involved in leukocyte rolling and recruitment during the early stages of activation, L-selectin acts as a guide for unstimulated leukocytes.

E and P-selectins upregulation appears to be involved in promoting ischemic inflammatory responses and increases injury due to ischemic stroke. In animal studies, mice overexpressing P-selectin had exacerbation of their infarcts, whereas treatment with antibodies or inhibitors against P and E-selectin were associated with improved neurological outcome[92].

P-selectin deficient mice demonstrated smaller infarct volume and improved survival compared with wild-type mice. Functional blockade of P-selectin using a monoclonal antibody also improved early reflow and stroke outcome, with reduced cerebral infarction volume even when the blocking antibody was administered after ischemia onset.

Nasal administration of E-selectin can induce immune tolerance

against brain antigens, reducing ischemic damage and even preventing a recurrence via regulatory T cell induction[93].

1.6.2.2 IMMUNOGLOBULINS

Members of the immunoglobulin superfamily include 5 molecules: ICAM-1 (intracellular adhesion molecule-1 o CD-54), ICAM-2 (intracellular adhesion molecule-2), VCAM-1 (vascular cellular adhesion molecule-1) PECAM-1 (platelet endothelial cell adhesion molecule-1) and MAdCAM-1 (mucosal vascular addresin cell adhesion molecule-1).

The most extensively immunoglobulins investigated in cerebral ischemia have been ICAM-1 and VCAM-1. ICAM-1 is expressed within the first hours following stroke and peaks at 12-24 hours, preceding leukocyte infiltration. Several studies have shown that blocking ICAM-1 with antibodies or inhibiting ICAM-1 RNA with antisense oligonucleotides, improves outcome after experimental stroke[94].

The role of VCAM-1 in stroke is less clear. Several studies showed that VCAM-1 mRNA increases following stroke, but other have failed to observe such significant changes. In a study of global cerebral ischemia in rats, potent leukotriene receptor antagonist, improved neurological deficits and reduced neuron death by inhibiting ischemia and inducing upregulation of VCAM-1 in the hippocampus of ischemic rats[95].

In clinical studies, increased ICAM-1 and VCAM-1 have been documented in plasma and cerebral spinal fluid of subjects with recent cerebral ischemic damage, and correlated to stroke severity [57,96].

However a phase III clinical trial of anti-ICAM therapy for stroke indicated that anti-ICAM therapy with *Enlimomab* was not an effective treatment for ischemic stroke. In fact, treatment significantly worsened outcome[97].

1.6.2.3 INTEGRINS

Integrins consist of a common subunit, β and a variable subunit, α. There are three subfamilies for the β subunits (denoted β_1, β_2 and β_3). Members of the β_1 subfamily bind collagen, laminin and fibronectin and are involved in extracellular matrix structure. β_2 integrins (CD18) are involved in leukocyte cell adhesion. β_3 integrins are involved in clot generation and stabilization.

Leukocyte integrins are activated by chemokines, cytokines and other chemoattractants. Intengrins must be expressed on the cell surface to recognize endothelial cell adhesion.

β_2 integrins contain a common β_2 domain with one of the three distinct α chains: CD11a or LFA-1 (lymphocyte associated antigen), CD11b or Mac-1 (macrophague 1 antigen) y CD11c. One of the α chains, CD 11b has been the most studied in stroke models. In vitro studies showed that hypoxia causes increase of neutrophil CD11b expression compared to normoxia, and this ischemic injury was regulated by aprotinin. Aprotinin reduced the upregulation of neutrophil CD11b and reduced the damage. Blocking CD11b as well as CD18 or both, reduced injury from experimental stroke and were associated with decreased neutrophil infiltration[98].

Few clinical studies examined the potential of anti-integrin

therapies in acute stroke patients. The HALT stroke trial used anti Mac-1 antibodies (Hu23F2G LeukoaArrest) in patients presenting within 12 hours after onset symptoms. The trial was stopped after enrolling 400 patients because of the lack of functional outcome improvement. However in preclinical studies, Hu23F2G was effective when administered 20 minutes after ischemia. The ASTIN trial[99] tested the recombinant neutrophil inhibitory factor (rNIF), a non-antibody small molecule, in acute stroke with a 6-hour time window. Patients presenting within 3 hours were also treated with tissue plasminogen activator or placebo. The trial was terminated prematurely after enrolling 966 patients because lack of efficacy. Although both compounds appeared to be effective in rodent stroke models[100], lack of an obvious effect in humans could be due to study design not in line with laboratory data (moment of treatment) or the inherent heterogeneity of clinical stroke.

1.6.3 Inflammatory mediators

The most important inflammatory mediators are: cytokines, chemokines, arachidonic acid metabolites, nitric oxide, reactive oxygen species and matrix metalloproteinases. Molecular markers of inflammation can be useful tools for the management of patients with ischemic stroke, both during the acute phase and to predict prognosis and prevent the risk of a new vascular event[101].

1.6.3.1 CYTOKINES

Cytokines are upregulated in the brain after a variety of insults, including stroke. Cytokines are expressed by immune system cells and

also by brain resident cells, including glia and neurons. The most studied cytokines related to inflammation in stroke are IL-1, TNFα, IL-4, IL-10 and TGFβ. Among those cytokines, IL-1 and TNFα exacerbate injury following stroke, while IL-10 and TGFβ may be neuroprotective[102] **(Table 1)**.

IL-1 can have two different isoforms (IL-1α and IL-1β) and there is also an endogenous inhibitor, IL-1 receptor antagonist (IL-1ra). It had been demonstrated that 15 or 30 minutes following stroke levels of mRNA of IL-1β increase, and levels of protein increase a few hours later[103]. Between six and twenty four hours after ischemia, there is a second peak of IL-1 expression.

PROINFLAMMATORY CYTOKINES	ANTI-INFLAMMATORY CYTOKINES
IL-1	IL-10
TNFα	TGFβ
IL-8	IL-4
IL-6	IL-13

Table 1. Proinflammatory and anti-inflammatory cytokines in ischemic stroke.

When IL-1β is administered to rats, there is an increased brain damage[104]. Moreover, deficient IL-1 mice had smaller infarcts compared with wild-type. Overexpression of IL-1ra or treatment with exogenous IL-1ra reduced infarct size[105], while IL-1ra deficient mice

exhibited a dramatic increase in ischemic damage.

IL-1 has two receptors, IL-1R1 and IL-1R2. Inactivating or knocking out the IL-1R1 decreased infarct size and preserved neurologic function[106, 107]. It is believed that deleterious effect produced by IL-1 is due to temperature increase, blood pressure increase, damage mediated by NMDA, microglial proliferation, araquidonic acid production and stimulation of nitric oxide synthesis.

IL-1 also acts over endothelium, promoting a prothrombotic state[108], leading to the expression of tisular factor, which activates instrinsic coagulation pathway, increases the synthesis of platelet activator factor and inhibits fibrinolisis. Moreover IL-1 promotes release of other cytokines such as IL-6, IL-8, TNFα or growth factors and also promotes itself production.

TNFα first increase is seen 1-3 hours after ischemia onset. TNFα has a biphasic pattern of expression, with a second peak between 24 and 36 hours. TNFα is produced by neurons, astrocytes, microglia and peripheric immune system.

TNFα inhibition reduces ischemic brain injury, while administration of recombinant TNFα protein in early stages of ischemic onset worsens brain damage[53]. However, TNFα can protect brain under some circumstances. It has been proposed that this cytokine is involved in ischemic tolerance[109]. These differences in experimental data might be due to different pathways of TNFα signals.

There are at least two TNFα receptors: TNFα receptor 1 and TNFα receptor 2. Most of TNFα effects are mediated by TNFα receptor 1,

which contains a death domain (DD) that interacts directly with TNFR1 and may act as a bifurcation point for signaling related to cell death or cell survival. TNFα can produce apoptosis through FADD (*Fas associated death domain)* and caspase 8. TNFα-receptor 2 activation, may lead to anti-inflammatory and antiapoptotic functions[110].

IL-10**,** an anti-inflammatory cytokine, acts inhibiting IL-1 and TNFα and also suppressing cytokine receptor expression and receptor activation. It is synthesized in the central nervous system and is upregulated in experimental stroke. Both exogenous administration and gene transfer of IL-10 in cerebral ischemia models appear to have beneficial effects[111, 112]. Patients with acute ischemic stroke have an elevated numbers of peripheral blood mononuclear cells secreting IL-10[113]. One study showed decreased levels in acute ischemic stroke patients compared to controls [114]. Some studies showed elevated IL-10 concentrations in cerebrospinal fluid and peripheral blood following ischemic stroke and found relationship between higher levels of IL-10 and good outcome after stroke[115-118]. Some studies also showed that serum levels of IL-10 facilitate the selection of ischemic stroke patients with clinical diffusion mismatch and perfusion diffusion mismatch for systemic thrombolysis[119]. High levels of interleukin-10 are associated with clinical diffusion mismatch[120].

IL-4 is a 19 kDa protein produced by thymic cells and T lymphocytes. It has anti-inflammatory activity trough a negative feedback that reduces cytokine production[115]. In ischemic stroke IL-4 inhibitory function is less important than IL-10[102, 121].

TGFβ1, (transforming growth factor β1) is a 25 kDa protein which

regulates and stimulates cell proliferation and differentiation and plays an important role in tisular repair[122]. TGFβ1 inhibits cytokine synthesis, bearing on ARNm traduction. It promotes expression of cytokine antagonists, that are competitive with cytokines in the union with receptors and also inhibits E-selectin synthesis by endothelial cells[123].

Autopsy studies in stroke patients showed an increase of TGFβ in the tissue around ischemic area (penumbra area). On the other side, TGFβ blood levels are lower in subjects with ischemic stroke than in controls, due to the accumulation of this cytokine in brain tissue[124].

TGFβ plays an important role as a protector factor in brain ischemia[125, 126]. To explain this positive effect, some mechanisms have been proposed: decrease of neutrophil adhesion to the endothelium surface[127], decrease of the release of oxygen and nitrogen derived products by macrophagues[128, 129], promotion of angiogenesis in the ischemic penumbra area and decrease of the expression and the efficacy of other cytokines such as TNFα[130]. Overexpression of TGFβ1 using an adenoviral vector, protected mouse brain from ischemic stroke and reduced the inflammatory response[131].

IL-6 is largely thought as a proinflammatory cytokine, but its role in ischemic stroke is far from clear. IL-6 deficient mice have similar size infarcts compared to wild-type, suggesting that it does not participate in ischemic pathogenesis[132,133]. However, other studies suggest either a beneficial or detrimental role in ischemic stroke. Clinical studies in stroke patients showed that higher levels IL-6 are related to poor outcome[134]. IL-6 levels on admission are associated with early clinical

deterioration[135].

IL-8 is the main cytokine involved in polymorfonuclear cells chemotaxis. IL-8 is produced by monocytes, fibroblasts and endothelial cells. It promotes integrin expression, improving leukocyte adhesion and activation, producing free radicals and increasing capilar permeability[136]. Some studies have shown high levels of IL-8 in stroke, which activate leukocytes in an early stage[137].

IL-13 is expressed in activated T lymphocytes. It acts similarly to IL-4 inhibiting proinflammatory cytokines production by monocytes and macrophagues[138].

1.6.3.2 CHEMOKINES

Chemokines are a family of regulatory polypetides with a role in cellular communication and inflammatory cell recruitment in host defense, such as regulate the migration of leukocytes in inflammatory and immune responses.

Expression of chemokines following focal ischemia is thought to have a deleterious role by increasing leukocyte infiltration. Based on the positions of cisteine residues, chemokines can be divided into four groups: C, CC, CXC, CX3C. Each chemokine acts trough specific and shared receptors belonging to the superfamily of G-protein-coupled receptors. Chemokine levels, such as monocyte chemoattractant protein 1 (MPC1) and macrophague inflammatory protein1α (MIP-1α) increase following ischemic stroke in animal models. Their inhibition or deficiency is associated with reduced injury following stroke[139]. Overexpression of MCP-1 in the brain exacerbates ischemic injury, and

is correlated with recruitment of inflammatory cells[140].

Fractalkine, a neuron expressed chemokine, acts through its G-protein-coupled receptor CX3C. Following ischemia, its expression has been localized in viable neurons in the infarct periphery as well as in some endothelial cells. Expression of its receptor, CX3CR1 (CXC3 receptor 1) was observed only in microglia/macrophagues, suggesting that fractalkine is involved in neuron-microglial signaling. Furthermore, fractalkine deficient mice have smaller infarct volume and lower mortality after transient focal cerebral ischemia, suggesting that this chemokine exacerbates cell death[141].

In addition to their chemotactic properties, chemokines were found to affect directly to blood brain barrier permeability. The addition of MCP-1, enhanced the permeability of an in vitro blood brain barrier model and caused alterations in tight junction proteins, suggesting that MCP-1 plays a role in opening the blood brain barrier [142]. Chemokines may also play a role in honing stem cells to regions of injury.

1.6.3.3 ARACHIDONIC ACID METABOLITES

The arachidonic acid cascade is initiated via phospholipase A_2 (PLA_2) release. Energy failure due to blood flow decrease in a brain area can result in calcium accumulation in brain cells. This high calcium concentration activates PLA_2 wich hydrolyses glycerophospholipids to release arachidonic acid. Following transient MCAO (middle cerebral artery occlusion), PLA_2 activity significantly increases[143] and produces arachidonic acid metabolites that contribute to post-ischemic brain inflammation following ischemia. PLA_2 deficient

mice had smaller infarcts and developed less brain edema with fewer neurological deficits than wild-type[144]. Arachidonic acid is metabolized through two different pathways via cyclooxygenase (COX) or lipoxygenase (LOX).

a) Cyclooxigenase pathway:

Arachidonic acid is released from brain phospholipids during ischemia and reperfusion and is converted to prostaglandin H2 (PGH_2) by COX. There are two isoforms of COX.

COX-1 is constitutively expressed in many cell types including microglia and leukocytes during brain injury. COX-1 deficient mice have increased vulnerability to brain ischemia. That would support a protective role of COX-1 due to an effect on cerebral blood flow maintenance. However, conflicting data exists. In transient global cerebral ischemia, pharmacologic inhibition of COX-1 increased the number of healthy neurons in the hippocampus. These discrepancies may be due to differences in the immune responses between focal and global cerebral ischemia models.

COX-2 is upregulated and present in the border of the ischemic territory. In postmortem specimens of ischemic stroke patients, COX-2 is not only present in regions around the ischemic injury, but also in remote areas. Several studies have showed that treatment with COX-2 inhibitors improved neurological outcome after stroke[145]. COX-2 deficient mice showed reduced injury after NMDA exposure, whereas COX-2 overexpression exacerbates brain injury[146]. COX-2 toxic effect is mediated by prostaglandin E_2 (PGE_2) rather than ROS, even though

COX-2 can generate both.

b) Lypoxygenase pathway

Arachidonic acid can be converted to 5-hydroxiperoxyeicosatetranoic-acid (5-HPETE) by 5-lypoxigenase (5-LOX), which is later metabolized to leukotriene A4 (LTA4).

During brain ischemia/reperfusion, biphasic arachidonic acid and LTA4 elevations have been documented and appear to be related to biphasic patterns of blood brain barrier disruption.

Pretreatment with AA-861, a 5-LOX inhibitor, resulted in significant attenuation of leukotriene C4 levels and reduction in brain edema and cell death[147]. However, 5-LOX deficient mice had similar infarct sizes 6 days after permanent and transient MCAO than wild type mice[148].

1.6.3.4 NITRIC OXIDE

Nitric oxide (NO) is an important signaling molecule involved in physiological processes such as neuronal communication, host defense, and regulation of vascular tone. This gas diffuses into cells membrane where it reacts with molecular targets. Three nitric oxide synthases (NOS) isoforms exist; endothelial NOS (type III), neuronal NOS (type I) and inducible NOS (type II). Among these isoforms, inducible NOS (iNOS) is especially relevant to inflammatory cells and may contribute to ischemic injury via nitric oxide. In the brain, ischemia-induced upregulation of iNOS mRNA and protein is associated with increase in iNOS enzymatic activity and NO production, that may cause DNA damage in cerebral ischemia through the formation of peroxynitrite. Moreover, activated astroglial cells

produce large amounts of NO after ischemia, to compensate for energy impairment and oxidative stress[149].

Increased NO in CFS is associated with greater brain injury and early neurological deterioration[150]. Inhibition of iNOS activity decreases glutamate release and ameliorates stroke outcome after experimental ischemia[151]. Protection by hypothermia is associated with reduced microglial generation of both NO and iNOS[152]. A study with the NO donor, glycerol trinitrate in patients with acute stroke and high blood pressure, showed that this treatment might be effective if started very early after stroke onset[153].

1.6.3.5 REACTIVE OXYGEN SPECIES (ROS)

Generation of reactive oxygen species (ROS) by inflammatory cells occurs via several enzyme systems. Superoxide is generated via COX, xantine oxidase and NADPH (nicotinamide adenine dinucleotide phosphate) oxidase. Myeloperoxidase and monoamine oxidase generate hypochlorous acid and H_2O_2.

Superoxide anion is the major one among all the oxidants in the brain parenchyma after MCAO, causing direct injury to ischemic brain or by reacting with NO to generate peroxynitrite. Peroxynitrites are actively involved in triggering cellular survival signals[154].

NADPH oxidase is an enzyme that, after an appropiate stimuli, cytosolic subunits are translocated to cell membrane where they interact with membrane bound subunits to transfer electrons from NADPH to oxygen to form superoxide. Some studies have shown that superoxide generated through this pathway promote damage over

blood brain barrier in ischemic stroke. Intraarterial treatment with superoxide dismutase, an oxygen free radical scavenger, improves neuronal function in the rat subjected to transient global ischemia[155].

Myeloperoxidase (MPO), is thought to mediate bactericidal effect through H_2O_2 (hydrogen peroxide) and hypoclorus acid. After focal cerebral ischemia, infarct size was increased in MPO deficient mice, suggesting a beneficial role. MPO deficient mice also have increased products of nitrosilation within the ischemic brain and suggested that MPO ´s protective effect may be due to its ability to scavenge nitrotyrosine (a product of peroxynitrite reactions) in the presence of glutathione[156].

Mitochondria represents both the main source and target of ROS and nitrogen species. Evidence suggests that disruption of mitochondrial bioenergetics and dynamics may have a critical role in the pathogenesis of these brain diseases. Drug therapies directed toward providing safer mitochondria are currently under both pre- and clinical investigations[157.]

1.6.3.6. MATRIX METALLOPROTEINASES

Matrix metalloproteinases (MMPs) are proteases that can break down extracellular proteins, such as collagen, and are involved in extracellular matrix remodeling as well as in neuroinflammatory response. MMPs are normally found in the cytosol in a pro or inactivated state, but are cleaved by proteases such as plasmin or other MMPs to their active state[158].

In experimental stroke models, MMP inhibition reduces infarct size,

brain edema and hemorrhage[159]. MMP-9 deficient mice had smaller infarcts compared to wild-type controls[160], however, such effect was not observed in MMP-2 deficient mice, suggesting that MMP-9 appears to have a more significant role in stroke compared to MMP-2.

Administration of a broad spectrum MMP inhibitor *GM6001,* was found to significantly decrease the migration of cells that extend from the subventricular zone into striatum in transient focal cerebral ischemia in mice[161].

Matrix metalloproteinases seem to play a different role in the later phases of cerebral ischemia and may participate in plasticity and recovery following stroke. MMPs are associated with factors involved in angiogenesis such as vascular endothelial growth factor (VEGF). Treatment with the MMP inhibitor FN-493, suppressed neurovascular remodelling, increased ischemic brain injury and impaired functional recovery at 14 days after ischemia[162].

MMP-9 levels were used as screening biomarkers for the prediction of parenchymal hematoma after thrombolytic therapy in acute ischemic stroke[163]. Prometalloproteinase 10 was associated with brain damage and poor clinical outcome[164].

1.6.4. Transcriptional regulation of inflammation

It is not totally known how cerebral ischemia upregulates gene expression. Activation of trascriptional factors has been documented in experimental stroke models.

1.6.4.1 NUCLEAR FACTOR KAPPA BETA (NF-κβ)

NF-κβ is a transcriptional factor composed of subunits of the Rel family, wich includes five Rel forms: cRel, RelA, RelB, NF-κβ1 and p50. The most common form is a heterodimer composed of RelA and p50.

Many genes involved in inflammation contain functional NF-κβ sites, such as TNFα, ICAM-1, COX-2, iNOS and IL-6. These genes are correlated to the anti-inflammatory effect of mild hypothermia following experimental stroke. However, the function of NF-κβ in stroke is still controversial. Mice deficient in NF-κβ p50 subunit are protected from experimental stroke[165], consisting with a death-promoting role of NF-κβ in focal ischemia. However, rats given diethyldithiocarbamate, a NF-κβ inhibitor, had enhanced neuronal DNA fragmentation and larger infarct sizes compared to controls, suggesting a beneficial role in ischemic stroke pathogenesis[166]. The reasons for these discrepancies are still not clear, but could be due to the cell type in which NF-κβ is activated, the experimental model studied or a lack of specificity of pharmacological inhibitors.

1.6.4.2 MITOGEN-ACTIVATED PROTEIN KINASE (MAPK)

Mitogen-activated protein kinases play an important role in the transduction of stress-related signals by a cascade of intracellular kinase phosphorylation and transcriptional factor activation that regulate inflammatory gene production among other functions. During cerebral ischemia, three interlinked signaling pathways have been documented: the stress-activated protein kinases/c-Jun N-terminal kinases, the p38 MAPKs and extracellular signal-regulated kinases.

Following brain ischemia in rodents, phosphorylated p38 MAPK, was detected in the hippoccampus within neuronal and microglial cells, suggesting its role in the endogenous inflammatory response. Furthermore, p38 MAPK inhibitors have been shown to reduce brain injury and neurological deficits in animal models of focal cerebral ischemia[167].

1.6.4.3 ACTIVATOR PROTEIN-1 (AP-1)

Fos families (*cFos, FosB*) and jun families (*cjun, junB, junD*) genes encode proteins that form heterodimers, called the AP-1. The Fos protein contains a DNA binding region and a leucine zipper. These dimers bind to specific DNA regions known as the AP-1 domain, which regulates the expression of target genes (collectively referred to as late response genes). Combinations of c-Fos and c-Jun family proteins form different dimers consisting of various subunits depending on the circumstance. The composition of the dimer may determine whether the late response gene is turned on or off. cFos was found to be upregulated as early as 30 minutes after stroke onset.

Inhibition of p38 MAP kinase resulted in the attenuation of c-Fos and c-Jun mRNA and AP-1 DNA binding by lypopolysaccharide, leading a neuroprotective effect in brain ischemia.

In human umbilical vein endothelial cells, a dose dependent lowering of mRNA expression of VCAM-1 and ICAM -1 was observed to inhibit AP-1 DNA binding activities[168]. Recent evidence also showed that selective induction of cFosB, can promote the proliferation of quiescent neuronal precursor cells, enhancing neurogenesis after

transient brain ischemia[169].

1.6.5 Inflammatory mechanisms in ischemic stroke: therapeutic approaches

Despite advances in the understanding of cerebral ischemia pathophysiology, therapeutic options for acute ischemic stroke remain very limited. Only one drug is approved for clinical use for the thrombolytic treatment of acute ischemic stroke in humans and that is intravenous recombinant tissue plasminogen activator (rt-PA) [171,172]. When delivered early following stroke, this treatment reduces neurological deficits and improves functional outcome of stroke patients. However, this treatment has important limitations, mainly the short therapeutic window in wich this treatment is useful and the complications arising from the treatment, such as hemorragic complications[172, 173].

During the last few years, there have been a lot of studies about possible therapeutic targets for the development of new drugs to treat ischemic stroke. Some of this neuroprotective treatments, act over inflammatory response[66, 174, 175]. Drugs acting in different stages of inflammation have been investigated in recent years **(Table 2)**.

Firstly, in order to contain ischemic cascade, some treatments such as hyperbaric oxygen or normobaric oxygen have been studied. Hyperbaric and normobaric oxygen therapies attempt to increase the partial pressure of oxygen to the tissue and thereby limit the damage caused by hypoperfusion. However, three clinical trials of hyperbaric oxygen therapy failed to show efficacy[176]. Normobaric, high-flow

oxygen therapy has shown to cause a transient improvement of clinical deficit and MRI abnormalities in a sub-group of patients with acute ischemic stroke. Further studies are needed to investigate the safety and efficacy of hiperoxia as stroke therapy[177].

Some compounds with antioxidant properties have been studied to decrease the oxidative stress following ischemic stroke. *Ebselen* and *resveratrol*, a substance present in dietetic products such as grapes or red wine, decrease brain damage after stroke in animals[178]. The use of a free radical scavenging U-78389-G failed to reduce cerebral lesion and mortality in rats after embolic cerebral infarct[179].

Other target for the treatment of ischemic stroke is the inhibition of proinflammatory cytokines, without affecting their positive effects. Proinflammatory cytokines in stroke are produced by macrophagues and glial cells. IL-20 inhibition with a specific antibody, decreased stroke size in experimental studies[180]. The use of an IL-1 receptor antagonist is safe and decrease the size of cortical strokes in animal models[181]. Both central and systemic administration of IL-10 to rats subjected to MCAO significantly reduced infarct size 30 minutes to three hours post MCAO[111, 112].

Following ischemic damage in animal models of ischemia, the levels of some chemokines increase, and their inhibition or deficiency has been associated with reduced injury[182]. Mice without chemokine receptor CCR2 are protected against ischemia-reperfusion injury[183].

Due to the key role of cellular adhesion molecules in ischemic stroke pathogenesis, those molecules can also be targets to develop

useful drugs in stroke treatment. Some studies with antiadhesion drugs have been studied in many inflammatory diseases where T lymphocytes play an important role.

There are antibodies against the target LFA1 (CD11a/CD18) and Mac 1 (CD11b/CD15) that act over leukocyte recruitment in cerebral veins following stroke. *Efalizumab* (Reptiva) is a humanized murine antibody against CD11a (subunit a of LFA1). In vitro studies showed multiple effects such as lymphocyte T activation, cutaneous T cell traffic and adhesion of T cells. *BIRT377* inhibits interaction between LFA1 and ICAM-1[184]. Genetic ablation of cellular adhesion molecules (CAM) resulted in reduced infarct size, which could be mimicked by treatment with anti-CAM antibodies. Both *Efalizumab* and *BIRT377* have been used in animal studies for ischemic stroke. Treatment with anti-ICAM1 antibodies, *Enlimomab*, has been proved in ischemic stroke in animal models[185]. Thus far, anti-CAM treatment has not been successful in patients with acute ischemic stroke, moreover, these studies showed that mortality rate and side effects are higher in the treated group compared to controls [186].

There is a clinical trial ongoing about the use of Natalizumab (an monoclonal antibody against integrin subunit α4) in acute ischemic stroke treatment. The primary objective of the study is to determine if one intravenous dose of Natalizumab reduces infarct volume from baseline to day 5 on MRI in subjects with acute ischemic stroke when given at less than 6 hours or at 6 to 9 hours when last seen normal (ACTION study).

Matrix metallproteinase 9 reduction in the first day following

stroke, decreases the size of the infarct on day 14. However, this beneficial effect disappears when the treatment is postponed to the third day following stroke and, when is delivered the seventh day following stroke, this treatment even worsens outcome. These studies suggest that MMP-9 inhibition could have a beneficial or a detrimental role depending on the moment when is delivered (therapeutic window) [141].

Additionally, some therapeutic strategies acting on T lymphocytes have been studied. These treatments, previously used for autoimmune diseases and degenerative diseases, are based on immune regulation, trying to downregulate the immune response mediated by T lymphocyte. *FTY720* is a therapeutic candidate for the modulation of inflammation in stroke. This drug causes a decrease in lymphocyte T level, but efficacy data are controversial. An animal study showed that administration of *FTY720* does not improve prognosis in ischemic stroke[187]; while other studies supported the beneficial effect of this molecule in stroke treatment[188]. On the other side, this molecule increases pneumonia risk, due to the inhibition of defensive function of peripheral T lymphocytes against bacteries. A recent study[189] shows that *FTY720* administration to the rats between the third and the seventh day following ischemic stroke improves functional outcome. It is believed that this effect is mediated through an increase in the expression of VEGFα. Treatment against T cells could prolong therapeutic window[190].

T lymphocyte receptor (TCR) recognizes an antigen, such as a small peptide through a MHC or in the surface of a antigen presenter cell.

The interaction between the receptor and MHC- peptide complex activate T lymphocytes. Drugs that act as antagonists of T cell receptors have been studied *in vitro*[191].

Statins are used in stroke treatment due to the beneficial effect decreasing levels of cholesterol and slowing atherosclerosis progression. Moreover, some studies propose that statins have an immunomodulatory effect, mainly atorvastatin and pravastatin. Firstly, statins inhibit MHC expression induced by INFϒ, decreasing T lymphocyte activation. On the other hand, statins block LFA1, limiting adhesion of T lymphocyte to ICAM-1, decreasing T lymphocyte activation. Both mechanisms inhibit differentiation of naive T lymphocytes to effector T lymphocytes, decreasing their direct action and cytokine release[192]. Atorvastatin treatment *in vitro* and *in vivo* induce transcription of FoxP3 (Fork head box Protein3), increasing amount of regulatory T cells[202].

Inflammatory response after stroke can increase temperature. High temperature after stroke is related with poor outcome. The relationship between brain damage and high temperature is greater the earlier the increase in temperature occurs[204]. Temperature increases in patients with stroke in the first 72 hours, with the harmful effect of high temperature occurring in the first 48 hours. The neuroprotective effect of low temperature occurs within the first 24 hours from stroke onset[205].

NEUROPROTECTIVE AGENT	MECHANISM	REFERENCE
IL-1RA HUMAN RECOMBINANT	Interleukin 1A receptor antagonist	[193]
ENLIMOMAB	Anti-ICAM-I antibody	[186]
TIRILAZAD	Lipidic peroxidation inhibitor	[194]
UK-279,276	Neutrophil inhibitor factor	[195]
CEROVIVE (NXY-059)	Nitrone-based free radical trapping agent	[196]
ACETAMINOPHEN	Antipyretic effect	[197]
MINOCYCLIN	Antibiotic and anti-inflammatory effect	[198]
GINSENOSIDE	Calcium channel antagonist	[199]
EDARAVONE (MCI-186)	Free radical scavenger	[200]
ONO-2506 (ARUNDUC ACID)	Astrocyte modulator	[201]

Table 2. Clinical trials of agents targeting inflammatory pathways in acute ischemic stroke. Adapted from[203].

1.6.6 Immunosuppression following stroke

A growing body of evidence currently indicates that central nervous system and immune system are two supersystems closely linked. This functional interaction could explain the existence of immunological manifestations as the result of central system damage and vice versa. Numerous studies have addressed the role of infection as a risk factor for stroke. Other studies proposed that stroke lead to an immunosuppression syndrome[206, 207].

Autopsy series indicate that death within the first week after stroke is attributable primarily to direct effects of brain damage, such as brain

edema with transtentorial herniation. Subsequently mortality is attributable to medical complications such as infection. The frequency and nature of the infectious complications following acute stroke have been addressed in several clinical studies using a wide range of different designs, methods of patient selection, and diagnosis **(Table 3)**. Most poststroke infections include urinary tract infections and respiratory infections, although respiratory infections prevail during the first few days after stroke.

AUTHORS	STUDY DESIGN	STROKE	N	% INFECTION	DAYS	REF
Davenport	Retrospective	Isch/hem	597	35	37	[210]
Johnson	Retrospective	Ischemic	297	8*	90	[211]
Langhome	Prospective	Isch/hem	311	65	35	[212]
Grau	Prospective	Ischemic	119	10**	2	[213]
Heuschmann	Retrospective	Ischemic	13440	6***	10,6	[214]
Hamidon	Prospective	Isch/hem	163	16	3	[215]
Hilker	Prospective	Isch/hem	124	15***	3	[216]
Weimar	Retrospective	Ischemic	3866	13,7	7	[217]
Vargas	Prospective	Isch/hem	229	27	7	[218]
Katzan	Retrospective	Isch/hem	14293	7***	30	[219]
Smithard	Prospective	Isch/hem	121	24***	7	[220]

* only serious complications were reported (life-threatening, hospitalization or death)
** this figure excludes additional 11% patients in whom infection was considered to precede ischemia
*** pneumonia only

Table 3. Major studies analyzing the infection rate in stroke patients[206].

Development of infections after acute stroke could be related to mechanisms related to stroke, such as application of invasive

maneuvers, decreased awareness or abnormal brain stem reflexes. But some data suggest that infection might also be explained by stroke induced immunological mechanisms.

Systemic infection after acute brain damage could be a symptom of central nervous system mediated decrease of immune competence, as described in patients with brain tumors, epilepsy or traumatic brain injury. All these conditions can induce an increase in autonomic nervous system, that affects innate and adaptative immune response and is responsible for the increase of susceptibility to bacterial infections[207]. Central nervous system modulates the activity of immune system through complex humoral and neural pathways that include the hypothalamic pituitary adrenal axis, the vagus nerve, and the sympathetic nervous system **(Figure 8)**.

The hypothalamic pituitary axis is a major part of the neuroendocrine system with important functions in health and disease, and with key elements located in the paraventricular nucleus of the hypothalamus, the anterior lobe of the pituitary gland and cortices of the adrenal gland[208]. Cytokines such as interleukin 1β, TNFα an IL-6, secreted by cells in different tissues and organs including the brain, can stimulate specialized neurons in the paraventricular nucleus to synthesize coriticotropin-releasing factor (CRF). Once released into the pituitary portal blood system, corticotropin releasing factor interacts within the anterior pituitary with a specific G protein-coupled receptor (corticotropin-releasing factor-F1 receptor), facilitating the secretion of adrenocorticotropin hormone precursor peptide proopomelanocortin, and adrenocorticotropin hormone (ACTH). ACTH

induces the secretion of glucocorticoids from the zona fasciculata of the adrenal cortex which suppresses the production of proinflammatory mediators, inducing IL-1β, IL-11, IL-12, interferon-ϒ, TNFα, chemokines (IL-8), prostaglandins and NO. Glucocorticoids also facilitate the release of anti-inflammatory mediators such as IL-4, IL-10 and TGFβ, and have strong anti-proliferative properties and apoptotic effects in immune cells[209]. In the end, cytokines can activate the release of glucocorticoids, which in turn suppress further cytokine synthesis in a classic negative feedback loop.

Indeed, there is another pathway of immunosuppression following stroke, the cholinergic neural pathway. The paraventricular nucleus-nucleus of the solitary tract pathway allows the synchronization of neuroendocrine responses with the cholinergic anti-inflammatory pathway to suppress the peripheral release of cytokines through macrophague nicotinic receptors.

The last pathway that has influence in immunodepression is the adrenergic pathway. The sympathetic nervous system also plays a crucial role in the communication between neural and immune structures. The sympathetic division originates in brain stem nuclei such as the locus coeruleus and the rostral ventrolateral medulla that give rise to preganglionic cholinergic efferent fibers. Postganglionic sympathetic fibers run paravertebral or prevertebral ganglia to release norepinephrine in different tissues, and parallel increases in brain norepinephrine concentrations and plasma corticosterone. Activation of the locus coeruleus leads to release norepinephrine from an extraordinarily dense network of neurons throughout the brain, and

from peripheral organs, resulting in enhanced arousal and vigilance, increased heart rate, vascular tone, and gastrointestinal motility, but also in the induction of pronounced immunological changes. The later effects mainly result from the inhibition of TH type 1 proinflammatory activities, giving way to predominance of TH type 2 anti-inflammatory activities[221, 222].

In mice, stroke induces a long lasting depression of the cell mediated immunity, including monocyte deactivation, lymphopenia, and a TH1/TH2 shift associated with spontaneus bacteriemia and pneumonia[223]. In patients, reported defects in immune function after stroke include reduced peripheral blood lymphocyte counts, impaired T and natural killer cell activity and reduced mitogen-induced cytokine reduction and proliferation in vitro.

Antibiotic therapy with moxifloxacin prevented infection in ischemic mice[224]. Nevertheless, the ESPIAS trial[225], early systemic prophylaxis of infection after stroke with levofloxacin and other similar studies were not able to prevent the incidence of infection in patients with nonseptic acute stroke. In this study, patients with stroke have a rapid increase of circulating cytokines in plasma with a low ratio of proinflammatory TNFα to anti-inflammatory IL-10, preceding the appearance of infection. Monocytes, neutrophils and total counts of white blood cells are also increased before infections. These observations caution about the potential risks of proinflammatory cytokine inhibition in patients with sepsis.

Immunosuppression following stroke could be an adaptive function, limiting inflammation induced by brain ischemia, but as

collateral effect could increase the infection risk[226].

Regulatory T lymphocytes play a role as immunosuppressor cells. In this way, some authors propose that Treg are implied in systemic immunosuppression following stroke, given their fact as regulators of immune system[227-228]. Acting over this subtype of lymphocytes (increasing Treg levels or Treg function) could potentially increase infections after ischemic stroke.

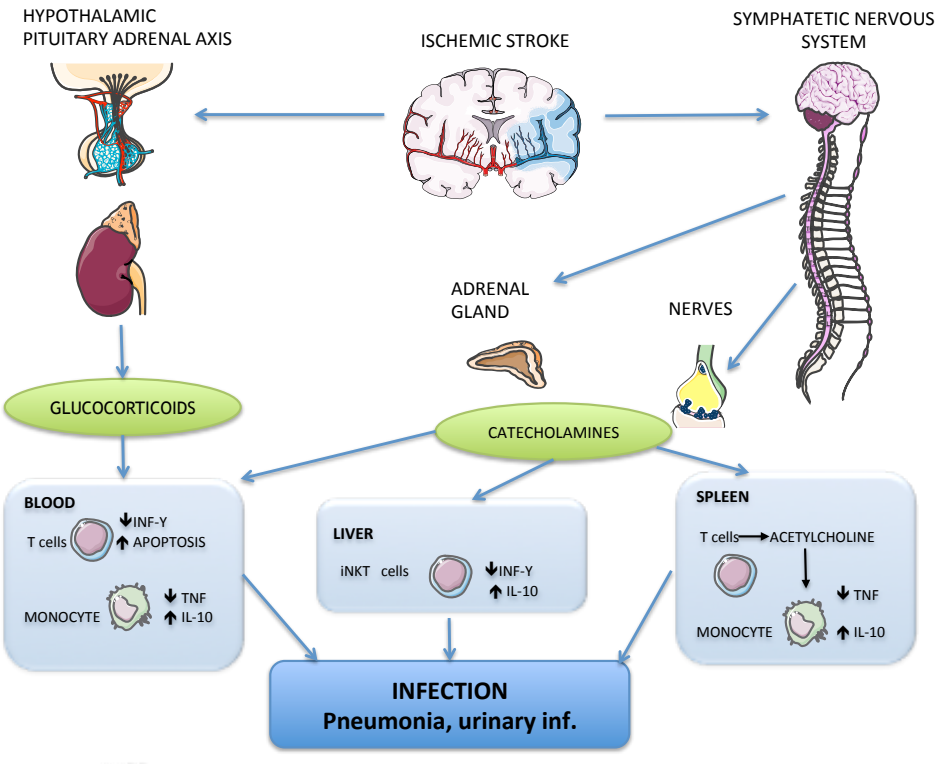

Figure 8. Immunosuppression following stroke. Central nervous system modulates immune system activity through different pathways, including the hypothalamic pituitary adrenal axis, the vagus nerve and the sympathetic nervous system. Modified from[206].

2 REGULATORY T LYMPHOCYTES

Some mechanisms have been proposed to be responsible for controlling and regulating immune system, minimizing or preventing reactivity against autoantigens or avoiding an excessive response against pathogens that leads to tissue damage.

Gershon and Kondo, in 1970, proposed that there is a subpopulation of T cells that could decrease immune response and also mediate a suppressor effect, different from T effector cells function[229].

Sakaguchi et al demonstrated that there is a subpopulation of suppressor T cells that express CD4 and CD25 and they named these cells regulatory T cells (Treg) [230]. They also proposed that Treg could be useful to limit excessive immunity produced by other T lymphocytes.

2.1 ONTOGENY OF REGULATORY T LYMPHOCYTES

Treg are a CD4+ lymphocyte subpopulation that proceeds from progenitor cells inside bone marrow. They are developed inside thymus through a positive and negative selection[231].

Their development needs interaction of T cell receptor (TCR) and major histocompatibility complex type II molecules. FoxP3 expression depends on the union of CD28 and their ligands CD80/CD86, and is necessary for these cells to survive, in spite of the recognition of a high affinity antigen.

Pro-T cells are the first T lymphocyte precursor **(Figure 9)**. If a cell shows a high reactivity against self-antigens (characterized by a high

affinity against MHC class II), this will cause death by apoptosis through a negative selection, because it shows a high risk of autoimmunity reaction.

Lymphocytes showing a low or moderate affinity against self-peptides, receive a stimulus of survival signals (positive selection). Some of these cells are differentiated in effector T lymphocytes (Th0) and released to the periphery. Other cells are differentiated in FoxP3+ cells and they are released like Treg to the periphery[232].

How Tregs, which reach functional maturity within the thymus, are generated has been the subject of considerable scientific debate. Recent reports proposed the "two-step" model of Treg development **(Figure 10)** [233, 234].

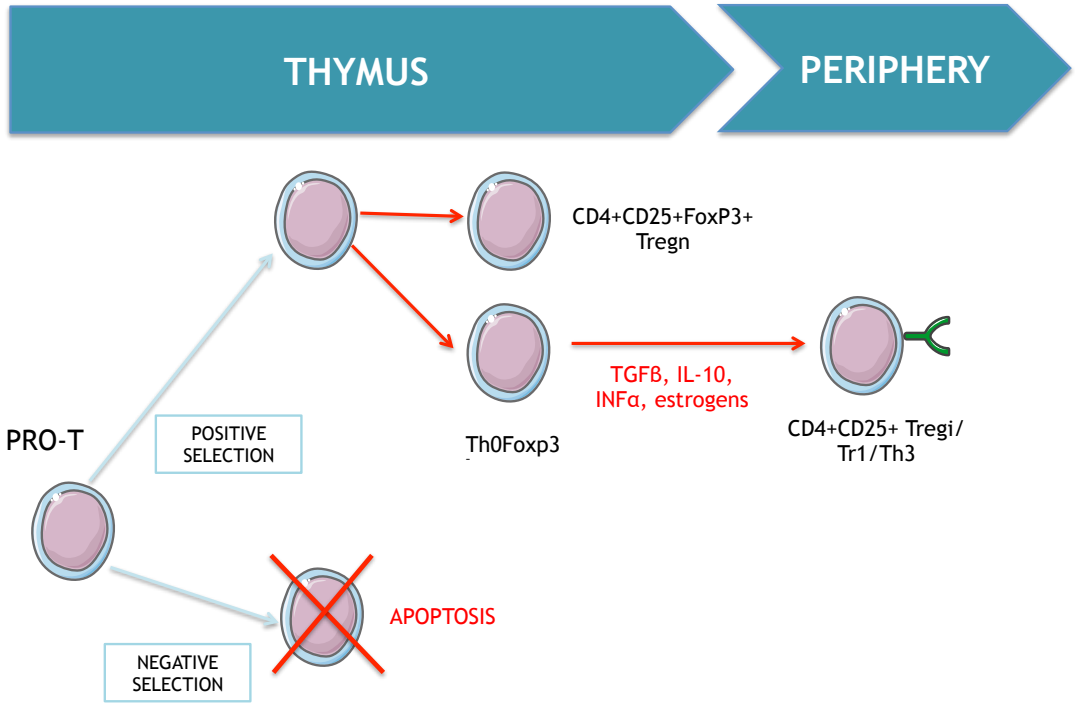

Figure 9. Treg ontogeny: Fairly autorreactive cells are FoxP3+ and are natural regulatory T cells. T cells with intermedious autorreactive avidity originated in thymus are FoxP3- and are differentiated to effector T cells (Th0). When in peripheral blood, Th0 can change into Treg cells, and are inducible T regulatory cells. Adapted from[233].

In the first step, thymocytes undergo TCR and coreceptor dependent selection that gives rise to the CD4+CD25+GITR hiFoxP3- Treg precursors. A subsequent TCR-independent IL-2/IL-15 dependent step results in FoxP3 expression, making the differentiation of Treg precursors into Treg. This two-step model is the currently prevailing framework through which thymic Treg development is analyzed[235]. TCR ligation eventually leads to activation of the transcription factors NF-AT (nuclear factor of activated T cells) and NF-κβ[236]. The activation of NF-AT depends on its dephosphorilation by the calcium dependent phosphatase calcineurin[237]. Both NF-AT and calcineurin pathways have been implicated in Treg development, but their precise role in this process is controversial. NF-AT is required for the induction of FoxP3 transcription in vivo [238].

Natural regulatory T cells are the 5-10% of all CD4+ T lymphocytes in human adults and mice[239]. These cells are produced inside the thymus during fetal development. These cells are specialized in prevention of autoimmune reactions.

Treg can also proceed from CD4+ naive T lymphocyte induced through antigenic stimulation in some conditions in peripheral blood by antigenic presentation and factors such as TGFβ, IL-10, INFα or estrogens. These cells are inducible Treg lymphocytes. Sundstedt et al showed that this Treg subtype can be induced by myelin basic protein (MBP) repetitive intranasal administration[240]. Inducible Treg, includes two similar subpopulations: TH3CD4+cells, that produce TGFβ, cells that could inhibit some autoimmune diseases and Tr1 (Treg CD4+ type1) that produce IL-10 and inhibits Th1 cells.

