Role of regulatory T-cells on ischemic stroke outcome: new clinical evidences

María Santamaría-Cadavid

Complejo Hospitalario Universitario de Santiago de Compostela

Emilio Rodríguez-Castro

Complejo Hospitalario Universitario de Santiago de Compostela

Manuel Rodríguez-Yáñez

Complejo Hospitalario Universitario de Santiago de Compostela

Susana Arias-Rivas

Hospital Clinico Universitario de Santiago de Compostela

Iria López-Dequidt

Hospital Clinico Universitario de Santiago de Compostela

María Pérez-Mato

Complejo Hospitalario Universitario de Santiago de Compostela

Manuel Rodríguez-Pérez

Complejo Hospitalario Universitario de Santiago de Compostela

Ignacio López-Loureiro

Complejo Hospitalario Universitario de Santiago de Compostela

Pablo Hervella

Complejo Hospitalario Universitario de Santiago de Compostela

Francisco Campos

Complejo Hospitalario Universitario de Santiago de Compostela

José Castillo

Complejo Hospitalario Universitario de Santiago de Compostela

Ramón Iglesias-Rey (✉ ramon.iglesias.rey@sergas.es)

Instituto de Investigacion Sanitaria de Santigao de Compostela <https://orcid.org/0000-0002-9985-9135>

Tomás Sobrino

Complejo Hospitalario Universitario de Santiago de Compostela

Research article

Keywords: Early Neurological Deterioration; Interleukin-10; Ischemic stroke; Neuroinflammation; Regulatory T-cells; Risk factors

Posted Date: October 23rd, 2019

DOI: <https://doi.org/10.21203/rs.2.16371/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at BMC Neurology on February 28th, 2020.
See the published version at <https://doi.org/10.1186/s12883-020-01648-w>.

Abstract

Background: Recent preclinical studies have shown that regulatory T (Treg) cells play a key role in the immune response after ischemic stroke (IS). However, the role of Treg-cells in human acute IS has been poorly investigated. Our aim was to study the relationship between circulating Treg-cells and outcome in human IS patients. **Methods** Methods: A total of 204 IS patients and 22 control subjects were recruited. The main study variable was good functional outcome at 3 months (modified Rankin scale ≤ 2) considering infarct volume, Early Neurological Deterioration (END) and risk of infections as secondary variables. The percentage of circulating Treg-cells was measured at admission, 48, 72h and at day 7 after stroke onset. **Results** Results: Circulating Treg-cell levels were higher in IS patients compared to control subjects. Treg-cells at 48h were independently associated with good functional outcome (OR, 3.5; CI: 1.9-7.8) after adjusting by confounding factors. Patients with lower Treg-cells at 48h showed higher frequency of END and risk of infections. In addition, a negative correlation was found between circulating Treg-cells at 48h ($r=-0.414$) and 72h ($r=-0.418$) and infarct volume. **Conclusions:** These findings suggest that Treg-cells may participate in the recovery of IS patients. Therefore, Treg-cells may be considered a potential therapeutic target in acute ischemic stroke.

Background

Stroke represents the second cause of death in Europe and developed countries. In addition to mortality, long-term morbidity remains as a significant problem since many stroke patients are dependent to carry out daily activities [1,2]. Although stroke causes enormous medical and economic burdens on society, thrombolysis with recombinant plasminogen activator (rtPA) remains as the only approved pharmacological treatment for ischemic stroke (IS). However, there is a narrow therapeutic window for the use of rtPA treatment (<4.5 h) due to side effects such as hemorrhagic transformation or treatment with previous anticoagulants [3]. Because of these limitations, rtPA is only available for a small percentage of IS patients in industrialized countries, being this situation worst in developing countries. Therefore, new and effective therapies are highly demanded in clinical practice.

Stroke triggers an acute immunological and inflammatory response in the brain that participates actively in the evolution of ischemic damage. In the last years, the interest in the role of inflammation in stroke pathogenesis has significantly increased, becoming an important target for future therapeutic drugs. In this regard, it is well known that decrease of blood flow in a brain area causes neuronal necrosis and leads to an immune response and to the invasion of inflammatory cells within the ischemic tissue, which mediates secondary brain injury [4-6]. However, although immune response contributes to brain tissue damage, therapeutic strategies based on immunosuppression have failed in clinical trials [7].

Alternatively to the use of immunosuppressant drugs, recently other mechanisms involved in the control and regulation of inflammatory response have been proposed in order to prevent brain damage after stroke. In this regard, regulatory T (Treg) cells are a subgroup of CD4 T lymphocytes that play an important role in maintaining immune homeostasis, preventing autoimmunity and inflammation. Due to

their immunomodulatory function, it has been proposed that Treg-cells may play an important role in the pathophysiology of IS [8].