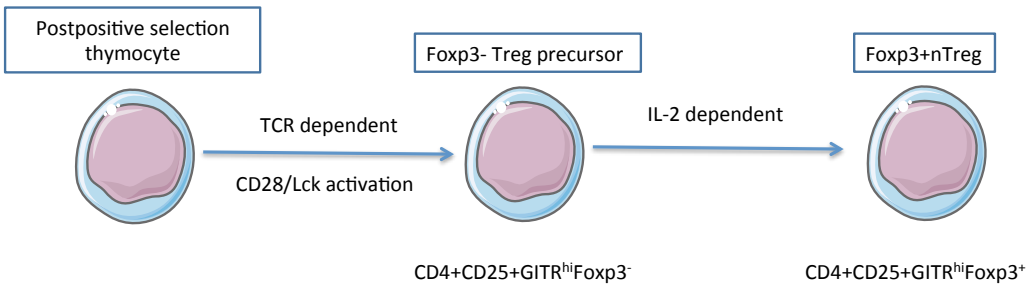

Figure 10. Treg development stages. Thymic Treg development is achieved in two stepts. Initially, pospositive selected thymocytes undergo TCR and CD28 dependent maduration into a FoxP3- Treg precursor population. A subsequent IL-2 dependent step leads to the development of FoxP3 expressing mature Treg. Adapted from[232].

There are other cells that could have a role in controlling and regulating immune system, different from these CD4+CD25+FoxP3+ cells:

- CD8+CD28-, NK (natural killer) cells, which activity lies on FAS/FASL contact.
- Tγδ lymphocytes.
- Tolerogenic dendritic cells stimulated with Galectin-1, which can be therapeutically administered.
- Some studies proposed have investigated the regulatory function of regulatory B cells and CD8+CD122+[241].

2.2 IDENTIFICATION OF REGULATORY T LYMPHOCYTES

Molecular markers are essential tools for defining and analyzing a subpopulation of immune cells. Several markers have been studied in order to characterization of Treg **(Figure 11)**.

The most widely used markers for Treg cells are:

- ***CD25:*** this subpopulation of T cells can be selected through the

positivity of a surface marker, the IL-2 receptor (CD25). Treg express constitutively high levels of CD25. They also depend on IL-2 presence for proliferation and survival. However, this marker is not exclusive of this cell subpopulation because it is also expressed in other activated T cells[242].

▪ ***CTLA-4 (cytotoxic T lymphocyte associated protein 4)*** [243, 244] this protein function is the regulation of homeostasis and peripheral tolerance, inhibiting T lymphocyte activation. Two mechanisms have been proposed for its function: negative signaling or competitive antagonism of costimulating pathway mediated by CD28-B7.

▪ ***GITR (glucocorticoid induced TNF receptor family related gen)*** [245].

▪ ***LAG-3 (lymphocyte activator gene-3)*** [246].

▪ ***FOXP3:*** FoxP3 is a transcription factor that is an exclusive marker for Treg, at rest and at activity phase. It is located in chromosome X and is shown by CD25**+** Treg and in some of the CD25-[247]**.**

It is though that this trascription factor is the one that manages the development and the function of Treg and it is useful as marker. FoxP3 blocks the activation of some trascription factors such as NF-κβ (nuclear factor κβ) and NF-AT (nuclear factor of activated T cells), which are essential for the cytokine genetic expression and for the normal function of T lymphocytes. FoxP3 also antagonizes CREB (cAMP-responsive -element- binding protein).

FoxP3 function in Treg is demonstrated by some findings. Firstly, the loss of FoxP3 activity produces a proliferation in autoagressive lymphocytes. On the other hand, FoxP3 overproduction causes severe

immunodeficience. Individuals with mutations in FoxP3 genes present autoimmune diseases such as: autoimmune endocrinophaty, type I diabetes mellitus, thyroiditis, allergy[248] and multiorganic autoimmune syndromes as XLAAD (X-linked autoimmunity allergic dysregulation syndrome) or IPEX (immunodysregulation, polyendocrinopathy, enteropathy, X linked syndrome). In humans these diseases could improve after a bone marrow transplant[249].

▪ ***CD127:*** Some studies showed that FoxP3 presence is directly related to IL7 receptor (CD127) abscense. FoxP3 is an intracellular protein and it requires fixation and permeabilization of the cell to identify it, so this marker cannot be used to separate human Treg cells for functional studies or in vivo expansion for cellular therapy, thereby limiting its use in human setting. That is the reason why some alternative methods have been investigated for Treg identification, like the absence of CD127 expression.

FoxP3 interacts with CD127 promotor, reducing CD127 expression in Treg. Some studies demonstrated that Treg will be those cells that do not show IL17 receptor (CD127). Treg should present the next surface markers: CD4+CD25+ and CD127-[250-252].

By the way, regulatory T cells are characterized by secreting TGFβ and IL-10 but there are other cells that can also secret these molecules[253]. Increases in TGFβ and IL-10 production could be used as markers for Treg identification.

On balance, to characterize Treg inside a heterogeneus group of cells, the most specific surface markers are CD4**+**, CD25**+** y FOXP3 **+**[254].

Like an alternative option, due to the problems to detect FoxP3 we should identify as Treg those which express CD4**+,** CD25**+**, CD127**-**[250].

Treg also show high levels of other surface markers as CD11a (LFA-1), CD44, CD54 (ICAM-1), CD103, CD45RB, CD122, CD134 (OX-40) and CD62 (L-selectin), Galectin-1, Neutropilin-1. These cells also show high levels of chemokine receptor CCR4 and CCR8[255].

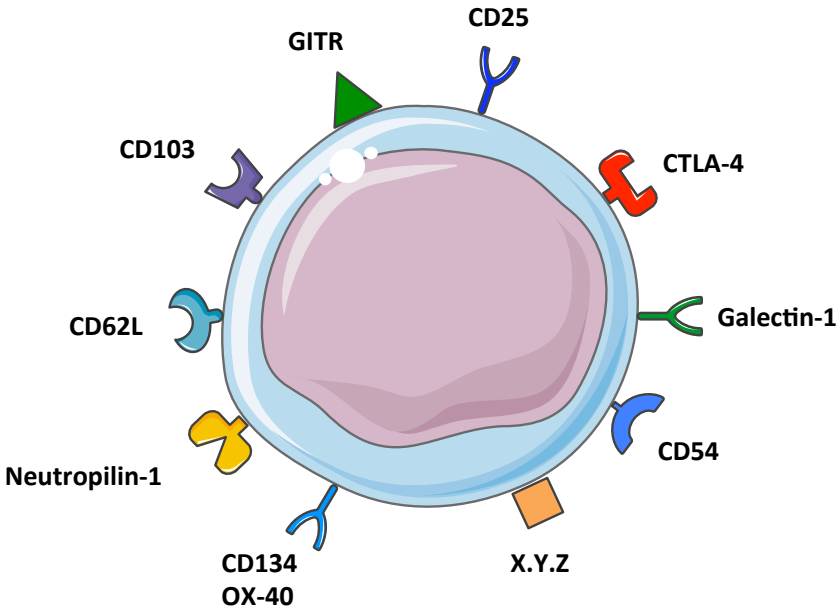

Figure 11. Surface molecules in Treg: Some of the surface molecules in Treg are CD4+,CD25+,CD103, Galectin-1, GITR, CTLA-4, Neutropilin 1, CD54, CD62L, CD134. Most of them decrease their expression after their activation and seem not to be involved in suppressor mechanisms of T cells. X, Y and Z markers are not defined yet. Adapted from[255].

2.3 MECHANISMS OF REGULATORY T LYMPHOCYTES

Initially, it was though that the main Treg function was to prevent autoimmune diseases through self-tolerance maintenance[256]. However, some years later, additional functions have been proposed. In this direction, participation of Treg has been proposed in:

1. Autoimmune disease prevention through self-tolerance maintenance[257].

2. Suppression of allergy and asthma[258].
3. Induction of tolerance against dietary antigens[259].
4. Induction of maternal tolerance to fetus[260].
5. Suppression of T cell activation triggered by weak stimuli[261].
6. Regulation of the effector class of the immune response[262].
7. Protection of commensal bacteria elimination by the immune system[263].
8. Excessive immune response regulation

The exact mechanism of action of these cells is not totally discovered. Different mechanisms have been proposed for their suppressor effect **(Figure 12)**. The main mechanisms proposed are:

a) ANTI-INFLAMMATORY CYTOKINE PRODUCTION: Mainly IL-10 and TGFβ. Soluble factors with antinflammatory effects have a direct action over effector T cells or over antigen presenter cells, leading to a tolerogenic response.

Treg secrete inhibitory cytokines to peripheral blood and to the brain, such as TGFβ, IL-10 and IL-35.

TGFβ, is an important factor for the inhibition of effector T cells through Treg[264]. TGFβ plays an important role in FoxP3 expression, in Treg differentiation and in their immunosuppressive effects.

IL-10 is other anti-inflammatory cytokine that has neuroprotective effects following cerebral ischemia. It could inhibit inflammatory response and limit inflammation that can be harmful for ischemic

tissue[231].

b) ELIMINATION MECHANISM THROUGH GRANZIMES AND PERFORINS and induction of apoptosis in effector cells[239].

c) METABOLIC DISRUPTION: Treg may also cause metabolic disruption, for example stimulating antigen presenter cells to produce enzymes that consume essential amynoacids, preventing naive/effector cell proliferation. TGFβ may induce the expression of FoxP3 in naive cells.

d) APC MATURATION AND FUNCTION INHIBITION: Treg are characterized by the expression of CTLA-4, a surface marker that is one of the most important component of immunosuppression by Tregs. CTLA-4 establishes a cell contact with its ligands, CD80 and CD86[231] and participates in the modulation of antigen presenter cells. Mice with CTLA-4 deficiency, show a decrease in immunosuppressor function in vivo and in vitro[265,266].

Dendritic cells and antigen presenter cells show an anti-inflammatory mediator called IDO (indoeleamina 2,3-dioxigenase), first enzyme in kyneurine pathway[267]. This substance can activate Treg under normal conditions to control inflammation.

e) COMPETITIVE MECHANISM: Treg consume growing and survival factors such as IL-2, leading to apoptosis of effector cells and the interference of their activation mechanism.

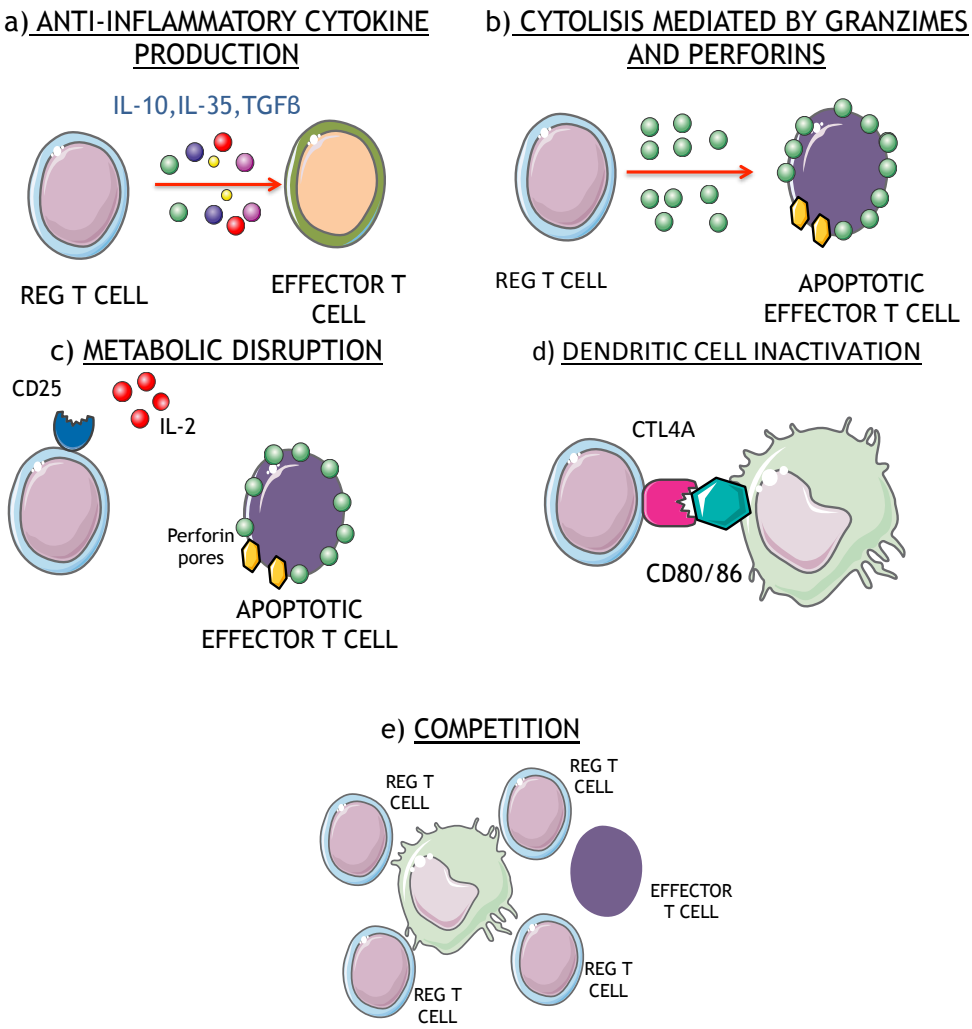

Figure 12. Suppressor mechanisms of Treg. Five mechanisms have been proposed: a) anti-inflammatory cytokine production, b) cytolisis mediated by granzimes and perforins, that activate caspases and osmotic lisis, c) metabolic disruption, d) through CTLA-4 union to CD80/86, inhibiting maturation and function of antigen presenter cells, e) competitive mechanism. Modified from[248].

2.4 ANGIOGENESIS AND REGULATORY T LYMPHOCYTES

Angiogenesis is likely to contribute to neurorepair, but the finality of the angiogenic response in acute ischemic stroke has not been fully elucidated[268]. T lymphocytes are involved in postischemic neovascularization. Mice which lack of all T cell subsets exhibit a

pronounced reduction in postischemic vessel growth[269].

Furthermore, some subsets of T lymphocytes have been suggested to play a role in vascular remodelling. Leukocytes trigger neovascularization through the release of several angiogenic/ arteriogenic factors, including vascular endothelial growth factor (VEGF) and proinflammatory cytokines, such as TNFα and IL-1β. Anti-inflammatory cytokines such as TGFβ and IL-10, are also able to modulate neovascularization process.

There are only few studies regarding the relationship between Treg and neovascularization following ischemia, and they showed contradictory results.

The first study has been done in mice with a hind limb ischemia model. They showed that profound Treg reduction with B7/CD28 deficient mice or anti-CD25 treatment, increased postischemic neovascularization. The vessel growth was higher in CD28 deficient mice than in mice treated with anti-CD25 antibodies. In this study, exogenous administration of Treg, reduced postischemic neovascularization. In conclusion, they showed that Treg play a key role in postischemic neovascularization, decreasing it[270].

The other study about postischemic vascularization[271], also used a hind limb ischemia model. Major changes in Treg number resulted in only moderate effects on postischemic neovascularization, in contrast with the previous study. In the second study, increasing Treg resulted in an attenuated blood flow recovery after ischemia induction. Depletion of CD25+ cells with anti-CD25 antibodies did not result in

any alterations of postischemic blood flow recovery as compared to controls. This study shows that only moderate effects on postischemic neovascularization were observed after major modulation of Treg.

Some authors also proposed that Treg cells contain growth factors that may be important in neurogenesis[272, 273], and these cells could play a role in neurorepair after stroke.

2.5 REGULATORY T LYMPHOCYTES ROLE IN DISEASES

Treg are the main responsible group of cells in charge of autotolerance, immunologic homeostasis and damage control after inflammation. Treg have been implicated in a lot of diseases and their decrease can predispose to some autoimmune diseases[274].

Treg are increased following renal ischemia and their depletion in this disease worsens outcome[275]. In renal ischemia Treg have a protector effect in acute phase and it is believed that the keystone of this process is IL-10, although TGFβ has also an effect at this level.

Treg have showed some positive effects in hepatic ischemia[276]. In addition, it has been demonstrated that regulatory T cells protect lymphopenic mice from intestinal inflammatory disease[277], from diabetes mellitus and can also protect them from transplant rejection[278].

On the other hand, some authors suggest that Treg can have a deleterious effect in the guest, due to their ability to inhibite the immune response against tumors[279].

With regard to central nervous system diseases, Treg play an

important role, specially in neurodegenerative diseases, such as multiple sclerosis, Parkinson´s disease or Alzheimer´s disease. It has ben demonstrated that in early stages of some neurodegenerative diseases there is a Treg dysfunction. This Treg dysfunction has been well described in multiple sclerosis patients[280]. In experimental allergic encephalitis, the transfer of Treg confers protection against disease induction and progression[281].

In the mice Parkinson´s disease model receiving MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropirydine), Treg transfer produces a neuroprotective effect, decreasing inflammation mediated by microglia and promoting expression of neurotrophic factors from astrocytes. It has been proposed that Treg disrupt the microglial proteoma, slowing down Parkinson´s disease progression[282].

Treg can also have beneficial or deleterious effects in neuronal survival following traumatic damage. Active or passive immunization with peptides from myelin, can confer protection in neuronal death following traumatic damage in spinal cord. Specific T cells against myelin basic protein (MBP) prevent cyst formation following traumatic spinal cord damage[283].

Briefly, it seems that Treg play an important role in central nervous system pathogenesis, but their exact mechanism of function is not totally known by the moment. It is necessary further investigation to fully know the role of Treg in neurodegenerative diseases.

Treg also play a role in atherosclerosis development, in vascular dysfunction, hypertension and in myocardial infarction.

Some authors consider atherosclerosis as an inflammatory disease. Immune cells, both from innate and adaptive arms are present throughout all stages of atherosclerosis injury development. Treg are found in atherosclerosis lesions in low number[285] and colocalised with both IL-10 and TGFβ expression[284, 285].

Using different mouse models it has been evident that Treg deficiency is linked to increased atherogenesis and injury inflammation[286], and that increased Treg presence and Treg/Th1 ratio reduced lesion formation[287].

Furthermore, inhibition of Treg production of IL-10 and TGFβ signaling clearly demonstrate atheroprotective effects[287]. Interestingly, recent studies showed that intravenous or oral anti-CD3 antibody administration induce Treg response and thus attenuates atherosclerotic lesion formation[288].

The significance and role of Treg in microvascular endothelial dysfunction and hypertension is an important question that remains unanswered. Treg play an important role in the regulation of endothelium dependent relaxation in coronary arterioles and in the regulation of arterial blood pressure[289]. Thus, the transfer of Treg, freslhy from normotensive mice into hypertensive mice three times a week for two weeks, significantly reduced arterial blood pressure and improved endothelin dependent relaxation in coronary arteries[289, 290].

In vitro results with cultured Treg isolated from normotensive mice showed that stimulation with angiotensin II for 24 hours significantly reduced the number of Treg by an apoptotic

mechanism[291].

It remains unclear, however, how Treg regulate arterial blood pressure and microvascular function. The transfer of Treg into hypertensive mice decreases inflammation as evidenced by the decrease in anti-inflammatory cytokines and macrophague infiltration into coronary arteries.

Microvessel from hypertensive mice incubated with conditioned media of Treg have significantly improved endothelium dependent relaxation responses mediated through an IL-10 dependent mechanism[292].

Ischemic injury in the myocardium, cause cell damage, and cells can die or react by releasing danger signals. The first hours or days there is a recruitment of neutrophils and monocytes within the myocardium. After day 4, inflammation begins to resolve. In mice with atherosclerosis, lack or delay of inflammation correlates with higher mortality. Recent studies showed an accumulation of Treg in infarcted tissue. After induced myocardial infarction in a model of genetic Treg ablation, infarct size was increased, inflammation was impaired and neutrophil and monocyte levels were augmented. Moreover the gene expression profile from macrophagues was biased towards inflammation, and factors typically associated with healing were decreased (TGF β, osteopontin and transglutaminase factor XIII). These results suggest that abscense of Treg leads to impaired transition toward resolution of inflammation, resulting in persistence of inflammatory macrophages and delayed healing[293]. When Treg were depleted with an anti-CD25 antibody 8 days before myocardial

infarction, survival was impaired which did not occur in FoxP3 DTR (dphteria toxin receptor) mice. Treatment with a superagonistic anti-CD28 antibody expanded Treg, improved infarct matrix repair and reduced infarct mortality[294].

3 REGULATORY T LYMPHOCYTES IN ISCHEMIC STROKE

Although deleterious effect of T lymphocytes on early stages following ischemic stroke is well studied, the specific function of some subpopulations, such as Treg in ischemic stroke is controversial. Recent studies showed uncertain results and, sometimes, contradictory results[295].

It is still unknown at which specific site Treg act as inmunomodulatory cell after stroke, but their kinetics and magnitudes of brain infiltration have been characterized in several studies using models of experimental middle cerebral artery oclussion (MCAO).

In one of the first studies systematically analizyng brain leukocyte invasion after transient brain ischemia by flow cytometry, a low number of CD25+FoxpP3 Treg, <5% of all CD4+ cells was observed within the first week after transient ischemia, but this percentage reaches 20% when a permanent ischemia model was used[296].

Stubbe et al[297] investigated cerebral Treg the first month after large hemispheric lesions. Although they detected only neligible amounts of FoxP3 early after MCAO, substantial Treg invasion occurred 14 and 30 days after MCAO.

Despite their delayed recruitment in the brain, Treg have been found in depletion and therapeutic paradigms to influence stroke outcome within the first days after ischemia. That data prompt a rational assumption that Treg might have an early function in the ischemic brain by targeting cells in the peripheral immune system[297].

On the other hand, it has been described that Treg can be part of

the inmmunodepression that occurs after ischemic stroke, acting against immune system[298]. That can suggest that Treg could potentially have a detrimental effect, increasing susceptibility to infections following ischemic stroke.

The precise mechanims of Treg action in ischemic stroke are not totally defined, but it is believed that there are different pathways of involvement in inmune system.

Nevertheless, studies of Treg function in animal models of ischemic stroke are contradictory. Some studies both in Treg depletion in experimental brain ischemia and in therapeutical enhancement of Treg function in brain ischemia have been published.

3.1 REGULATORY T CELL DEPLETION IN EXPERIMENTAL BRAIN ISCHEMIA

There are some studies in animal models regarding Treg depletion in experimental brain ischemia **(Table 4).** Methodologically, 2 principally different approaches have been used to deplete Treg: antibody-mediated cell lysis, using CD-25 specific antibodies; or the use of transgenic mice with diphteria toxin receptor transgene under the control of the FoxP3 promoter for inducible T reg depletion.

Using Treg-depletion paradigms about half of the experiments performed revealed an increase in infarct volume[112, 300, 308] whereas the other half did not detect any effect on stroke outcome[112, 297, 299, 301,308] and one study even observed a reduction of infarct size in Treg-deficient mice[302].

In one of the first studies[112], using a mice model of acute ischemic

stroke, authors found that Treg helped to reduce brain injury. Liesz et al, investigated the effect of CD4+CD25+FoxP3 cells on infarct volume after focal brain ischemia in mice by in vivo depletion of this T cell subpopulation with a antiCD25-specific antibody. In their previous experiments, anti-CD25 resulted in a depletion of at least half of FoxP3 cells and a 90% reduction of CD25+ cells in lymphatic tissue, but did not affect other leukocytes.

To model acute ischemic stroke, the researchers used standard procedures for middle cerebral artery occlusion (MCAO) in mice. Permanent MCAO distal to lenticuloestriates arteries was induced 48 hours after injecting either CD25-specific monoclonal antibody or PBS. Between days 3 and 7 after MCAO, infarct size in the PBS group remains almost unchanged whereas infarct volume is significantly increased in Treg depleted mice. When middle cerebral artery occlusion lasts for almost 90 minutes and induced extensive lesions that extended through approximately 70% of the hemisphere including cortical and subcortical structures, Treg depletion did not increase infarct volume.

In this study, Treg cell depletion elevates proinflammatory cytokines (TNFα, INFγ and IL-1β) and had a minor effect of postischemic cerebral expression of IL-10 and TGF β (anti-inflammatory cytokines).

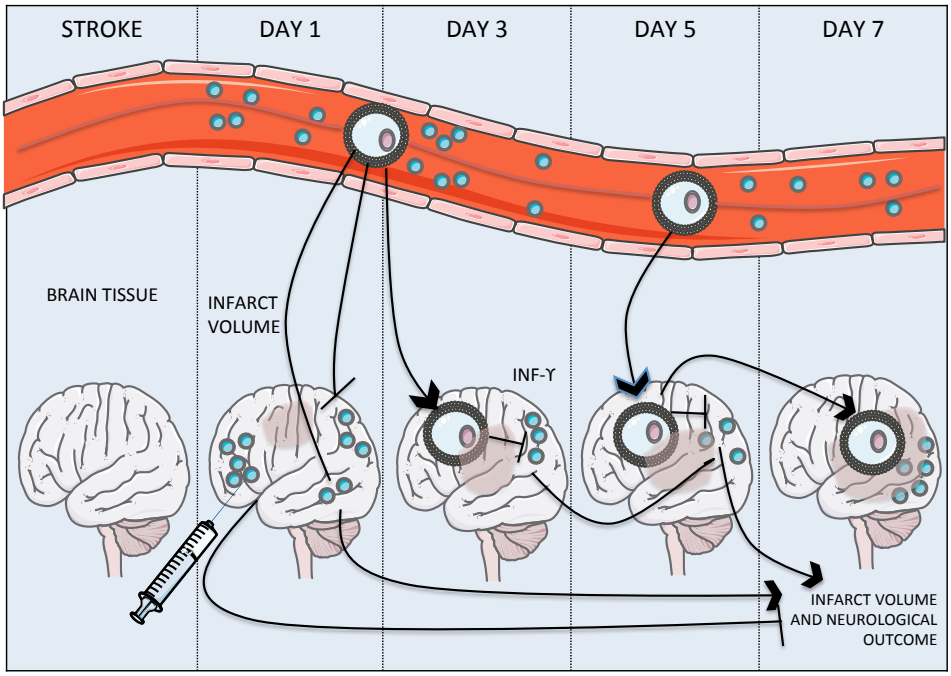

Figure 13. Treg reduce proinflammatory cytokines production (TNFα, INFY , e IL-1ß in brain and blood), limits infiltration of neutrofils and modulate microglia activation in ischemic tissue. IL -10 inyected in intracerebroventricular space reduces infart size. Adapted from[303].

The authors also investigated what happens with brain infarcts in an animal model when cerebral proinflammatory cytokines were depleted (TNFα and INFY) and anti-inflammatoy cytokines (IL-10) were administered to the brain. Their findings suggest that IL-10, a cytokine related to Treg, mediates the protective effect of Treg following stroke.

On the other side, early intracerebroventricular administration of IL-10 diminished the size of brain infarctions at day seven in Treg depleted mice, and lowered the cerebral expression of proinflammatory cytokines such as TNFα and IL-1β **(Figure 13)**. Adoptive transfer of CD4+CD25+ cells to lymphocyte deficient mice also reduced infarct volume, but that did not occur if the cells were

obtained from IL-10 deficient mice, supporting IL-10 role in ischemic stroke.

Liesz et al in another study[308], used a mice model of FoxP3-KO animals. Treatment with diphtheria toxin in these animals reduced CD4+FoxP3+ cells and significantly increased infarct volume 7 days after MCAO. Levels of IL-1β, IL-17, TNFα and INFϒ were augmented after Treg depletion.

STUDY	MODEL	LESION	ENDPOINT	DEPLETION MODEL	DEPLETION EFFICACY	INFARCT VOLUME
LIESZ ET AL [112]	DISTAL PERMANENT	10%	7 DAYS	ANTI-CD25	90%	INCREASED
				ADOPTIVE CELL TRANSFER	NA	INCREASED
	90 MIN TRANSIENT	55%	7 DAYS	ANTI-CD25	90%	NO EFFECT
LIESZ ET AL [308]	DISTAL PERMANENT	10%	7 DAYS	FOXP3-KO	85%	INCREASED
	60 MIN TRANSIENT	55%	3 DAYS	FOXP3-KO	85%	NO EFFECT
	30 MIN TRANSIET	10%	7 DAYS	ANTI-CD25	90%	INCREASED
XI ET AL [300]	90 MIN TRANSIENT	40%	3 DAYS	ANTI-CD25	65%	INCREASED
REN ET AL [301]	60 MIN TRANSIENT	55%	7 DAYS	FOXP3-KO	65%	NO EFFECT
LI ET AL [299]	60 MIN TRANSIENT	50%	3 DAYS	ANTI-CD25	90%	NO EFFECT
STUBBE ET AL [297]	30 MIN TRANSIENT	50%	27 DAYS	ANTI-CD25	75%	NO EFFECT
KLEINSCHNITZ ET AL [302]	60 MIN TRANSIENT	55	7 DAYS	FOXP3-KO	90%	REDUCED

Table 4: Summary of studies regarding T reg depletion paradigms. Modified from[241].

Xie et al[300] also showed an increase in infarct volume after treatment with anti-CD25 after 90 minutes of transient MCAO. Other authors have not found this relationship between Treg depletion and infarct volume. Ren et al[301], accomplished near a complete depletion of FoxP3 expression in the peripheral blood, lymph nodes and spleen by treating DEREG (depletion of regulatory T cell) mice with two daily injections of diphteria toxin prior to induction of MCAO in mice. In their study, depletion of FoxP3 Treg did not affect infarct volume in cortex or striatum in mice compared with control rats. Stubbe et al neither found changes in their study whith regard to infarct volume after Treg depletion[297]. They also showed an accumulation of Treg in the ischemic hemisphere until day 30 following stroke.

There is also one study that showed a negative relationship between Treg and stroke size[302]. In this study Kleinschnitz et al, performed a selective depletion of Treg (using DEREG mice treated with diphteria toxine once daily for 3 consecutive days before MCAO) and that dramatically reduced stroke size 24 hours after a severe insult. Furthermore, adoptive transfer of purified T reg in RAG1-/- mice showed a deleterious effect in a model of ischemic stroke, enhancing stroke progression. They also proposed a mechanism to explain the deleterious effect of Treg on stroke. According to these authors, in contrast to primary resting T cells, natural Tregs have a higher adhesive propensity, predestining them to interact functionally with activated cerebral endothelial cells and platelets. This interaction causes microvascular dysfunction, leading to increased thrombus formation and subsequently impaired cerebral reperfusion after tMCAO. In contrast, mechanisms related to the “immunologic”

functions of Tregs do not appear to be of major importance in stroke pathophysiology.

3.2 THERAPEUTIC ENHANCEMENT OF REGULATORY T CELL FUNCTION IN EXPERIMENTAL BRAIN ISCHEMIA

Despite the discrepancies arising from studies investigating the effects of Treg depletion, a rapidly increasing number of reports have tested Treg augmentation for stroke therapy. These studies have investigated different approaches to increase Treg number and function **(Table 5).** The most intuitive approach might be the adoptive cell transfer of purified Treg and the second most widely used approach was the administration of CD28 supergonist (CD28SA) that induces in vivo expansion of Treg and amplification of their suppressive function[304, 305].

A pathomechanistically different approach used in earlier studies was the paradigm of muscosal immunization with mucosal administration of cerebrovascular antigens, such as myelin oligodendrocyte glycoprotein or selectins which induce expansion of autoantigen specific Treg[93, 306, 307.] All these studies showed a reduction in infarct volume in animal models of brain ischemia.

Liesz et al, described the use of histone deacetylase inhibitors (HDACi) to boost the number and function of Treg[308]. By using specific histone deacetylase inhibitors, they achieved to increase the number of Treg and boost their immunosuppressive capacity. This treatment reduced the final brain lesion volume and improved mice behavioral deficits by attenuating cerebral production of proinflammatory

cytokines and activation of encephalitogenic T cells. They showed that main mediator of Treg function was the immunosuppressive cytokine IL-10.

Li et al[299], showed in their study that the administration of therapeutic doses of exogenous Treg after brain ischemia exerted potent protection against brain damage in transient focal MCAO models in mice. Treg improved both short and long term neurological outcome in the animals. Reduced infarct volume at 7 and 28 days after MCAO were also seen in treated animals. They showed that Treg inhibited cerebral inflammation in the brain as early as 1 day after ischemia. They found a decrease in the production of IL-6, IL-1β IL-17, IL-17 y TNFα. IL-10 and TGFβ were also upregulated at 24 hours post-MCAO. They proposed that Treg may lead their protective effect through MMP-9. Treg inhibited MMP-9 production by neutrophils as early as first day following ischemia. They also propose a Treg role in mantaining the integrity of tight junction complexes and the impermeability of blood brain barrier.

The same group in 2013[309], found that Treg therapy preserves immune homeostasis after cerebral ischemia. Adoptive transfer of therapeutic dose of Treg at 2 hours after MCAO elevated the number of functional Treg in the periphery. A small number of Treg had also infiltrated into the brain. Rats with Treg treatment exhibited significantly lower levels of IL-6, IL-1β and TNFα. Plasma concentrations of IL-10 exhibited a slight increase in the Treg treated group. Moreover Treg treatment corrects the long-term lymphopenia observed after ischemic brain injury. Results also suggest that Treg

treatment improves cell-mediated immunity functions after MCAO, with a balanced TH1/TH2 response. Treg treatment did not exacerbate poststroke immunosuppression, on the contrary, it improved immune status after MCAO.

On the other side, Kleinschnitz et al[302], transfered purified Treg to Rag 1 $^{-/-}$ mice. Reconstitution of Treg in these animals was able to reverse the reduction in stroke size previously observed, underlining that Treg represent a major detrimental T-cell population in acute ischemic stroke.

In a recent study[311], role of Treg in ischemic stroke is studied by Treg expansion with CD28 monoclonal antibody. Mice were treated with CD28 monoclonal antibody injected 3 hours after MCAO. Treated animals showed a higher expression of Treg compared with control. Mean amount of IL-6, TNFα and INFγ found in serum was rather low in CD28 treated animals compared with control. Circulating IL-10 serum levels were significantly increased in treated animals. Administration of CD28SA after MCAO significantly reduced infarct volume at day 7 compared with control-treated mice. Administration of CD28SA 6 hours after MCAO still reduced infarct size 7 days after MCAO. Infarct volume was significantly reduced in CD28SA treated mice when a longer middle cerebral artery was performed (60 min of occlusion). Treatment with CD28SA induces IL-10 production in Treg in the periphery and in the brain after MCAO. In IL-10 knockout mice treated with CD28SA infarct size did not differ, supporting an essential role of IL-10 in this model.

Overall, these findings suggest that Treg reduce the magnitude of

stroke progression through local IL-10 mediated effect.

In other recent publication, expansion of Treg by CD28SA treatment in vivo [312], leads to an increase of Treg in mice. Stroke volume in the treated group was significantly larger compared with control animals. In Rag 1 -/- animals, CD28SA treatment had no impact in stroke volume, so a direct effect of the CD28SA on clinical outcome in the abscense of T and B cells could be excluded. CD28SA treatment administered 3 days before ischemia, induced significantly larger infarcts in DEREG mice containing Treg, while this was no longer in the group of DEREG without Treg. They demonstrated a higher number of occluded vessels and glycoprotein IX positive platelets in CD28SA treated mice. Based on these results, they proposed that Treg promote stroke progression boosting "thrombo-inflammation" (interplay between thrombotic and inflammatoy processes).

The controversial results of the different studies can be explained by the different methods used for Treg depletion or Treg administration in the different studies (some studies use antibodies whereas other studies use genetic mice models) and by the different stroke models in the studies, producing brain ischemia with different size after MCAO[295].

It seems that stroke severity might predict the net biological effect of Treg, because most of studies only report benefit of Treg role in small to moderate sized lesions[241].

STUDY	MODEL	LESION	ENDPOINT	ENHANCEMENT MODEL	INFARCT VOLUME
CHEN ET AL [306]	RAT: DISTAL PERMANTENT	20%	48 HOURS	MUCOSAL IMMUNIZATION	REDUCED
GEE ET AL [307]	RAT: 3 H TRANSIENT	NA	28 DAYS	MUCOSAL IMMUNIZATION	REDUCED
ISHIBASHI ET AL [93]	RATS: DISTAL, PERMANET	20%	28 DAYS	MUCOSAL IMMUNIZATION	REDUCED
LI ET AL [299]	RATS; 2 H TRANSIENT MOUSE: 60 MIN TRANSIENT	40% 35%	3 DAYS	ADOPTIVE TRANSFER	REDUCED
LI ET AL [309]	MOUSE:60 MIN TRANSIENT	35%	3 DAYS	ADOPTIVE TRANSFER	REDUCED
LI ET AL [310]	MOUSE: 60 MIN TRANSIENT	35%	3 DAYS	ADOPTIVE TRANSFER	REDUCED
LIESZ ET AL [308]	MOUSE: DISTAL PERMANTET TRANSIENT	10%	7 DAYS	HDACI	REDUCED
NA ET AL [311]	MOUSE:DISTAL, PERMANENT MOUSE: 60 MIN TRASIENT	10% 50%	7 DAYS 3 DAYS	CD28SA CD28SA	REDUCED REDUCED
XIE ET AL [300]	RATS: 90 MIN TRANSIENT	45%	28 DAYS	mTORinh	REDUCED
KLEINSCHNITZ ET AL [302]	MOUSE:60 MIN TRANSIENT	55%	3 DAYS	ADOPTIVE TRANSFER	INCREASED
SCHUMANN ET AL [312]	MOUSE: 60 MIN TRANSIENT	40%	3 DAYS	CD28SA	INCREASED

Table 5: Summary of studies regarding T reg enhancement paradigms. Modified from[241].

In a recent published meta-analysis performed with the basic studies evaluating Treg role in stroke, the overall effect size estimation revealed an odds ratio favoring the respective intervention for each interventional paradigm and for all the studies in total. However, the large heterogenety between the included studies regarding study design, experimental model, and species (rats and mice) has to be taken into account[241].

3.3 MECHANISMS OF REGULATORY T CELL FUNCTION IN POSTSTROKE NEUROINFLAMMATION

Basic immunologic research on Treg function has identified several mechanisms by which Treg suppress immune reactions. These can be mainly divided into mechanisms acting on lymphocyte activation and mechanisms inhibiting antigen-presenting cells **(Figure 14).**

The most prominent mechanisms of Treg function in vivo are the secretion of anti-inflammatory cytokines, mainly IL-10 and TGFβ. Other mechanisms implied in Treg function are the expression of immunosuppressant molecules, such as CTLA-4, CD39 o PD1, compsumption of vital cytokines (IL-1, IL-2) and the secretion of cytolitic molecules (granzymes or perforin) [239].

In the context of experimental brain ischemia, several studies have demonstrated that IL-10 is a critical neuroprotective cytokine regulating poststroke neuroinflammation[112, 311].

Treg are the main productors of IL-10**,** but this cytokine could also be produced by regulatory B cells and microglia/monocytes. The property of IL-10 as an important tissue protective effector mechanism of Treg was verified in some of the previous stroke studies. Accordingly, strategies increasing lymphocyte-derived IL-10 production[112, 308, 311] or therapeutic IL-10 administration[111, 112, 311, 314] have been shown to improve stroke outcome.

The transcriptional factor E4BP4 and the expression of the gen 3 of lymphocyte activation are essential for the regulation of IL-10 and their production by T lymphocytes[298, 315]. TGFβ is also an important

cytokine in the production of IL-10 by T lymphocytes and is highly expressed in brain tissue. TGFβ enables cell differentiation in T lymphocytes producers of IL-10.

Alternative Treg-related mechanisms have been found to act in ischemic brain such as TGFβ and IL-35[300]. TGFβ is expressed in brain after ischemia and can have an anti-inflammatory effect, protecting neurons from hipoxic, cytotoxic and apoptotic effect. In vivo, recombinant TGFβ1 administration is related with the upregulation of BCL-2 (B-cell lymphoma 2) family proteins, with an antiapoptotic effect: BCL2 and BCL-XL[315].

The role of Treg in the PD-1 (programmed death 1) /PD-L1 (programmed death ligand-1) pathway was also proposed as an important mechanism of Treg function in ischemic stroke. Whereas the immune regulatory function of PD-1 limits ischemic brain lesions and blood-brain barrier damage, PD-L1 seems to have a detrimental effect in stroke[309].

The beneficial effect of Treg at later stages of ischemic stroke was frequently related to the reduced invasion of T cells, suppression of effector T-cell proliferation[112, 299], suppressed cytokine production and reduced microglial/monocyte activation[112, 300, 307].

Some authors proposed that Treg acts through MMP-9 (matrix metalloproteinase 9) in ischemic stroke pathogenesis. One recent study shows that Treg decrease MMP-9 levels, a protein that is responsable of the rupture of blood brain barrier following ischemic stroke, avoiding excessive inflammatory response blocking the pass of

cells through blood brain barrier. In this study, the administration of Treg did not show a increase in IL-10 and TGFβ levels[299].

Other authors[300, 312] proposed a "thromboinflammatory mechanism". Some studies showed that deleterious effects observed with Treg might be independent of their immunologic function but rather attributable to an impact on secondary microthrombosis.

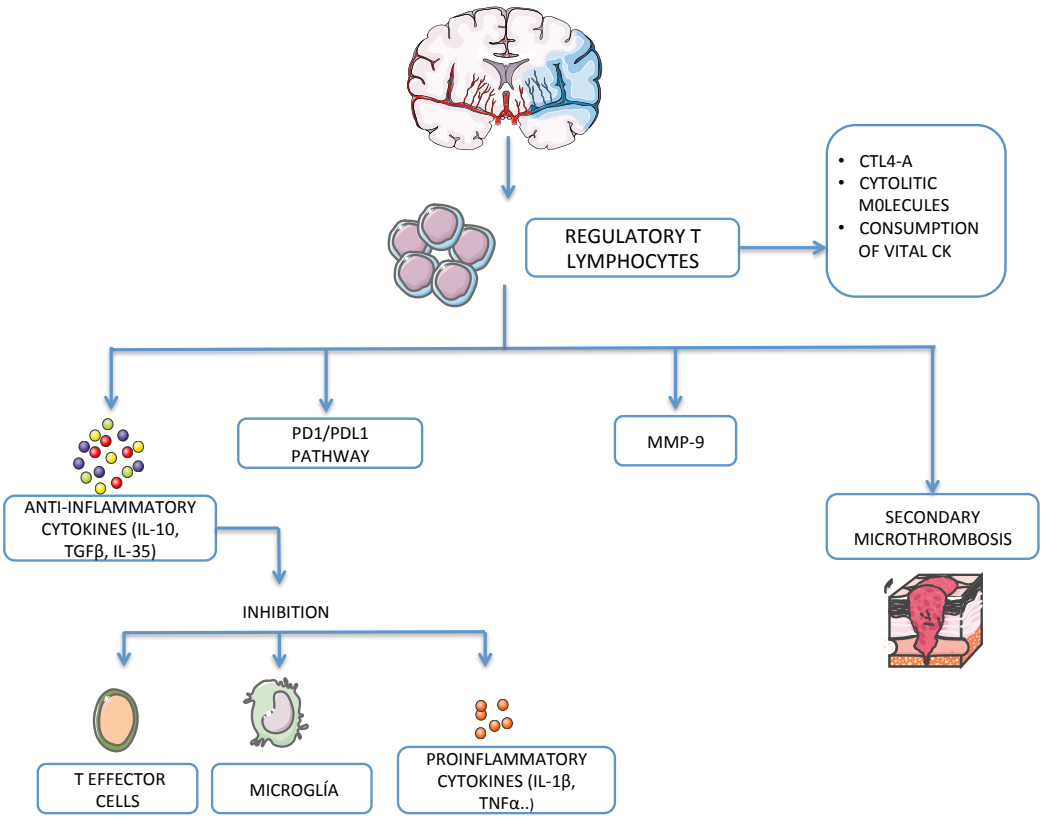

Figure 14. Potential pathways of T reg function in ischemic stroke. The main mechanisms studied in ischemic stroke are: anti-inflammatory cytokine production, PD1/PDL1 pathway, MMP-9 and secondary microthrombosis.

3.4 HUMAN STUDIES ABOUT REGULATORY T CELLS ROLE IN STROKE

In contrast with all the studies performed in animal models of brain ischemia, only a few studies have investigated Treg role in patients experiencing acute ischemia. The first study specifically analyzing Treg function after stroke by Hug et al has found that Treg function is preserved in the context of poststroke immunosuppression in contrast to the dysfunction of effector cell populations[316]. A second study has found reduced suppressive capacities of poststroke Treg in female but not in male patients, proposing sex-specific effects in postroke peripheral immunity[317]. Although the latter study has detected a robust increase on Treg cell counts after stroke in accordance with experimental studies, Li et al reported a significant reduction of peripheral T reg in patients with artherosclerotic stroke[318].

Yan et al[319] studied 77 patients with acute ischemic stroke and 64 control subjects. The percentage of Treg was increased compared to control subject at all times following stroke (day 1, day 7 and week 3 after stroke). There was no significant influence of stroke severity (as determined by NIHSS) on the percentage of Treg. In this study there was a very slight, but not significant increase in the levels of Treg at day 1 in patients who had been on statins prior to stroke.

A report analyzed the association of Treg counts in a cohort of 700 participants of the Malmo Diet and Cancer study with the prospective incidence of stroke. Although low Treg counts at baseline were associated with an increased risk of myocardial infarction, this association was not present for stroke[320].

Urra et al[64] showed Treg decreased after stroke and did not show a relevant association with the development of infections or stroke outcome.

Overall, clinical data are supporting the experimental finding of substantial peripheral immunomodulation after stroke, which is also affecting the Treg population, yet specific changes might depend on stroke entity, severity and patient characteristics[241].

Treg are likely to contribute to poststroke immunosuppression. After severe injuries, Treg play a role in downregulation of innate immune response and that fact could also occurs after stroke. Lymhopenia and increased apoptosis of TH lymphocytes, Treg, B lymphocytes and impaired natural –killer cell activity were observed early after stoke[206, 207, 223] and could be related to infectious complications after stroke. Patients with stroke have a rapid increase of circulating cytokines in plasma with a low ratio of proinflammatory TNFα to anti-inflammatory IL-10 preceding the appearance of infection[321, 322]. Treg and increased IL-10 levels could be implied in immunosuppression after stroke, whereas animal studies do not support this role. Li et al[299, 309] proposed that Treg treatment corrects the long-term lymphopenia observed after ischemic brain injury and improves cell-mediated immunity functions after MCAO, with a balanced TH1/TH2 response. Regarding theses studies, Treg treatment did not exacerbate poststroke immunosuppression, on the contrary, it improved immune status after MCAO. In humans, only small studies did not found relationship between infection development after stroke and Treg levels[64].

JUSTIFICATION

Ischemic stroke is due to the decrease of blood brain flow that causes metabolic and biochemical changes in neurons and glial cells, leading to cell necrosis[18]. Stroke is the fourth leading cause of death in the United States. More than 140,000 people die each year from stroke and is the leading cause of serious, long-term disability in the United States. Each year, approximately 795,000 people suffer a stroke. About 610,000 of these are first attacks, and 185,000 are recurrent attacks[3]. For every 100000 habitants in Spain, 187 people have a stroke each year. Most of them, 80%, are ischemic strokes[4].

The high incidence of stroke and disability following stroke causes high health care costs, productivity loss due to mortality and morbidity, and informal care costs. The only drug approved for clinical use in the treatment of acute ischemic stroke is intravenous tissue plasminogen activator, rTPA[170, 171]. The short therapeutic window and the complications related to this treatment cause important limitations in acute ischemic stroke treatment[172, 173].

There are different mechanisms implied in stroke pathogenesis. In the last years there is more evidence about the role of inflammation in stroke pathogenesis. Decrease of blood flow in a brain area, causes neuronal necrosis and leads to an immune response and the activation of inflammatory cells infiltrating ischemic tissue[54]. Inflammatory response is characterized by accumulation of inflammatory cells and mediators in ischemic brain and systemic changes in peripheral inflammatory response. Those facts contribute to tissue injury following stroke[58, 59]. Although it is known that immune response contribute to tissue damage, attempts to improve stroke outcome by

immunosuppression strategies have been so far unsuccessful[203].

Some mechanisms to control, regulate immune system and to prevent reactivity against autoantigens and brain damage have been proposed in last years. Regulatory T cells are a subgroup of CD4 T lymphocytes that play an important role in maintaining immune homeostasis, preventing autoimmunity and inflammation. In the last decade, some publications proposed that Treg play an important role in immune response, modulating T effector cells function. Due to this immunomodulatory function, some authors have proposed that Treg play an important role in physiopathology after ischemic stroke.

There are some studies in animals that evaluate Treg depletion in experimental brain ischemia, through antibody-mediated lysis, using anti-CD25 specific antibodies [112,297,299,300,310] or using transgenic mice with a diphtheria toxin receptor transgene under the control of the FoxP3 promoter for inducible Treg depletion[112, 301, 302]. There are also some studies testing Treg augmentation in animal models of brain ischemia, through adoptive cell transfer of purified Treg to wild-type animals to increase circulating Treg numbers[299, 302, 309, 310] or with the administration of a CD28 superagonist which induces in vivo expansion of Treg and amplification of their suppressive function[311, 312]. In addition, histone deacetylase inhibition and treatment with mammalian target of rapamicin inhibitor have previously been shown to boost Treg by increasing FoxP3 expression in T cells[308]. Most of experimental studies support an overall positive effect of Treg in brain ischemia and propose that they can constitute a cell population capable of modulating immunologic pathways in stroke. However,

results from these studies are different, and sometimes controversial due to the vast variability regarding study design, experimental model, used species (rat and mice) and the distinct properties of the commonly used ischemia models. On the other hand, no studies have evaluated the possible effect of Treg on neurogenesis and angiogenesis mechanisms after brain ischemic injury.

Despite animal studies exploring Treg role in brain ischemia, only few studies have investigated Treg in patients with ischemic stroke. The first study specifically analyzing Treg function after stroke, performed by Hug et al, has found that Treg function is preserved in the context of poststroke immunosuppression in contrast to the dysfunction of effector cell populations[316]. A second study has found reduced suppressive capacities of Treg after stroke in female but not in male patients, proposing sex-specific effects in postroke peripheral immunity[317]. Li et al reported a significant reduction of peripheral Treg in patients with artherosclerotic stroke[318].

Yan et al[319] found that percentages of Treg were increased compared to control subjects at all times following stroke (day 1, day 7 and week 3 after stroke). In this study, there was no significant influence of stroke severity (as determined by NIHSS) on the percentage of Treg.

A report analyzed the association of Treg counts in a cohort of 700 participants of the Malmo Diet and Cancer study with the prospective incidence of stroke. Although low Treg counts at baseline were associated with an increased risk of myocardial infarction, this association was not present for stroke [320].

Urra et al[64] showed significant higher levels of Treg in stroke patients (hemorragic and ischemic stroke) compared to controls. In the same study, they did not find a relevant association with the development of infections or stroke outcome.

However, these studies include a low number and heterogeneous group of patients and the results were inconclusive. Treg could be involved in inflammation following ischemic stroke, but the relationship between Treg levels and functional long-term outcome is not well established. No studies have investigated temporal profile of Treg during the acute phase of stroke, and nothing is know about the association of Treg and IL-10 in stroke patients. IL-10 is an anti-inflammatory cytokine, considered as one of the main effector mechanisms of Treg after stroke. The association of Treg and infarct volume or the presence of neurological deterioration has not been studied yet.

Regulatory T cells can constitute a potential therapeutic target to improve functional outcome in patients with ischemic stroke. Several studies demonstrated that Treg levels can be regulated. These cells can be *in vivo* stimulated through anti-CD28 antibodies[235, 304, 305], and also can be depleted by anti-CD25 antibodies[112, 299, 300, 310]. An active increase of Treg levels during the acute phase could improve functional outcome in patients with ischemic stroke through an immunomodulatory effect. Due to their potential immunosupressor role, some authors proposed a relationship between high levels of Treg and infectious complications development in acute phase of ischemic stroke. This fact could be a disadvantage for a treatment based on

increasing Treg level. However, the relationship between Treg level, immunosuppression and risk of infection after stroke is not well studied.

Due to the controversial results both in animal and clinical studies about Treg role in brain ischemia, it would be useful to further study the effect of Treg on animals, the clinical relevance and their association with brain and peripheral inflammation. It would be also important to study other potential mechanisms, different from inflammation, which can be implicated in the effect of Treg and have not been previously investigated, such as neurogenesis and angiogenesis. It would be also useful to study Treg evolution in human after ischemic stroke and its relationship with stroke outcome, infarct volume and infectious complication development.

HYPOTHESIS

Our hypothesis is that adoptive transfer of exogenous Treg and in vivo stimulation of endogenous Treg reduce infarct volume in a transient focal ischemia animal model, and the depletion of Treg through antibodies plays a detrimental role, increasing infarct volume. We also hypothesized that this effect of Treg is mediated by a dual mechanism, reducing inflammatory response and increasing neurogenesis and angiogenesis processes.

In clinical practice we hypothesized that higher levels of Treg in peripheral blood of patients with ischemic stroke is associated with good functional outcome at three months. We also think that patients with higher Treg levels would have smaller infarcts and lower neurological deterioration. This effect of Treg is mediated by lower inflammatory response, determined by higher levels of anti-inflammatory cytokines, and is not associated with higher incidence of infectious complications.

OBJECTIVES

To demonstrate our hypothesis we will perform an experimental and a clinical study.

1 EXPERIMENTAL STUDY

1.1 PRIMARY OBJECTIVE

- To analyze the effect of exogenous administration of Treg and endogenous stimulation of Treg (by CD28 superagonist administration) on infarct volume in an animal model of brain ischemia.

1.2 SECONDARY OBJETIVES

- To evaluate the effect of Treg depletion by anti-CD25 antibodies administration on infarct volume in an animal model of brain ischemia.
- To quantify the presence of Treg (by FoxP3 marker), proinflammatory cytokines (IL1β and TNFα) and other markers of active inflammation (CD68 and CD11b) in ischemic hemisphere and contralateral hemisphere in an animal model of brain ischemia after administration of exogenous Treg.
- To quantify the presence of Treg (by FoxP3 marker) and proinflammatory cytokine (IL-1β) in brain tissue after endogenous Treg stimulation and after Treg depletion in an animal model of brain ischemia.
- To determine neurogenesis and angiogenesis markers in ischemic and contralateral hemisphere after brain ischemia in an animal model after administration of exogenous Treg.

- To analyze long-term effects of exogenous Treg administration in an animal model of brain ischemia.

2 CLINICAL STUDY

2.1 PRIMARY OBJECTIVE

- To analyze the relationship between percentage of Treg in peripheral blood and functional outcome at three months in patients with first ischemic stroke.

2.2 SECONDARY OBJECTIVES

- To compare percentage of Treg and IL-10 levels in peripheral blood in patients with acute ischemic stroke and healthy controls.
- To study the temporal profile of Treg and IL-10 levels in acute phase of ischemic stroke.
- To analyze the relationship between percentage of Treg in peripheral blood and infarct volume in patients with first ischemic stroke.
- To evaluate the relationship between percentage of Treg in peripheral blood and early neurological deterioration in patients with first ischemic stroke.
- To study the relationship between percentage of Treg and IL-10 levels in peripheral blood of patients with acute ischemic stroke.
- To study the relationship between percentage of Treg in

peripheral blood and the risk of infection during the acute phase of ischemic stroke

MATERIALS AND METHODS

1 EXPERIMENTAL STUDY

To evaluate the effect of the administration of Treg on infarct volume we previously performed an *in vitro* study to obtain and expand Treg and check the immunosuppressing properties of these cells. Then cells were administered to an animal model of brain ischemia.

The local Animal Care Committee according to the European Union (EU) rules (86/609/CEE, 2003/65/CE and 2010/63/EU) approved all experimental protocols.

1.1 *IN VITRO* STUDIES

1.1.1 Purification and expansion of regulatory T cells.

Male Sprague-Dawley rats weighted between 280 and 330 g were used to obtain Treg. Animals were housed individually, in stable environmental conditions (environmental temperature of 23°C, relative humidity of 40% and a light-dark cycle of 12 h), and free access to food and water. Single-cell suspensions were obtained from neck and mesenteric lymph nodes of the rats.

For the extraction of neck lymph nodes, the next steps were followed:

1. Anesthetize the rat.
2. Make an incision in the middle of the neck, expose sub-maxillary gland and extract lymph nodes located in front of parotid glands **(Figure 15)**.
3. Extract lymph nodes, immerse then in ethanol 40-50% and transfer immediately to PBS (Phosphate Buffered Saline) 2%.

4. Make an abdominal incision through linea alba and locate mesenteric fat (large intestine). Extract mesenteric lymph nodes, immerse them in ethanol 40-50% and transfer to PBS 2% **(Figure 16)**.
5. Locate the lymph nodes in a cell strainer and mash them.
6. Centrifuge the nodes (300xG) and mix them with PBS (1:100 dilution).

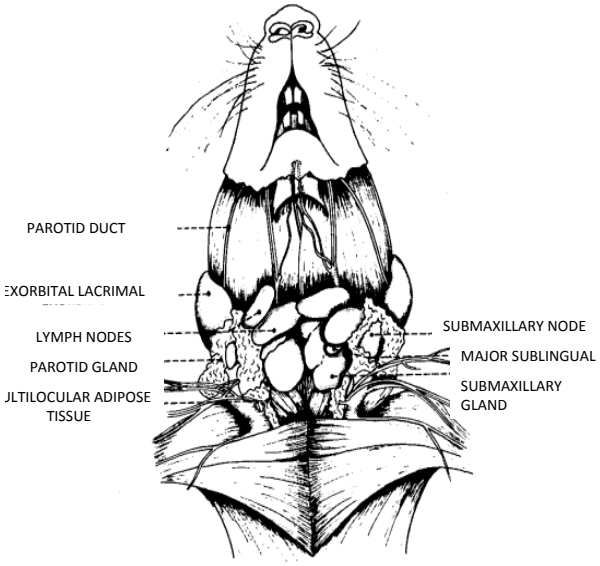

Figure 15. Rat neck diagram. Neck lymph nodes are located in front of parotid glands.

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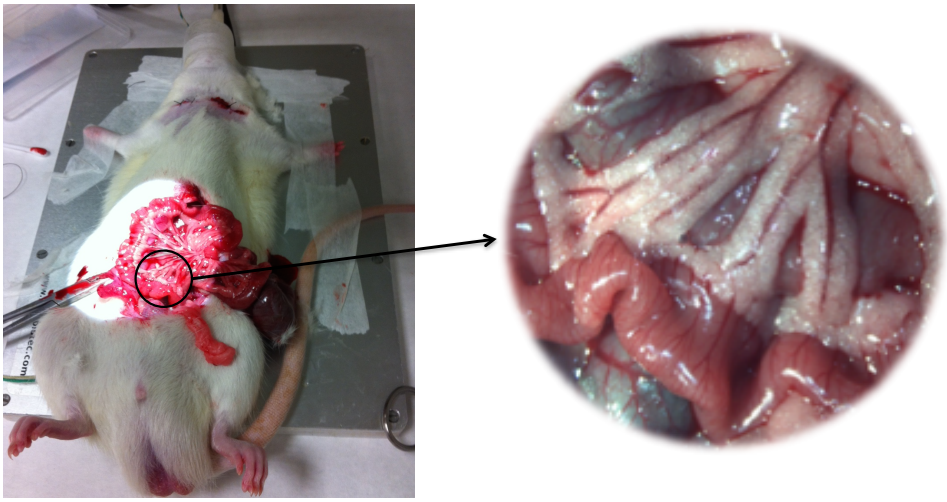

Figure 16. Real image of mesenteric lymph nodes extraction. (A) Image of rat surgery for mesenteric lymph nodes extraction. (B) Zoom image of mesenteric lymph nodes.

Once the neck and mesenteric lymph nodes were removed and the tissue dissaggregated, extracted cells were incubated with 200 µl Fluorescence IsoTioCyanate (FITC)-conjugated anti-CD4 and 200 µl Phycoeritrin (PE)-conjugated anti-CD25 antibodies (BD Biosciences, NJ, USA), both at 10 µL/10^6 cells **(Figure 17).**

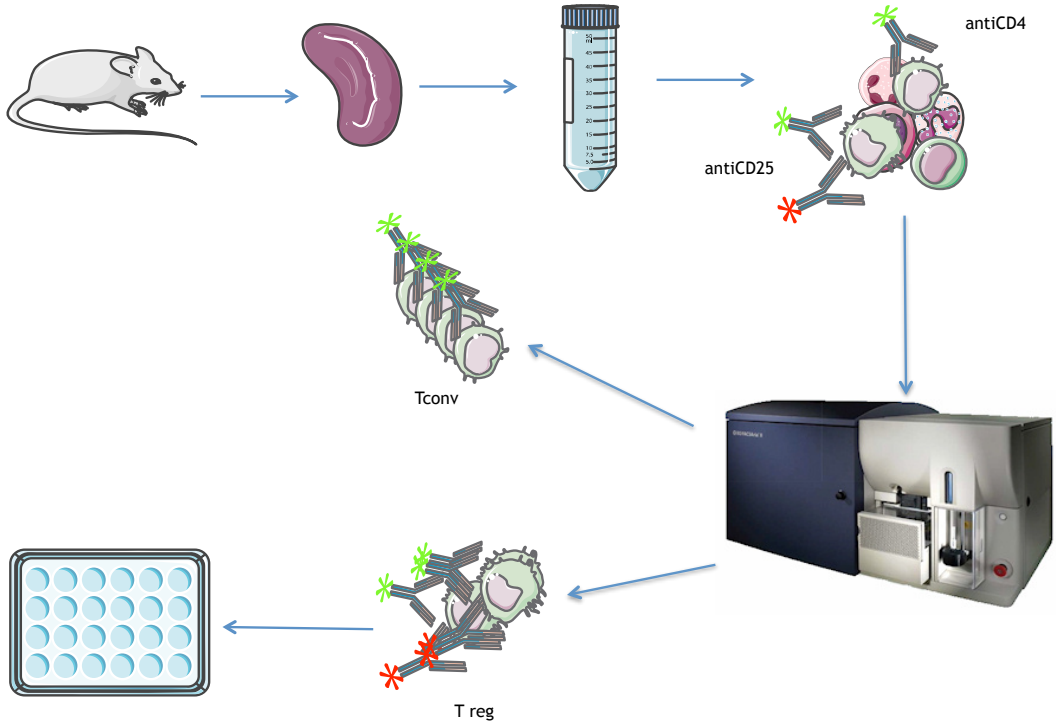

Figure 17. Single-cell suspensions were obtained from the neck and mesenteric lymph nodes, incubated with FITC (Fluorescence IsoTioCyanate)-conjugated anti-CD4 and PE (Phycoeritrin)-conjugated anti-CD25 antibodies. CD4+CD25+ (Treg) and CD4+CD25- cells (Tconv) were sorted using FACSAria I cell sorter.

CD4+CD25+, regulatory T cells (Treg) and CD4+CD25- cells, conventional T cells (Tconv) were sorted using FACSAria I cell sorter (BD Biosciences, NJ, USA), achieving 84.7±4.8% and 97.4 ±0.9% of purity, respectively **(Figure 18)**.

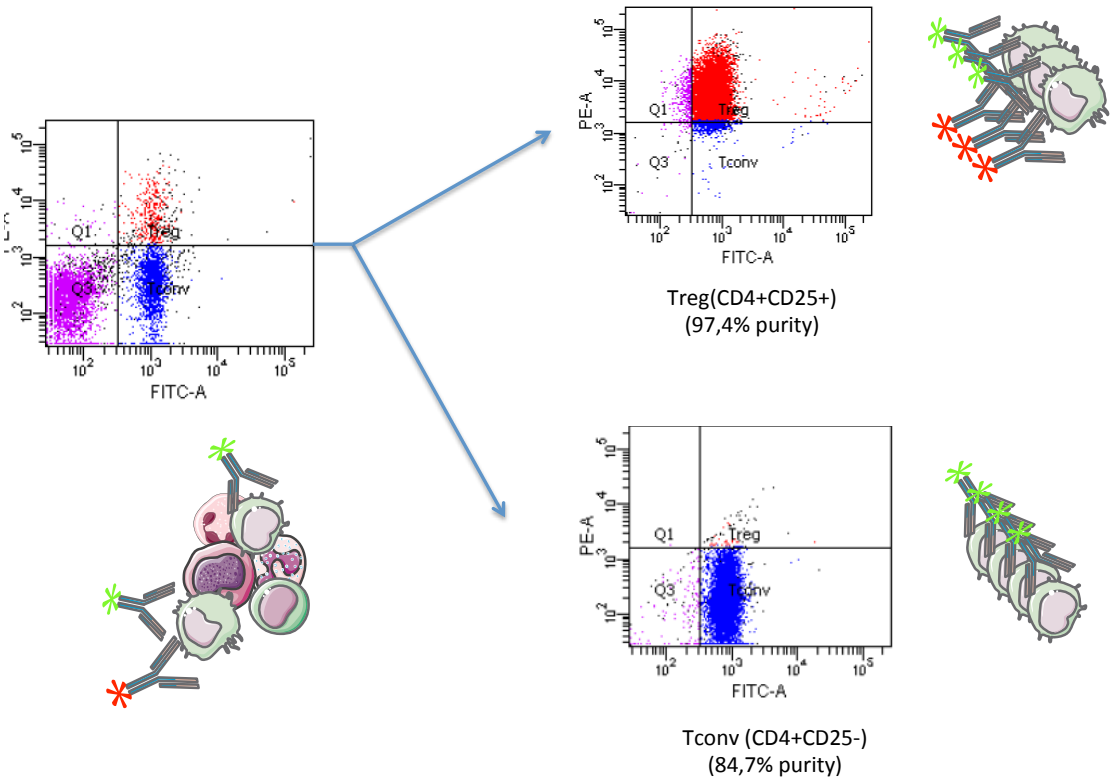

Figure 18. CD4+CD25+ (Treg) and CD4+CD25- cells (Tconv) were sorted using FACSAria I cell sorter (BD Biosciences, NJ, USA).

Treg were expanded in vitro as previously described[323] and Tconv were used in co-cultures for in vitro suppression assays. Treg were suspended in *x-vivo* 15 ™ medium, supplemented with 15% fetal bovine serum, 30 µM of mercaptoethanol, non-essential amino acids, sodic piruvate, glutamine, penicillin and streptomicin. For costimulation anti-CD28 (clone JJ316; BD Biosciences, USA) (5 µg/mL), and recombinant human IL-2 (300 U/ml) were added in solution after 2 days of incubation. The cells were used the day 5 after culture.

1.1.2 Verification of immunosuppressing properties of expanded regulatory T cells

In vitro supression assays were performed to verify immunosuppressing properties of expanded Treg. Freshly isolated

Tconv were stained with carboxyfluorescein succinimidyl ester diacetate (CFSE) dye (5 μM: Invitrogen, Carlsbad, California, USA) and $5x10^4$ cells per well were stimulated to proliferate with 2 μg/mL of anti-TCR mAb (BD Biosciences, USA) and 10 μg/ml of antiCD28 mAb (clone JJ316; BD Biosciences, USA).

To determine the immunosuppressing capacities of Treg, different numbers of regulatory T cells were added (control, $1.25x10^4$, $2.5x10^4$ and $5x10^4$). Tconv and Treg were co-cultured for 3 days, when cells were analyzed by flow cytometry. Proliferation was measured by determining the dilution of CFSE fluorescence[324].

1.2 ANIMAL STUDIES

1.2.1 Brain ischemia rat model

Transient focal cerebral ischemia was induced in male Sprague-Dawley rats weighted between 280 and 330 g by intraluminal occlusion of the middle cerebral artery, performed as previously described[325] **(Figure 19).** Animals were housed individually, in stable environmental conditions (environmental temperature of 23°C, relative humidity of 40% and a light-dark cycle of 12 h), and free access to food and water.

Surgical procedures were performed under sevoflurane anesthesia (6% induction and 4% maintenance in a gas mixture of 70% NO_2 and 30% O_2). During surgery, all animals were subjected to temperature control, maintaining rectal temperature at 37±0.5°C by a thermostat-controlled electric pad (NeosBiotec, Spain). Animal heads were located

out of the thermal pad, over a porexpan plate, to avoid the direct contact between the pad and head.

Transient focal ischemia (45min) was induced by intraluminal occlusion of the middle cerebral artery (MCA), following the method described by Longa et al[325]. Commercial sutures (Doccol, MA, USA) with a silicon-rubber coated head of 350 µm in diameter and 1.5 mm in length were used to perform the occlusion. Cerebral blood flow was monitored with a Periflux 5000 laser-Doppler system (PerimedAB, Sweden) by placing a Doppler probe (model 411, PerimedAB, Sweden) in the parietal bone surface near the sagital crest, under the temporal muscle.

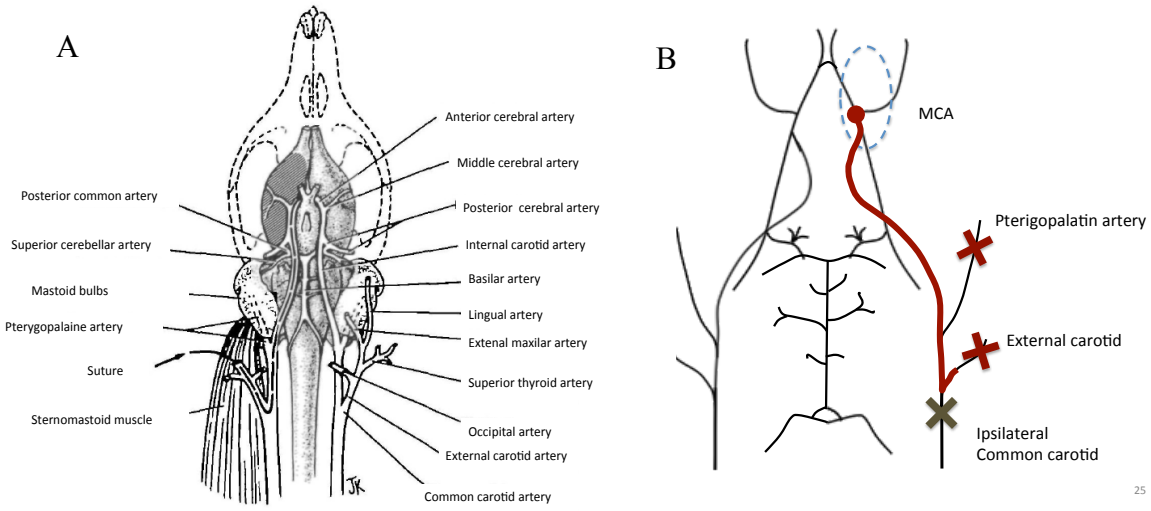

Figure 19. MCAO (middle cerebral artery occlusion) surgery procedure (A) Vascular anatomy of the rat. Adapted from[325] (B) Brain ischemia rat model: A suture was introduced into the external carotid artery lumen through a puncture and advanced within the internal carotid artery lumen blocking the origin of MCA, occluding sources of blood flow. After 45 minutes of ischemia the suture was pulled back to perform reperfusion.

Reperfusion was performed 45 min after the occlusion onset. Animals were subsequently randomized in one of the experimental groups performed, by using computer-generated random numbers. Only rats with a ≥75% reduction in hemispheric cerebral blood flow (measured by laser Doppler flow) were included in the study.

1.2.2 Experimental studies

To determine the role of Treg cells in ischemic pathology, 4 different experimental conditions were tested to evaluate different effects:

- Study 1: Effect of exogenous Treg administration
- Study 2: Effect of stimulation of endogenous Treg
- Study 3: Effect of inhibition of Treg
- Study 4: Evaluation of neurogenesis and angiogenesis processes

Study 1: Effect of exogenous Treg administration

The objective of the first study was to assess the role of the adoptive transfer of exogenous Treg after MCAO model.

Twenty rats were assigned to control (n=10) or Treg treated group (n=10). Rats in control group received an intravenous intraiugular injection **(Figure 20)** of 1 mL of PBS, two hours after ischemia induction. Rats in treated group received an injection of $3x10^6$ expanded Treg cells in 1 ml of PBS. Infarct volumes were analyzed by magnetic resonance imaging (MRI) at days 1,3,7 and 10 after ischemia onset. At day 10 animals were sacrificed, and the brains were used to analyze IL-1B, FoxP3, CD11b and CD68 positive cells **(Figure 21)**.

Western Blot was used for FoxP3 determination, ELISA analysis for IL-1β and TNFα and immunohistochemistry for CD11b and CD68 positive cells evaluation.

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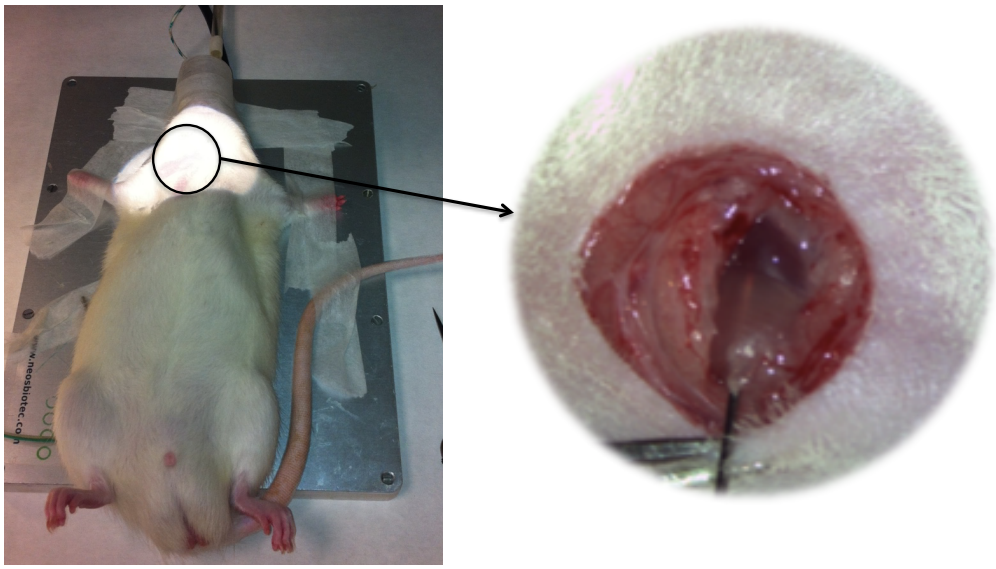

Figure 20. Real image of intravenous intraiugular injection of treatments in the rat (A) General view of the rat during the experiment (B) Zoom image of intraiugular injection.

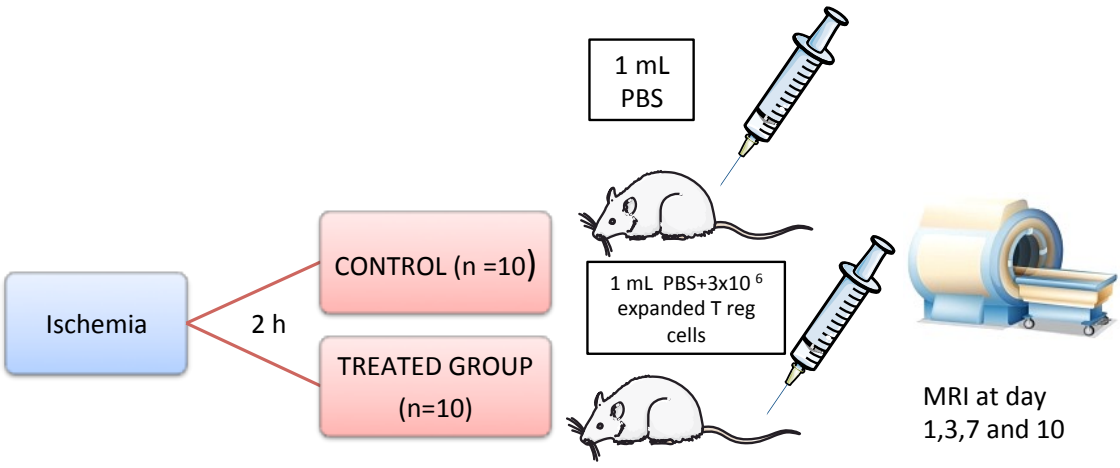

Figure 21. First experiment. The control group received an intravenous injection of 1 mL of PBS and the treated group received an intravenous injection 1 mL of PBS with $3x10^6$ expanded regulatory T cells.

Study 2: Effect of stimulation of endogenous Treg

A promoter of endogenous Treg was tested in the second experimental study. With that purpose, rats received an intravenous injection of 1 mL of PBS (Control group, n=8) or 500 mg/animal of anti-CD28 monoclonal antibody (clone JJ316; BD Biosciences, USA) in 1 mL of PBS (Treated group, n=8), four days before ischemia. CD28A have shown to increase Treg level in animal models three days after treatment[304, 305, 326, 327]. To verify the expansion of Tregs after CD28SA treatment, we determined Treg levels (CD4+FoxP3+ cells) in peripheral blood by flow cytometry, at day 1, 2, 3 and 4 after treatment administration. Infarct volumes were analyzed by magnetic resonance imaging (MRI) at days 1, 3, 7 and 10 **(Figure 22)**. At day 10 animals were sacrificed, and the brains were used to analyze FoxP3 levels and IL-1β. Western Blot was used for FoxP3 determination, ELISA analysis was used for IL-1β determination.

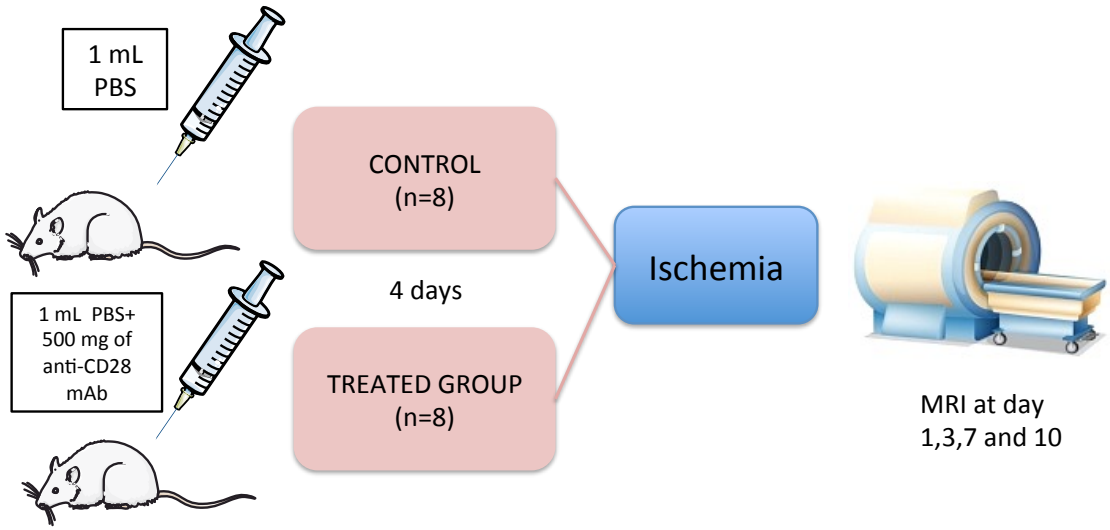

Figure 22. Second experiment. Treated group received an intravenous injection of 500 mg/animal of antiCD28 antibodies in 1 mL of PBS four days before ischemia, the control group received an intravenous injection of 1 mL of PBS four days before ischemia.

Study 3: Effect of inhibition of Treg

In the third study, an inhibitor of Treg was tested. Treated group (n=8) received 500 mg/animal of anti-CD25 antibodies (clone OX39; BD Biosciences, USA) in 1 mL of PBS four days before ischemia. As control animals we used the same group as used in study 2 (rats received an intravenous injection of 1 mL of PBS four days before ischemia). Anti-CD25 antibodies have previously showed to decrease Treg levels 48 hours after treatment administration[112, 300, 310] .To verify the depletion of Treg after anti-CD25 antibody treatment, we determined Treg levels (CD4+FoxP3+ cells) in peripheral blood by flow cytometry at day 1, 2, 3 and 4 after treatment administration. Infarct volumes were analyzed by magnetic resonance imaging (MRI) at days 1,3,7 and 10 **(Figure 23)**. At day 10 animals were sacrificed, and the brains were used to analyze FoxP3 levels and IL-1β. Western Blot was used for FoxP3 determination and ELISA analysis was used for IL-1β determination.

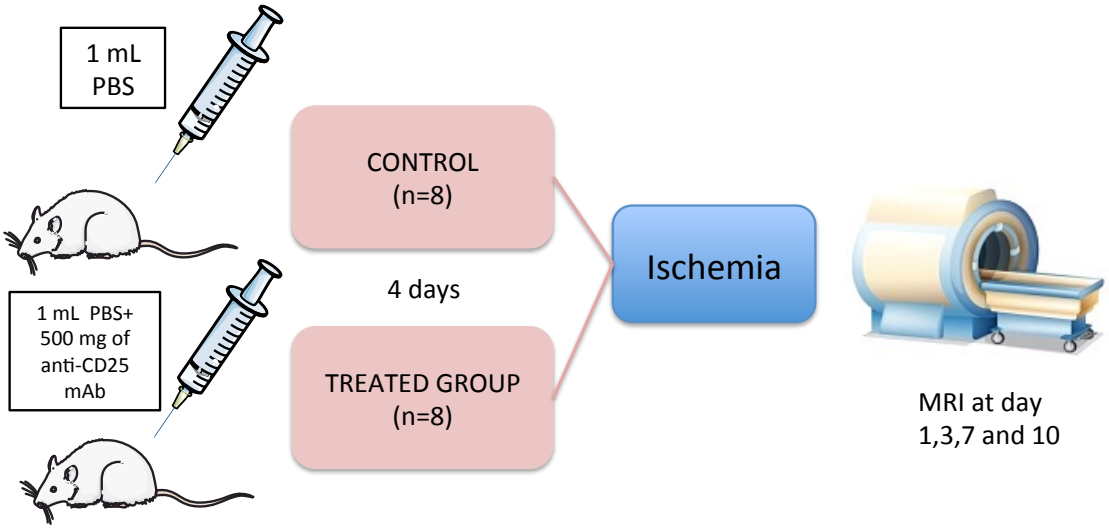

Figure 23. Third experiment. Treated group received an intravenous injection of 500 mg/animal of anti-CD25 antibodies in 1 mL of PBS four days before ischemia, the control group received an intravenous injection of 1 mL of PBS four days before ischemia.

Study 4: Evaluation of neurogenesis and angiogenesis processes

In the fourth experimental study, the long time effects of Treg on neurogenesis and angiogenesis processes were tested. Control group (n=8) received an intravenous injection of 1mL of PBS, two hours after the induction of ischemia, and treated group (n=8) received an injection of $3x10^6$ expanded Treg in 1 mL of PBS. Infarct volume was analyzed by MRI at days 7,14, 21 and 28.

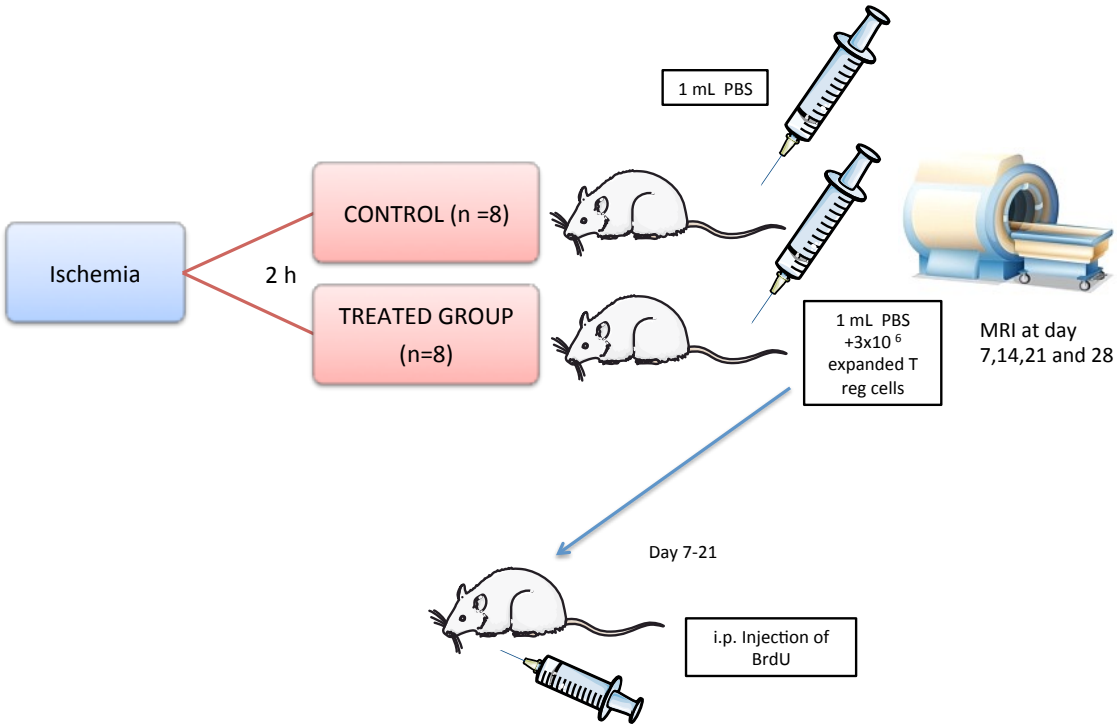

Figure 24. Fourth study. Treated group received an intravenous injection of 1 mL of PBS and $3x10^6$ expanded Treg cells and control group received an intravenous injection of 1 mL of intravenous PBS. Between days 7 and 21 all animals received an intraperitoneal injection of BrdU.

All animals received an intraperitoneal injection of BrdU (bromodeoxyuridine) between days 7 and 21, (Sigma-Aldrich, Buchs SG, Switzerland) (50 mg/kg). At day 29, animals were sacrificed. Brains were used to analyze the presence of neurogenesis and

angiogenesis **(Figure 24).** BrdU, NeuN (neuronal nuclei) and NCAM (neuronal cell adhesion molecule) were considered as neurogenesis markers[328-330]. CD31 was used to demonstrate the presence of endothelial cells and to evaluate the degree of angiogenesis[331,332].

1.2.3 Technical procedures

1.2.3.1 MAGNETIC RESONANCE (MRI) PROTOCOL

MRI was used to assess infarct volume and brain edema. Images were acquired at 9.4 Tesla MRI (Bruker Biospec, Ettlingen, Germany) **(Figure 25).** T2-weighted images were obtained using RARE (Rapid Acquisition with Refocused Echoes) pulse sequences with a train of n= echoes. Two imaging settings were used with the following parameters:

1) Effective echo time TE eff= 4 ms, repetition time TR=3,5 s, signal average n=2, field of view FOV= 19.2x19.2 mm2, matrix 192x192 points, in-plane resolution of 0.1x0.1 mm2, 14 consecutive slices of 1 mm thickness and a acquisition time of 5 min 36 s.
2) Effective echo time TE eff= 60 ms, repetition time TR=2.0 s, signal average n=1, field of view FOV= 28.8x28.8 mm2, matrix 192x192 points, in-plane resolution of 0.15x0.15 mm2, 14 consecutive slices of 1 mm thickness and a acquisition time of 1 min 12 s.

Images were analyzed and processed by a scientist blinded to the study using Bruker Paravision 5.1 software and Image-J (Rasband, W.S., ImageJ, U.S National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2011).

After measuring the volume of both brain hemispheres, edema formation was quantified by the difference in hemispheric volumes using the following equation: Edema (%)= 100*[1- (V1/V2)], with V1 and V2 representing the contralateral and ipsilateral hemisphere volumes, respectively.

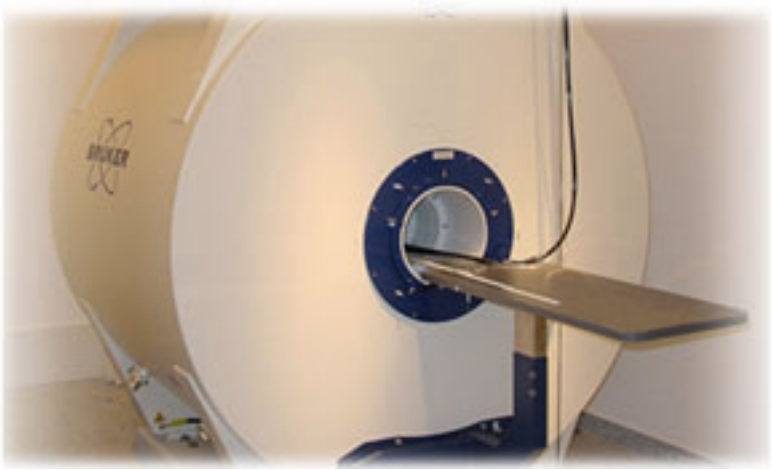

Figure 25. 9.4 Tesla MRI (Bruker Biospec, Ettlingen, Germany).

1.2.3.2 WESTERN BLOT AND ELISA ANALYSES

FoxP3, IL-1β and TNFα were analyzed in animal brains. FoxP3 is a transcription factor that is an exclusive marker for Treg and IL-1β and TNFα are proinflammatory cytokines. Western blot analyses were performed to quantify FoxP3 expression. ELISA tests were performed to quantify IL-1β and TNFα in rat brains following ischemia.

Brains were extracted, and the infarcted and contralateral hemispheres were separated, sonicated with RIPA buffer (Sigma-Aldrich, St.Gallen, Switzerland) and centrifuged at 18000 g for 30 min at 4°C. Protein concentrations of the supernantants were calculated

using the Bradford method.

Western blots were performed using 50µg of protein, separated with 10% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel) and blotted onto PVDF (polyvinylidene fluoride) membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% fat dehydrated milk in Tris Buffered Saline (TBS)(Sigma-Aldrich, MO, USA) for 2 hours and incubated overnight at 4°C with mouse anti rat FoxP3 (1:75 dilution, Santa Cruz Biotechnology, Heidelberg, Germany) and rabbit anti-rat actin (1:3000,Abcam, Cambride, UK). The membranes were incubated with goat anti mouse IgG-Cy3 and goat anti-rabbit IgG-Cy5 (1:3000 both, GE Lifesciences, Munich, Germany) for 2 h and were digitized using a FXProplus scanner (Bio-Rad, Hercules CA, USA). The intensity of bands was analyzed with Quantity One software (Bio-Rad).

ELISA tests for IL-1β and TNFα were acquired from GE Lifesciences (Munich, Germany), and the quantification of these proteins was performed according to the manufacturer´s instructions.

1.2.3.3 FLOW CYTOMETRY DETERMINATION

Flow cytometry in peripheral blood was performed to determinate levels of Treg in rats after treatment administration (CD28SA and anti-CD25 antibodies). For the analysis of Treg, 50 µL of peripheral blood of treated rats were used. Lymphocytes were separated by their forward and side scattering signal characteristics. Peripheral blood was incubated with 10 µL of anti- CD4 antibody tagged with FITC (BD PharmigenTM,USA), and anti-Foxp3 antibody tagged with Alexa Fluor

(BD PharmigenTM, USA). Finally sample was loaded on the FASCAria flow cytometer. Treg were identified as CD4+FoxP3+, and levels were expressed in percentages of CD4+FoxP3 over total CD4+ cells.