Several preclinical studies have tested the therapeutic role of Treg-cells in cerebral ischemia, finding that their depletion causes larger infarct volumes [9], while their exogenous administration mediates a protective effect [10]. However, few clinical studies have investigated the role of Treg-cells in IS patients [11-16]. These studies showed controversial results and included a small number and a heterogeneous group of patients. Moreover, the relationship between circulating Treg levels with infarct volume, patient's outcome or early neurological deterioration (END) is not yet well established. In addition, no studies have investigated the temporal profile of Treg-cells during acute phase of IS. Similarly, there is no data about the association of Treg-cells and interleukin-10 (IL-10), a cytokine with anti-inflammatory properties, which is considered as the main effector mechanisms of Treg-cells. These data would help to establish the potential use of Treg-cells as a therapeutic target able to improve functional outcome in IS patients.

In this clinical study, we have analyzed if higher circulating levels of Treg-cells are associated with better functional outcome in IS patients. Likewise, if higher levels of these cells determine smaller infarct volumes and less frequency of END has been also analyzed. Finally, we have studied the correlation between circulating Treg-cells and serum levels of IL-10.

Methods

Patient's Characteristics

Between April 2013-July 2014, consecutive ischemic stroke patients within 12 hours from symptoms onset were prospectively evaluated to be included in the study. A cohort of control subjects matched by gender and age was included.

Inclusion criteria were: hospitalized patients with first-episode of IS within 12 hours from symptoms onset; age >18 years; previously independent for their daily living activities (modified Rankin Scale (mRS) ≤ 1).

Exclusion criteria were: presence of intracerebral hemorrhage confirmed by neuroimaging; previous IS; cancer or severe systemic disease that determine a life expectancy lower than 6 months; infections during the last 30 days before admission; chronic inflammatory disease; pregnancy; renal replacement therapy; treatment with steroids, immunosuppressive and immunomodulatory drugs or antibiotics during the last 30 days before admission; periodontal disease; and fever in the previous 72h (axillary temperature over 38°C). Patients with active infection (axillary temperature > 37.5°C and leukocyte levels >15000/ μ L or <4000/ μ L), cough and spitting, voiding dysfunction, diarrhea and clinical signs of endocarditis or meningitis.

On the other hand, a cohort of subjects without any neurological, inflammatory or infectious disease was included as control group. The selection of these control subjects was made by inviting the patient's

relatives to participate in the study. Control subjects were matched to patients by gender and age.

Clinical variables and Neuroimaging studies

All patients were admitted in the Stroke Unit of University Clinical Hospital of Santiago de Compostela and treated according to the guidelines of the Cerebrovascular Diseases Study Group of the Spanish Society of Neurology [17]. Medical history recording demographic data, potential vascular risk factors, blood counts, biochemistry and coagulation tests, 12-lead ECG, chest radiography, carotid and transcranial ultrasonography and Computed Tomography (CT) or Magnetic Resonance Imaging (MRI) were performed at admission.

To evaluate neurologic deficit, the National Institute of Health Stroke Scale (NIHSS) was performed at admission, 24, 48 and 72 hours, at discharge, and at 3 months. END was defined as an increase of 4 points or more in NIHSS assessment between baseline and any other NIHSS evaluation during the first 72 hours. Functional outcome was evaluated at discharge and at 3 months by mRS. NIHSS and mRS were evaluated by internationally certified neurologists. Stroke etiology was classified according to TOAST criteria [18].

We evaluated the incidence of any infection during the hospitalization period. A protocol has been implemented in order to evaluate the presence of infections during the acute phase of stroke. The following tests were performed in those patients who showed an axillar temperature $>37.5^{\circ}\text{C}$ in two different determinations separated by 1 hour, or one axillar temperature determination $>38^{\circ}\text{C}$: blood counts, biochemistry analysis and blood culture; physicians made a clinical suspicion regarding the infection origin, and if it was possible. During the etiological examination of the infection origin, empiric antibiotherapy was started according to clinical suspicion. Once antibiogram was obtained, specific antibiotic treatment was started in case of positive cultures.

To evaluate infarct volume, a control CT was performed between 4th-7th days after IS. Infarct volume was quantified in cubic centimeters (cm^3) and was assessed according to the formula $0.5 \times A \times B \times C$, where A and B correspond to higher diameters in perpendicular direction and C to the number of 10 mm slices where infarct volume is present [19]. All neuroimaging evaluations were made by the same neuroradiologist blinded to clinical and laboratory data.

Quantification of Treg-cells

Circulating levels of Treg-cells were measured by flow cytometry according to methods and using the markers described elsewhere [20-22]. Previously to patient's inclusion, we selected 20 IS patients who matched inclusion/exclusion criteria to evaluate the optimal temporal profile for the quantification of Treg-cells during the acute phase of IS. Blood samples were collected with an evacuated tube system (Vacutainer) in EDTA tubes at baseline, 24, 48, 72 hours and at days 4, 5 and 7. According to this temporal profile, later we obtained blood samples in those more relevant time-points for Treg-cell evaluation (admission, 48 and 72 hours and day 7).

Blood samples were processed within 3 hours after collection by a single researcher who had no knowledge of the patients' clinical, biochemical or radiological results. Circulating Treg-cells were analyzed for the expression of specific surface antigens with direct flow cytometry (BD FACS Aria IIu, BD, Franklin Lakes, NJ, USA). In brief, 50 µL of peripheral blood were labelled with 10 µL of FITC-conjugated anti-CD4 (BD, Franklin Lakes, NJ, USA), 10 µL of PE-conjugated anti-CD25 (BD, Franklin Lakes, NJ, USA), and 10 µL of Alexa Fluor® 647-conjugated anti-CD127 (BD, Franklin Lakes, NJ, USA) monoclonal antibodies. We considered Treg as CD4+/CD25+/CD127- staining cells in the lymphocyte gate. In all analyses, 2.5×10^5 events were acquired, scored using a FACS Aria IIu analyzer (BD, Franklin Lakes, NJ, USA), and processed using the PC FACSDiva software program (BD, Franklin Lakes, NJ, USA). Treg-cell count was expressed as percentage of Treg-cells over total analyzed lymphocytes.

IL-10 determination

Blood samples, drawn from all patients at admission, and at 24 ± 6 , 48 ± 12 , and 72 ± 12 hours, were collected in glass chemistry test tubes, centrifuged at 3000 rpm during 10 minutes, and immediately frozen and stored at -80°C . Serum levels of IL-10 were measured using an immunodiagnostic IMMULITE 1000 System (Siemens Healthcare España, Madrid, Spain). Determinations were performed in an independent laboratory blinded to clinical and neuroimaging data.

Outcome Variables

The primary endpoint was good functional outcome ($\text{mRS} \leq 2$) at 3 months. Infarct volume and the presence of END were evaluated as secondary outcome variables. The development of infections during hospitalization was recorded as safety variable. Finally, we analyzed the correlation between circulating Treg-cells and serum levels of IL-10 in order to investigate the possible mechanism of action of Treg-cells.

Statistical analysis

Sample size was calculated using the statistical EPIDAT 3.1 software (http://www.sergas.es/MostrarContidos_N3_T01.aspx?IdPaxina=62714), considering that those patients within the highest quartile regarding Treg-cell levels during the first week after stroke achieve a 25% more frequency of good outcome at 3 months compared with those with Treg-cell levels in the lowest quartile. The minimum calculated sample size was 172 patients in order to obtain a statistical power of 80% with a significant difference level of 0.05.

Results were expressed as percentages for categorical variables and as mean (SD) or median and range (25th and 75th percentiles) for the continuous variables depending on whether their distribution was normal or not. The Kolmogorov-Smirnov test was used for testing the normality of the distribution. Proportions were compared using the chi-square or Fisher test, while the continuous variables between groups were compared with the Student's t or the Mann-Whitney tests depending on whether their distribution was normal or not, respectively. In case of more than 3 groups, variables were compared

using ANOVA test. Bivariate correlations were performed using Pearson's (normally distributed variables) or Spearman (variables without normal distribution) coefficients.

ROC curves were used to establish the best cut-off point for Treg-cell levels that optimally predicted good functional outcome.

The independent association of circulating Treg-cell levels with good functional outcome at 3 months and the risk of infections was assessed by logistic regression analysis; while their independent influence on infarct volume was assessed by multiple linear regression models. Each logistic regression analysis or multivariable linear regression model was adjusted for those significant variables in the bivariate analysis. Residual plots were examined to detect potential non-linear relationships between the outcome variable and continuous independent variables. Results were expressed as adjusted odds ratios (ORs) or Beta estimate with the corresponding 95% confidence intervals (95% CI). A p-value <0.05 was considered to be statistically significant in all tests. The statistical analysis was conducted in SPSS 20.0 (IBM, Chicago, IL, USA) for Mac.