Flow cytometry was also used to determinate neurogenesis and angiogenesis markers after exogenous Treg administration. As neurogenesis markers, we used BrdU (bromodeoxiuridine), NeuN (neuronal nuclei) and NCAM (neuronal cell adhesion molecule). BrdU is a uridine analogue, which incorporates into the DNA during cells division, was used to detect newly generated nucleus[328]. NeuN is a mature neuronal marker protein that detects the neuronal character of the nucleus[328]. NCAM is an adhesion molecule that is expressed in neurogenic sites in postnatal brain[329, 330]. As angiogenesis marker we used CD31 or PECAM (platelet endothelial cell adhesion molecule). CD31 is a molecule used to demonstrate the presence of endothelial cells and evaluate the degree of angiogenesis[331, 332].

Brain hemispheres were separated and homogenized using a cell strainer. Cells were washed and stained by incubation (15 min) with NCAM-PE (10 µL per 10^6 cells, Miltenyi Biotec, Bergisch Gladbach, Germany) or CD31-PE (2 µL per 10^6 cells, BD Bioscences, San Jose, CA, USA) antibodies. Then, cells were suspended in 100 µL of Cytofix-cytoperm (BD Biosciences, San Jose, CA, USA) and stained for BrdU using a APC BrdU Flow Kit from BD Pharmigen (BD Biosciences, San José CA, USA), according to the manufacturer´s instructions. Finally, cells were stained with NeuN-Alexa Fluor 488 (10 µL per 10^6 cells, Chemicon Millipre, Temecula, CA, USA) and 50,000 cell events were acquired in BD FACS AriaI (BD Biosciences, San José CA, USA); the

number of double positive NeuN-BrdU, NCAM-BrdU or CD31-BrDU cells were counted.

1.2.3.4 IMMUNOHISTOCHEMISTRY

Immunochemistry of inflammatory cells was performed on three animals per group to determine the markers of active inflammation Cd11b and CD68[333, 334].

Extracted brains were cut into 3-mm thick coronal slices and immersed in a 20% sucrose solution for 2 h at 4°C. Brain slices were embedded in OCT (optimum cutting temperature) compound (Sakura Finetek Inc., Torrance, CA) and stored at -80°C. For immunostaining, 10 μm coronal sections were obtained using a cryosat. Sections were incubated with 3% H_2O_2 and 10% methanol in PBS, to block endogenous peroxidase. Sections were incubated overnight at 4°C with primary antibodies against CD68 and CD11b, following by incubation with a biotin conjugated secondary anti-rabbit antibody for 1 h and streptavidin-conjugated peroxidase for 30 min. The slices are visualized under an IX-51 microscope (Olympus Life and Material Science Europe GMHB, Hamburg, Germany), which was attached to a DS-U2 LCD camera. All slides were evaluated by a blinded, trained observer. Cells were counted in five representative fields of 192 μm^2 in the infarcted area.

2 CLINICAL STUDY

We developed a prospective analysis, including consecutive patients with diagnosis of ischemic stroke within 12 hours from the symptom onset that met all the inclusion criteria and none of the exclusion criteria listed below. All patients were admitted in the acute Stroke Unit and treated by the same team according to Guidelines of the Cerebrovascular Diseases of Stroke Unit of Santiago de Compostela[335]. A control group was included to compare Treg and IL-10 levels between healthy population and stroke patients.

The Local Ethical Committee of the Clinical University Hospital of Santiago de Compostela approved the protocol before patients and control subjects were included. Healthy controls and patients gave signed informed consent before inclusion in the study. When patients were not able to sign, direct relatives gave signed informed consent.

Clinical, neuro-radiological and molecular variables were included in a database to study the influence of Treg level on prognosis. Functional outcome at 3 months was considered as main variable. As secondary variables we analyzed the presence of neurological deterioration and final infarct volume. The development of infections was recorded as safety variable. To investigate the possible mechanism of action of Treg in clinical practice, we analyzed the correlation of these cells with IL-10 levels.

Clinical and laboratory staffs were blinded respect to each other´s data.

2.1 SUBJECT SELECTION

2.1.1 Patient selection

We prospectively included consecutive patients with ischemic stroke that met all the following inclusion criteria and none of the exclusion criteria:

INCLUSION CRITERIA:

1. Hospitalized patients with first episode of ischemic stroke within 12 hours from symptoms onset.
2. Age >18 years.
3. Previously independent for daily living activities (defined as modified Rankin Scale≤1).
4. Signed the informed consent.

EXCLUSION CRITERIA:

1. Presence of intracerebral haemorrhage in admission neuroimagen.
2. Previous ischemic stroke.
3. Cancer or a severe systemic disease that determine a life expectancy lower than 6 months.
4. Inflammatory disease in the last 30 days before admission (arthritis, prostatitis, neuritis, myositis).
5. Chronic inflammatory diseases (vasculitis, connective tissue diseases, inflammatory central nervous system diseases, intestinal inflammatory diseases, pelvic inflammatory disease, psoriasis, venous ulcers or chronic obstructive pulmonary disease).

6. Pregnancy.
7. Renal replacement therapy or receiving treatment with steroids, immunosuppressor drugs, immunomodulatory drugs or antibiotics in the last 30 days.
8. Periodontal disease or bacterial endocarditis.
9. Fever in the previous 72 hours, defined as temperature determination over 38 Celsius degrees (ºC).
10. Active infection (temperature over 37.5 ºC and one of the following criteria):
    a. Leucocyte levels over 15.000/μL or lower than 4.000/μL.
    b. Cough and spitting.
    c. Voiding syndrome.
    d. Diarrhea.
    e. Clinical signs of endocarditis.
    f. Clinical signs of meningitis
11. Inclusion in other clinical trial.

2.1.2 Control selection

We included a group of control subjects without any neurological, inflammatory or infectious disease. The selection of these subjects was made inviting the relatives of patients included in the study to participate. Control subjects were matched by sex and age with the patients included in the study.

2.2 PATIENT EVALUATION

All patients were admitted in the Stroke Unit of Santiago de Compostela Clinical University Hospital. Once the patient was admitted

in the Emergency Department, demographic data and medical history was recorded. The initial evaluation of stroke patients included the following complementary tests: blood sample for blood count, biochemistry and coagulation tests, 12-lead ECG, brain computerized tomography, chest radiography and carotid and transcranial ultrasonography.

The initial data collected were:

1. Age.
2. Gender.
3. Latency time, defined as the time between symptoms onset and the arrival to the hospital.
4. Medical history: hypertension, diabetes mellitus, atrial fibrillation, peripheral arteriopathy, ischemic cardiopathy and toxic habits.
5. Treatment before the ischemic event.
6. Intravenous or intraarterial thrombolysis.
7. Body temperature in the axillary hole.
8. Blood pressure measured by non-invasive techniques.
9. Neurologic examination in the admission to the Hospital.

Patients with previous disability were excluded from the study. To evaluate the degree of dependence in the daily activities before the stroke onset, modified Rankin Scale (mRS) was used. The modified Rankin Scale (mRS) is a commonly used scale for measuring functional status in people who have suffered a stroke or other causes of neurological disability, and it has become the most widely used clinical outcome measure for stroke clinical trials **(Table 6)** [336]. This scale

varies from 0 to 6, where 0 is a patient without any symptom and 6 is a death patient. The degrees of disability are listed in **table 6**. We only included patients with previous independence, defined as mRS ≤1.

MODIFIED RANKIN SCALE (mRS)	
0	No symptoms
1	No significant disability. Able to carry out all usual activities, despite some symptoms.
2	Slight disability. Able to look after own affairs without assistance, but unable to carry out all previous activities.
3	Moderate disability. Requires some help, but able to walk unassisted.
5	Moderately severe disability. Unable to attend to own bodily needs without assistance, and unable to walk unassisted.
5	Severe disability. Requires constant nursing care and attention, bedridden, incontinent.
6	Dead

Table 6. Modified Rankin Scale (mRS) [336].

2.2.1 Stroke etiological classification

Once the initial evaluation of patients was performed, echocardiogram and/or ECG monitoring were performed for stroke etiological classification. Stroke was classified in four groups according

to TOAST criteria[8]:

- Atherothrombotic
- Cardioembolic
- Lacunar
- Undetermined

2.2.2 Neurogical evaluation

To evaluate neurologic deficit the National Institute of Health Stroke Scale (NIHSS) was used[337-341] **(Appendix).** NIHSS was assessed at inclusion of patients, every 24 hours during the first 72 hours, at 7 days, at discharge and at 3 months. NIHSS is a scale used for the evaluation of basic neurologic functions in acute stroke phase. It is compound by 11 items, allowing a quickly examination of mainly functions: cortical function, cranial nerves, motor function, sensibility, coordination and language. This scale classifies stroke in different groups according to its severity: 0: no deficit, 1: minimal deficit, 2-5: minor deficit, 6-15 moderate deficit, 15-20 important deficit, >20: severe deficit[341].

Modified Rankin Scale was assessed at discharge and at 3 months for functional outcome evaluation. NIHSS and mRS were evaluated by internationally certified neurologists.

2.3 STUDY VARIABLES

2.3.1 Primary endpoint

The main variable of the study was functional outcome at 3 moths. To evaluate functional outcome mRS was used. We classified patients

in two groups according to the score of mRS:

- Group 1: Patients with good outcome. We considered in this group independent patients, defined as mRS score ≤2.
- Group 2: Patients with poor outcome. In this group we included patients with functional dependency, defined as mRS > 2. This group included death patients (mRS = 6).

2.3.2 Secondary endpoints

We also considered secondary variables to evaluate the effect of Treg on patients with ischemic stroke. These variables were the presence of early neurological deterioration and final infarct volume.

2.3.2.1 EARLY NEUROLOGICAL DETERIORATION

We defined the presence of early neurological deterioration (END) as an increase of 4 points or more in NIHSS assessment between baseline and any other NIHSS evaluation during the first 72 hours.

2.3.2.2 INFARCT VOLUME

To evaluate final infarct volume, a control computerized tomography was performed between 4 to 7 days after ischemic stroke. Infarct volume is expressed in cubic centimeters and was assessed according to the formula 0,5 x a x b x c, where “a” and “b” correspond to higher diameters in perpendicular direction and “c” to the number of 10 mm slices where ischemic stroke is present[342].

2.3.3 Safety endpoints

We considered the presence of any infection during the

hospitalization as a safety variable. A protocol to evaluate the presence of infections during the acute phase of stroke has been implemented. When the patient showed an axillar temperature over 37,5 °C in two different determinations separated by 1 hour or one axillar temperature determination over 38 °C, the following tests were performed:

1. Urgent blood sample for blood count and biochemical study and blood culture were obtained.
2. A clinical suspicion regarding the infection origin was made, and if it was possible, samples were obtained to perform a specific culture (urine, feces, sputum).
3. If a respiratory infection was suspected, chest X-ray was performed. If a respiratory infection was due to bronchoaspiration, the patient bed was elevated between 45 and 60 degrees and the patient received suerotherapy and oral diet was stopped.

After the etiological study of the infection, empiric antibiotherapy was started according to clinical suspicion. Once an antibiogram was obtained, in case of positive cultures, specific antibiotic treatment was started.

Data regarding fever presence, infection origin and antibiotic treatment has been recorded.

2.4 LABORATORY TESTS

2.4.1 Regulatory T lymphocytes determination

Previously to patient inclusion, we selected 20 patients with ischemic stroke that met the same inclusion/exclusion criteria to evaluate temporal profile of Treg during the acute phase of ischemic stroke. Blood samples were collected at baseline, day 1, day 2, day 3, day 4, day 5 and day 7. According to this temporal profile we later selected blood samples determinations that were more significant for Treg evaluation in all patients.

To identify regulatory T cells from a heterogeneous cell sample (blood sample from the patient) we need to identify some cell specific markers. Some studies showed that FoxP3 is the most specific marker for Treg. Treg are usually identified as CD4+ CD25+ and FoxP3+ cells[254]. FoxP3 is an intracellular protein, so it is difficult to identify it without the destruction of cell membrane. It cannot be used to separate human Treg for functional studies or in vivo expansion for cellular therapy, thereby limiting its use in the human setting. There are some studies that propose that FoxP3 presence is directly related to the absence of IL-7 receptor (CD127). FoxP3 interacts with CD127 promotor, decreasing CD127 expression in Treg[250-252]. Regarding these studies, other possibility to detect Treg is the identification of these cells as CD4+ CD25+ and CD127-. Thus, the CD127 biomarker can be used to selectively enrich human Treg for in vitro functional studies and, potentially, in vivo therapy.

For the analysis of Treg, 50 µL of peripheral blood were used. Blood samples were collected with an evacuated tube system (Vacutainer) and the blood tubes (BD Vacutainer ® SST) were mixed with ethylenediaminetetraacetic acid (EDTA). Treg were analyzed by flow cytometry, using FACSAria I cell sorter (BD Biosciences, NJ, USA). Fifty µL of peripheral blood were incubated with 10 µL of anti-CD4 antibody tagged with FITC (BD Pharmigen™, USA), 10 µL of anti-CD127 tagged with Alexa Fluor® 647(BD Pharmigen™, USA) and 10 µL of anti-CD25 tagged with PE (BD Pharmigen™, USA). Then 500 ml of erythrocyte lysis buffer was added and the sample was kept at room temperature for 5 min. Finally, sample was loaded on the FASCAria flow cytometer. Lymphocytes were separated by their forward and side scattering signal characteristics. Data acquisition was performed until 50,000 events (cells passing through the system detection chamber) were recorded. CD4+/CD25+/CD127- immune selective-fluorescent probe was used to quantify regulatory T cells (according to the pre-defined minimun signal threshold level).

Due to the differences in total lymphocyte counts between acute ischemic stroke patients, to compare Treg levels between different samples we used percentage of Treg over total analyzed lymphocytes, instead of total Treg count.

2.4.2 IL-10 determination

For IL-10 determination, the same temporal profile than in case of Treg was used. Blood samples were collected with an evacuated tube system (Vacutainer) and the blood tubes (BD Vacutainer ® SST) were

mixed with EDTA. Blood samples were kept frozen under 80 ºC for further determination of IL-10 levels.

IL-10 levels were determined by ELISA test acquired from GE Lifesciences. The quantification of these proteins was performed according to the manufacturer´s instructions.

3 STATISTICAL METHOD

3.1 Sample size

To estimate the sample size of the clinical study, we used the program EPIDAT 3.1[343]. Assuming that patients with Treg level during the first week after stroke in the higher quartile achieve a 25% better outcome at 3 moths compared with those with Treg in the lower quartile, it would be necessary to include 172 patients to obtain a statistical power of 80% with a significant difference level of 0,05.

3.2 Statistical analysis

The statistical analysis was performed using the statistic program IBM®SPSS® statistics v.20 for Mac.

To identify the variables that followed a normal distribution, the Kolmogorov-Smirnov test was used. Continuous variables with normal distribution were expressed as mean (SD) and those variables not normally distributed were expressed as median [quartiles].

Proportions between groups were compared by chi-square test. Student's T test was used to compare continuous variables with normal distribution between 2 groups. In case of variables with no-normal distribution, Mann-Whitney U test was used to compare 2

groups. In case of more than 3 groups, variables were compared using ANOVA test.

ROC curves were configured to establish cut-off points of Treg levels that optimally predicted good outcome of patients with ischemic stroke. Spearman correlation was used to correlate Treg levels and infarct volume and Pearson correlation to correlate Treg and IL-10 levels.

In order to assess variables that were independently associated with functional outcome, infarct volume, and the presence of infections, a multivariate logistic regression analysis was performed. Odds ratios were adjusted by significant variables in the bivariate analysis. The results were expressed as adjusted odds ratios (OR) with corresponding 95% confidence intervals (95% CI).

Values of p below 0.05 were considered to be statistically significant in all tests.

RESULTS

1. EXPERIMENTAL STUDY

1.1 VERIFICATION OF IMMUNOSUPPRESSING PROPERTIES OF EXPANDED REGULATORY T CELLS

Once Treg were purified and expanded, a study was performed to verify their immunosuppressing properties. For this purpose, in vitro suppression assays combining fresh conventional Tcells (Tconv) with expanded Treg were performed. Treg cells were co-cultured for 3 days at different concentrations: 0:1, 1:4, 1:2 and 1:1 (Treg/Tconv cell ratio).

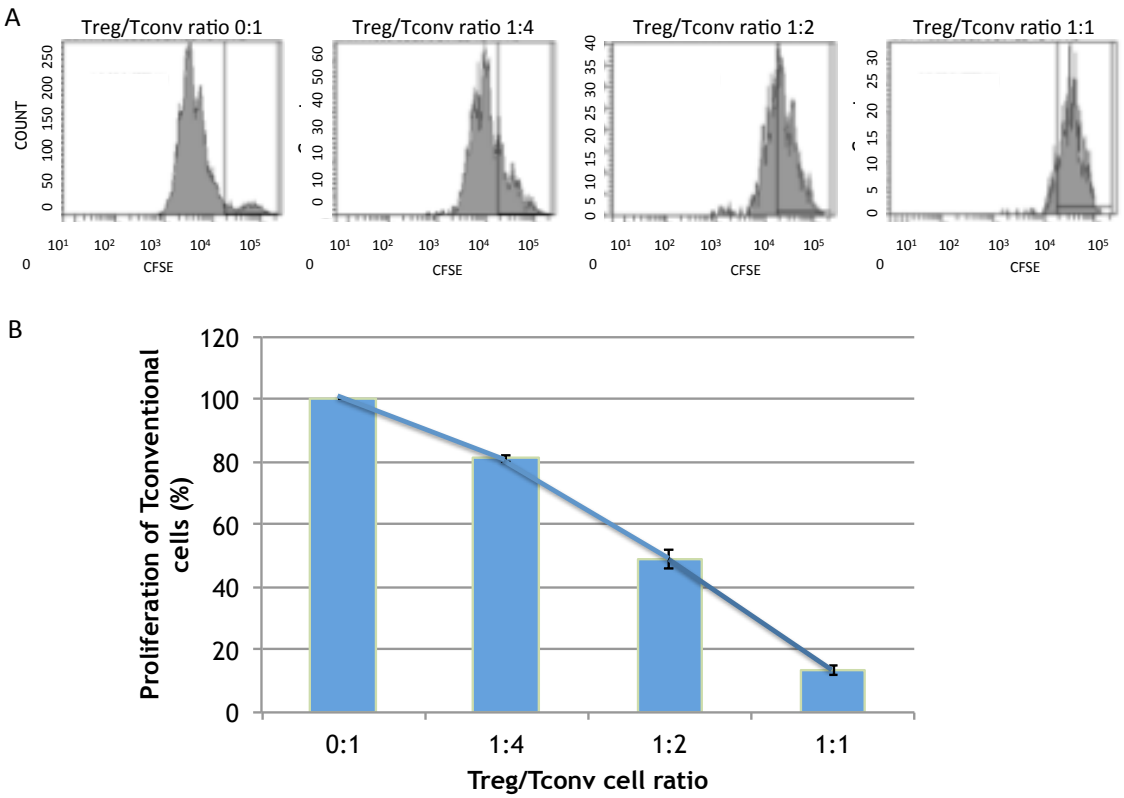

Figure 26. Suppression of the proliferation of conventional T cells by expanded regulatory T cells. (A) Mono-parametric representation of CFSE fluorescence of Tconv cells co-cultured with Treg cells at the indicated ratios. High fluorescence is indicative of low proliferation ratio (Treg/Tconv ratio 1:1), while low fluorescence is indicative of high proliferation (Treg/Tconv ratio 0:1). (B) Proliferation of T conventional cells co-cultured with different concentrations of T reg.

When Treg were co-cultured at a 1:1 ratio, a 86.4±3.0% suppression of Tconv cell proliferation was achieved. Proliferation was measured by determining dilution of CFSE fluorescence **(Figure 26).**

1.2 EFFECT OF EXOGENOUS REGULATORY T CELLS ADMINISTRATION

1.2.1 Effect of exogenous regulatory T cells administration on infarct volume and brain edema

Exogenous Treg were administered 2 hours following ischemia. Animals treated with Treg showed smaller lesions at day 3 compared to controls (198.01±29.61 vs 349.84±48.59 mm^3; p=0.027). Similar results were observed at day 7 (149.16±19.43 vs 227.24±30.64 mm^3; p=0.049) and day 10 after occlusion (120.38±12.94 vs 186.71±26.88 mm^3; p=0.041) **(Figure 27).**

In addition, rats treated with Treg also showed a reduction in brain edema at day 3 following occlusion (11.87±1.30 vs 18.46±2.44 %; p= 0.042).

Figure 28 represents 4 consecutive MRI slices from controls and animals treated with Treg acquired 10 days after induction of the infarct, showing smaller infarct volume in treated animals. **Figure 29** represents MRI slices from controls and treated animals at day 1, 3, 7 and 10 after MCAO, showing smaller lesions in treated animals at day 3, 7 and 10. **Figure 30** represents temporal evolution of brain edema.

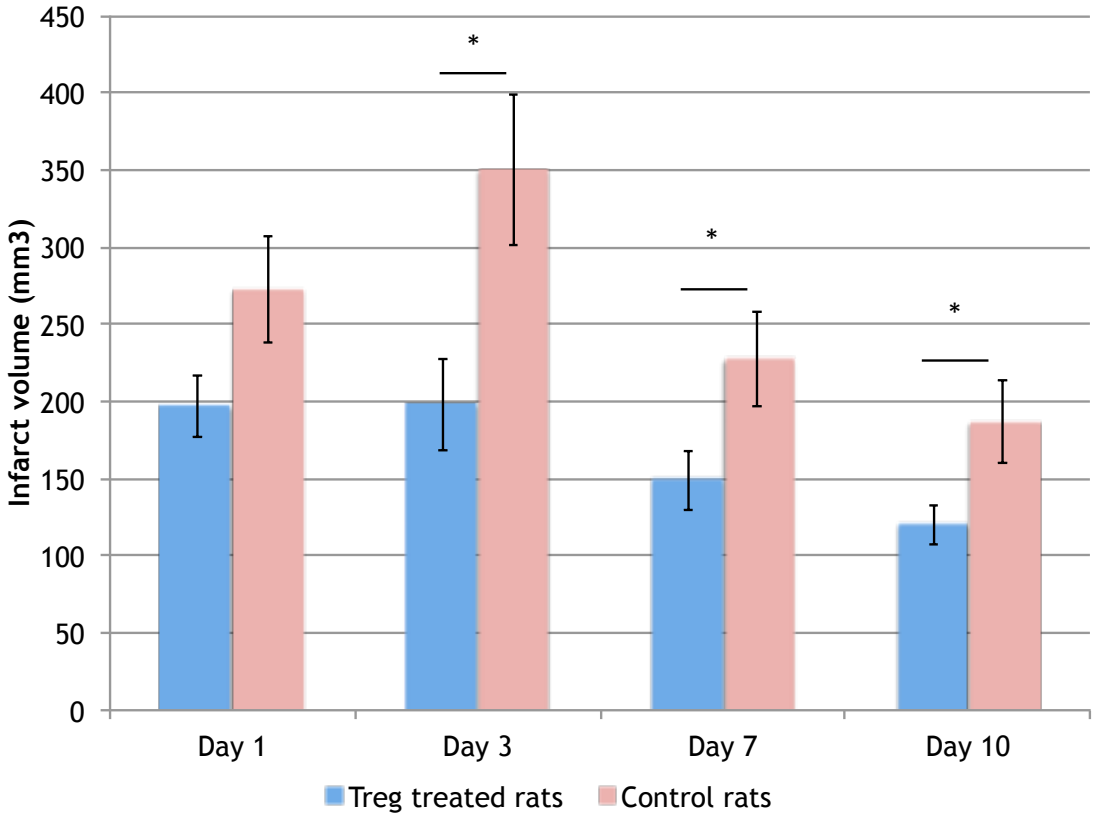

Figure 27. Temporal evolution of infarct volume: infarct volumes were smaller in treated group versus control at day 3, 7 and 10. Data are shown as mean ± SEM, *p<0.05 (n=10/group).

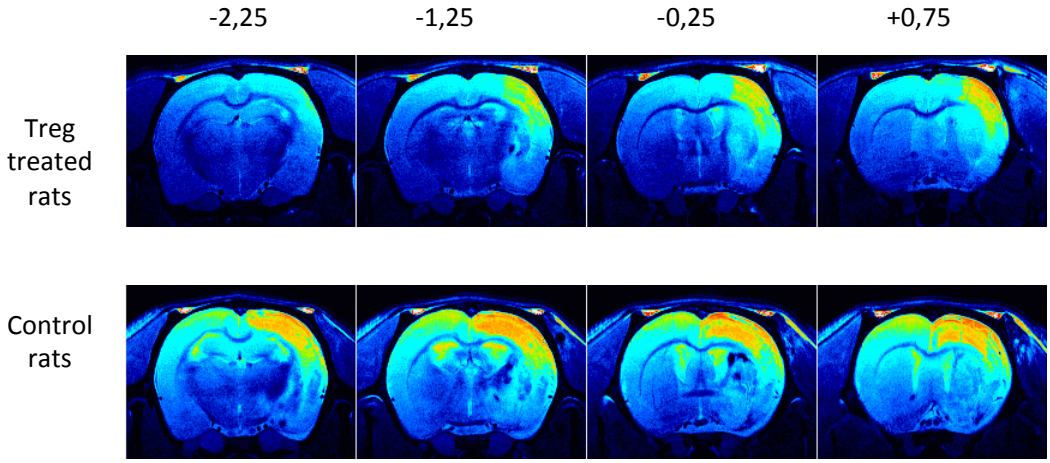

Figure 28. Consecutive MRI slice from control and Treg treated animals. T2 weighted images of the ischemic brain of a control rat and a treated animal, acquired 10 days after induction of the infarct.

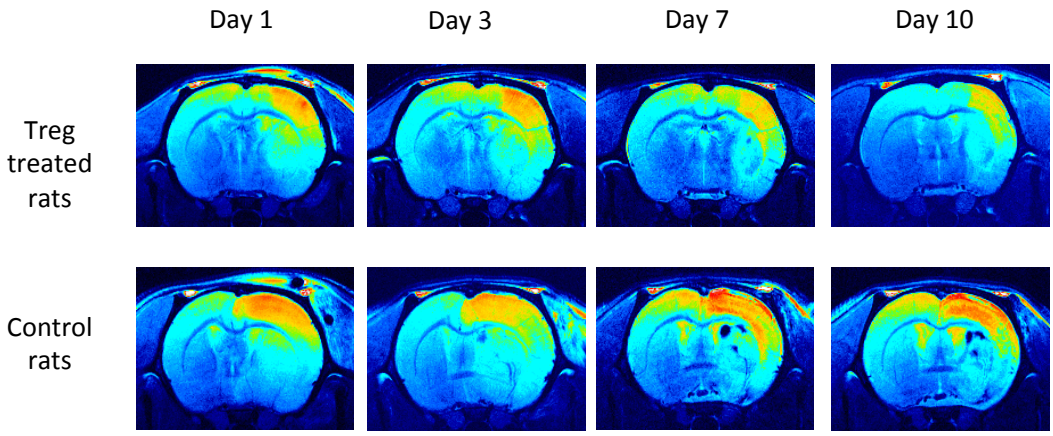

Figure 29. Representative MRI slices from control and Treg treated animal at day 1, 3, 7 and 10 after stroke are represented, showing smaller lesions in Treg treated animal at day 3, 7 and 10.

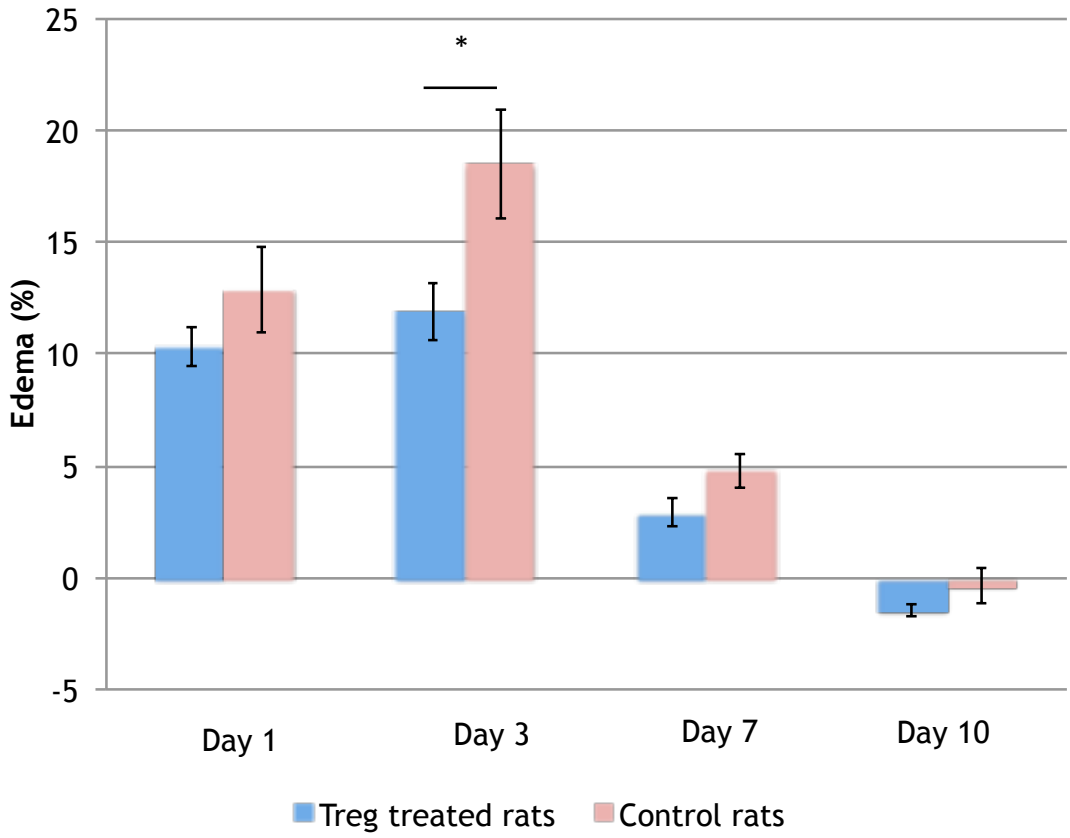

Figure 30. Temporal evolution of brain edema: edema measured in control and Treg-treated rats at days 1,3,7 and 10 post-MCAO. Rats treated with Treg showed a reduction in brain edema at day 3 following occlusion. Data are shown as mean ± SEM, *p<0.05 (n=10/group).

1.2.2 Evaluation of the presence of regulatory T cells in brain tissue after exogenous administration

To evaluate the presence of Treg in brain of rats, we analyzed the expression of FoxP3 by western blotting in the contralateral and infarcted hemispheres. FoxP3 expression in the infarcted hemisphere was higher in treated group compared to control group (3.5±0.3 vs 2.3±0.3; p=0.015) **(Figure 31).**

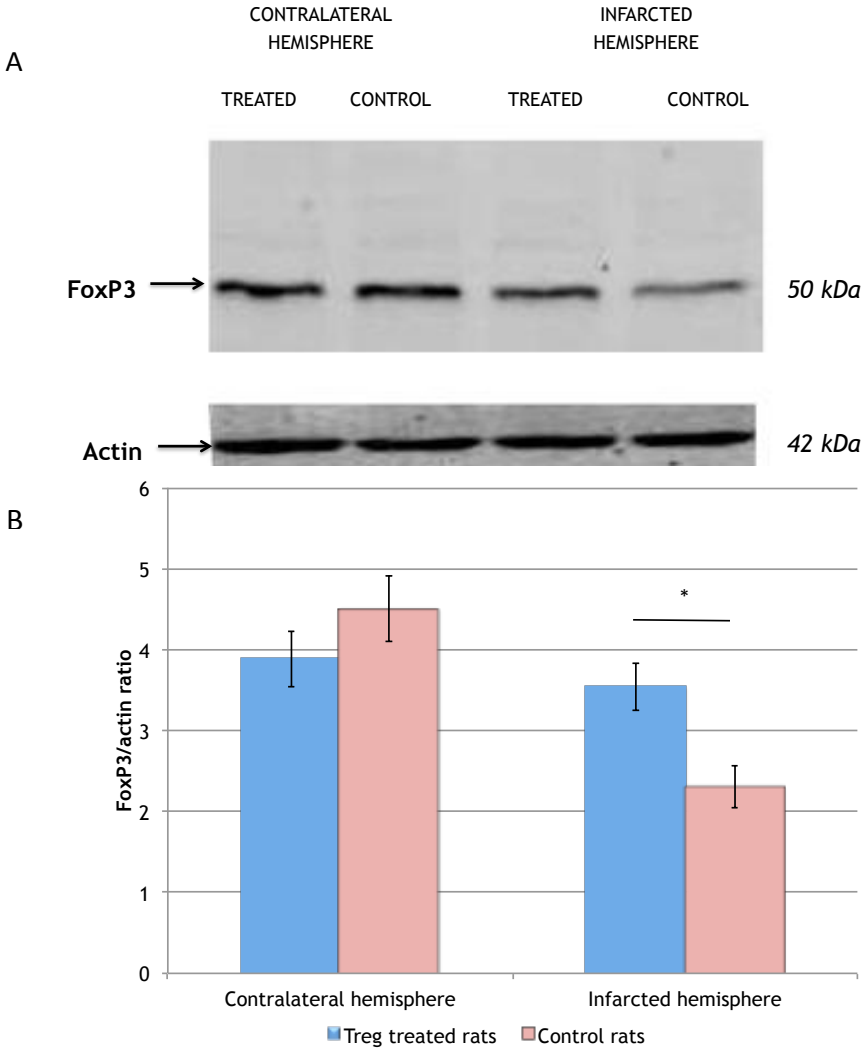

Figure 31. FoxP3 expression by Western blot (A) Western blot image of FoxP3 levels and actin control (B) Levels of FoxP3/actin ratio in both hemispheres. FoxP3 expression in the infarcted hemisphere was higher in treated compared to control group. Data are shown as mean ± SEM, *p<0.05 (n=10/group).

1.2.3 Evaluation of exogenous regulatory T cells administration effect on proinflammatory markers

Because FoxP3 was overexpressed in treated rats, and considering Treg immunosuppressing function, we analyzed levels of the proinflammatory markers IL-1β and TNFα in both brain hemispheres to verify this function. IL-1β levels were lower in the infarcted hemisphere in treated compared with control animals (19.46±1.02 vs 25.92±2.58 pg/ml of total protein; p=0.048), indicating that in treated animals there are lower inflammatory marker levels in brain than in control animals **(Figure 32).**

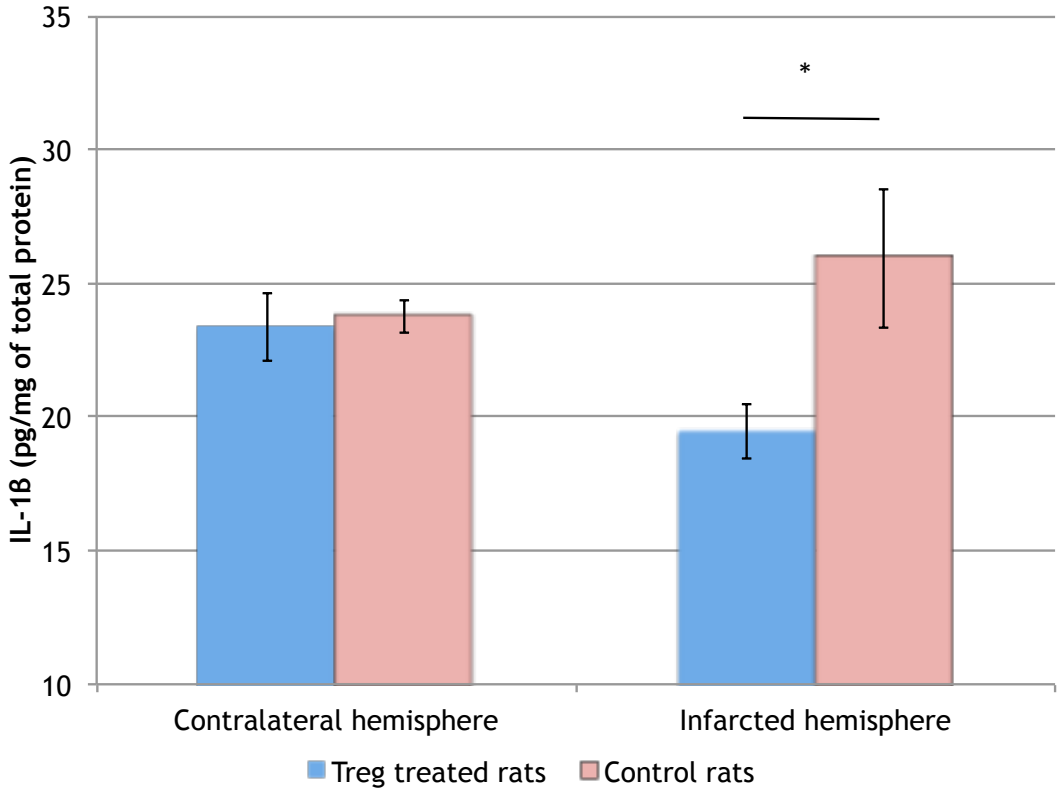

Figure 32. Level of IL-1β in the contralateral and infarcted hemisphere in Treg treated rats and control rats determined by ELISA. IL-1β levels were lower in the infarcted hemisphere in treated animals compared with control animals. Data are shown as mean ± SEM, *p<0.05 (n=10/group).

TNFα levels were also lower in the infarcted hemisphere of treated compared with control animals (17.6±0.5 vs 18.9±0.6 pg/ml of total protein), but this difference is not statistically significant **(Figure 33).**

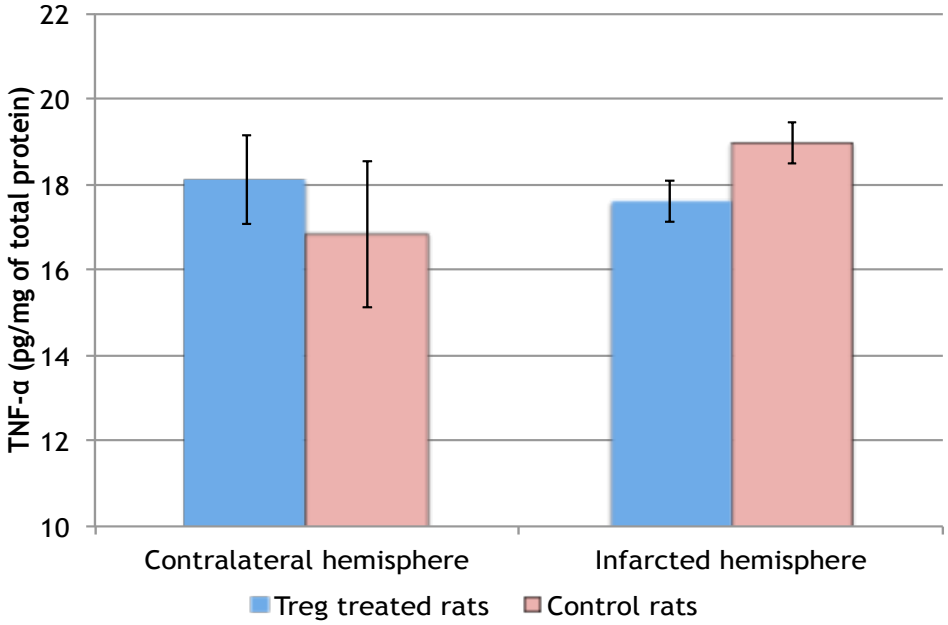

Figure 33. Levels of TNFα in the contralateral and infarcted hemisphere in Treg treated and control rats determined by ELISA. No significant differences were found between treated and control animals both in contralateral and infarcted hemisphere. Data are shown as mean ± SEM, (n=10/group).

1.2.4 Evaluation of active inflammation after exogenous regulatory T cells administration

To evaluate the presence of active inflammation, immunochemistry for CD11b and CD68 was performed as previously described. Treated animals showed lower count of CD68+ cells (n=17.93±1.07 vs 28.8±0.7; p=0.05) and CD11b+ cells (n=18.23±1.04 vs 24.85±055; p=0.018) in infarcted hemisphere compared to control animals **(Figure 34 and 35)**, indicating that treated animals showed lower inflammatory activity in infarcted hemisphere.

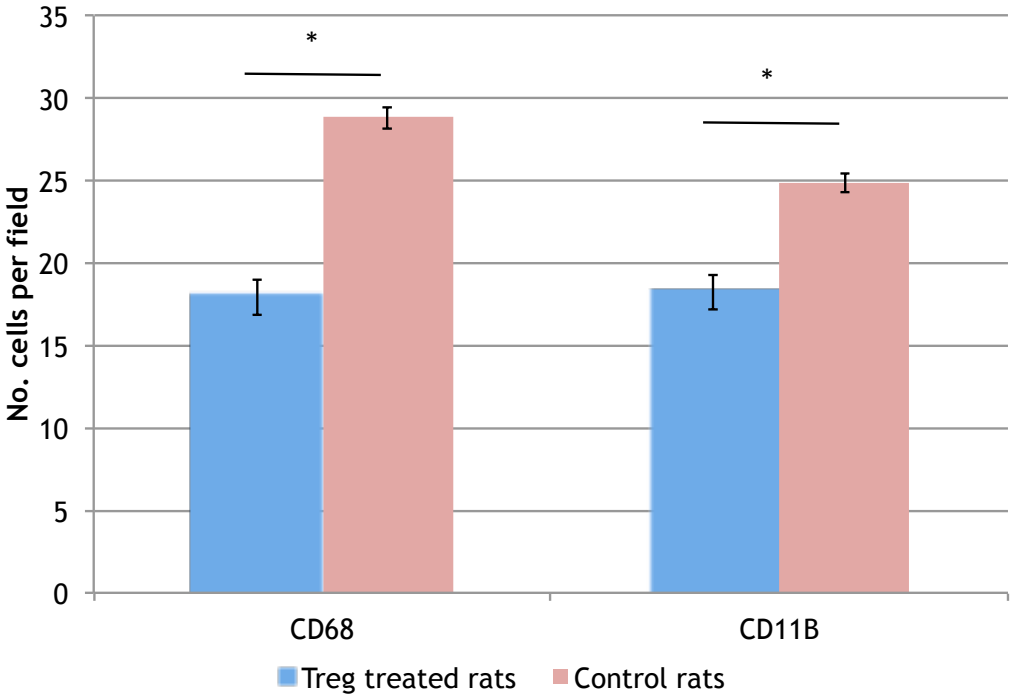

Figure 34. Analysis of CD68+ and CD11b+ cells in the brain of Treg treated and control rats. Treated animals showed lower count of CD68+ cells and CD11b+ cells compared to control animals. Data are shown as mean ± SEM, *p<0.05 (n=3/group).

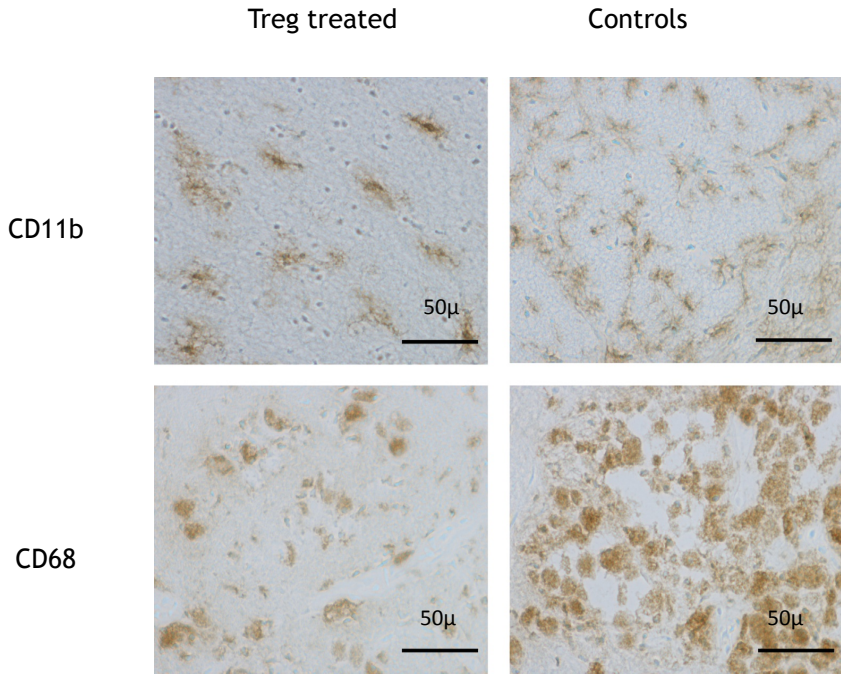

Figure 35. Inmunochemistry staining for CD11b and CD68 in T reg treated rats and control rats. Treg treated animals showed lower count of CD68 and CD11B compared to control animals.

1.3 EFFECT OF STIMULATION OF ENDOGENOUS REGULATORY T CELLS

An additional study was performed to evaluate the role of Treg in brain ischemia. A group of animals was treated with CD28-superagonists (CD28SA), which have demonstrated to induce endogenous Treg cell proliferation.

1.3.1 Effect of CD28SA administration on peripheral regulatory T cells

CD28SA was administered 4 days before ischemia. To evaluate Treg expansion after treatment, flow cytometry in peripheral blood was performed, analyzing the percentage of CD4+ FoxP3+ cells over CD4+ cells after CD28SA administration.