Results

Twenty-two control subjects were included. No differences were found between controls and IS patients regarding age, sex, previous history of hypertension, diabetes, dyslipidemia, coronary disease, peripheral artery disease, and alcohol or tobacco consumption. IS patients showed more prevalence of atrial fibrillation than control subjects (42.2 vs. 0%, $p<0.0001$).

On the other hand, 335 IS patients admitted in the Stroke Unit within the first 12 hours from stroke onset were consecutively evaluated to include in the study. 204 patients who fulfilled inclusion criteria and did not fulfill any exclusion criteria were included. 103 patients (50.5%) were males. Mean age was 71.7 ± 10.6 years. The NIHSS score at admission was 8 [4, 12] and infarct volume was $50.8\pm88.4\text{ cm}^3$. Regarding stroke etiology, we found 93-cardioembolic (45.6%), 26-atherothrombotic (12.7%), 7-lacunar (3.4%) and 78-undetermined (38.2%).

Circulating levels of Treg-cells (0.0222 ± 0.0177 vs. $0.0013\pm0.0009\%$; $p<0.0001$) as well as IL-10 serum levels (6.9 ± 1.7 vs. $1.8\pm0.1\text{ pg/mL}$; $p<0.0001$) at admission were higher in IS patients than in control subjects.

The temporal pattern of Treg-cell and IL-10 levels are shown in **Figure 1**. We found that circulating Treg-cells were significantly higher at 48, 72 hours and day 7 in relation to the baseline measurement. Based on these results, we evaluated this temporal profile in the complete cohort of IS patients included in the study.

Primary endpoint: influence of Treg-cells on functional outcome

Patients with lower mRS scores at 3 months showed higher levels of Treg-cells at 48, 72 hours as well as at day 7, but not at admission (**Figure 2**). **Table 1** shows the baseline clinical characteristics, vascular risk factors, stroke subtype, biochemical/cellular parameters and Treg-cell levels of patients with good (n=87; 36.2%) and poor outcome (n=117; 63.8%) at 3 months. We found that patients with good outcome had higher levels of Treg-cells at 48 hours ($p<0.0001$), 72 hours ($p<0.0001$) and 7 days ($p=0.001$), but not at admission ($p=0.962$).

ROC analysis showed that Treg-cell levels at 48 hours $\geq 0.0550\%$ predicted good functional outcome 3 months with a specificity of 97% and a sensitivity of 95% (area under the curve: 0.990; 95% CI: 0.997-1.000; $p<0.0001$). Similarly, Treg-cell levels at 72 hours $\geq 0.0650\%$ predicted good outcome at 3 months with a specificity of 95% and a sensitivity of 93% (area under the curve: 0.964; 95% CI: 0.886-1.000; $p<0.0001$).

In the logistic regression analysis, Treg-cell levels at 48 hours were independently associated with good functional outcome at 3 months (OR 3.5; 95% CI: 1.9-7.8; $p<0.0001$) after adjustment by age, previous history of hypertension, dyslipemia, atrial fibrillation, leukocyte counts, glucose and fibrinogen levels, high-sensitive C-reactive protein levels, basal NIHSS and cardioembolic stroke. Treg-cell levels at 72 hours were independently associated with good functional outcome at 3 months (OR 1.7; 95% CI: 1.1-3.1; $p=0.016$) after adjustment by the same variables.

Early neurological deterioration (END)

END was observed in 13 patients (6.4%). Circulating Treg-cell levels at 48 hours ($0.0132\pm 0.0125\%$ vs. $0.0411\pm 0.026\%$; $p<0.0001$) and 72 hours ($0.0096\pm 0.0061\%$ vs. $0.0453\pm 0.0234\%$; $p<0.0001$) were lower in patients who suffered END. Due to the small number of patients with END it was not possible to perform a logistic regression analysis.

Infarct volume

Infarct volume was measured in 195 patients. We found a negative correlation between infarct volume and circulating levels of Treg-cells at 48 hours ($r= -0.414$; $p<0.0001$) and 72 hours ($r= -0.418$; $p<0.0001$). No correlation has been found between infarct volume and Treg-cell levels at baseline and at day 7.

In the multivariate analysis, Treg-cell levels at 72 hours (B: -648.9; 95% CI: -1251.2 to -46.8; $p=0.035$), but not at 48 hours (B: -545.4; 95% CI: -1036.5 to 291.6; $p=0.199$) were independently associated with infarct volume after adjustment by age, previous history of hypertension, dyslipemia, atrial fibrillation, leukocyte counts, glucose and fibrinogen levels, high-sensitive C-reactive protein levels, basal NIHSS and cardioembolic stroke.

Risk of infections

Twenty-six patients (12.7%) developed infections during the hospitalization period: 17 (65.4%) had respiratory infections, 6 (23.1%) urinary infections and in 3 patients (11.5%) the origin was unknown.

Infection complications during hospitalization were associated with higher temperature at 24 hours ($38.1 \pm 0.3^\circ\text{C}$ vs. $36.6 \pm 0.5^\circ\text{C}$; $p < 0.0001$) and at 48 hours ($37.9 \pm 0.5^\circ\text{C}$ vs. $36.7 \pm 0.5^\circ\text{C}$; $p < 0.0001$), and with greater neurological deficit at admission (NIHSS 14 [11, 20] vs. 9 [5, 18]; $p = 0.033$). The presence of infections during the hospitalization was associated with poor functional outcome at 3 months; patients with infection showed higher scores of mRS at 3 months (5 [4, 6] vs. 3 [1, 4]; $p < 0.0001$).

Circulating Treg-cell levels at 48 hours were lower in patients with infections ($0.0189 \pm 0.009\%$ vs. $0.0425 \pm 0.0280\%$; $p < 0.0001$). Similar results were found for Treg-cell levels at 72 hours ($0.0168 \pm 0.0105\%$ vs. $0.0473 \pm 0.0238\%$; $p < 0.0001$).

In the logistic regression analysis, lower Treg-cell levels at 48 hours (OR: 0.35; 95% CI: 0.00-0.57; $p = 0.001$) and 72 hours (OR: 0.24; 95% CI: 0.02-0.51; $p < 0.0001$) were independently associated with infections during hospitalization after adjusting by age, previous history of hypertension, dyslipemia, atrial fibrillation, leukocyte counts, glucose and fibrinogen levels, high-sensitive C-reactive protein levels, basal NIHSS and cardioembolic stroke.

Circulating Treg-cells and serum levels of IL-10

We found a positive correlation between Treg-cell and IL-10 levels for the 4 time points analyzed. However, this association was stronger the later after stroke during the first 7 days (**Figure 3**).

Discussion

This study evaluated the relationship between circulating levels of Treg-cells (defined as CD4+/CD25+/CD127) and brain injury in IS patients. Circulating Treg-cell levels at 48 and 72 hours were independently associated with good functional outcome at 3 months. This favourable effect on the primary endpoint was supported by positive effects on infarct volume, END and reduction of infections during hospitalization.

Results showed higher levels of IL-10 in patients with ischemic stroke. A previous study comparing IL-10 serum levels between stroke patients and healthy population [23] has reported contrary results, finding lower levels of IL-10 in stroke patients compared to controls. Our results suggest that levels of Treg-cells and IL-10 increase during the acute phase, and could exert a pathophysiological role in IS.

Previous studies in animal models of cerebral ischemia showed an increase of Treg-cell infiltration brain tissue at days 14 and 30 after MCAO [24]. In our study we used peripheral blood samples to determinate the temporal pattern of Treg-cell levels during the acute phase of IS. We found that circulating levels of Treg-cells increase during the first 3 days from stroke onset, showing a subsequently but not significant decrease at day 7. Therefore, these results represent a clear clinical evidence of the potential role of Treg-cells during the first phase of acute IS. Our results differ from those found in previous small and heterogeneous studies that described a decrease in circulating Treg-cell levels at the second day after stroke, followed by a significant increase at day 7 [16].

We found that higher levels of Treg-cells during the acute phase of IS were independently associated with good functional outcome at 3 months. Our results disagree to those reported by Urra et al. [16] that did not find relationship between Treg-cell levels and functional outcome of IS patients. The fact that this study included ischemic and hemorrhagic stroke patients could explain this difference. To the best of our knowledge, this is the first prospective study that specifically analyzed the association between circulating Treg-cells during the acute phase of IS and long-term outcome.

Our results showed that patients with END had lower levels of Treg-cells during the acute phase of stroke. The sample size of patients who suffered END was not enough to perform a multivariate analysis to determinate if the effect of Treg-cells on END could be a direct cause or it acts as a surrogate marker. Lower IL-10 levels were associated with clinical worsening [25], but we did not found previous studies that have analyzed the relationship between END and Treg-cells, so this aspect should be investigated in further studies.

We studied the relationship between Treg-cell levels and infarct volume, since it has not been previously reported in literature. We found that higher levels of Treg-cells were related with smaller infarct volume. These results also suggest a potential beneficial role of Treg-cells in acute IS, probably by decreasing inflammation which is reflected in a reduction of infarct volume.