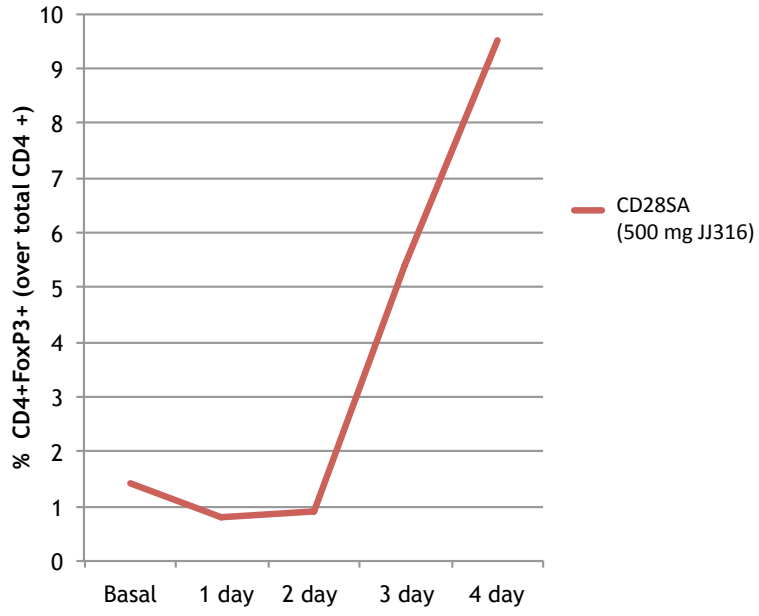

Figure 36. %CD4+FoxP3+ cells over total CD4+ after CD28SA treatment. Treatment was administrated to the rats 4 days before ischemia. Treg levels increased at day 4 after CD28SA treatment.

An increase of circulating Treg (%CD4+Foxp3+/total CD4+) was observed the fourth day after CD28SA administration compared to basal moment (9.5±1.1 vs 1.4±0.4%, p<0.001) **(Figure 36).** MCAO was performed 4 days after CD28SA treatment.

1.3.2 Effect of CD28SA administration on infarct volume and brain edema

MRI evaluated infarct volume at day 1, 3, 7 and 10 after ischemia. Infarct volume was slightly lower at day 3 and 7 in animals treated with CD28SA compared with control group. Infarct volume at day 10 was significant lower in treated animals compared with control group (160.58±12.2 vs 181.27±17.3 mm^3, p<0.001) **(Figure 37)**.

We also analyzed the percentage of change of infarct volume at days 3, 7 and 10 compared to the initial infarct volume. Animals treated with CD28SA showed a significant reduction in infarct volume at day 10 compared with control group (-33.37±4.14 vs -22.12±2,91%, p=0.034) **(Figure 38).**

We also observed that edema was significantly lower at day 7 (0.95±0.33 vs. 2.8±0.35%, p=0.02) and at day 10 (-0.21±0.4 vs 0.61±0.5 %, p=0.02) in treated animals compared to control **(Figure 39).**

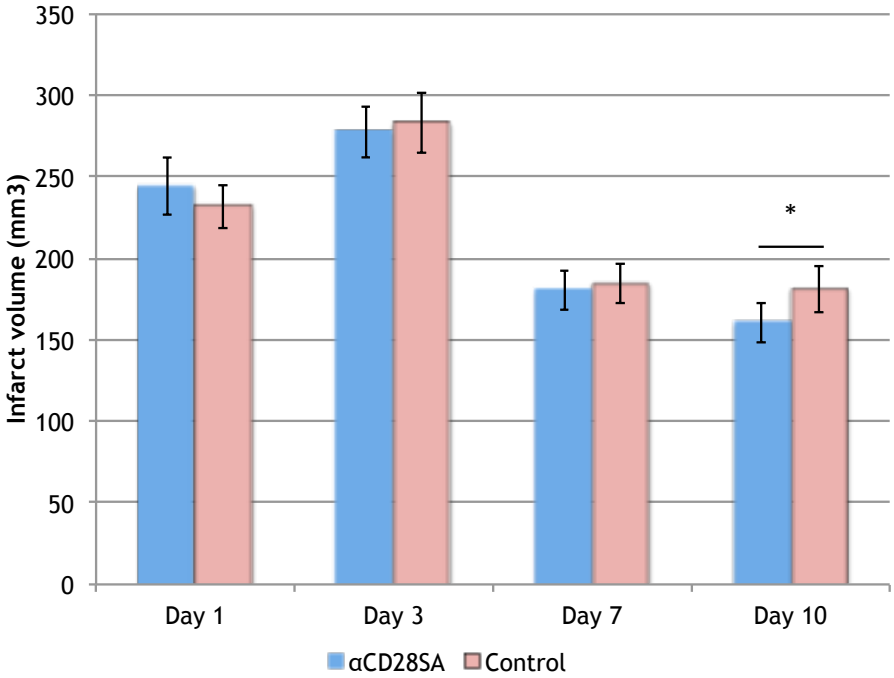

Figure 37. Temporal evolution of infarct volume (expressed as total volume) in CD28 superagonists treated and control rats. Infarct volume at day 10 was significant lower in treated animals compared with control group. Data are shown as mean ± SEM, *p<0.05 (n=8/group).

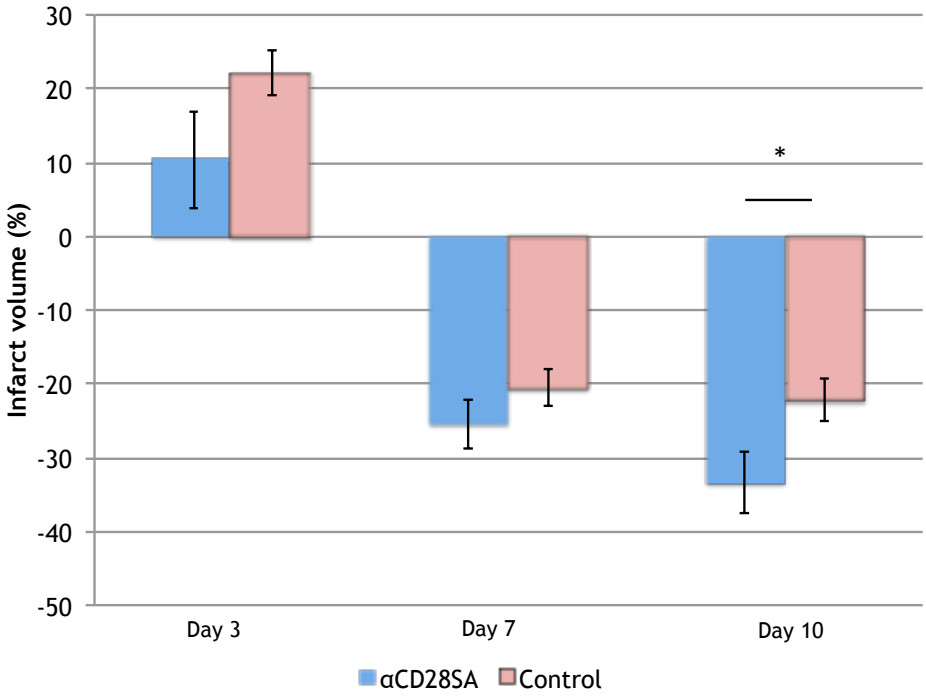

Figure 38. Temporal evolution of infarct volume (expressed as the percentage of change from day 1) in CD28SA treated and control rats Animals treated with CD28SA showed a significant reduction in infarct volume at day 10 compared with control group. Data are shown as mean ± SEM, *p<0.05 (n=8/group).

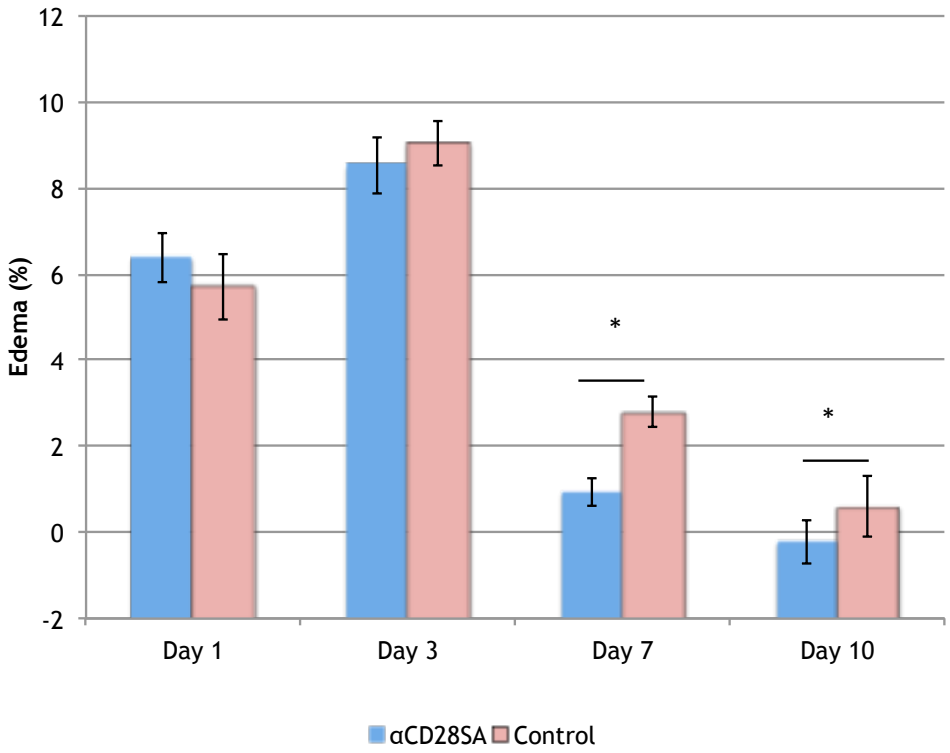

Figure 39. Temporal evolution of edema volume in CD28SA treated and control rats. Edema was significantly lower at day 7 and 10 in treated animals compared to control. Data are shown as mean ± SEM, *p<0.05 (n=8/group).

1.3.3 Effect of CD28SA administration on the presence of regulatory T cells in brain tissue

Western blot analyses were performed to quantify FoxP3 expression in contralateral and infarcted hemisphere in control and CD28SA treated animals. Treated animals have higher FoxP3 levels both in infarcted (1.8±0.1 vs 1.49±0.5) and contralateral hemisphere (2.1±0.4 vs 1.7±0.5) compared to control, although differences are not statistically significant **(Figure 40).**

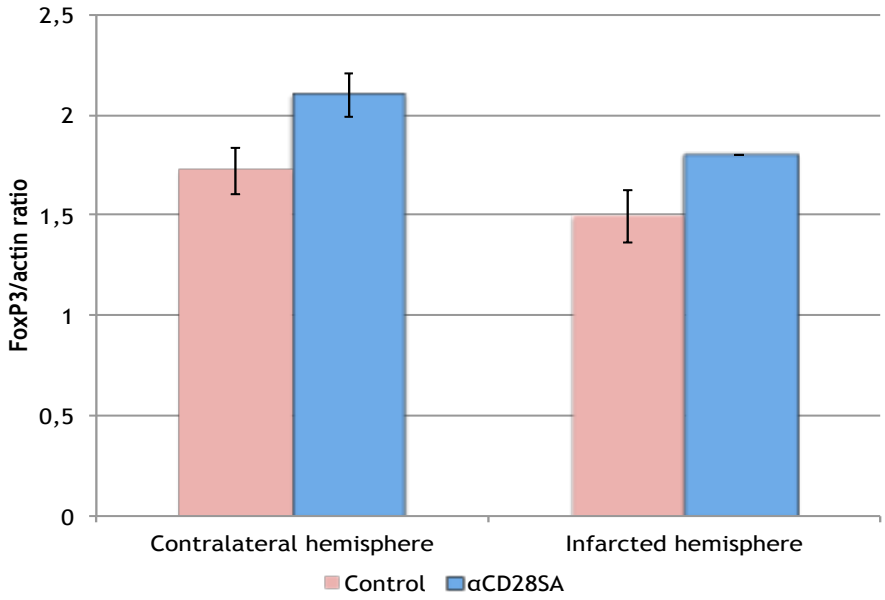

Figure 40. FoxP3 levels measured by Western blot in contralateral and infarcted hemisphere in control and CD28SA treated animals. No significant differences were found between treated and control animals.

1.3.4 Effect of CD28SA administration on proinflammatory cytokines

IL-1β levels were analyzed both in contralateral and infarcted hemisphere of treated and control animals. There were no significant differences between levels of IL-1β in infarcted hemisphere of CD28SA treated and control animals (47.84±1.01 vs 47.78±1.26 pg/mg of total protein). IL-1β levels were higher in contralateral hemisphere in treated animals compared to control (52.99±3.18 vs 44.94±2.14, p=0.042 pg/mg of total protein) **(Figure 41).**

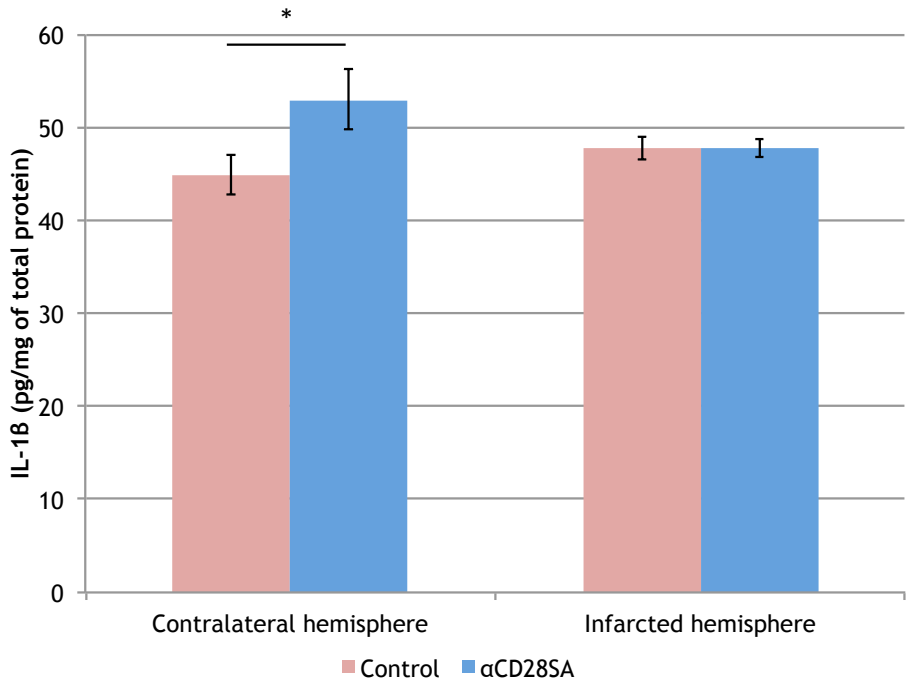

Figure 41. IL-1β levels in contralateral and infarcted hemisphere in CD28SA treated animals and control. IL-1β levels were higher in contralateral hemisphere in treated animals compared to control. Data are shown as mean ± SEM, *p<0.05 (n=8/group).

1.4 EFFECT OF INHIBITION OF REGULATORY T CELLS

A group of animals was treated with anti-CD25 antibodies, which have demonstrated to deplete Treg in peripheral blood.

1.4.1 Effect of anti-CD25 antibodies administration on peripheral regulatory T cells

Anti-CD25 antibodies were administered four days before ischemia. To evaluate Treg expansion after treatment, flow cytometry in peripheral blood was performed, analyzing the percentage of CD4+FoxP3+ cells over CD4+ cells after anti-CD25 antibodies treatment.

Although we found a significant reduction of circulatory Treg after anti-CD25 administration from basal moment to day 4 after treatment (1±0.2 vs 0.8±0.3%, p<0.001), we achieved less than 30% of depletion in Treg levels on day 4 after treatment **(Figure 42).**

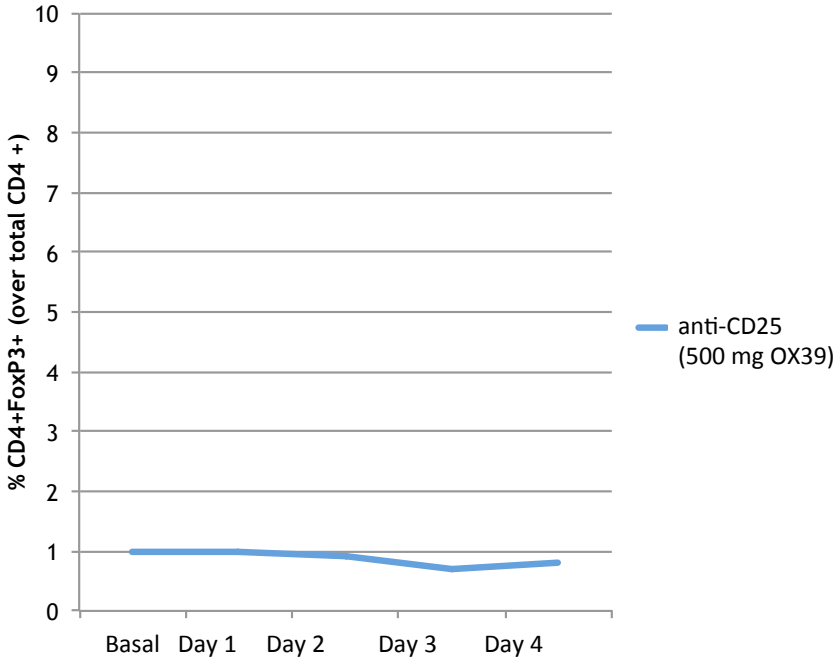

Figure 42. % CD4+FoxP3+ cells (measured by flow cytometry) in peripheral blood over total CD4+ cells evolution after anti-C25 administration. Only a slight decrease in Treg on day 3 and 4 after treatment was observed.

1.4.2 Effect of anti-CD25 administration on infarct volume and brain edema

We did no find any difference in infarct volume **(Figure 43)** or edema **(Figure 44)** in animals treated with anti-CD25 compared with control animals after MCAO.

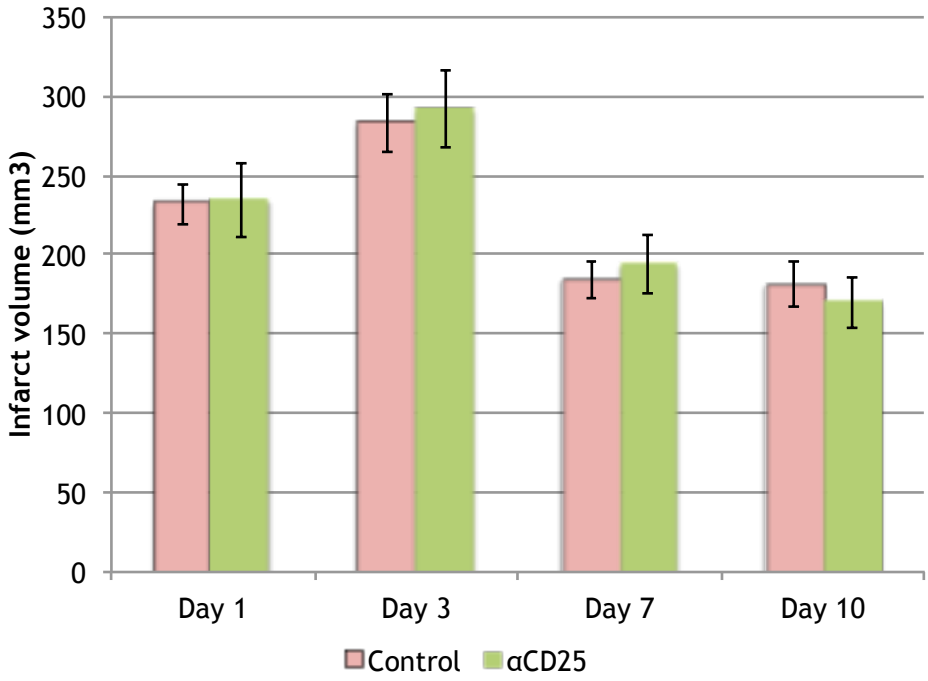

Figure 43. Temporal evolution of infarct volume (expressed as total volume) in anti-CD25 treated rats and control rats. No significant differences were found between treated and control animals. Data are shown as mean ± SEM, (n=8/group).

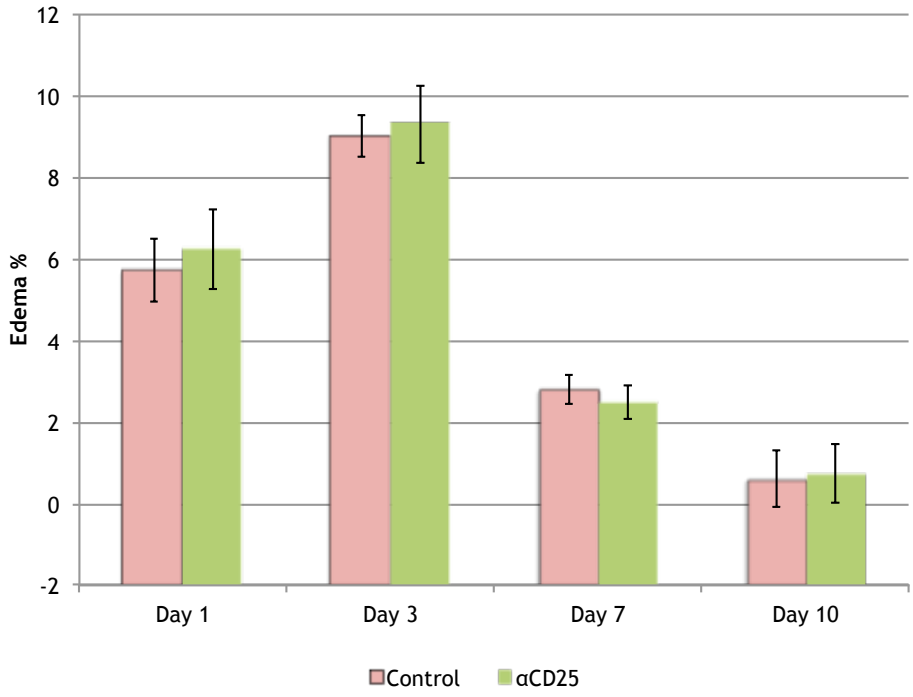

Figure 44. Temporal evolution of edema volume in anti-CD25 treated and control rats. No significant differences were found between treated and control animals. Data are shown as mean ± SEM, (n=8/group).

1.4.3 Effect of anti-CD25 antibodies administration on the presence of regulatory T cells in brain tissue

Western blot analyses were performed to quantify FoxP3 expression in contralateral and infarcted hemisphere in control and in anti-CD25 antibody treated animals. There were no significant differences between treated and control animals in FoxP3 levels both in contralateral (1.72±0.12 vs 1.29±0.25) and infarcted hemisphere (1.49±0.13 vs 1.19±0.26) **(Figure 45).**

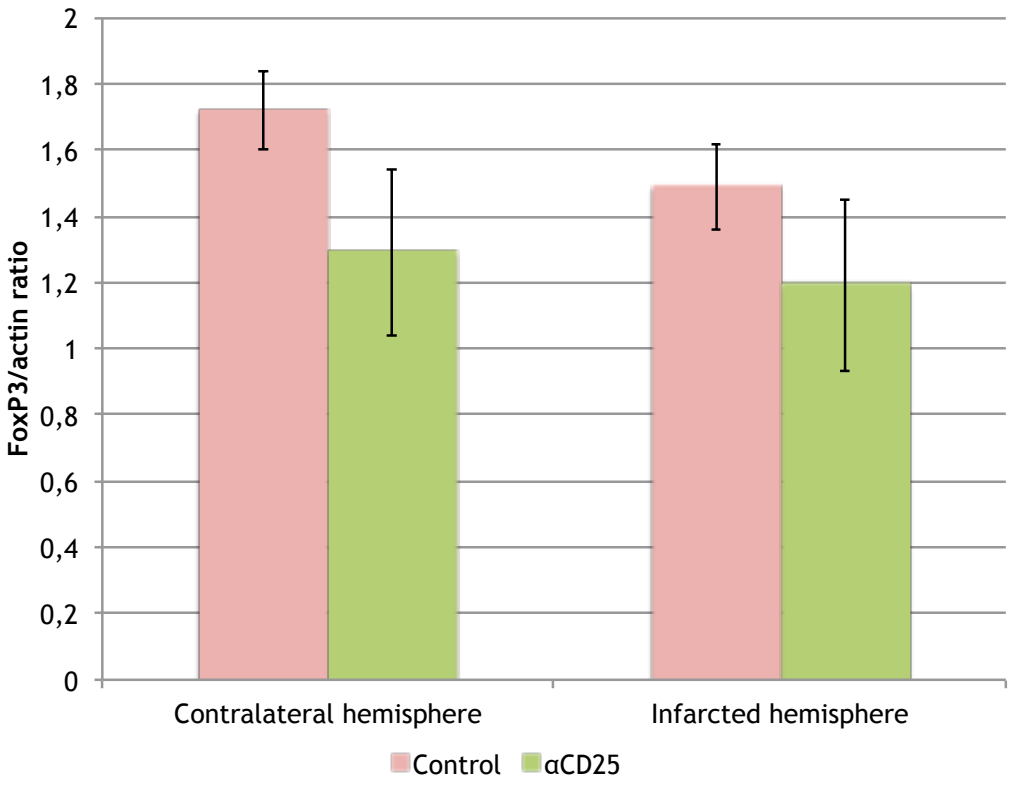

Figure 45. FoxP3/actin ratio in contralateral and infarcted hemisphere in anti-CD25 treated animals and control. No significant differences were found between treated and control animals.

1.4.4 Effect of anti-CD25 antibodies administration on proinflammatory cytokines

IL-1β levels were analyzed both in contralateral and infarcted hemisphere of anti-CD25 antibody treated and control animals. IL-1β levels were slightly higher in the infarcted hemisphere of treated animals compared to control, but there were no significant differences between levels of IL-1β in infarcted hemisphere of anti-CD25 treated and control animals (47.78±2.14 vs 55.12±3.75 pg/mg of total protein) or in contralateral hemisphere (44.94 ± 2.14 vs 44.18±2.51 pg/mg of total protein) **(Figure 46).**

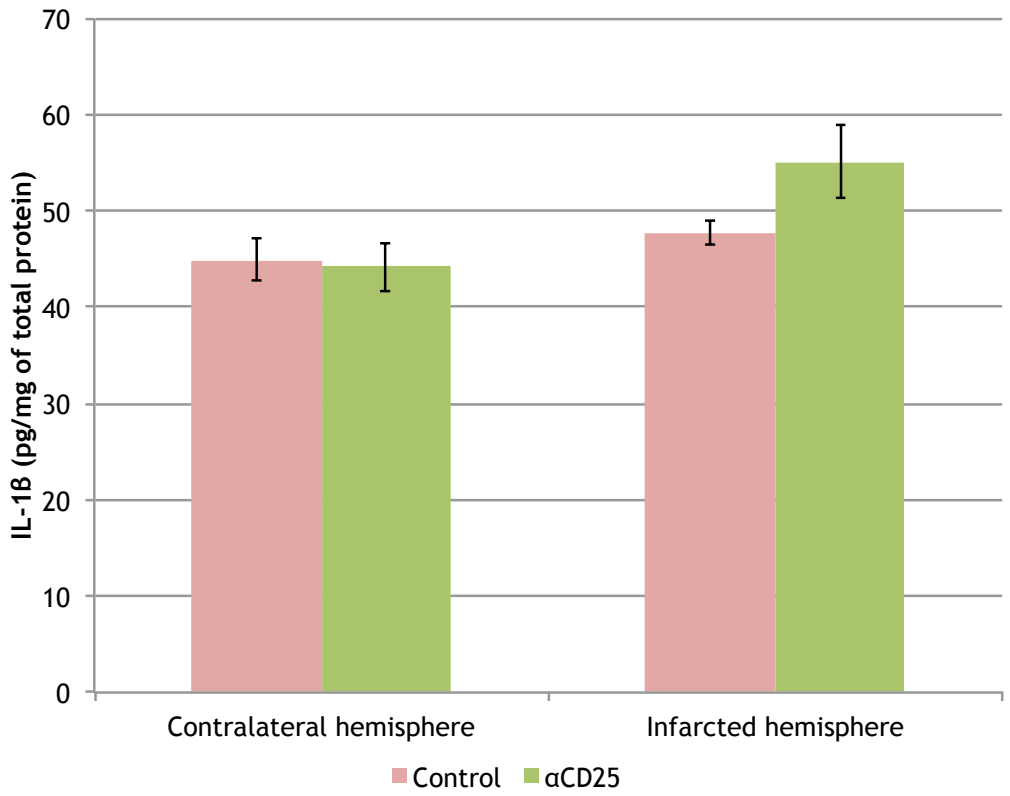

Figure 46. IL-1ß levels in contralateral and infarcted hemisphere in anti-CD25 treated and control animals. No significant differences were found between treated and control animals. Data are shown as mean ± SEM, (n=8/group).

1.5 LONG TERM EFFECT OF EXOGENOUS REGULATORY T CELLS ADMINISTRATION

1.5.1 Evaluation of neurogenesis and angiogenesis processes

To evaluate neurogenesis and angiogenesis, we analyzed brains of animals that received exogenous Treg and compared them with brains of control animals. We analyzed by flow cytometry the percentage of NeuN+BrdU+ and NCAM+BrdU+ cells to quantify neural proliferation, and the percentage of CD31+BrDu+ cells to assess angiogenesis.

Treatment with exogenous Treg cells did not impair endogenous cell proliferation in any case. There was a slightly higher proliferation in treated group in the analysis of NeuN+BrdU+, NCAM+BrdU+ and CD31+BrDu+ in both hemispheres, but differences were not statistically significant **(Figure 47,48,49,50,51,52).**

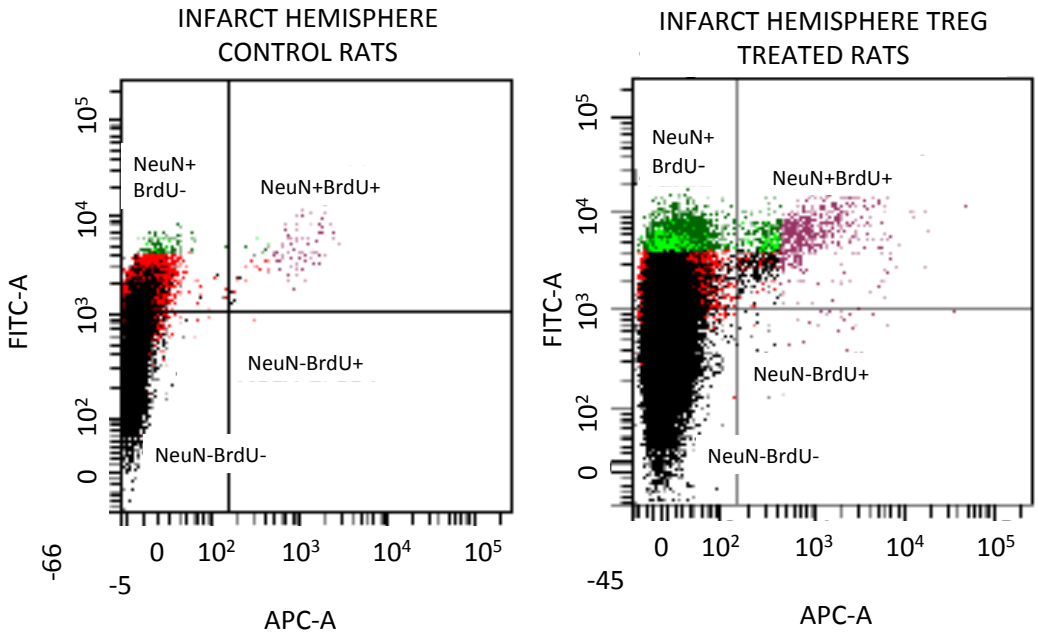

Figure 47. Flow cytometry of NeuN and BrdU in infarcted hemisphere of control and T regulatory treated rats.

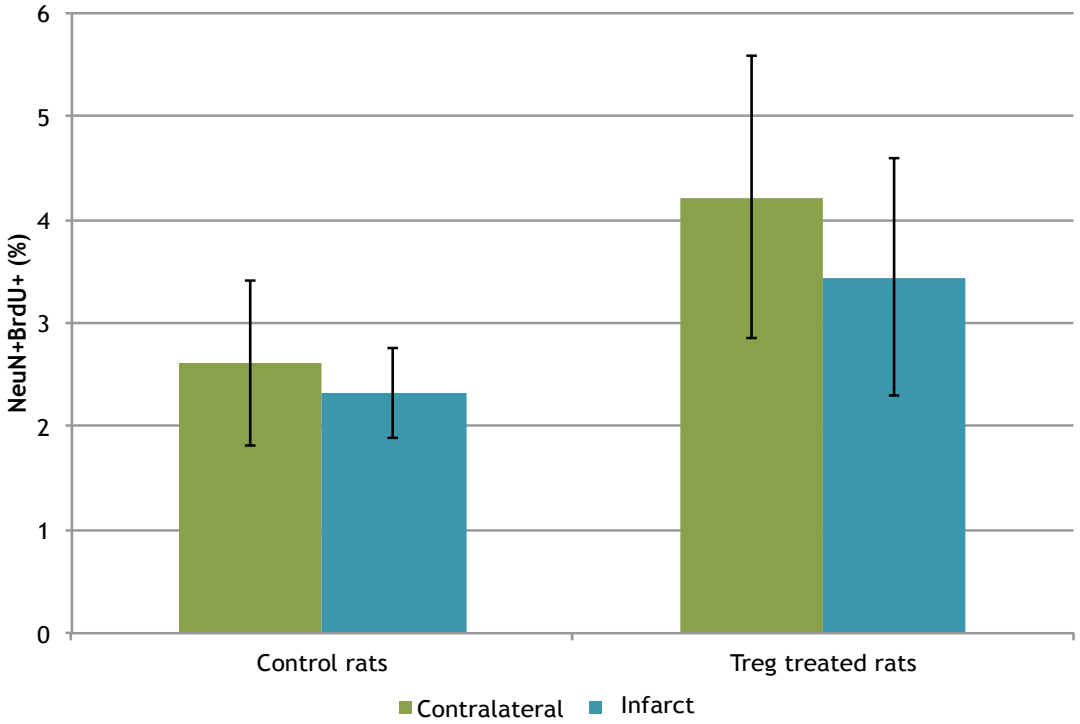

Figure 48. NeuN-BrdU percentage in Treg treated rats and control rats. Differences seen between Treg treated and control animals are not statistically different. Data are shown as mean ± SEM, (n=8/group).

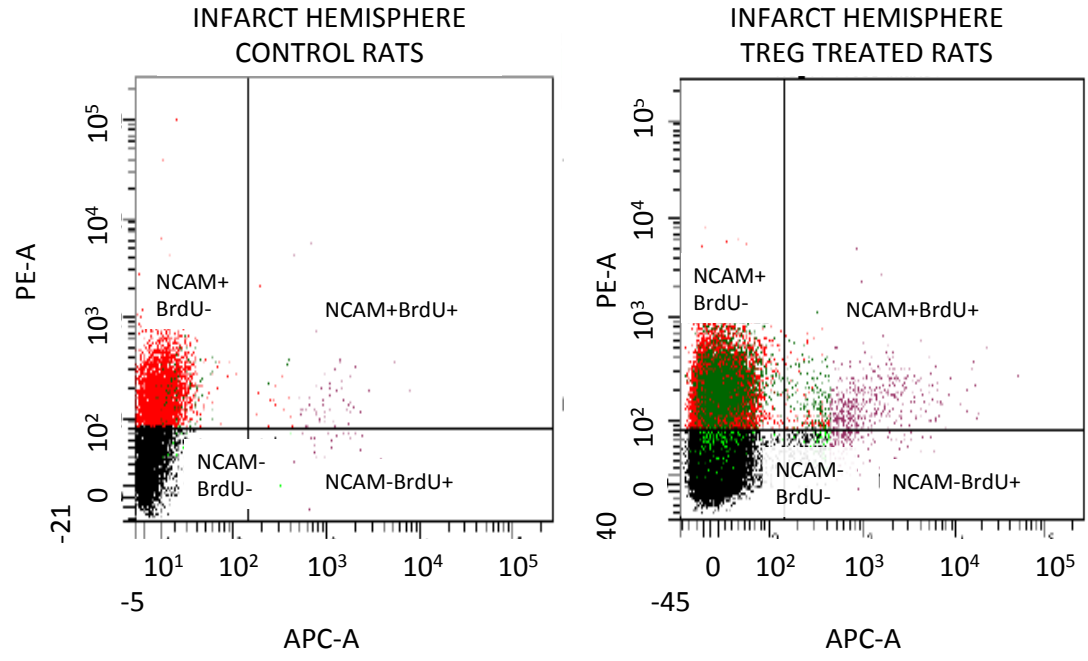

Figure 49. Flow cytometry of NCAM and BrdU in infarcted hemisphere of control and T regulatory treated rats.

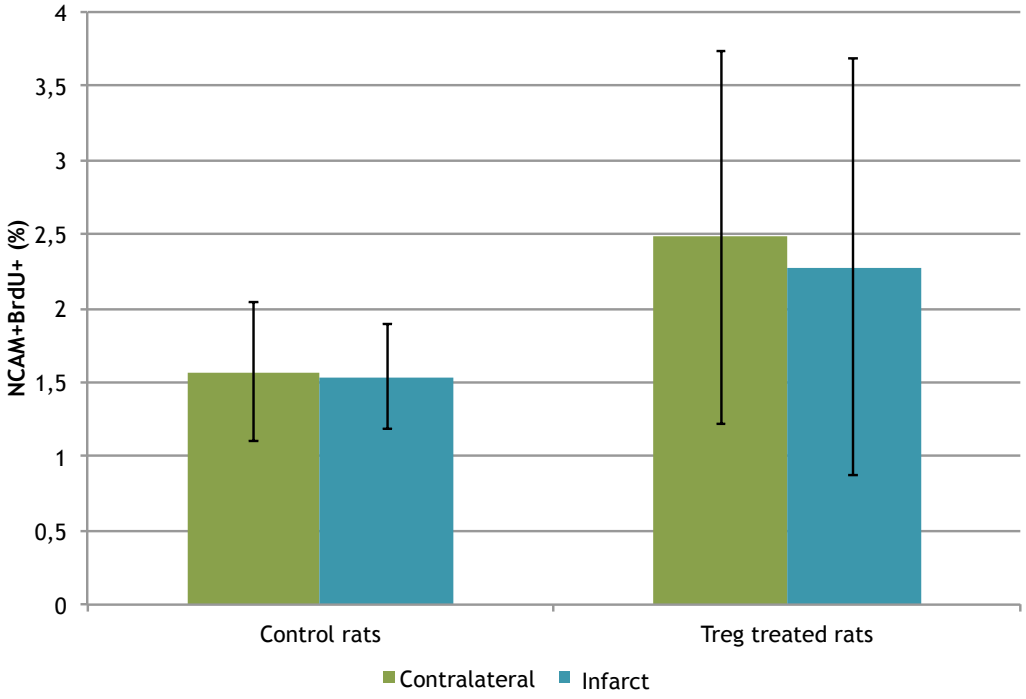

Figure 50. NCAM-BrdU percentage in Treg treated rats and control rats. Differences seen between Treg treated and control animals are not statistically different. Data are shown as mean ± SEM, (n=8/group).

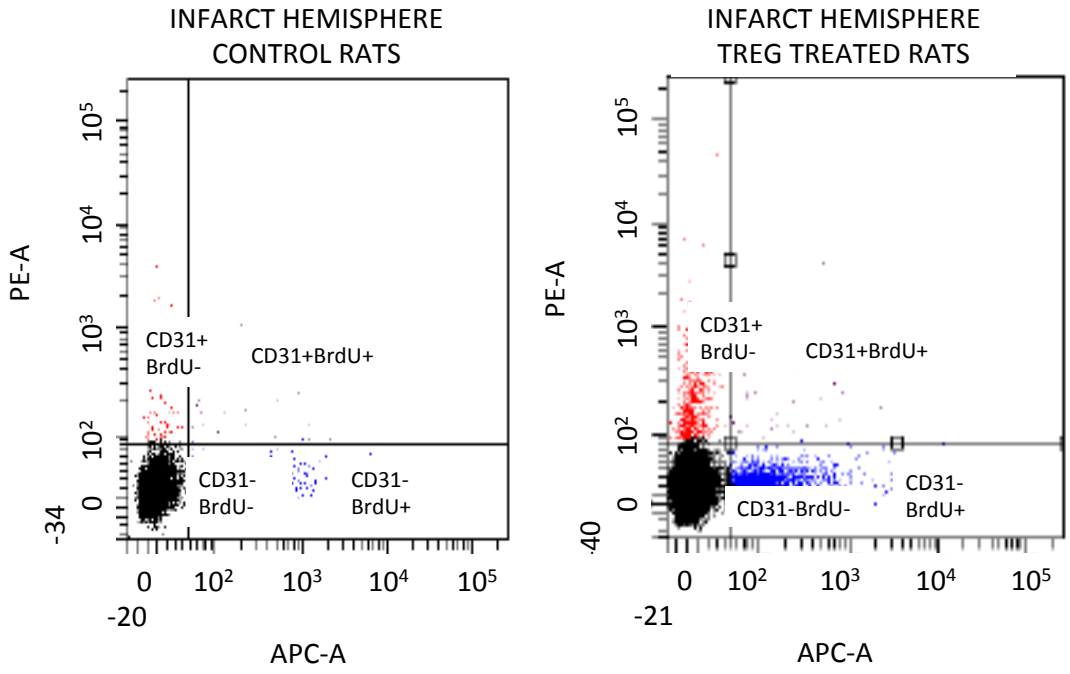

Figure 51. Flow cytometry of CD31and BrdU in infarcted hemisphere of control and Treg treated rats.

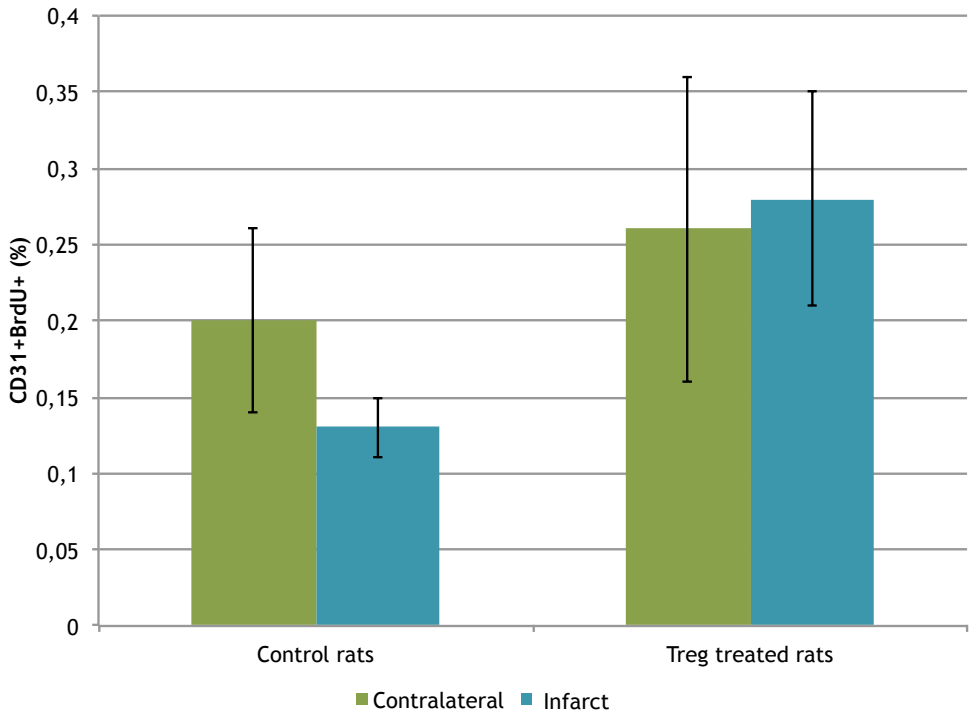

Figure 52. CD31-BrdU percentage in Treg treated rats and control rats. Differences seen between Treg treated and control animals are not statistically different. Data are shown as mean ± SEM, (n=8/group).

1.5.2 Evaluation of long-term effect of exogenous regulatory T cells administration on infarct volume

To evaluate long-term effect of treatment with exogenous Treg, infarct volume was measured after the induction of ischemia at day 7, 14, 21 and 28. The temporal evolution of lesion volume for control and treated animals is represented in **Figure 53**. Treated animals showed smaller brain infarcts compared with control animals both at day 7 (88.42±17.63 vs 138.56±10.92 mm^3; p=0.026), day 14 (75.68±11.66 vs 114.62±10.17 mm^3; p=0.023), day 21 (71.13±12.21 vs 103.99±9.11 mm^3; p=0.045) and day 28 (66.61±9.48 vs 100.87±11.64 mm^3; p=0.046) **(Figure 54).**

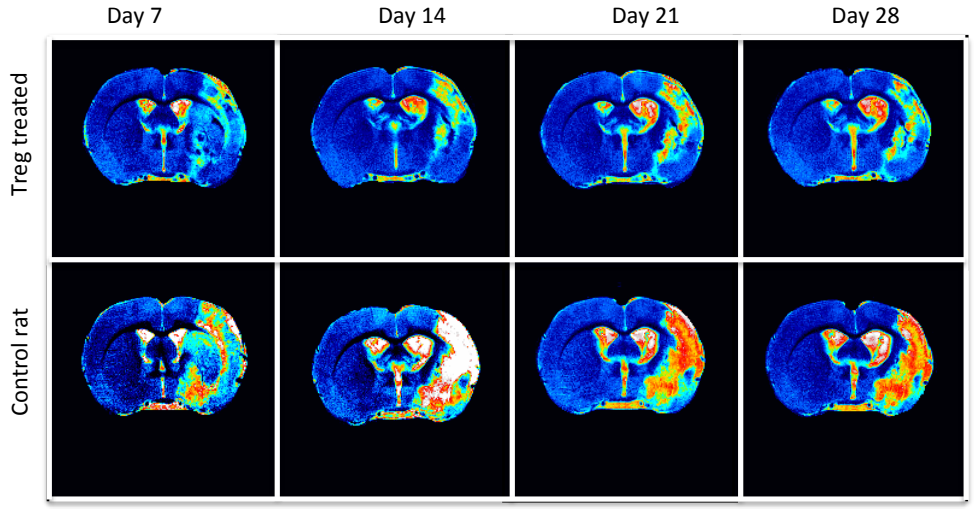

Figure 53. T2 weighted MR images showing the temporal evolution of the lesion for control and treated animals. Smaller infarct volume was found in Treg treated animals at day 7,14, 21 and 28 after MCAO.