Several mechanisms have been proposed for Treg-cells in stroke [26,27] such as the production of anti-inflammatory cytokines, the elimination through granzymes and perforins and metabolic mechanisms. In the context of experimental cerebral ischemia, several studies have demonstrated that IL-10 is a key neuroprotective cytokine regulatory of post-stroke neuroinflammation [9,28]. In the brain, Treg-cells together with B regulatory (Breg) cells and microglial/monocytes represent the main sources of IL-10. Previous studies in animal models of cerebral ischemia have confirmed the role of IL-10 as a mediator of the protective effect mediated by Treg-cells [28,29]. In fact, preclinical strategies directed towards the increase of lymphocyte IL-10 production [29,30] or exogenous IL-10 administration have been shown to improve outcome [28]. Therefore, we studied the relationship between Treg-cells and IL-10 levels in IS patients. We found a strong correlation between IL-10 and Treg-cell levels at admission, 48 and 72 hours, and day 7, supporting the possible anti-inflammatory role of Treg-cells by increasing IL-10 levels, as it has been demonstrated in preclinical studies [28,29]. Previous clinical studies have established a positive association between higher IL-10 levels during the acute phase and good outcome in IS [25, 31-35]. Nevertheless, no studies had previously investigated the role of IL-10 as a possible action mechanism of Treg-cells in acute phase of IS in humans.

Other point of interest in this study was to evaluate the effect of Treg-cells on the risk of systemic infections. Systemic infections are a frequent complication during the acute phase of stroke (7-35%, depending on the series) [36], and its presence worsens long-term outcome [36-42]. Some authors proposed that stroke may induce a systemic immunosuppression that could increase the risk of infections [38]. However, the underlying mechanisms that result in widespread immunosuppression after stroke and subsequent systemic infections is unknown. A study has observed lymphopenia and

increased apoptosis of Th lymphocytes, cytotoxic T lymphocytes and B lymphocytes at early phases of stroke [36]. Increased levels of cortisol and metanephrine have been also related with the risk of infections after stroke [43,44]. It has been described that during the first hours after stroke, pro-inflammatory cytokines are up-regulated (IL-6, IL-1, TNF α , IL-8, MCP-1, etc) [45]. This inflammation stimulates both hypothalamic-pituitary-adrenal axis and sympathetic nervous system, which suppresses immune cell function and can be related to systemic downregulation of the immune system [36].

Treg-cells are a subpopulation of cells with immunosuppressive effects [46,47], so these cells could be related with the risk of infections during the acute phase of stroke. Nevertheless, preclinical studies in animal models [48] demonstrated that exogenous administration of Treg-cells does not exacerbate immunosuppression after cerebral ischemia, this study even found that exogenous Treg-cells administration may improve immune status after induction of ischemia. Other clinical studies [16] found no association of Treg-cell levels with the development of infections after stroke, so this aspect is still unclear. In our study we tried to establish the relationship between circulating Treg-cell levels and development of infections during hospitalization.

We found that the presence of infections during the acute phase of stroke was associated with poor long-term outcome, as previously reported [36]. Patients with systemic infections showed higher body temperature at 24 and 48 hours, suggesting that infections were early developed after ischemic stroke. Interestingly, both lower Treg-cell levels at 48 and 72 hours were independently associated to development of infections after stroke. In fact, most of the infections in our patients were detected during the early phase of stroke (first-second day), when Treg-cells have not achieved their highest levels. In this regard, previous preclinical studies in animal models of cerebral ischemia have demonstrated that Treg-cells exogenous administration improves immune system function, reducing the risk of spontaneous infections after MCAO [31,48]. Our results confirm this effect described in animal models of cerebral ischemia, suggesting a possible protective role of Treg-cells in the risk of infections, or at least not a deleterious effect.

Finally, our study has some weaknesses: First, we used CD4+, CD25+ and CD127- as membrane markers for Treg-cells. Most authors proposed that FoxP3 is the most specific marker for Treg-cells, but FoxP3 is an intracellular protein, so it cannot be used to isolate human Treg-cells for functional studies or in vivo expansion for cellular therapy. We used the non-expression of CD127, which has previously demonstrated to be directly related with FoxP3 levels [20,22]. Second, we determined Treg-cell levels in peripheral blood but we have not demonstrated that these cells are infiltrating the brain tissue after IS. For this objective, invasive techniques are needed to confirm the presence of Treg-cells in ischemic lesion region. Three, Treg-cell levels were only determined during the acute phase of IS. We did not evaluate long-term temporal profile of these cells. Finally, we have only evaluated final infarct volume (no brain edema), because we did not perform halftime neuroimaging studies unless the patient developed END.