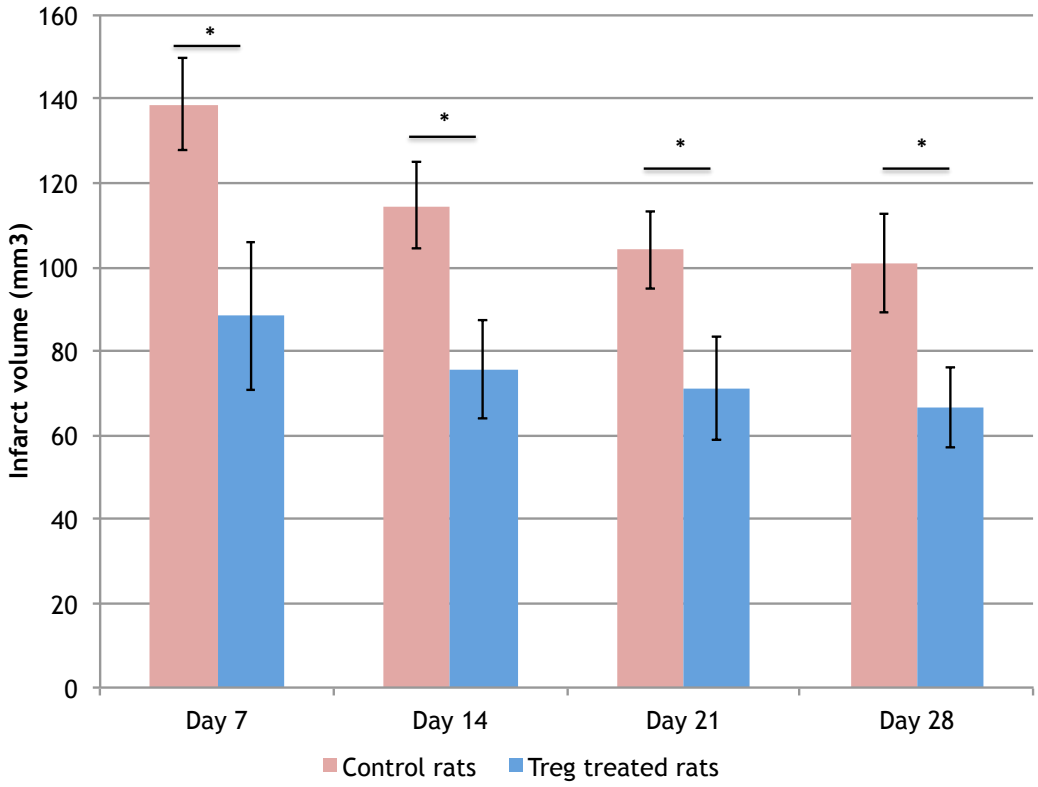

Figure 54. Infarct volume measured for control and Treg treated rats at days 7,14, 21 and 28 post-MCAO. Treated animals showed smaller brain infarcts compared with control animals both at day 7, 14, 21 and 28. Data are shown as mean ± SEM, *p<0.05 (n=8/group).

2 CLINICAL STUDY

2.1 DESCRIPTIVE ANALYSIS

2.1.1 Patient sample

Three hundred and thirty-five patients with ischemic stroke within 12 hours from stroke onset were consecutively evaluated between April 2012 and July 2013. One hundred and thirty-one patients were excluded from the study. Sixty-one patients (46.6%) had previous disability (previous modified Rankin scale score higher than 1), 18 patients (13.7%) were included in other clinical trial, 13 patients (9.9%) suffered an inflammatory process within the last 30 days before admission, 24 patients (18.3%) had previous chronic inflammatory diseases, 7 patients (5.3%) were in renal replacement or receiving immunosuppressive drugs and 8 patients (6.1%) refused to participate in the study. Finally, 204 patients who fulfilled inclusion criteria and did not fulfill any exclusion criteria were included.

Over the 204 patients included, 9 patients died within the first 48 hours following stroke onset (4.4%), 2 patients died between 48 and 72 hours (0.9%) and 8 patients died between 72 hours and 7 days (3,9%). At 48 hours 195 samples were included for the analysis, at 72 hours 193 patients were included and at 7 days 185 patients **(Figure 55).**

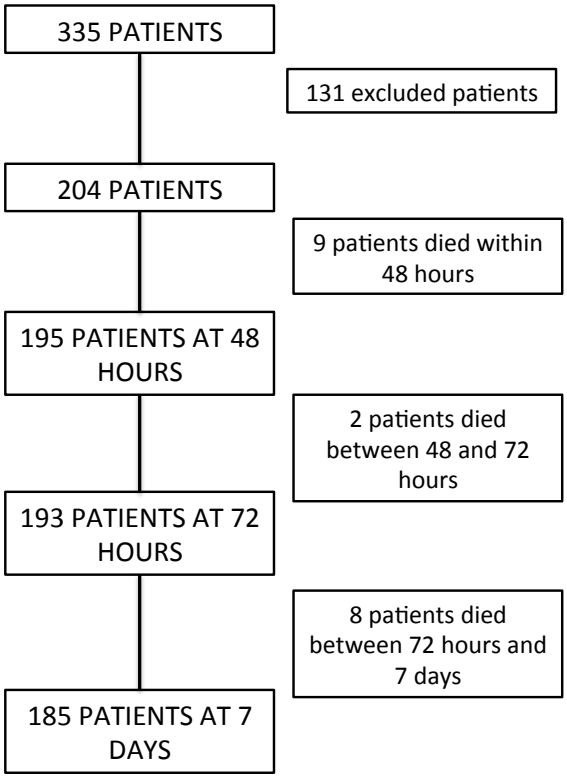

Figure 55. Lost patients during monitoring. One- hundred thirty-one patients were excluded of the study. Nineteen patients died in the first 7 days after inclusion.

2.1.2 Control sample

Twenty-two healthy controls without any previous neurological disease, matched by sex and age, were included during the period of study.

No differences regarding age, sex, previous history of hypertension, diabetes, dyslipidemia, coronary disease, peripheral artery disease, and alcohol and tobacco consumption were found between patients with ischemic stroke and controls. We only found that patients with ischemic stroke had more prevalence of atrial fibrillation **(Table 6).**

Variable	Patients n = 204	Controls n = 22	p
Female gender, n (%)	101(49.5)	12(54.5)	0.446
Age (years)	71.7 ± 10.6	75.0 ± 9.3	0.161
Previous hypertension, n (%)	122 (59.8)	9 (40.9)	0.255
Previous diabetes, n (%)	47 (23.0)	2 (9.1)	0.176
Previous dyslipidemia, n (%)	77 (37.7)	6 (27.3)	0.364
Previous atrial fibrillation, n (%)	86 (42.2)	0 (0)	<0.0001
Previous coronary disease, n (%)	8 (3.9)	0 (0)	0.344
Previous peripheral artery disease, n (%)	2 (1.0)	0 (0)	0.641
Enolism, n (%)	22 (10.8)	4 (18.2)	0.294
Smokers, n (%)	44 (21.6)	4 (18.2)	0.480
Statin consum, n (%)	73 (36.3)	6 (27.3)	0.277

Table 6. Collected data in control and ischemic stroke patients.

2.1.3 Basal characteristics of patients

One hundred and three patients (50.5%) were men. Mean age was 71.7±10.6 years. The mean NIHSS score at admission was 8 [4,12] and infarct volume was 50.79±88.43 cc.

Regarding stroke etiology, according to TOAST criteria, we found 93 cardioembolic strokes (45.6%), 26 atherothrombotic strokes (12.7%), 7 lacunar strokes (3.4%) and 78 undetermined strokes (38.2%) **(Figure 56).**

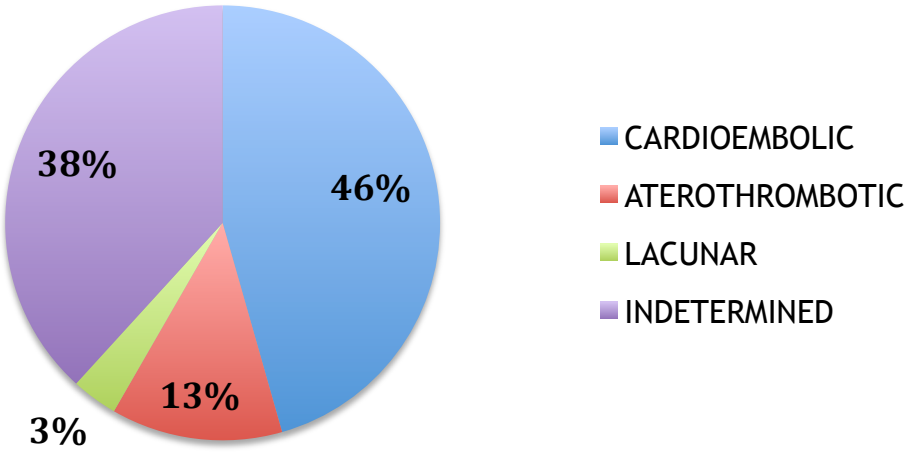

Figure 56. TOAST classification in stroke patients

2.2 COMPARATIVE ANALYSIS BETWEEN CONTROLS AND PATIENTS

2.2.1 Differences in regulatory T cells between controls and patients

To investigate if ischemic stroke increases the percentage of Treg, we compared blood samples obtained at admission of stroke patients with blood samples of healthy controls. We found that the percentage of Treg was higher in patients with ischemic stroke that in healthy controls (0.0222±0.0177 vs 0.0013±0.0009%, $p<0.001$) **(Table 7)(Figure 57)**.

2.2.2 Differences in IL-10 levels between controls and patients

To investigate if ischemic stroke modifies IL-10 levels, we compared blood samples obtained at admission of stroke patients with

blood samples of healthy controls. We found that IL-10 levels were higher in patients with ischemic stroke that in healthy controls (6.9± 1.7 vs 1.8± 1.1 pg/mL, p<0.001) **(Table 7)(Figure 58).**

Variable	Patients n = 204	Controls n = 22	p
Regulatory T cells (% total lymphocytes) admission	0.0222 ± 0.0177	0.0013 ± 0.0009	<0.0001
IL-10 (pg/mL) admission	6.9 ± 1.7	1.8 ± 1.1	<0.0001

Table 7. %Treg and IL-10 (pg/mL) at admission samples comparing stroke patients and controls.

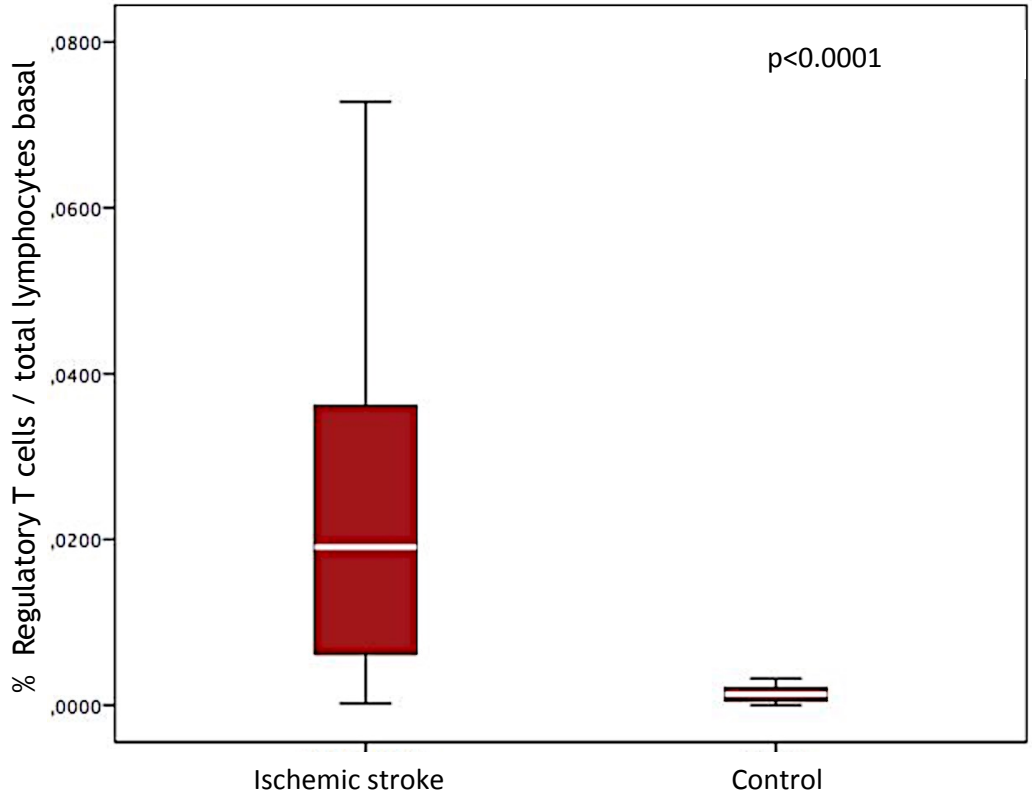

Figure 57. % Treg at admission comparing ischemic stroke patients vs control group (p<0.0001).

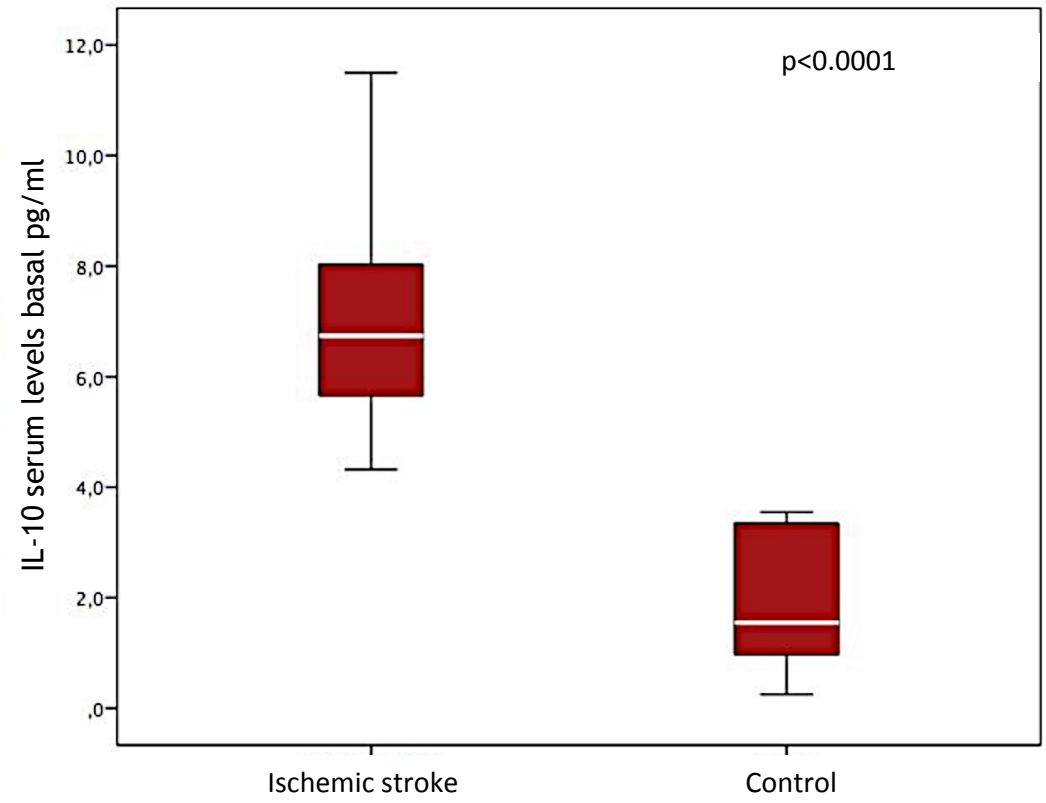

Figure 58. IL-10 serum levels (pg/mL) at admission comparing ischemic stroke patients vs control group (p<0.0001).

2.3 TEMPORAL PROFILE OF MOLECULAR MARKERS DURING ACUTE PHASE OF STROKE

A preliminary study including 20 patients was performed to analyze temporal profile of Treg during the acute phase of stroke and later select the time frame for blood sample in the complete cohort of study. We also analyzed temporal profile of IL-10 during the acute phase of stroke.

2.3.1 Temporal profile of percentage of regulatory T cells

We analyzed the percentage of Treg over total lymphocytes during the acute phase of stroke at baseline, day 1, day 2, day 3, day 4, day 5 and day 7. Compared to basal sample, we found that Treg were

statistically different at 48 h (0.023±0.017 vs 0.051±0,002, p= 0.01), 72 h (0.023±0.017 vs 0.017±0.009, p<0.001) and day 7 (0.023±0.017 vs 0.031±0.018, p= 0.048) **(Figure 59)**. Based on these results we selected baseline, 48 h, 72 h and 7 days as the most representative samples, and we obtained blood samples at these times in the complete cohort of the study.

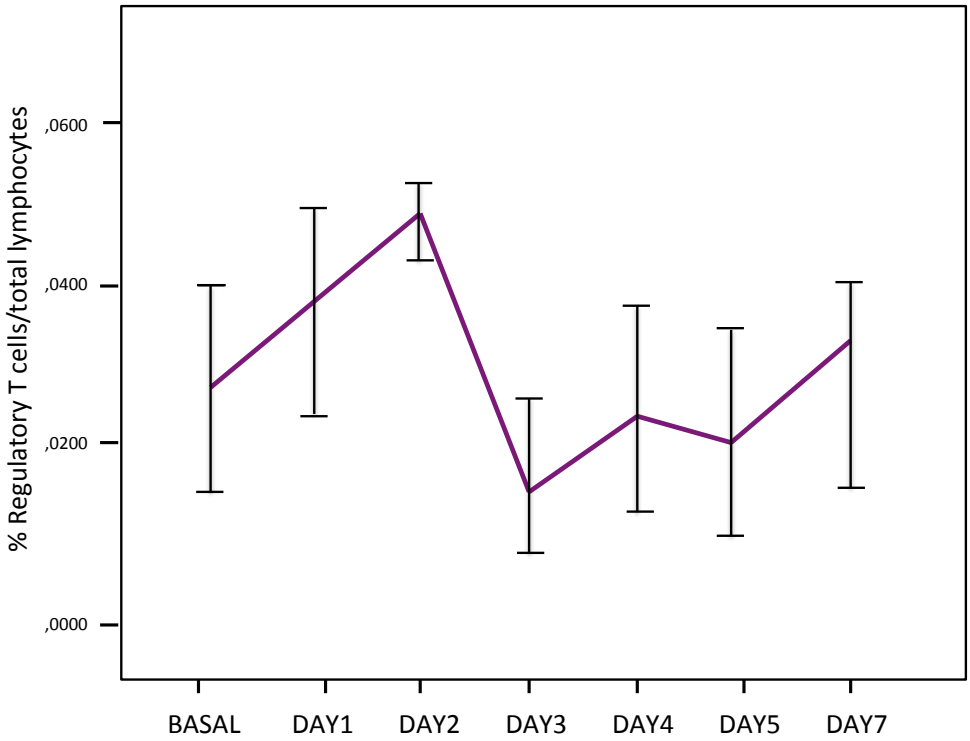

Figure 59. Temporal pattern of percentage of Treg between admission moment and day 7. Compared to basal sample, we found that Treg were statistically different at 48 h, 72 h and 7 days (p<0.05).

We analyzed the percentage of Treg in peripheral blood at admission, 48 h, 72 h and 7 days in the complete sample of patients (n=204). We found that the percentage of Treg increase during the first 72 hours and decrease later at day 7 after stroke **(Table 8)(Figure 60).**

Variable	Patients n = 204	p
Regulatory T cells (% total lymphocytes) at admission	0.0222 ± 0.0177	<0.0001
Regulatory T cells (% total lymphocytes) at 48h	0.0463 ± 0.0310	
Regulatory T cells (% total lymphocytes) at 72h	0.0467 ± 0.0256	
Regulatory T cells (% total lymphocytes) at 7 days	0.0321 ± 0.0239	

Table 8. Temporal pattern of % Treg during the first 7 days following stroke (at admission, 48 hours, 72 hours, 7 days) (p<0.0001).

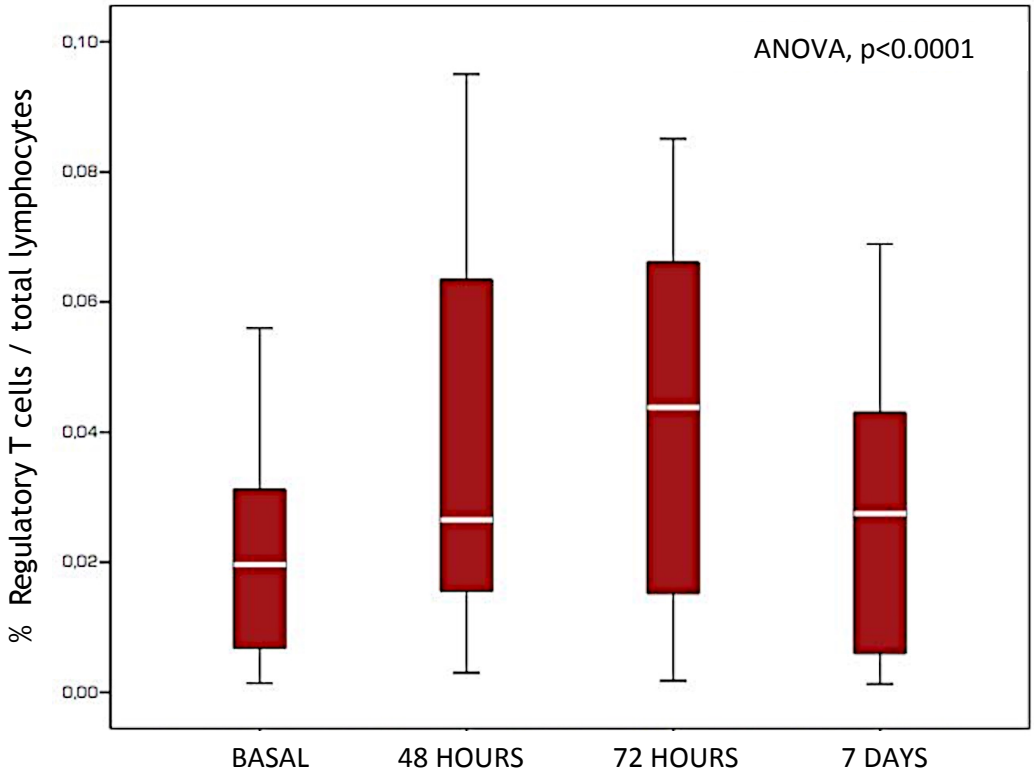

Figure 60. Temporal pattern of % Treg during the first 7 days following stroke (at admission, 48 hours, 72 hours, 7 days) (p<0.0001).

2.3.2 Temporal profile of serum IL-10

Based on the selected time frame of Treg, we also determined the temporal profile of IL-10 in the complete sample of patients at baseline, 48 h, 72 h and 7 days. Similar to Treg, IL-10 levels increase during the first 72 hours after stroke, and decrease again at day 7 **(Table 9)(Figure 61).**

Variable	Patients n = 204	p
IL-10 (pg/mL) at admission	6.9 ± 1.7	<0.0001
IL-10 (pg/mL) 48 h	11.9 ± 7.0	
IL-10 (pg/mL) 72 h	13,4 ± 8.8	
IL-10 (pg/mL) 7 day	5.7 ± 3.6	

Table 9. Temporal pattern of IL-10 (pg/mL) during the first 7 days following stroke (at admission, 48 hours, 72 hours, 7 days) (p<0.0001).

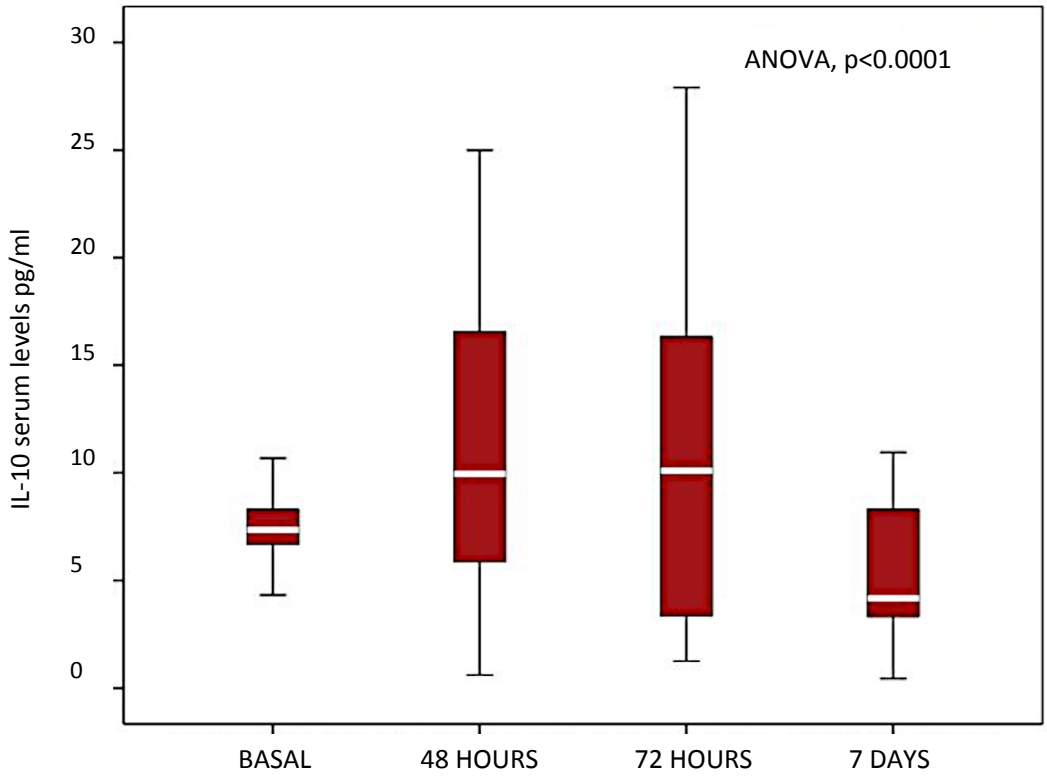

Figure 61. Temporal pattern of IL-10 serum levels (pg/mL) at admission moment, 48 hours, 72 hours and 7 days (p<0.0001).

2.4 PRIMARY ENDPOINT: INFLUENCE OF REGULATORY T CELLS ON FUNCTIONAL OUTCOME

We analyzed % Treg in relationship with stroke outcome at 3 months after stroke (measured using modified Rankin Scale at 3 months). We found that patients with lower mRS score at 3 months had higher levels of Treg at 48 hours, 72 hours and 7 days after stroke. No differences in percentage of Treg at admission were found regarding mRS at 3 months **(Figures 62,63,64,65).**

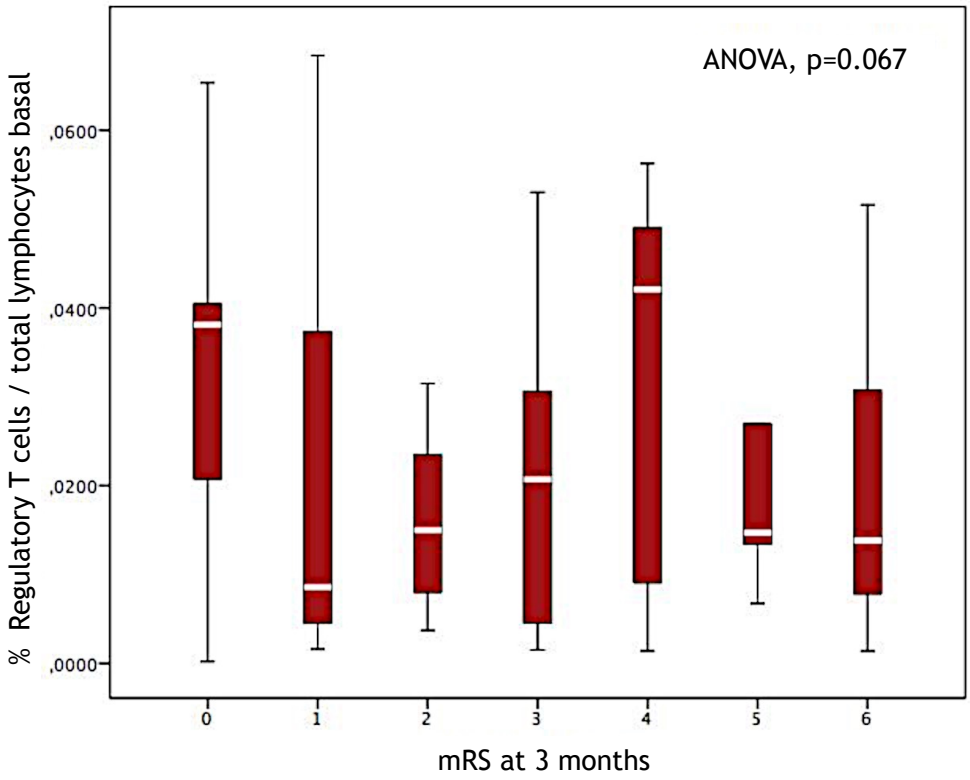

Figure 62. Relationship between % Treg at admission moment and mRS at 3 months (p=0.067).

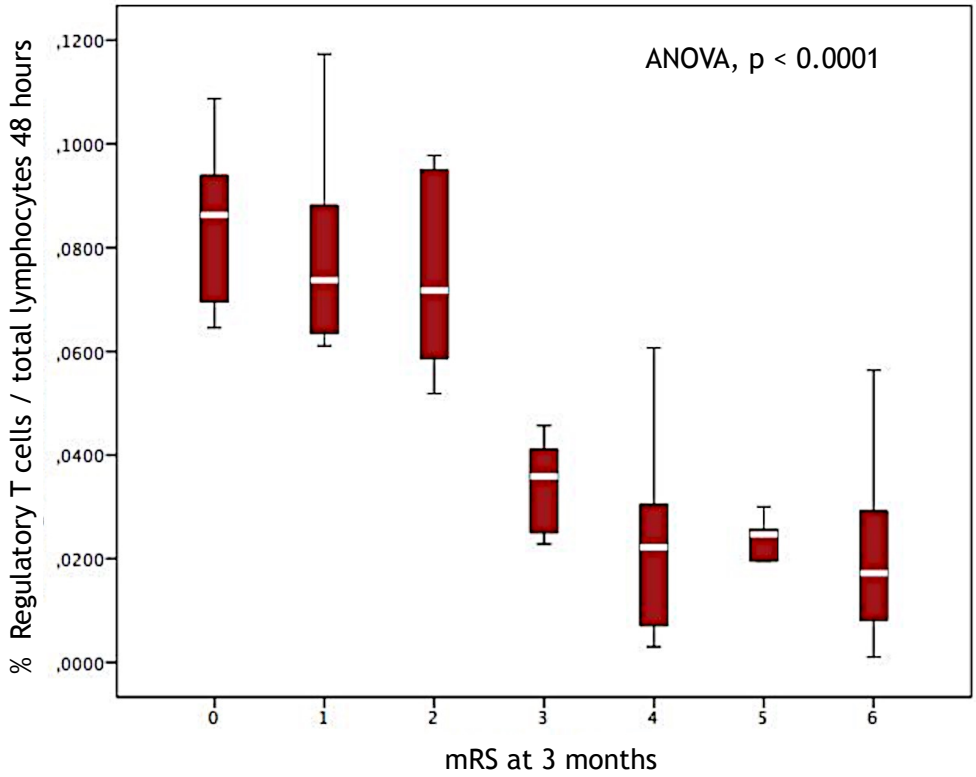

Figure 63. Relationship between % Treg at 48 hours and mRS at 3 months (p<0.0001).

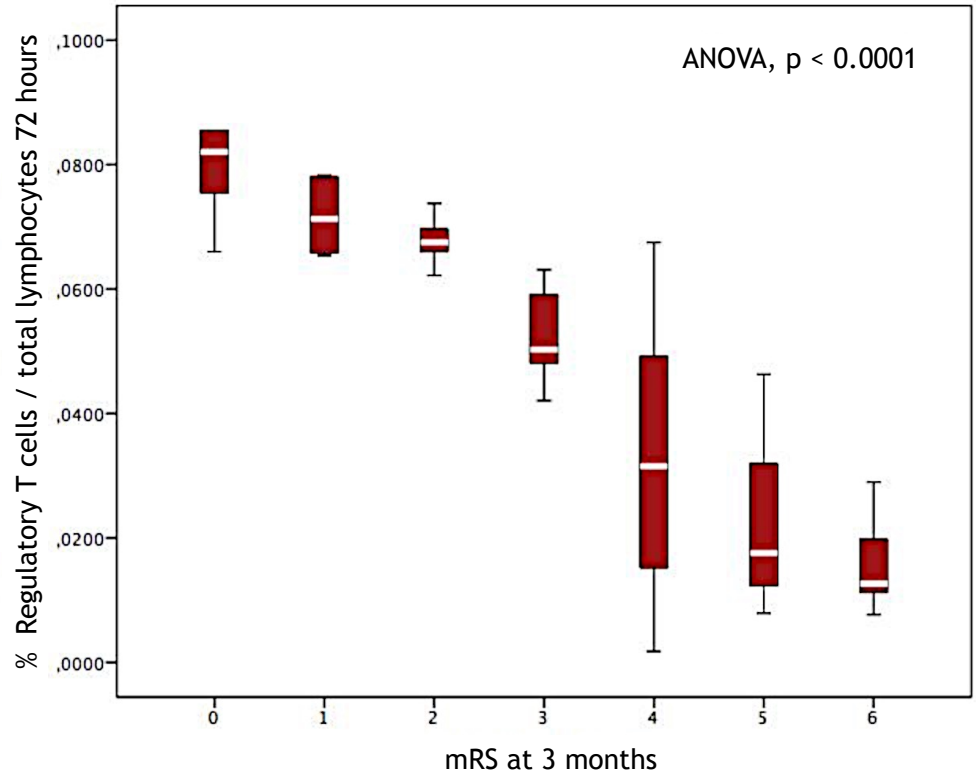

Figure 64. Relationship between % Treg at 72 hours and mRS at 3 months (p<0.0001).

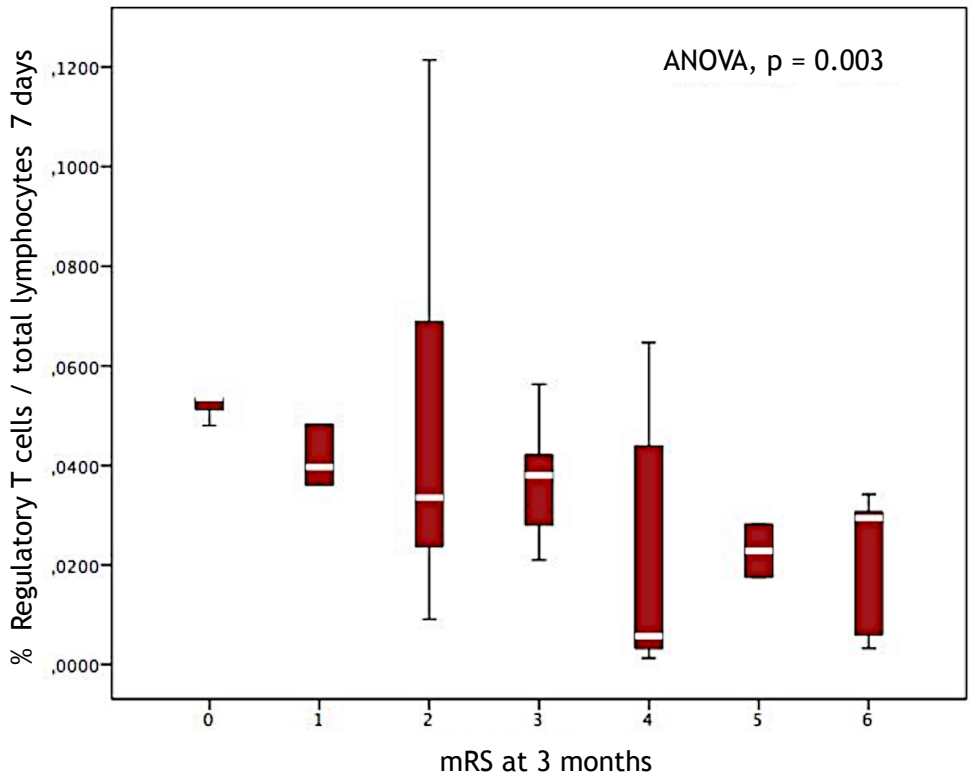

Figure 65. Relationship between % Treg at 7 days and mRS at 3 months (p=0.003).

2.4.1 Univariate analysis of primary endpoint: functional outcome at 3 months

To analyze those variables associated with functional outcome at 3 months after ischemic stroke, a univariate analysis was performed. Patients were classified according to the presence of good outcome (mRS≤2 at 3 months; n=87) or poor outcome (mRS>2 at 3 months; n=117).

Variable	Good outcome n = 87	Poor outcome n = 117	p
Age (years)	57.4 ± 8.4	76.3 ± 7.3	<0.0001
Female gender, n (%)	36(42.7)	67(57.3)	0.548
Previous hypertension, n (%)	32(36.9)	74(63.2)	0.030
Previous diabetes, n (%)	31(36.2)	75(64.1)	0.197
Previous dyslipidemia, n (%)	28(32.5)	79(67.5)	0.016
Previous atrial fibrillation, n (%)	40(46.7)	63(53.8)	<0.0001
Previous ischemic cardiopathy, n (%)	65(75.0)	29(24.7)	0.059
Previous peripheric arteriopathy, n (%)	0(0)	100 (100)	0.220
Alcohol consumption, n (%)	39(45.5)	64(54.7)	0.475
Smoking, n (%)	47(54.5)	53(45.3)	0.052
Previous statin consumption, n (%)	33(37.9)	73(62.4)	0.184
Leukocyte at admission (x 10^3/mmc)	8.1 ± 1.7	8.7 ± 3.8	<0.0001
Glucose at admission (mg/dL)	132.5 ± 39.3	155.8 ± 89.2	<0.0001
Fibrinogen at admission (mg/dL)	380.0 ± 64.9	400.4 ± 101.8	<0.0001
CRP(C reactive protein) (mg/L)	1.5 ± 1.7	3.2 ± 4.8	<0.0001
Recanalization therapy, n (%)	55.6	44.4	0.433
Basal NIHSS	4 [2, 7]	13 [7, 18]	<0.0001
TOAST			0.003
- Cardioembolic, n (%)	27 (31.0)	66 (56.4)	
- Atetrothrombotic, n (%)	13 (14.9)	13 (11.1)	
- Lacunar, n (%)	3 (3.4)	4 (3.4)	
- Undetermined,n (%)	44 (56.4)	34 (43.5)	
T regulatory cells (% over total lymphocytes) at admission	0.0224±0.0177	0.0197±0.0112	0.962
T regulatory cells (% over total lymphocytes) 48 hours	0.0715±0.0133	0.0231±0.0173	<0.0001
T regulatory cells (% over total lymphocytes) 72 hours	0.0709±0.0064	0.0273±0.0183	<0.0001
T regulatory cells (% over total lymphocytes) 7 days	0.0515±0.0189	0.0209±0.0172	0.001

Table 10. Univariate analysis for functional prognosis of stroke patients after 3 months.

Older age, (57±8.4 years vs 76.3±7.3 years; p<0.0001), previous history of diabetes (36.9% vs 63.1%; p=0.03), atrial fibrillation (46.7% vs 53.8%; p<0.0001), higher leukocyte count at admission (8.1±1.7 x 103/mmc vs 8.7±3.8 x 10^3/mm; p<0.0001), higher glucose levels at admission (132.5±39.3mg/dl vs 155.8±82.9 mg/dl; p<0.0001), higher fibrinogen at admission (380.0±64.9 mg/dl vs 400.4± 101.8 mg/dl; p<0.001), higher CRP (C Reactive protein) (1.5±1.7 mg/dl vs 3.2±4.8 mg/dl; p<0.0001), higher NIHSS score at admission (4 [2,7] vs 13

[7,18]; p <0.0001), and TOAST criterial (p=0.003) were associated with poor outcome at 3 months **(Table 10).** Regarding the percentage of Treg, we found that patients with good outcome had higher levels of Treg at 48 h (0.0715±0.0133 % vs 0.0231±0.0173 %; p<0.0001), 72 hours (0.0709±0.0064% vs 0.0273±0.0183 %; p<0.0001) and 7 days (0.0515±0.0189% vs 0.0209±0.0172%; p=0.001) **(Table 10)(Figure 66).**

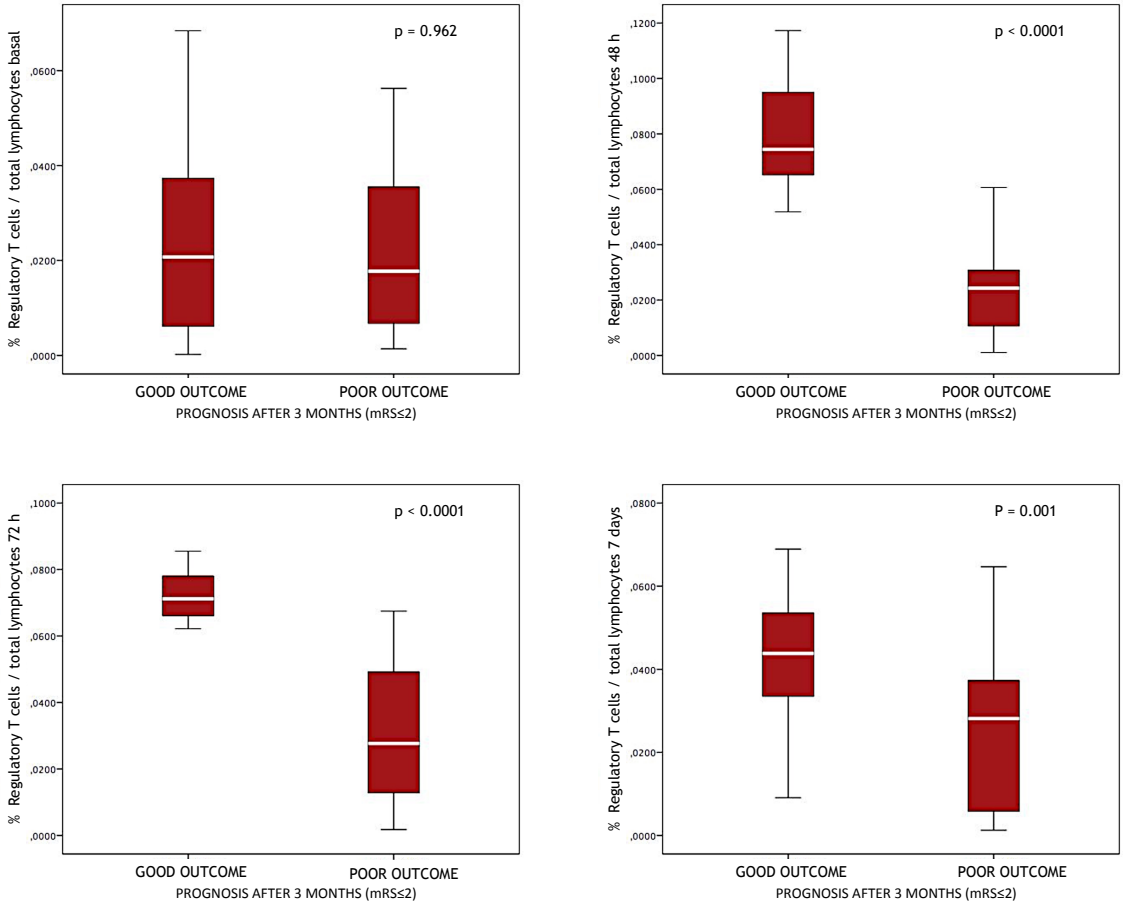

Figure 66. Comparison of % Treg between good outcome group (mRS≤2) and poor outcome (mRS>2) at admission moment, at 48 hours, at 72 hours and at 7 days.

2.4.2 Multivariate analysis of primary endpoint: functional outcome at 3 months

ROC curves were performed to establish the best cut-off point of Treg percentage at 48 hours, 72 hours and 7 days to predict functional outcome at 3 months. A percentage of Treg at 48 hours >0.0550 was associated with good outcome at 3 months (area under the curve: 0.990, CI 95%: 0.997-1.000, p<0.0001, specificity: 97%, sensitivity: 95%). A percentage of Treg at 72 hours >0.0650 was associated with good outcome at 3 months (area under the curve: 0.964, CI 95%: 0.886-1.000, p<0.0001, specificity: 95%, sensitivity: 93%) **(Figure 67)**. ROC curve for the percentage of Treg at day 7 was not significant (area under the curve: 0.711, CI 95%: 0.600-0.823, p=0.088).

Using percentage of Treg as a categorical variable, we found that Treg at 48 h was independently associated with good outcome (OR 3.5, CI 95% 1.9-7.8, p<0.0001) after adjustement by all significant variables associated with stroke outcome (age, previous history of hypertension, dyslipemia and atrial fibrillation, leukocyte, glucose, fibrinogen and C reactive protein at admission, basal NIHSS and TOAST criteria). Treg at 72 h was independently associated with good outcome (OR 1.7, CI 95% 1.1-3.1 p=0.016) after adjustement by the same variables.

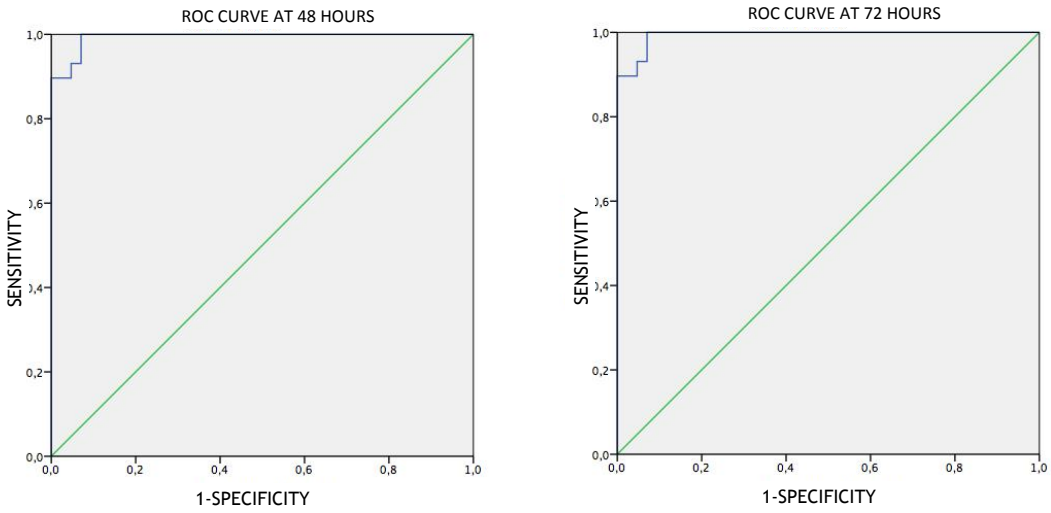

Figure 67. ROC curves of % Treg at 48 h and at 72 h to predict good outcome. The cut-off point associated with good prognosis at 3 months was 0.0550 at 48 h and 0.0650 at 72 h (p<0.0001).

2.5 SECONDARY ENDPOINTS

2.5.1 Early neurological deterioration

Early neurologic deterioration (END), defined as an increase of 4 or more points on NIHSS score within the first 72 hours after stroke onset occurred in 13 patients (6.4%). The percentage of Treg at 48 h (0.0132±0.0125 % vs 0.0411±0.0266 %; p<0.0001) and 72 h (0.0096±0.0061 % vs 0.0453±0.0234 %; p<0.0001) was lower in patients with early neurological deterioration **(Table 11) (Figure 68).**

Variable	No END n = 191	END n = 13	p
Regulatory T cells (% over total lymphocytes) at admission	0.0214±0.0167	0.0100±0.0088	0.103
Regulatory T cells (% over total lymphocytes) 48 hours	0.0411±0.0266	0.0132±0.0125	<0.0001
Regulatory T cells (% over total lymphocytes) 72 hours	0.0453±0.0234	0.0096±0.0061	<0.0001
Regulatory T cells (% over total lymphocytes) 7 days	0.0284±0.0200	0.0114±0.0070	0.008

Table 11. % Treg between patients with END or without END.

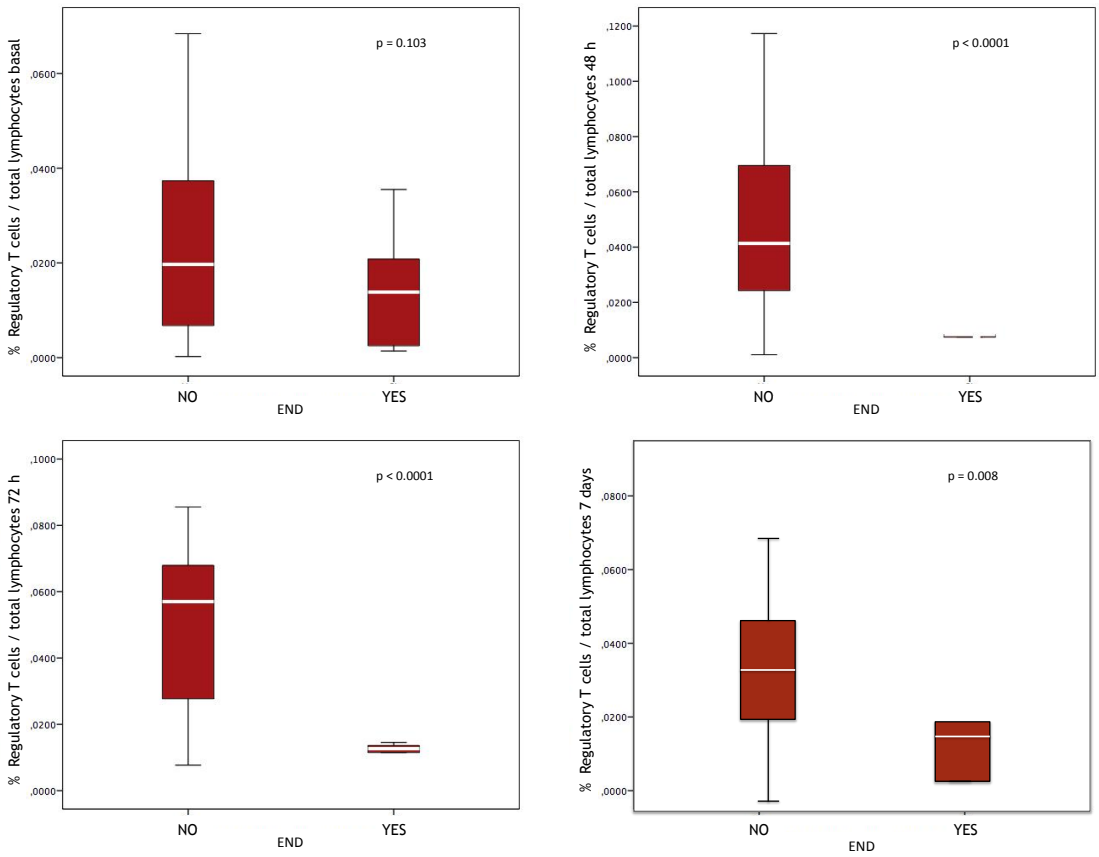

Figure 68. % Treg in patients without END versus patients with END at admission, at 48, 72 hours and 7 days.

2.5.2 Infarct volume

Infarct volume has been measured in 195 patients (9 patients died before control CT was performed). The mean volume was 50.79±88.43 cc.

2.5.2.1 UNIVARIATE ANALYSIS: VARIABLES ASSOCIATED WITH INFARCT VOLUME

To analyze those variables associated with infarct volume, a univariate analysis was performed. The variables associated with infarct volume were: age (Spearman coefficient 0.229, p=0.001), previous history of hypertension (Spearman coefficient 0.217, p=0.002), previous history of atrial fibrillation (Spearman coefficient 0.384, p<0.001), leukocyte count at admission (Spearman coefficient 0,307, p<0.001), glucose level at admission (Spearman coefficient 0.226, p=0.001), fibrinogen level at admission (Spearman coefficient 0.179, p=0.013), basal NIHSS (Spearman coefficient 0.569, p<0.001) and TOAST classification (Pearson coefficient -0.035, p<0.001) **(Table 12).**

Regarding molecular markers, we found that the percentage of Treg at 48 and 72 hours were inversely associated with infarct volume. Higher levels of Treg at 48 (Spearman coefficient -0.414, p<0.001) and 72 hours (Spearman coefficient -0.418, p<0.001) were associated with lower infarct volume. No correlation between infarct volume and percentage of Treg at baseline and 7 days was found **(Figures 69, 70, 71, 72).**

Independent variables	Spearman or Pearson coefficient	p
Gender	0.039	0.583
Age	0.229	0.001
Previous hypertension	0.217	0.002
Previous diabetes	0.059	0.402
Previous atrial fibrillation	0.384	<0.0001
Previous ischemic cardiopathy	-0.069	0.323
Previous peripheral artery disease	0.132	0.060
Alcohol consumption	-0.041	0.557
Smoking	-0.036	0.228
Previous statin consumption	0.085	0.228
Leukocyte at admission (x 10^3/mmc)	0.307	<0.0001
Glucose at admission (mg/dL)	0.226	0.001
Fibrinogen at admission (mg/dL)	0.179	0.013
CRP(C reactive protein) (mg/L)	-0.069	0.336
Basal NIHSS	0.569	<0.0001
Recanalization therapy(%)	-0.002	0.972
TOAST	-0.035	<0.0001
T regulatory cells (% over total lymphocytes) at admission	-0.025	0.728
T regulatory cells (% over total lymphocytes) 48 hours	-0.414	<0.0001
T regulatory cells (% over total lymphocytes) 72 hours	-0.418	<0.0001
T regulatory cells (% over total lymphocytes) 7 days	-0.157	0.185

Table 12. Univariate analysis for infarct volume

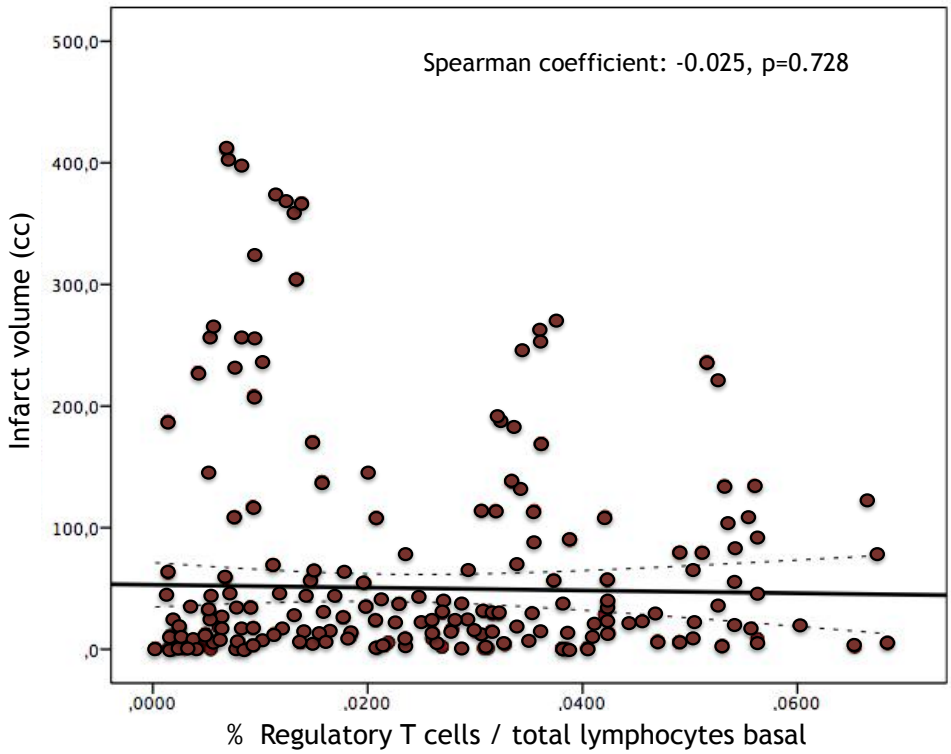

Figure 69. Correlation between %Treg at admission and infarct volume (cc). Spearman correlation coefficient :-0.025, p =0.728.

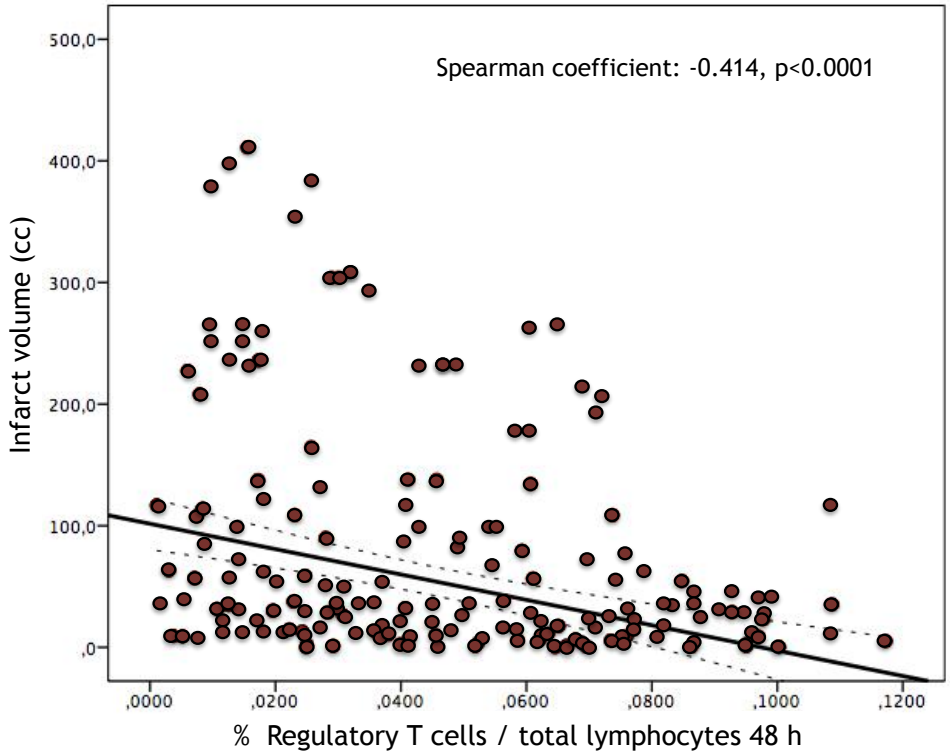

Figure 70. Correlation between %Treg at 48 hours and infarct volume (cc). Spearman correlation coefficient:-0.414, p < 0.0001.

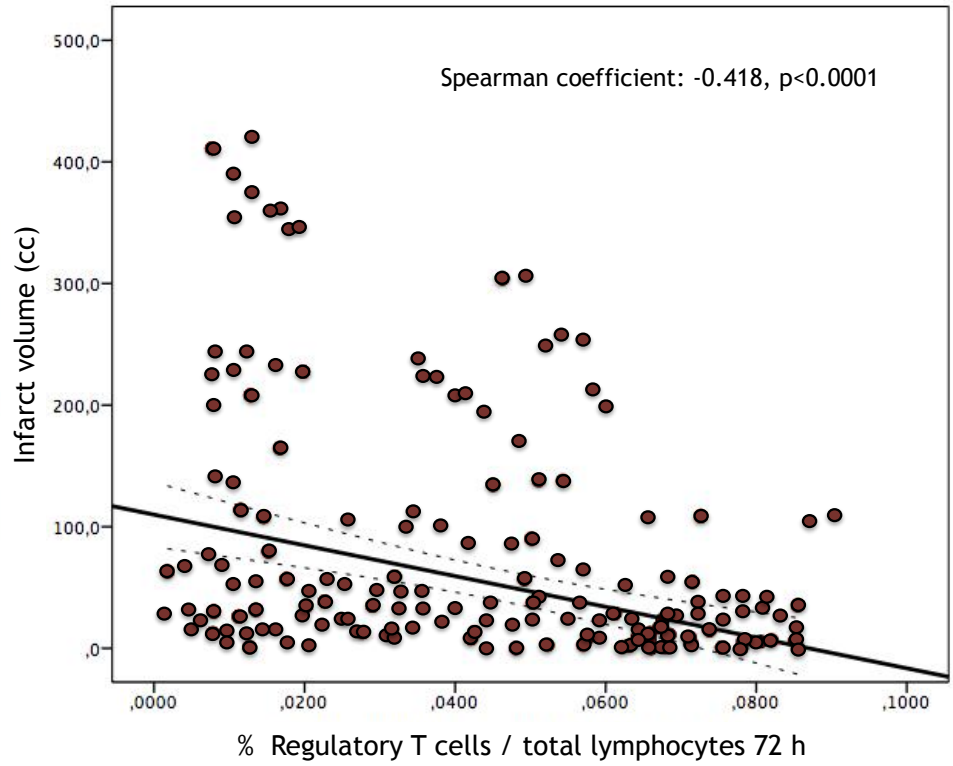

Figure 71. Correlation between %Treg at 72 hours and infarct volume (cc). Spearman correlation coefficient:-0.418, p < 0.0001.

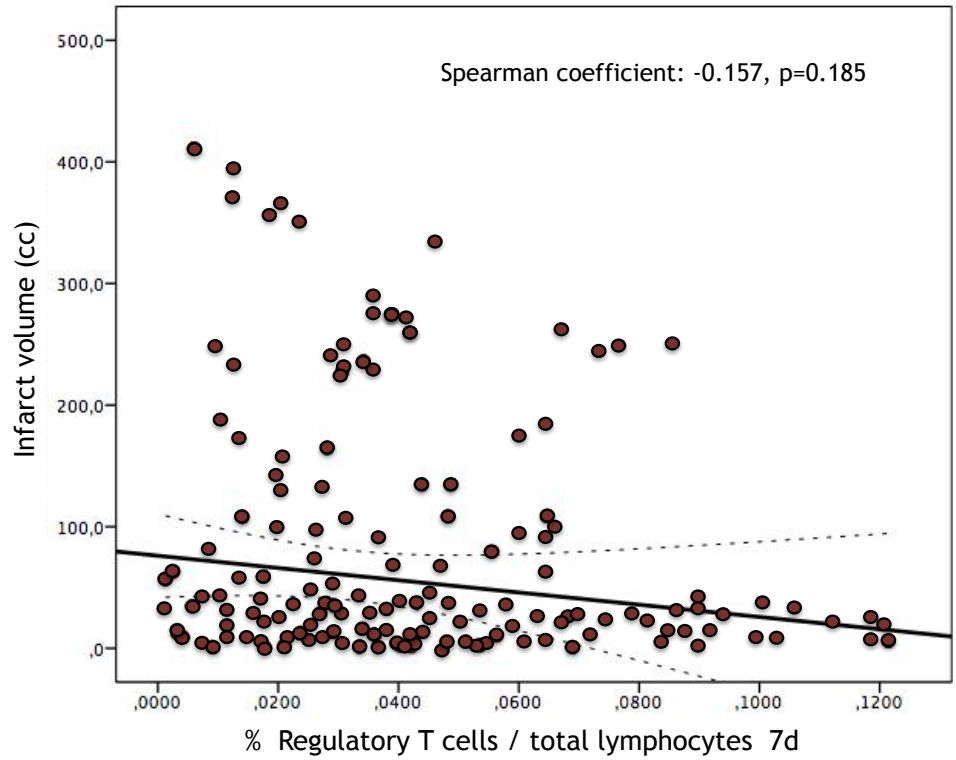

Figure 72. Correlation between %Treg at 7 days and infarct volume (cc). Spearman correlation coefficient=-0.157, p= 0.185.

2.5.2.2 MULTIVARIATE ANALYSIS. DEPENDENT VARIABLE: INFARCT VOLUME.

A multivariate analysis adjusted by significant variables (age, previous history of hypertension, dyslipidemia and atrial fibrillation, leukocyte count, glucose and fibrinogen at admission, NIHSS at admission and TOAST classification) was performed to evaluate those variables associated with infarct volume.

Two regression models were performed to analyze the relationship between infarct volume and %Treg. The first model included basal NIHSS and %Treg at 48 hours. We found that basal NIHSS is the only independent factor related to infarct volume (B: 2.88; IC 95%: 0.58, 5.18, p=0.014). In a second model we included basal NIHSS and %Treg at 72 hours. In this model basal NIHSS (B: 3.54; IC 95%: 1.10, 5.99,

p=0.005) and percentage of Treg at 72 hours (B: -673.38; IC 95%: -1267.75,-79.02; p=0.027) were independently associated with infarct volume.

2.6 RELATIONSHIP BETWEEN TREG AND IL-10 SERUM LEVELS

Using a correlation model, we found a positive correlation between the percentage of Treg and IL-10 levels. At admission, higher percentage of Treg was associated with higher levels of IL-10 (Spearman correlation coefficient=0.194, p=0.012) **(Figure 73)**. Similar results were found at 48 hours (Spearman correlation coefficient=0.687, p<0.0001) **(Figure 74)**, 72 hours (Spearman correlation coefficient=0.793, p<0.0001) **(Figure 75)** and 7 days (Spearman correlation coefficient=0.841, p<0.001) **(Figure 76)**.

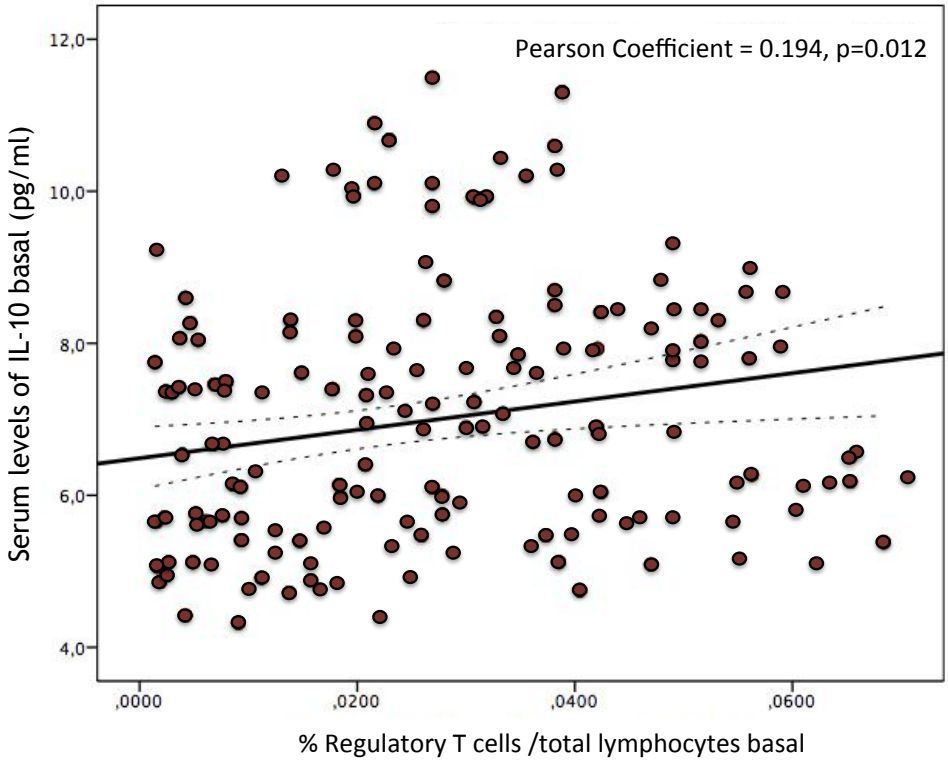

Figure 73. Relationship between serum levels of IL-10 and % Treg at admission moment (Pearson coefficient=0.194, p=0.012).

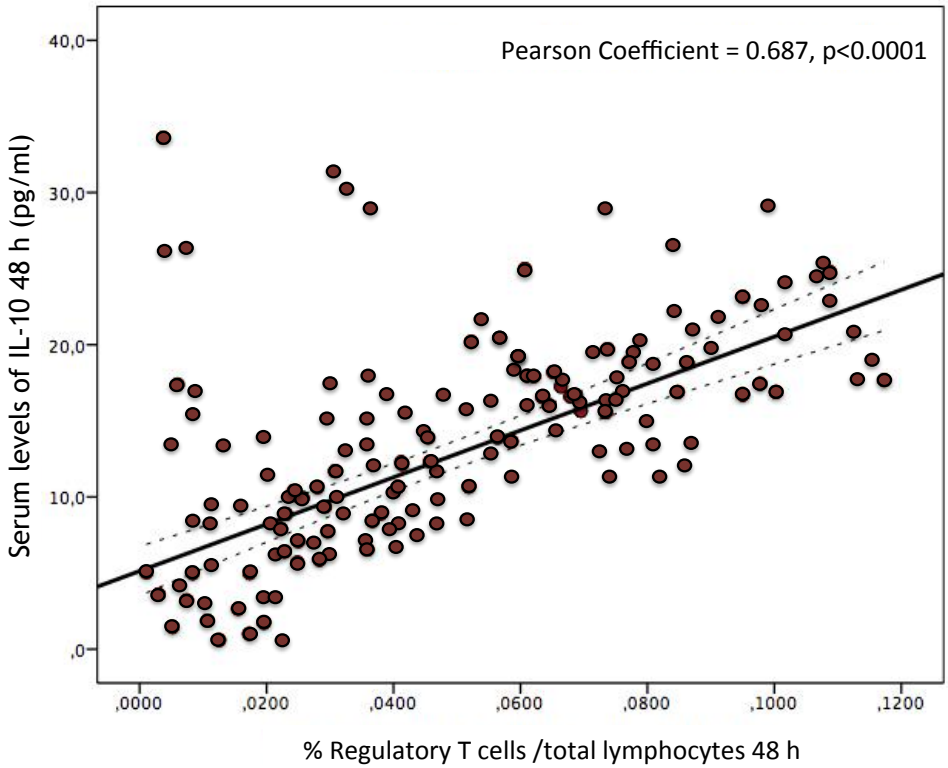

Figure 74. Relationship between serum levels of IL-10 and % Treg at 48 h (Pearson coefficient =0.687, p< 0.0001).

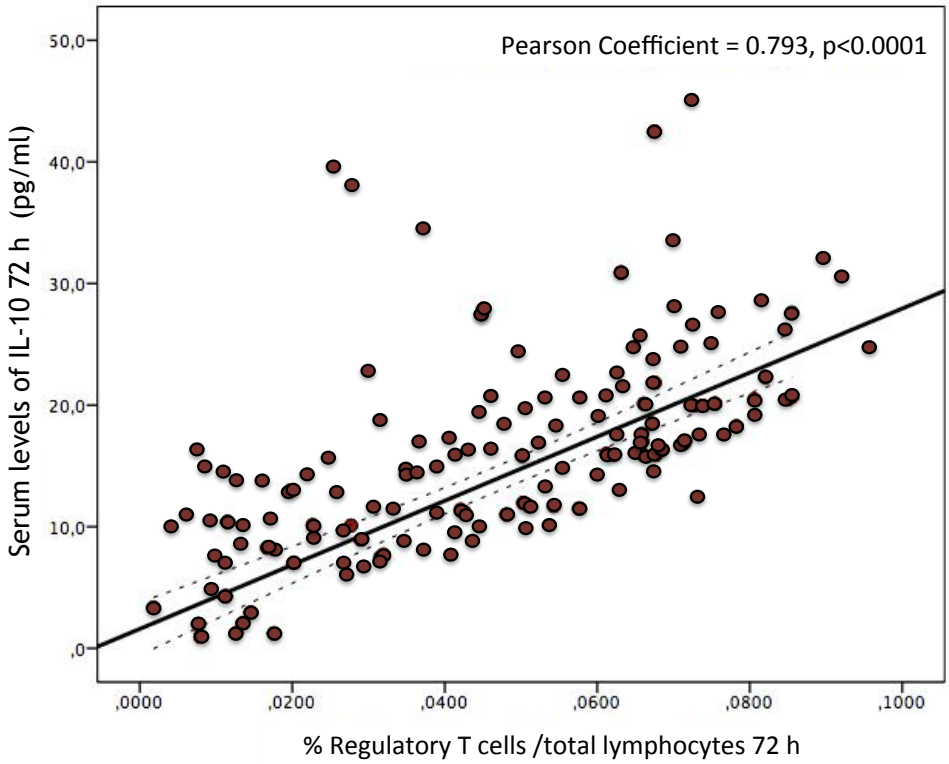

Figure 75. Relationship between serum levels of IL-10 and % Treg at 72 hours (Pearson coefficient=0.793, p < 0.0001).

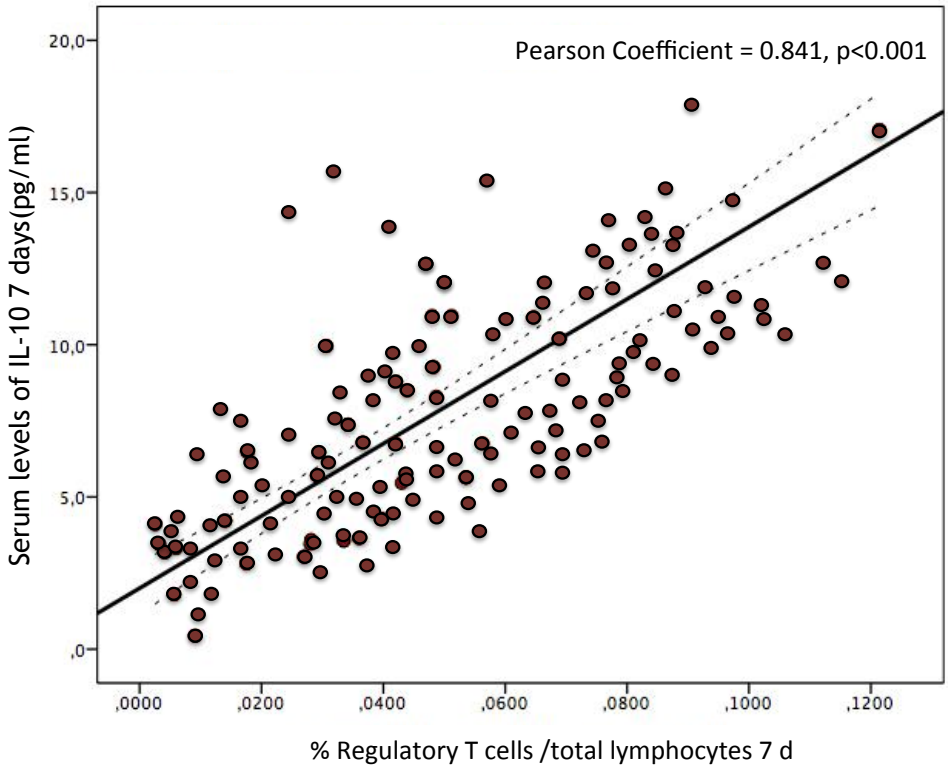

Figure 76. Relationship between serum levels of IL-10 and % Treg at 7 days (Pearson coefficient= 0.841, p < 0.0001).

2.7 SAFETY VARIABLE: RISK OF INFECTIONS

Twenty-six patients (12.7%) developed infections during hospitalization. Seventeen patients (65.4%) had respiratory infection, 6 patients (23.1%) urinary infection and in 3 patients (11.5%) the origin was unknown.

Infectious complications during hospitalization were associated with higher temperature at 24 hours (38.04±0.29 ºC vs 36.63±0.48 ºC, p <0.0001) and at 48 hours (37.94±0.48 ºC vs 36.66±0.48 ºC, p < 0.0001). These results show that infections occur early after stroke, within the first 48 hours **(Figure 77).**

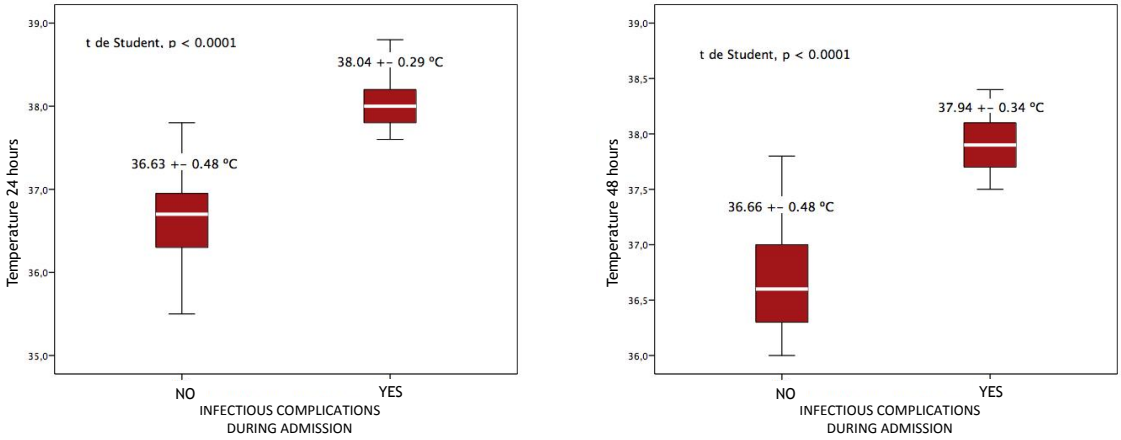

Figure 77. Temperature in patients with and without infections at 24 and 48 hours. Higher temperature was found in patients with infectious complications at 24 and 48 hours (p < 0.0001).

The presence of infections during the hospitalization was associated with poor outcome at 3 months. Modified Rankin Scale at 3 months was higher in patients with infection compared with those without (5 [4,6] vs 3 [1,4], p<0.0001) **(Figure 78)**.

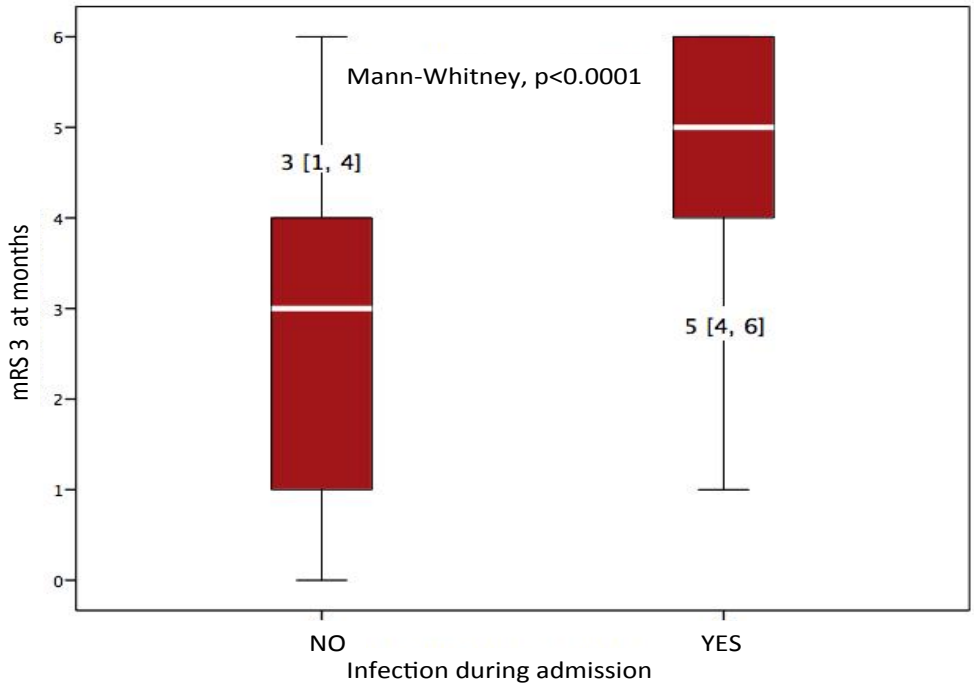

Figure 78. Relationship between infections during admission and mRS at three months (p < 0.0001).

2.7.1 Univariate analysis: variables associated with infections

The presence of infections during hospitalization was associated with stroke severity (determined by NIHSS score at admission). Patients with infections had higher NIHSS score at admission compared with patients without infections ((14 [11, 20]) vs 9 [5, 18]), p=0.033) **(Table 13).**

The percentage of Treg at 48 hours was lower in patients with infections compared with those without infections (0.0189±0.009 % vs 0.0425 ± 0.0280 %; p<0.0001). Similar results were found at 72 hours 0.0168±0.0105 % vs 0.0473±0.0238 %; p<0.0001) (**Figure 79).**

Independent variables	No infection n = 177	Infection n = 26	p
Age (years)	71.11 ± 11.03	74.92 ± 7.64	0.094
Female gender, n (%)	89 (50.3)	13 (50)	1.000
Previous diabetes, n (%)	42 (23.7)	4 (15.4)	0.249
NIHSS at admission	9 [5, 18]	14 [11, 20]	0.033
Regulatory T cells (%over total lymphocytes) at admission	0.0196 ± 0.0080	0.0228 ± 0.0196	0.607
Regulatory T cells (%over total lymphocytes) at 48 hours	0.0425 ± 0.0280	0.0189 ± 0.0090	< 0.0001
Regulatory T cells (%over total lymphocytes) at 72 hours	0.0473 ± 0.0238	0.0168 ± 0.0105	< 0.0001
Regulatory T cells (%over total lymphocytes) at 7 days	0.0306 ± 0.0196	0.0102 ± 0.0089	0.021

Table 13. Factors influencing infectious complications during admission

2.7.2 Multivariate analysis. Dependent variable: presence of infections

A multivariate analysis adjusted by significant variables (previous history of hypertension, dyslipidemia and atrial fibrillation, leukocyte

count, glucose levels and fibrinogen at admission, NIHSS at admission and TOAST classification) was performed.

The percentage of Treg at 48 hours (OR: 0.35 IC 95%: 0.00-0.57, p=0.001) and 72 hours (OR: 0.24 IC 95%: 0.02-0.51; p<0.0001) were inversely associated with infections during hospitalization, indicating that higher levels of Treg are associated with lower risk of infection.

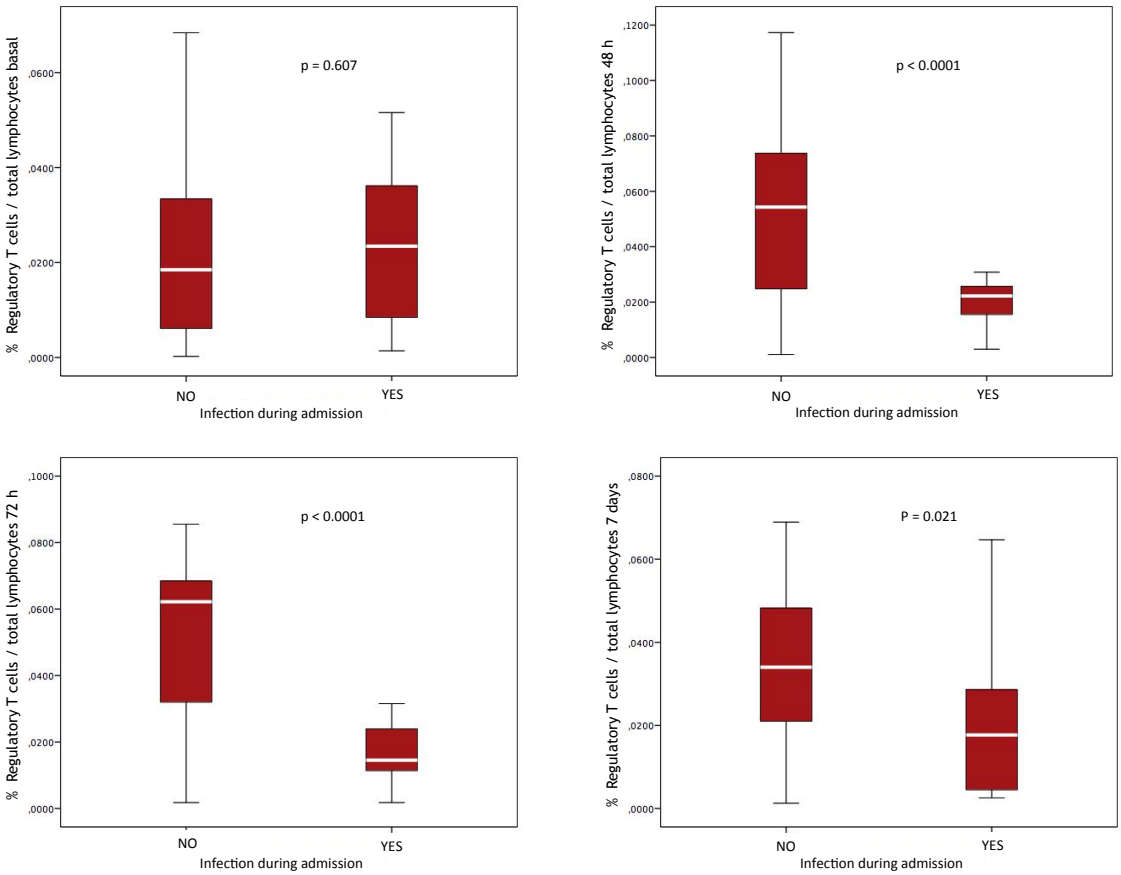

Figure 79. % Treg at admission, at 48 hours, at 72 hours and at 7 days in patients with and without infectious complications during admission.

DISCUSSION

In our study we have found that Treg exert an anti-inflammatory effect during the acute phase of brain ischemia, which lead to a better outcome.

To evaluate the role of Treg in brain ischemia we initially performed an animal study. The first aim of this study was to evaluate the effect of the administration of exogenous Treg on a rat model of MCAO. The first step was to obtain and expand Treg cells, and verify the immunosuppressive effect. To obtain Treg, we used lymph nodes of rats. CD4+ and CD25+ cells were separated using flow cytometry and further cultured and expanded in vitro to obtain Treg as previously was described[302, 310, 324]. Before the administration of these cells in an animal model of brain ischemia, we had to demonstrate their immunosuppressive effect in an in vitro model. For this purpose we co-cultured the Treg obtained with Tconv. Previous studies have shown that the immunosuppressive effect of Treg can be demonstrated by inhibiting the proliferation of Tconv, using CFSE dilution method[324]. This immunosuppressive effect was observed in our study when Treg were co-cultured with Tconv, indicating that our cells were functional.

Once we obtained Treg and demonstrated that the cells were functional, we investigated the effect of the administration of these cells after ischemia induction on a MCAO animal model. Treg were intravenously administered 2 hours after induction of ischemia. Previous studies administering exogenous Treg performed the treatment at different times, while some of them administered Treg 2 hours after ischemia[299, 309, 310], other studies administered them before ischemia[302]. In our study, we decided to administer exogenous Treg 2

hours after ischemia induction, because that will allow us to evaluate a more translational treatment that could potentially be used in humans after acute stroke. To evaluate infarct volume we performed MRI sequential studies, since it permit to evaluate infarct volume at different times without sacrificing the animal. Brain infarct was smaller in animals that received Treg compared with the control group, and this effect was observed during the first days (3, 7 and 10 days), but also was maintained at longer periods (14, 21 and 28 days), indicating a possible neuroprotective effect.

The effect of Treg administration on infarct volume is controversial in literature. Previous studies showed that administration of exogenous Treg exerted potent protection against brain damage in MCAO models[299, 309, 310], indicating a possible anti-inflammatory effect as the mechanism responsible. They found that Treg administration decreases proinflammatory and increases anti-inflammatory cytokines, such as IL-10 and TGF-β. Other recent study[302] have found opposite results, showing greater infarcts in mice that received exogenous Treg. The authors propose that Treg promote ischemic brain damage by causing microvascular dysfunction and thrombosis, not by an immunologic mechanism. The large heterogeneity between studies regarding design, experimental model, animal species and the moment when treatment is administered can explain these differences in results. Other explanation can be that the biological effect of Treg may be different according to stroke severity, since it has been demonstrated that Treg had more relevance in smaller compared with greater infarcts[241]. According to previous studies[299, 309, 310] our findings suggest a neuroprotective effect of exogenous Treg administration

after MCAO[344], since this treatment is related with smaller infarct volume both at short and long term after ischemia.

Once we demonstrated the beneficial effect of Treg administration on infarct volume we would like to know if this effect was produced locally. For this purpose we evaluated the presence of Treg in rat brains by analyzing the expression of FoxP3 by Western Blot both in infarcted and contralateral hemispheres. Compared to control group, we found higher levels of FoxP3 in infarcted hemisphere of treated animals. FoxP3 is a specific marker of Treg, so increased FoxP3 in infarcted hemisphere of treated animals indicates the infiltration of Treg into the brain, where they perform their neuroprotective effect. Previous studies have demonstrated an increase in FoxP3 expression in brain after ischemia. One of them[297] has found higher levels of Treg (CD4+FoxP3+) in ischemic hemisphere and peripheral lymphatic organs after MCAO on day 14 and 30 compared with day 7. Other study found that this expression could occur earlier, finding FoxP3 expression in the brain at 24 hours after MCAO[302]. After exogenous Treg transfer, Li et al[310] found an increase of Treg in spleen, lymph nodes, bone marrow, lung, liver and blood but not in brain. The same authors in a later study found that only a small number of Treg, detected by flow cytometry, infiltrated the brain, but the majority of the transferred Treg remained in the peripheral blood and organs for one week after exogenous Treg administration in an animal brain ischemia model.

To evaluate the possible mechanism implicated in the effect of Treg on infarct volume reduction we determined inflammatory markers in

brain tissue after exogenous Treg administration. We initially analyzed proinflammatory cytokines. IL-1β and TNFα levels were determined both in contralateral and infarcted brain in Treg treated and control animals. Lower IL-1β levels were found in Treg treated animals compared to controls in the infarcted hemisphere, but not in the contralateral one. TNFα was also analyzed in rat brains, but differences were not found, either in infarcted or contralateral hemisphere. Markers of active inflammation were later analyzed. CD11b+ and CD68+ cells were evaluated in infarcted hemisphere of Treg treated and control animals, finding a lower number of CD68+ and CD11b+ cells in treated animals compared to control. CD11b+ and CD68+ are markers of active inflammation, and IL-1β is a proinflammatory cytokine. Lower levels of IL-1β, CD11b+ and CD68 in infarcted brain of treated animals suggest a local anti-inflammatory effect of exogenous Treg administration in brain tissue. Previous studies have found increased levels of proinflammatory cytokines (IL-6, IL- 17, IL1β and TNFα) in the ischemic hemisphere 24 hours after MCAO, and this cytokine increase was significantly blunted after exogenous Treg administration[299]. However, other studies have not found differences in pro- and anti- inflammatory cytokines levels in the ischemic hemisphere between DEREG mice (transgenic mice with depletion of regulatory T cells) with or without Tregs[302].