Conclusions

We found an independent association between Treg-cell levels and good functional outcome at 3 months in IS patients. Treg-cell levels increase after stroke and this increase was closely associated to protective effects; higher levels of these cells were associated to better functional outcome, smaller infarct volume, lower risk of END and infections during hospitalization. Treg-cells were also correlated with IL-10 levels, supporting that this anti-inflammatory cytokine may play an important role in the beneficial effects of these cells after ischemic stroke. Therefore, Treg-cells may be considered a potential therapeutic target in acute ischemic stroke. Both exogenous administration and endogenous stimulation of Treg-cells exert beneficial effects on animal models of cerebral ischemia. Finally, due to the potential relevance of Treg-cells in stroke and other diseases, new therapeutic strategies able to increase these cells could be developed in the future.

Declarations

Ethics approval and consent to participate

This research was carried out in accordance with the Declaration of Helsinki of the World Medical Association (2008), approved by the Ethics Committee of the Servizo Galego de Saúde. Informed consent was obtained from each patient or their relatives after full explanation of the procedures.

Consent for publication

Not applicable.

Availability of data and materials

The datasets analyzed during the present study will be available from the corresponding author upon reasonable request based on the guidelines of the Ethics Committee of the Servizo Galego de Saúde.

Competing interests

The authors report no conflicts of interest.

Funding

SAF2014-56336-R and SAF2017-84267-R; GRC2014/027 and IN607A2018/3, PIE13/00024; PI17/01103, RETICS-INVICTUS-PLUS (RD16/0019) and European Union FEDER. F. Campos (CP14/00154), T. Sobrino (CPII17/00027), I. López-Loureiro (IFI15/00111), M. Rodríguez-Pérez (IN606A-2018/031).

Authors' contributions

MS-C, JC and TS designed and coordinated the study, participated in the analyzed the data and manuscript preparation. MS-C, ER-C, IL-D, SA-R, MR-Y and MP-M collected the data, and have been involved in the statistical analysis. RI-R, MR-P, PH, MP-L and IL-L have been involved in the statistical analysis, interpretation and processed data. MS-C, JC, PH, TS and FC participated in the data analysis,

statistical treatment, and have been involved in revising the manuscript for important intellectual content. All authors have critically read and approved the submitted manuscript.

Acknowledgements

Not applicable.

References

1. Mozaffarian D, Benjamin EJ, Go AS, et al; American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Heart disease and stroke statistics–2015 update: a report from the American Heart Association. *Circulation*. 2015; 131:e29-e322.
2. Rodríguez-Castro E, López-Dequít I, Santamaría-Cadavid M, et al. Trends in stroke outcomes in the last ten years in a European tertiary hospital. *BMC Neurol*. 2018;18:164.
3. Wahlgren N, Ahmed N, Dávalos A, Hacke W, Millán M, Muir K, et al; SITS investigators. Thrombolysis with alteplase 3-4.5 h after acute ischaemic stroke (SITS-ISTR): an observational study. *Lancet*. 2008; 372:1303-1309.
4. Wang Q, Tang XN, Yenari MA. The inflammatory response in stroke. *J Neuroimmunol*. 2007; 184:53-68.
5. Rosenberg GA. Ischemic brain edema. *Prog Cardiovasc Dis* 1999; 42:209-216.
6. Siesjö BK, Siesjö P. Mechanisms of secondary brain injury. *Eur J Anaesthesiol*. 1996; 13:247-268.
7. del Zoppo GJ. Acute anti-inflammatory approaches to ischemic stroke. *Ann N Y Acad Sci*. 2010; 1207:143-148.
8. Chen S, Wu H, Klebe D, Hong Y, Zhang J, Tang J. Regulatory T cell in stroke: a new paradigm for immune regulation. *Clin Dev Immunol*. 2013;2013:689827.
9. Liesz A, Suri-Payer E, Veltkamp C, Doeber H. Regulatory T cells are key cerebroprotective immunomodulators in acute experimental stroke. *Nat Med*. 2009; 15:192-199.
10. Brea D, Agulla J, Rodríguez-Yáñez M, Barral D, Ramos-Cabrera P, Campos F, et al. Regulatory T cells modulate inflammation and reduce infarct volume in experimental brain ischaemia. *J Cell Mol Med*. 2014; 18:1571-1579.
11. Hug A, Liesz A, Muerle B, et al. Reduced efficacy of circulating costimulatory cells after focal cerebral ischemia. *Stroke*. 2011; 42:3580-3586.
12. Yan J, Greer JM, Etherington GP, Cadigan GP, Cavanagh H, Henderson RD, et al. Immune activation in the peripheral blood of patients with acute ischemic stroke. *J Neuroimmunol*. 2009; 206:112-117.
13. Li Q, Wang Y, Yu F, Wang YM, Zhang C, Hu C. Peripheral Th17/Treg imbalance in patients with atherosclerotic cerebral infarction. *Int J Clin Exp Pathol*. 2013; 6:1015-1027.
14. Yan J, Read SJ, Henderson R, Hull R, O'Sullivan J, McCombe P, Greer JM. Frequency and function of regulatory T cells after ischemic stroke in humans. *J Neuroimmunol*. 2012; 243:89-94.