To ensure the effect of Treg on protection after stroke we performed a second study evaluating the effect of endogenous stimulation of Treg. We used CD28 superagonists (CD28SA), which had previously demonstrated to induce endogenous Treg proliferation[304, 305, 326, 327]. Firstly we verified whether CD28SA expands peripheral

Tregs in an animal model of MCAO. For this purpose we determinated CD4+FoxP3+ cells in peripheral blood after CD28SA administration, and we found a significant increase in Treg levels at day 3 and 4 after MCAO. We used FoxP3 as a marker, because it is currently accepted as the most definitive marker of Treg[254]. Similar results were observed in previous studies[235, 302], finding an increase in Treg levels in peripheral blood 3 days after CD28SA administration. Since Treg increases at 3rd-4th day after CD28SA administration, to evaluate the effect of endogenous Treg stimulation, we administrated CD28SA 4 days before MCAO. We found a significant reduction in infarct volume and edema in treated rats at day 10 compared to the control group. Recent studies used CD28SA to evaluate the effect of Treg in animal models of brain ischemia, with contradictory results. One of them found that CD28SA treatment reduces the magnitude of stroke progression[311]. However, other found an increase in infarct volume after CD28SA treatment, suggesting a dual mechanism through thrombotic and inflammatory processes[312]. Our results show that CD28SA administration induces an endogenous Treg stimulation, and lower infarct volume, confirming the neuroprotective effect not only of exogenous administration but also of endogenous stimulation of Treg.

To evaluate the presence of Treg in brain tissue after CD28SA administration, we analyzed FoxP3 levels by Western Blot in treated and control animals, both in contralateral and infarcted hemispheres. We found that animals treated with CD28SA did not show higher FoxP3 levels in brain compared to control, indicating that endogenous stimulated Treg are not present in brain tissue. Other study using CD28SA in animal brain ischemia models[311], have found that the

number of Treg was significantly higher in brains of CD28SA treated mice compared with controls, and Treg were located mainly in the peri-infarct area of ischemic brains in both treated and control animal. Differences in our results with this study could be explained by the moment of CD28SA administration, the method of detection of Treg and the animal model. In our study, we administrated the treatment 4 days before ischemia to a rat MCAO model and evaluated FoxP3 level by Western Blot, while this study administrated it 3 hours after ischemia in a mouse MCAO model and used flow cytometry to identify Treg. Schumann et al[312] found in their study that Treg were predominantly located within the vessel lumina and did not transmigrate into the brain parenchyma.

The benefit of CD28SA administration in infarct volume reduction associated with the lack of differences in FoxP3 levels in treated and control group in our study suggest pleiotropic effects of endogenous Treg. Our results indicate that the stimulation of endogenous Treg can exert a beneficial effect after brain ischemia without infiltrating brain tissue. This effect could be explained by local direct modulation of neuroinflammation in brain parenchyma, but also by a peripheral modulation of immune response, as some authors proposed[299]. This peripheral effect of Treg must induce early neuroprotection, either by releasing neuroprotective mediators into peripheral circulation or targeting other peripheral cells that influence in infarct volume.

We also analyzed IL-1β levels; both in infarcted and contralateral hemisphere of CD28SA treated animals and controls. No differences in IL-1β levels were found between brain tissue of treated and control

animals, suggesting that endogenous Treg exert their effect on peripheral system, without any changes in inflammatory markers in brain tissue.

To confirm the protective effect of Treg, we developed a third experiment to evaluate the effect of Treg depletion. For this purpose we used an in vivo depletion model with an anti-CD25 antibody, which resulted in Treg depletion in previous studies[112, 297, 299, 300].

In contrast to previous studies[112, 297, 299] we found only less than 30% of decrease in CD4+FoxP3+ cells after anti-CD25 antibodies treatment in our study. Liesz et al[112] found at least half of FoxP3 cells depletion after anti-CD25 treatment, and other studies[299] found even higher levels of Treg depletion. Some authors proposed that injection of anti-CD25 monoclonal antibodies result in functional inactivation, but not depletion of CD25+ cells. According to our results, these authors found that injection of anti-CD25 antibodies only produces alteration in CD25 surface expression, but not in FoxP3 expression, failing to influence in the number of CD4+FoxP3+ cells[344].

Treatment with anti-CD25 antibodies did not influence in infarct volume or brain edema after brain ischemia in our study. This can be explained because we did not achieve a sufficient Treg depletion to obtain significant differences between treated and control animals. Some studies of MCAO have found greater infarct volumes after anti-CD25 antibodies administration[112, 300, 301], whereas other studies[297, 299] showed no effect of this antibody regarding infarct volume. Our results are concordant with latter studies, showing no differences in infarct volume, probably due to an insufficient depletion of Treg. Other

studies that used genetic models of animals with Treg depletion also found contradictory results. While some studies found a negative relationship between Treg depletion and infarct volume[301, 303], others found a reduction of infarct size after Treg depletion[272].

IL-1β levels were also analyzed both in contralateral and infarcted hemisphere of animals treated with anti-CD25 antibodies. IL-1β levels were slightly higher in infarcted hemisphere of treated animals compared to control, but there were no significant differences between levels of IL-1β in infarcted hemisphere of anti-CD25 antibodies treated animals and control. Again, these results could be explained because in our study we did not achieve a sufficient Treg depletion after anti-CD25 antibodies administration.

We finally tried to evaluate the effects of Treg in neurogenesis and angiogenesis. To determinate neurogenesis and angiogenesis processes we used flow cytometry techniques. In contrast to inmunohistochemical techniques, cytometry studies the entire brain, not an individual slice of brain and the measurement is more objective. The inconvenience of this method is the impossibility of determining the specific neurogenesis areas. To evaluate neurogenesis, we analyzed the percentage of double positive NeuN-BrdU and NCAM-BrdU cells, as previously described[328-329], in brain tissue of animals treated with exogenous Treg and controls at 28 days. BrdU, a uridine analogue, which incorporates into the DNA during cells division, was used to detect newly generated nucleus[328]. NeuN is a mature neuronal marker protein that detects the neuronal character of the nucleus[328]. NCAM is an adhesion molecule that is expressed in neurogenic sites in postnatal

brain[329, 330]. To evaluate angiogenesis we determined percentage of double positive CD31-BrDU cells in animal brains. CD31 is a molecule used to demonstrate the presence of endothelial cells and evaluate the degree of angiogenesis[331-332]. In our study we found no differences in neurogenesis (comparing NeuN and NCAM levels) or angiogenesis (comparing CD31 levels) in animals treated with exogenous Treg compared to control animals.

Regarding angiogenesis, few studies proposed a deleterious role of Treg in postischemic neovascularization. One of the studies found a decreased neovascularization after Treg administration and increased neovascularization after Treg depletion in a hind limb ischemia model in animals, proposing a negative effect of Treg[270]. Other study did not show differences after major changes in Treg levels, following Treg expansion and Treg depletion[271]. Both studies were performed in hind limb ischemia models[270, 271]. Our results suggest that Treg treatment did not impair angiogenesis in brain ischemia in an animal model.

Some authors proposed that Treg cells contain growth factors that may be important in neurogenesis[93, 272, 273]. In our study Treg treated animals showed slightly higher levels of neurogenesis markers, but results were not statistically significant, so we have not found clear effects in neurogenesis after exogenous administration of Treg.

Once we demonstrated the effect of Treg on infarct volume reduction in animal models, we tried to evaluate the effect of these cells on clinical practice. For this purpose we analyzed Treg levels in patients with ischemic stroke. Treg levels were expressed as the percentage of Treg over total lymphocytes in peripheral blood. Due to

the variability of lymphocyte levels in patients after acute stroke, we used percentage instead total counts of Treg to minimize differences between samples.

Before to evaluating the effect of Treg on stroke outcome, we analyzed whether Treg are modified by ischemic stroke. For this, we compared the percentage of Treg during the acute phase in patients with ischemic stroke with healthy subjects matched by age and sex. Our results are in concordance with previous studies showing significant higher percentages of Treg in ischemic stroke patients at the moment of admission compared to controls[319]. We also compared IL-10 levels, an anti-inflammatory cytokine important in Treg function, in stroke patients and healthy controls, finding higher levels of IL-10 in patients with ischemic stroke. We only found a previous study comparing IL-10 levels in stroke patients and healthy population[114] and they found contrary results, finding lower levels of IL-10 in stroke patients compared to controls. Our results suggest that Treg and IL-10 levels increase during the acute phase of stroke, and could exert a physiopathological role in ischemic stroke.

We also tried to establish a temporal profile of Treg during the acute phase of stroke. Previous studies in animal models showed an increase of Treg within brain tissue later, at day 14 and 30 after MCAO[297]. Unfortunately, it is not possible to determinate Treg in patient brains without invasive techniques, so we have to use peripheral blood samples to establish a temporal pattern. Previous small and heterogenous studies showed a decrease in percentage of Treg in peripheral blood the second day following stroke and a

significant increase on day 7 after stroke[64]. In our study we found that the percentage of Treg increases from stroke onset to day 3 after stroke, and later decreases. These results could propose a potential role of Treg in the first moment after acute ischemic stroke, mainly within the first three days after stroke.

To evaluate the effect of Treg on patients with ischemic stroke we studied the percentage of Treg in peripheral blood during the acute phase of stroke and functional outcome at 3 months. We found that higher levels of Treg during the acute phase of stroke were associated with better functional outcome at 3 months. Using percentage of Treg as a categorical variable we found that percentage of Treg at 48 hours > 0.055% was associated with good outcome with a specificity of 95% and a sensitivity of 93%, and percentage of Treg at 72 hours >0.065% predicts good outcome with a specificity of 97% and a sensitivity of 95%. This relationship between higher percentage of Treg and good outcome at three months support the beneficial role of Treg in acute phase of ischemic stroke. There are only few studies evaluating relationship between levels of Treg and outcome after stroke. One study[64] did not find relationship between Treg levels and functional outcome at three months. However, this study has a relatively small size and included ischemic and hemorrhagic stroke. To the best of our knowledge this is the first study that specifically correlates percentage of Treg during the acute phase of ischemic stroke and long-term outcome.

Another objective of the study was to analyze the effect of Treg levels on the development of early neurological deterioration. Early

neurological deterioration following ischemic stroke is a serious event associated with higher mortality and poor functional outcome. In our study we found that patients with early neurological deterioration showed lower levels of Treg during the acute phase of stroke. Unfortunately, our sample is not big enough to perform multivariate analysis regarding early neurological deterioration, since only 13 patients developed it, so we cannot analyze if the effect of Treg on avoiding neurological deterioration is direct, or acts as a surrogate maker. In previous studies, lower plasma concentrations of IL-10 were associated with clinical worsening[115], but we did not found in the literature previous studies regarding relationship between early neurological worsening and Treg levels, so this aspect should be investigated in further studies.

Finally, we studied in our patients the relationship between percentage of Treg during the acute phase of ischemic stroke and infarct volume, since it has not been previously reported in literature. We found that higher levels of Treg were related with smaller infarct volume. These results also could suggest a beneficial role of Treg in acute ischemic stroke, probably by decreasing inflammation within the infarcted area and decreasing infarct volume.

In the clinical model we also investigated the possible immunosuppressive mechanism of Treg. Several mechanisms have been proposed in Treg action[248, 241]: anti-inflammatory cytokine production, elimination through granzimes and perforins, metabolic disruption and competitive mechanisms. One of the main and more studied mechanisms is the production of IL-10. IL-10 is an anti-

inflammatory cytokine with neuroprotective effects after cerebral ischemia. In the context of experimental brain ischemia, several studies have demonstrated that IL-10 is a critical neuroprotective cytokine regulating postroke neuroinflammation[111, 112]. The main sources of cerebral IL-10 are Treg, Breg and microglial/monocytes. Previous studies have verified the effect of IL-10 as a mediator in the protective effect of Treg on animal models of brain ischemia[112, 308]. Accordingly, strategies increasing lymphocyte IL-10 production[308, 311] or therapeutic IL-10 administration have been shown to improve stroke outcome[112]. In this sense, we studied the relationship between Treg and IL-10 levels in patients with ischemic stroke. We found a direct relationship between serum IL-10 levels and percentage of Treg at admission, 48 hours, 72 hours and 7 days, supporting that the possible anti-inflammatory role of Treg is mediated by increasing IL-10 levels, as has been found in animal studies[112, 299]. Previous studies have found a positive relationship between higher IL-10 seric levels in acute phase of ischemic stroke and good outcome[115-119], but no studies have previously investigated the role of IL-10 as a mechanism of action of Treg in acute phase of ischemic stroke in humans.

Other point of interest in this study was to evaluate the effect of Treg on the risk of systemic infections. Systemic infections are frequent during the acute phase of stroke (7-35%, depending on the series) [206], and it presence worsens long-term outcome[210-230]. Some authors proposed that stroke could induce a systemic immunosuppression that could increase the risk of infections[206]. The underlying mechanisms that result in widespread immunosuppression after stroke and concurrent systemic infections are not well

understood. Some studies have observed lymphopenia and increased apoptosis of TH lymphocytes, cytotoxic T lymphocytes and B lymphocytes early after stroke[206]. Increased cortisol and metanephrine have been also related with the risk of infections after stroke[209, 222]. During the first hours after stroke, proinflammatory cytokines are up-regulated (IL-6, IL-1, TNFα, IL-8, MCP-1, etc) [101,102,115, 224]. This inflammation stimulates both hypothalamic-pituitary-adrenal axis and sympathetic nervous system, which suppress immune cell function and can be related to systemic downregulation of the immune system[206].

Treg are a subpopulation of cells with an immunosuppressive effect[227-228], so these cells can be implicated in the risk of infections development during the acute phase of stroke. Some studies in animal models[310] indicate that exogenous Treg administration does not exacerbate immunosuppression after stroke, even found that can improve immune status after induction of ischemia. Few studies[64] with small number of patients found no association with the development of infections after stroke in humans, but this aspect is still unclear. In our study we tried to establish the relationship between percentage of Treg in acute ischemic stroke and development of infections during admission. We found that the presence of infections during the acute phase of stroke was related with poor long-term outcome, as previously has been reported[206]. Patients with systemic infections developed higher temperature at 24 and 48 hours, suggesting that infections appear early after ischemic stroke. Higher percentage of Treg after stroke was not related with higher risk of infections. By contrast, percentage of Treg at 48 and 72 hours was inversely related to development of infections after stroke. In fact, most of the infections

in our patients occur at an early phase after stroke (first-second day), when Treg have not achieved their highest levels.

We found that Treg levels were not associated with the risk of infection. These results do not support the immunosuppressive effect of these cells as the reason of infections development after stroke. One explanation of these results is that Treg have different effects, not only immunosuppressive but also immunomodulatory. Previous studies in animal models have proved that Treg administration improves immune system after brain ischemia[299, 310]. Treg correct low levels of lymphocytes, improve cellular immune functions and reduce the risk of spontaneous infections after MCAO. Our results confirm this effect observed in animals, suggesting a possible protective role of Treg in the risk of infections, or at least not a deleterious role of these cells.

Finally a number of limitations need to be considered. In our work, regarding the experimental study, we used CD4+CD25+ cells to obtain Treg and expanded them in vitro as performed in previous studies. This in vitro stimulation was shown to induce Treg[324], but some authors proposed that other CD4+ T cell subpopulations apart from Treg are also involved after this treatment in stroke development[302]. However, to assess the immunosuppressive function of the expanded cells we performed in vitro studies previous to animal Treg administration. In addition, in our study we could not extract clear conclusions about depletion of Treg in an animal model of brain ischemia, because we did not achieve a complete depletion of these cells after anti-CD25 administration. It would be useful to support the neuroprotective role of Treg after brain ischemia to deplete Treg and

find an increase in brain infarct. To determinate if Treg infiltrates brain tissue in infarcted hemisphere after exogenous Treg administration we used FoxP3. FoxP3 is the most specific marker of Treg, but some authors proposed that FoxP3 could be expressed by other types of lymphoid or myeloid cells. For that reason, part of FoxP3 detected in brain could derive from other cells different of Treg[247]. To evaluate the anti-inflammatory effects of Treg increase we determined proinflammatory cytokine levels in brain tissue. Treg could also exert neuroprotection through peripheral means, by increasing anti-inflammatory cytokines or by decreasing proinflammatory cytokines in peripheral blood. Peripheral blood cytokines levels were not analyzed in our animal study, nevertheless were analyzed in clinical study.

With regard to the clinical study the first limitation is that we used CD4+CD25+CD127- as markers for Treg. Most authors proposed that FoxP3 is the most specific marker of Treg, but FoxP3 is an intracellular protein, so it cannot be used to separate human Treg cells for functional studies or in vivo expansion for cellular therapy, limiting its use in human setting. For that reason we used CD127 lack, that has previously demonstrated to be directly related with FoxP3 levels[250-252] and is a more translational option regarding future studies using Treg for cellular therapy. In our study, we only determinated Treg in peripheral blood, we have not demonstrated that these cells are infiltrating the brain tissue after stroke, however, invasive techniques would be need to determinate Treg in infarcted tissue. One of the secondary endpoints of our study was to assess the levels of percentage of Treg in patients who developed early neurological

deterioration, but we only found 13 patients with early neurological deterioration in our sample. Thus, we cannot assure that lower Treg levels are independently associated with early deterioration or they act as a surrogate marker. The low presence of early neurological deterioration could be explained due to the fact that treatment of acute stroke in a specialized Stroke Unit improves short-term evolution in our patients. Another potential limitation of our study is that we only determined Treg in acute phase of ischemic stroke (until the first 7 days after stroke). We have not evaluated long-term temporal profile of these cells, so we cannot establish their potential role in neurorepair after ischemic stroke. We have only determined IL-10 as a possible mechanism of Treg function. Other anti-inflammatory cytokines such as TGFβ and IL-35 could also be implied in Treg role after ischemic stroke. Moreover, reduction of proinflammatory cytokines could also have a role in Treg action after ischemic stroke. In the clinical study we have not evaluated brain edema as a marker. Brain edema could be related to inflammatory processes. We have only evaluated final infarct volume, because we did not perform halftime neuroimaging studies unless the patient developed neurological worsen.

In summary, we have found that Treg play an important beneficial role in ischemic stroke. The administration of Treg in an animal model of brain ischemia decreases infarct volume. This effect can be explained by a dual mechanism, both systemic and local. In brain tissue Treg exert an immunosuppressive effect, and in peripheral blood could act as immunomodulatory cells. On the other hand, Treg does not impair processes of neurogenesis or angiogenesis, so these cells can be considered as an important therapeutic target in ischemic stroke.

We also proved the beneficial effect of Treg on clinical practice. We have found that Treg levels increase after stroke. High levels of Treg during the acute phase of stroke induce a neuroprotective effect; since they are associated with better functional outcome, lower infarct volume and lower risk of early neurological deterioration. Treg were correlated with IL-10 levels, supporting that this anti-inflammatory cytokine can play an important role in the beneficial effect of Treg after ischemic stroke. Moreover, we found that higher levels of Treg had not negative effects during the acute phase of stroke, since no increase in the overall risk of infection was observed.

Treg constitute a potential therapeutic agent in acute ischemic stroke. Both exogenous administration and endogenous stimulation, exert a beneficial effect on animal models, and no deleterious effects were observed. Due to the relevance of Treg in stroke and other diseases[241, 274-294], it would be interesting to develop in the future new therapeutic strategies to increase these cells.

Gershon proposed using Treg for immunotherapy decades ago; however, clinical implementation of protocols employing Treg immunotherapy has proved challenging[346]. Based on our and other studies, the use of Treg cells as therapeutic agents in acute ischemic stroke in humans could potentially be developed in different ways in the future.

The first one is the use of Treg in adoptive transfer of autologous or donor-derived Treg. In autologous Treg adoptive transfer, immune cells from the patients are modified in vitro, expanded and reintroduced to the person. These expanded Treg should not be

rejected by the immune system and Treg therapy can reestablish immunological tolerance and has lasting therapeutic effects on mice. The problem of acute stroke treatment with adoptive transfer of autologous cells, is the therapeutic window for Treg administration. To the best of our knowledge, there are no studies investigating the best timing of Treg delivery for improving outcome after ischemic stroke in humans. Animal studies designs in brain ischemia are heterogenous, administrating Treg from 2 hours to 24 hours after induced ischemia. Most of these studies have shown reductions in infarct volume, whether Tregs were administered at 2 hours or at 24 hours after ischemia. It would be interesting to develop new methods to generate large amounts of Treg cells for clinical use in a shorter time and to investigate the therapeutic window of autologous Treg as a treatment in acute stroke phase. Treg might take over different tasks in stroke and their net biological effects probably depend on the stage of the insult and the timing of application. A combination therapy involving Treg transfer and actual stroke treatment (intravenous and intraarterial thrombolysis) could also be considered. Based on our study results, higher levels of Treg at 48 and 72 hours are related with better long-term outcome, so these cells could potentially be administered in later stages of this disease, as a strategy in neuroprotection and neurorepair. Protocols in clinical practice for adoptive transfer of Treg from a donor have also been reported[347]. Peripheral or banked umbilical cord blood may serve as Treg source, as proposed by some authors[348]. Some clinical trials regarding Treg cell therapy, with autologous or donor-derived cells have been used in

other diseases (prevention of graft versus host disease after allogeneic bone marrow transplantation and other autoimmune diseases) [346].

Other pharmacological agents have been reported to be capable of expanding and activating Treg cells in mice models. These include administration of immunosuppressive cytokines as TGFβ and IL-10 in acute phase of ischemic stroke or the histone deacetylase inhibitor, which induces FoxP3 increase. Some clinical trials using recombinant IL-10 and IL-10 homologs have been used in humans in other diseases[349]. Agents such as the immunosuppressor drug rapamycin and antibodies to CD3, CD4, CD8 or CD40L (CTLA-Ig fusion protein) inhibit T cells activation and selectively activate and expand Treg in vivo. Some of these, are being tested in the clinic in other diseases, CTLA-Ig fusion protein for rheumatoid arthritis and allograft rejection and a humanized antibody to CD3 for type I diabetes[350]. Potentially, administration of these agents in acute or subacute phase of ischemic stroke could exert a beneficial role. Further studies are needed to assess the efficacy and adverse effects of these treatments in ischemic stroke.

Treatments to induce in vivo expansion of Treg (as CD28SA) could also be a therapeutic weapon in ischemic stroke. CD28SA increases Treg levels 3 days after administration, and this increase has a beneficial effect on stroke outcome in mice. However, a phase I clinical trial of TGN1412 (a super-agonistic anti-CD28 antibody) in healthy volunteers[351] caused massive cytokine storm and multi-organ dysfunction. That fact, highlights the risks of drugs designed to modulate T cell activity without selectively targeting specific T cell

subsets. The development of new drugs that expand Treg in vivo in a shorter time than CD28SA, with a selective effect, could be an interesting therapeutic weapon in ischemic stroke to be developed in the future.

However, further studies are necessary to clarify the mechanism of action of these cells after ischemic stroke, especially regarding their immunomodulatory effects, to evaluate the role of Treg as a therapeutic weapon in ischemic stroke.

CONCLUSIONS

1. EXPERIMENTAL STUDY

1.1 Exogenous administration of in vitro expanded Treg decreases infarct volume and edema in an animal model of brain ischemia, both in acute phase and long-term period.

1.2 When Treg are administered after ischemia, these cells are present in infarcted brain.

1.3 Treg administration exerts an anti-inflammatory effect within the brain tissue, decreasing proinflammatory cytokines (IL-1β) and other markers of active inflammation (CD68 and CD11b).

1.4 Exogenous Treg administration does not impair neurogenesis and angiogenesis after brain ischemia in animal models.

1.5 The administration of CD28SA induces Treg stimulation in animal models.

1.6 The endogenous stimulation of Treg also decreases infarct volume and brain edema in animal models of ischemia.

1.7 Endogenous Treg stimulation does not increase Treg presence and does not modify proinflammatory markers in brain tissue after brain ischemia.

1.8 The administration of anti-CD25 antibodies does not achieve enough depletion of Treg in an animal model, and does not modify infarct volume or brain edema.

2. CLINICAL STUDY

2.1 Patients with ischemic stroke have higher Treg and IL-10 levels in peripheral blood compared with healthy controls.

2.2 Percentage of Treg and IL-10 levels increase during the first 3 days after acute ischemic stroke and decrease at day 7.

2.3 Higher percentages of Treg in acute phase of ischemic stroke in humans are associated with good functional outcome at 3 months.

2.4 High levels of Treg are associated with lower infarct volume in acute phase of ischemic stroke.

2.5 Higher percentages of Treg in acute phase of ischemic stroke reduce the risk of early neurological deterioration.

2.6 There is a correlation between percentage of Treg in acute phase of ischemic stroke and IL-10 serum levels.

2.7 Higher percentages of Treg in acute phase of ischemic stroke do not increase the risk of infection after ischemic stroke.

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APPENDIX

1. NATIONAL ISTITUTE OF HEALTH STROKE SCALE (NIHSS) [338]

1a. Level of Consciousness:.	0: Alert; keenly responsive. 1: Not alert; but arousable by minor stimulation to obey, answer, or respond. 2: Not alert; requires repeated stimulation to attend, or is obtunded and requires strong or painful stimulation to make movements (not stereotyped). 3:Responds only with reflex motor or autonomic effects or totally unresponsive, flaccid, and areflexic.
1b. LOC Questions: The patient is asked the month and his/her age.	0:Answers both questions correctly. 1:Answers one question correctly. 2: Answers neither question correctly
1c. LOC Commands: The patient is asked to open and close the eyes and then to grip and release the non-paretic hand. If the patient does not respond to command, the task should be demonstrated to him or her (pantomime).	0 = Performs both tasks correctly. 1 = Performs one task correctly. 2 = Performs neither task correctly.
2. Best Gaze: Only horizontal eye movements will be tested.	0 = Normal. 1 = Partial gaze palsy; gaze is abnormal in one or both eyes, but forced deviation or total gaze paresis is not present. 2 = Forced deviation, or total gaze paresis not overcome by the oculocephalic maneuver
3. Visual: Visual fields (upper and lower quadrants) are tested by confrontation, using finger counting or visual threat, as appropriate.	0 = No visual loss. 1 = Partial hemianopia. 2 = Complete hemianopia. 3 = Bilateral hemianopia (blind including cortical blindness).
4. Facial Palsy: Ask – or use pantomime to encourage – the patient to show teeth or raise eyebrows and close eyes. Score symmetry of grimace in response to noxious stimuli in the poorly responsive or non-comprehending patient.	0 = Normal symmetrical movements. 1 = Minor paralysis (flattened nasolabial fold, asymmetry on smiling). 2 = Partial paralysis (total or near-total paralysis of lower face). 3 = Complete paralysis of one or both sides (absence of facial movement in the upper and lower face).

5. Motor Arm: The limb is placed in the appropriate position: extend the arms (palms down) 90 degrees (if sitting) or 45 degrees (if supine). Drift is scored if the arm falls before 10 seconds. **5a. Left Arm 5b. Right Arm**	0 = No drift; limb holds 90 (or 45) degrees for full 10 seconds. 1 = Drift; limb holds 90 (or 45) degrees, but drifts down before full 10 seconds; does not hit bed or other support. 2 = Some effort against gravity; limb cannot get to or maintain (if cued) 90 (or 45) degrees, drifts down to bed, but has some effort against 3 = No effort against gravity; limb falls 4 = No movement UN = amputation or joint fusion
6. Motor Leg: The limb is placed in the appropriate position: hold the leg at 30 degrees (always tested supine). Drift is scored if the leg falls before 5 seconds. **6a. Left Leg** **6b. Right Leg**	0 = No drift; leg holds 30-degree position for full 5 seconds 1 = Drift; leg falls by the end of the 5-second period but does not hit bed. 2 = Some effort against gravity; leg falls to bed by 5 seconds, but has some effort against gravity. 3 = No effort against gravity; leg falls to bed immediately. 4 = No movement. UN = Amputation or joint fusion
7. Limb Ataxia: This item is aimed at finding evidence of a unilateral cerebellar lesion. Test with eyes open.	0 = Absent. 1 = Present in one limb. 2 = Present in two limbs. UN = Amputation or joint fusion
8. Sensory: Sensation or grimace to pinprick when tested, or withdrawal from noxious stimulus in the obtunded or aphasic patient.	0 = Normal; no sensory loss. 1 = Mild-to-moderate sensory loss; patient feels pinprick is less sharp or is dull on the affected side; or there is a loss of superficial pain with pinprick, but patient is aware of being touched. 2 = Severe to total sensory loss; patient is not aware of being touched in the face, arm, and leg.

9. Best Language: A great deal of information about comprehension will be obtained during the preceding sections of the examination. For this scale item, the patient is asked to describe what is happening in the attached picture, to name the items on the attached naming sheet and to read from the attached list of sentences..	0 = No aphasia; normal. 1 = Mild-to-moderate aphasia; some obvious loss of fluency or facility of comprehension, without significant limitation on ideas expressed or form of expression. 2 = Severe aphasia; all communication is through fragmentary expression; great need for inference, questioning, and guessing by the listener. 3 = Mute, global aphasia, no usable speech or auditory comprehension
10. Dysarthria: If patient is thought to be normal, an adequate sample of speech must be obtained by asking patient to read or repeat words from the attached list.	0 = Normal. 1 = Mild-to-moderate dysarthria; patient slurs at least some words and, at worst, can be understood with some difficulty. 2 = Severe dysarthria; patient's speech is so slurred as to be unintelligible in the absence of or out of proportion to any dysphasia, or is mute/anarthric. UN = Intubated or other physical barrier
11. Extinction and Inattention (formerly Neglect):	0 = No abnormality. 1 = Visual, tactile, auditory, spatial, or personal inattention or extinction to bilateral simultaneous stimulation in one of the sensory modalities. 2 = Profound hemi-inattention or extinction to more than one modality; does not recognize own hand or orients to only one side of space.

2. RESUMEN

Las enfermedades cerebrovasculares son consecuencia de una alteración en la circulación cerebral que ocasiona un déficit transitorio o definitivo del funcionamiento de una o varias partes del encéfalo. Constituyen una causa importante de mortalidad y morbilidad. La oclusión de una arteria cerebral, produce una reducción del flujo sanguíneo del área de irrigación correspondiente (isquemia cerebral), que es más grave en el foco isquémico que en la periferia, donde se establece la denominada área de penumbra. El impacto de la isquemia cerebral depende de la duración y de la reducción del flujo sanguíneo.

Durante la isquemia cerebral, se desencadenan una secuencia de mecanismos moleculares a corto y a largo plazo que producen una alteración energética, la despolarización de la membrana y un aumento de los niveles de glutamato. Muchos de estos mecanismos desembocan en la muerte neuronal, que puede ser necrótica o apoptótica. La muerte celular tras la isquemia cerebral desencadena una respuesta inflamatoria muy intensa que incrementa el daño cerebral y que se caracteriza por la acumulación de células (leucocitos procedentes de la sangre periférica y de la microglía residente) y de mediadores inflamatorios (citoquinas, quimioquinas, metabolitos del ácido araquidónico, especies reactivas de oxígeno…). Diversas evidencias muestran que la inflamación y la respuesta inmune juegan un importante papel en el pronóstico de los pacientes que han sufrido un ictus. Por este motivo, el estudio del proceso inflamatorio que se produce en el ictus isquémico puede conducir al descubrimiento de nuevas dianas terapéuticas anti-inflamatorias a través de la manipulación del sistema inmune.

Los linfocitos T reguladores son un subtipo de linfocitos T CD4+ que desempeñan un importante papel en el control y en la regulación del sistema inmune a través de su efecto inmunosupresor, inhibiendo la función de los linfocitos T efectores. Actúan por múltiples mecanismos, como la producción de citoquinas anti-inflamatorias (IL-10, TGFβ o IL-35), la eliminación directa de células efectoras por apoptosis mediante granzimas y perforinas, la alteración metabólica de las células T efectoras o la inhibición de la función y maduración de células presentadoras de antígenos. Se ha demostrado que los linfocitos T reguladores están implicados en diversas enfermedades como la isquemia renal, la esclerosis múltiple, la enfermedad de Parkinson, el infarto de miocardio o en el desarrollo de la aterosclerosis.

Aunque el efecto deletéreo de los linfocitos T en los estadíos iniciales del ictus isquémico está bien establecido, la función específica de determinadas subpoblaciones, como la de los linfocitos T reguladores, es controvertida. Existen múltiples estudios sobre la función de los linfocitos T reguladores tras la isquemia cerebral en modelos animales, que valoran el efecto de la depleción y del aumento de estas células en la evolución del ictus isquémico. Para producir la depleción se emplean fundamentalmente dos métodos: mediante anticuerpos anti-CD25 y, utilizando ratones transgénicos con un receptor de toxina diftérica, en los que se puede inducir la depleción de estas células. En la mayoría de estos estudios se ha demostrado un efecto negativo como consecuencia de la depleción de linfocitos T reguladores, en cambio en otros se ha puesto de manifiesto un efecto

positivo y, por lo tanto, un potencial efecto deletéreo de estas células tras la isquemia cerebral.

Para evaluar el efecto del incremento de linfocitos T reguladores tras la inducción de una isquemia cerebral en animales, se utilizan tres métodos para aumentar estas células : la administración de células T reguladoras exógenas; el tratamiento con superagonistas anti-CD28, que inducen la expansión endógena de linfocitos T reguladores y la inhibición de la histona deacetilasa y posterior tratamiento con un inhibidor de la rapamicina. Tambien se han obtenido resultados contradictorios en los estudios en los que se ha llevado a cabo un aumento de linfocitos T reguladores. Sin embargo, en la gran mayoría de ellos, se obtiene un efecto positivo tras el aumento de los mismos, por lo que se deduce que estos linfocitos podría constituir una población celular sobre la que potencialmente se podría actuar para modular la respuesta inflamatoria tras el ictus isquémico. Se han propuesto varios mecanismos para explicar el efecto de los linfocitos T reguladores tras el ictus. El principal y el más estudiado, es la producción de citoquinas anti-inflamatorias, sobre todo la IL-10 y el TGFβ. Tambien se ha destacado la importancia de la vía de PD-1 y PD-L1 y/o la disminución de niveles de MMP-9.

Sin embargo, algunos estudios proponen un efecto para los linfocitos T reguladores, independiente de los mecanismos anti-inflamatorios, que se basa en la inducción de microtrombosis en el tejido cerebral isquémico, con el consiguiente efecto negativo local. Por otra parte, y debido al papel inmunosupresor de los linfocitos T reguladores, algunos autores plantean que estas células pueden afectar

a la inmunosupresión que se produce tras el ictus, aumentando el riesgo de infecciones, lo que podría limitar su potencial terapéutico.

Como se puede observar, existen múltiples estudios que evalúan el papel de los linfocitos T reguladores tras la inducción de la isquemia cerebral en animales de experimentación, en cambio son escasos los estudios que valoran su función en pacientes con ictus isquémico. Recientemente, un estudio prospectivo en pacientes, muestra que bajos niveles de linfocitos T reguladores se asocian a un aumento del riesgo de infarto de miocardio, pero no se ha encontrado una relación entre los niveles de estos linfocitos y el riesgo de ictus isquémico. Algunos autores han detectado un aumento del porcentaje de linfocitos T reguladores en pacientes tras un ictus isquémico en comparación con pacientes sanos, que se podría relacionar con un efecto beneficioso de estas células. Sin embargo el tema sigue siendo objeto de controversia, entre otras razones, porque son pocos los estudios realizados y además su tamaño muestral es pequeño y las características de los pacientes incluidos son heterogéneas. Asimismo, no se ha demostrado la existencia de una asociación entre los niveles de linfocitos T reguladores y el pronóstico a largo plazo del ictus isquémico ni con la aparición de infecciones en la fase aguda.

En este estudio nos planteamos como hipótesis que tanto la administración exógena como la estimulación endógena de linfocitos T reguladores, disminuyen el volumen del infarto en un modelo animal de isquemia cerebral transitoria y que su depleción desempeña un papel negative, aumentando el tamaño del infarto. Asimismo, se postula que el efecto de los linfocitos T reguladores tras la isquemia

cerebral, es producido por un mecanismo dual, disminuyendo la respuesta inflamatoria y aumentando los procesos de neurogénesis y angiogénesis. Por otro lado, también se propone que, en pacientes con ictus isquémico, los niveles más elevados de linfocitos T reguladores en la fase aguda se asocian con un mejor pronóstico funcional a largo plazo, con infartos de tamaño más pequeño, con un menor grado de deterioro neurológico precoz y con menor respuesta inflamatoria, determinada por niveles más elevados de la citoquina anti-inflamatoria (IL-10) y que además, un elevado número de células T reguladoras en la fase aguda del ictus no se asocia con una incidencia mayor de complicaciones infecciosas.

En este trabajo hemos realizado un estudio experimental en animales y un estudio en pacientes con ictus isquémico en fase aguda. Con animales de experimentación, se han llevado a cabo cuatro subestudios. En el primero, hemos analizado el efecto de la administración exógena de células T reguladoras en un modelo animal de isquemia cerebral. Se ha analizado el efecto de este tratamiento sobre el tamaño del infarto y el edema cerebral, demostrando una disminución del tamaño del infarto y del edema en animales que han recibido tratamiento exógeno con linfocitos T reguladores. Para analizar su mecanismo de acción y evaluar su efecto anti-inflamatorio, se ha analizado el efecto de la administración de estas células en: la expresión cerebral de FoxP3 (un marcador específico de linfocitos T reguladores), el nivel de citoquinas proinflamatorias (IL-1β y TNFα) y de marcadores de la inflamación activa (CD11B y CD68) a nivel cerebral. Se ha comprobado que se produce un aumento de los niveles de FoxP3 en el tejido cerebral de los animales tratados respecto a los

controles y una disminución de citoquinas proinflamatorias (IL-1β) y otros marcadores de la inflamación, demostrando la presencia de estas células a nivel cerebral y un efecto anti-inflamatorio local tras la administración de los linfocitos T reguladores. En el segundo experimento se han administrado superagonistas anti-CD28, de los que se ha demostrado, en estudios previos, que producen una expansión endógena de linfocitos T reguladores. En nuestro estudio, se ha demostrado que el tratamiento con CD28SA aumenta los niveles periféricos de linfocitos T reguladores. Se ha analizado el efecto de este tratamiento en el tamaño del infarto y del edema cerebral y sobre los niveles cerebrales de FoxP3 e IL-1β. Se ha demostrado que en los animales tratados, disminuye el tamaño del infarto cerebral y del edema, pero no presentan niveles más elevados de FoxP3 ni disminución de IL-1β a nivel cerebral. En el tercer experimento, se ha llevado a cabo un tratamiento con anti-CD25, que ha demostrado en estudios previos inducir la depleción de linfocitos T reguladores y se ha analizado su efecto sobre el tamaño del infarto, el edema cerebral y sobre los niveles cerebrales de FoxP3 e IL-1β. En nuestro estudio, la administración de anti-CD25 sólo ha conseguido una depleción parcial de linfocitos T reguladores en sangre periférica por lo que no se han observado cambios en el tamaño del infarto ni en el edema, ni tampoco variaciones en los niveles de FoxP3 e IL-1β a nivel cerebral. En el último experimento, se han analizado los efectos de la administración exógena de linfocitos T reguladores en el tamaño del infarto cerebral a largo plazo y en los marcadores de neurogénesis (NeuN y NCAM) y de angiogénesis (CD31). El efecto positivo en cuanto a la reducción del infarto cerebral tras la administración de linfocitos T reguladores se

mantiene a largo plazo, por lo que se propone un efecto neuroprotector de estas células. Además, hemos encontrado que la administración exógena de linfocitos T reguladores no modifica los procesos de neurogénesis ni de angiogénesis.

Por otro lado, hemos llevado a cabo un estudio clínico en el que se han incluido 204 pacientes con ictus isquémico de menos de 12 horas de evolución. En estos pacientes se han analizado los niveles de linfocitos T reguladores y de IL-10 en el momento del ingreso, a las 48, a las 72 horas y a los 7 días tras el ictus. En primer lugar, se han comparado los niveles de linfocitos T reguladores e IL-10 de estos pacientes con los de controles sanos, comprobando que los pacientes con ictus isquémico presentan, en la fase aguda, niveles más elevados tanto de linfocitos T reguladores como de IL-10 que los controles. Tambien se ha establecido un perfil temporal de ambos parámetros, comprobando que tanto los niveles de linfocitos T reguladores como los de IL-10 aumentan los tres primeros días tras el ictus y disminuyen al séptimo día. Posteriormente, se han tratado de relacionar los niveles de linfocitos T reguladores con el pronóstico funcional de los pacientes a largo plazo, con el tamaño del infarto y con el desarrollo de deterioro neurológico precoz. Un buen pronóstico funcional tres meses después del ictus isquémico se ha relacionado con un aumento de los linfocitos T reguladores a las 72 horas. Asimismo, niveles más elevados de linfocitos T reguladores a las 48 y 72 horas se han relacionado con un menor tamaño del infarto cerebral y un riesgo más bajo de deterioro neurológico precoz. Dado que el aumento de IL-10 se ha propuesto como uno de los principales mecanismos responsable del efecto beneficioso de los linfocitos T reguladores, se han correlacionado los

niveles de esta citoquina en sangre periférica con los de linfocitos T reguladores, comprobándose que los niveles de IL-10 muestran asociación con los niveles de linfocitos T reguladores en la fase aguda del ictus. Por último, se ha evaluado la relación entre los niveles de linfocitos T reguladores y el riesgo de infección tras el ictus isquémico, y no se han encontrado niveles más elevados de linfocitos T reguladores en pacientes que desarrollaron una infección respecto a los que no la desarrollaron. Estos datos muestran, en nuestro estudio, que niveles más elevados de linfocitos T reguladores no se asocian a un mayor riesgo de infección tras el ictus.

En resumen, en modelos animales de isquemia cerebral tanto la administración exógena como la estimulación endógena de linfocitos T reguladores disminuyen el tamaño del infarto cerebral y el edema, sugiriendo un efecto neuroprotector de estas células. Además, tras su administración exógena se ha detectado un aumento de las mismas y un efecto anti-inflamatorio a nivel cerebral. La administración de linfocitos T reguladores no tiene efectos en la neurogénesis o en la angiogénesis en modelos de isquemia cerebral. En pacientes, los niveles de linfocitos T reguladores aumentan en la fase aguda del ictus isquémico, sobre todo en los tres primeros días. Niveles más elevados de linfocitos T reguladores se asocian con un mejor pronóstico a largo plazo, menor volumen de infarto y menor deterioro neurológico precoz y no se ha detectado una asociación con el aumento de infecciones tras el ictus. Asimismo, los niveles de linfocitos T reguladores se correlacionan con los niveles de IL-10 en fase aguda.

En base a nuestros resultados y a estudios previos, los linfocitos T reguladores constituyen un nuevo abordaje terapéutico en el tratamiento del ictus agudo, disminuyendo la respuesta inflamatoria que se produce tras la isquemia, sin que se haya observado un aumento de complicaciones infecciosas con niveles elevados de estas células.

En conclusión, el presente estudio demuestra, a nivel de un modelo animal de isquemia cerebral, que:

1. La administración exógena de linfocitos T reguladores disminuye el volumen del infarto y el edema en modelos animales de isquemia cerebral, tanto a corto como a largo plazo.

2. Cuando los linfocitos T reguladores se administran tras la isquemia, estas células están presentes en el tejido cerebral.

3. La administración exógena de linfocitos T reguladores produce un efecto anti-inflamatorio en el tejido cerebral, disminuyendo los niveles de citoquinas proinflamatorias (IL-1β) y otros marcadores de inflamación activa (CD68 y CD11b).

4. La administración exógena de linfocitos T reguladores no afecta a la neurogénesis ni a la angiogénesis en modelos animales de isquemia cerebral.

5. La administración de superagonistas de CD28 induce la estimulación de linfocitos T reguladores en modelos animales.

6. La estimulación endógena de linfocitos T reguladores disminuye el volumen de infarto y el edema cerebral en modelos animales de isquemia.

7. La estimulación endógena de linfocitos T reguladores no aumenta la presencia de estas células ni modifica marcadores proinflamatorios en el tejido cerebral tras la isquemia.

8. La administración de anticuerpos anti-CD25 no produce una depleción completa de linfocitos T reguladores en modelos animales y su administración no modifica el tamaño del infarto cerebral ni del edema.

En el estudio clínico, concluimos que:

1. Pacientes con ictus isquémico presentan niveles más elevados de linfocitos T reguladores e IL-10 en sangre periférica comparados con controles sanos.

2. Los porcentajes de linfocitos T reguladores y los niveles de IL-10 aumentan durante los primeros 3 días tras el ictus isquémico y disminuyen al séptimo día.

3. Porcentajes más elevados de linfocitos T reguladores en la fase aguda del ictus isquémico se asocian con mejor pronóstico funcional a los 3 meses.

4. Niveles más elevados de linfocitos T reguladores en fase aguda se asocian con volúmenes de infarto más pequeños.

5. Niveles más elevados de linfocitos T reguladores en fase aguda se asocian con menor riesgo de deterioro neurológico precoz.

6. Existe una correlación entre el porcentaje de linfocitos T reguladores en la fase aguda de ictus isquémico y los niveles séricos de IL-10.

7. Niveles más elevados de linfocitos T reguladores en fase aguda del ictus isquémico no se relacionan con un aumento del riesgo de infecciones tras el ictus agudo.