15. Wrigen M, Björkbacka H, Andersson L, Ljungcrantz I, Fredrikson GN, Persson M, et al. Low levels of circulating CD4+FoxP3+ T cells are associated with an increased risk for development of myocardial infarction but not for stroke. *Arterioscle Thromb Vasc Biol.* 2012; 32:2000-2004.
16. Urra X, Cervera A, Villamallor M, Planas AM, Chamorro A. Harms and benefits of lymphocyte subpopulations in patients with acute stroke. *Neuroscience.* 2009; 158:1174-1183.
17. Alonso de Leciñana M, Egido JA, Casado I, Ribó M, Dávalos A, Masjuan J, et al. Guidelines for the treatment of acute ischaemic stroke. *Neurologia.* 2014; 29:102-122.
18. Adams Jr HP, Bendixen BH, Kappelle J, Biller J, Love BB, Gordon L, et al. Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. TOAST. Trial of Org 10172 in Acute Stroke Treatment. *Stroke.* 1993; 24:35-41.
19. Pedraza S, Puig J, Blasco G, Daunis-Estadella J, Boada I, Bardera A, et al. Reliability of the ABC/2 method in determining acute infarct volume. *J Neuroimaging.* 2012; 22:155-159.
20. Liu W, Putman AL, Xu Yu Z, Szot GL, Lee MR, Zhu S, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+Treg cells. *J Exp Med.* 2006; 20:1701-1711.
21. Peters JH, Preijers FW, Woostenenik R, Hilbrands LB, Koenen HJ, Joosten I. Clinical grade Treg: GMP isolation, improvement of purity by CD127 depletion, Treg expansion, and Treg cryopreservation. *PLoS One.* 2008; 3:e3161.
22. Seddiki N, Santner-Nonan B, Martninson J, Zaunders J. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med.* 2006; 203:1693-700.
23. Perini F, Marro M, Alecci M, Galloni E, Marchi M, Tosjo V. Temporal profile of serum anti-inflammatory and pro-inflammatory interleukins in acute ischemic stroke. *Neurol Sci.* 2001; 22:289-296.
24. Stubbe T, Ebner F, Ritcher D, et al. Regulatory T-cells accumulate and proliferate in the ischemic hemisphere for up to 30 days after MCAO. *J Cereb Blood Flow Metab.* 2013; 33:37-47.
25. Vila N, Castillo J, Dávalos A, Esteve A, Planas AM, Chamorro A. Levels of anti-inflammatory cytokines and neurological worsening in acute ischemic stroke. *Stroke* 2003; 34:671-675.
26. Otsubo K, Kanegane H, Kamachi Y, et al. Identification of FOXP3-negative regulatory T-like (CD4(+)/CD25(+)/CD127(low)) cells in patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome. *Clin Immunol.* 2011; 141:111-120.
27. Liesz A, Hu X, Kleinschnitz C, Offner H. Functional role of regulatory Lymphocytes in stroke. Facts and controversies. *Stroke.* 2015; 46:1422-1430.
28. Spera PA, Ellison JA, Fenersten GZ, Barone FC. IL-10 reduces rat brain injury following focal stroke. *Neurosci Lett.* 1998; 251:189-192.
29. Liesz A, Zhou W, Na S, Hammerling GJ. Boosting regulatory T cells limits neuroinflammation in permanent cortical stroke. *J Neurosci.* 2013; 33:17350-17362.
30. Na SY, Marckso E, Liesz A, Hunig T, Velktamp R. Amplification of Regulatory T-cells using a CD28 superagonist reduces brain damage after ischemic stroke in mice. *Stroke.* 2015; 46:212-220.

31. Li P, Gan Y, Sun BL, Zhang F, Lu B, Gao Y, et al. Adoptive regulatory T-cell therapy protects against cerebral ischemia. *Ann Neurol* 2013; 74:458-471.
32. Protti GG, Gagliardi RJ, Forte WC, Spravieri SR. IL-10 may protect against progressing injury during acute phase of ischemic stroke. *Arq Neuropsiquiat*. 2013; 71:846-851.
33. Singh HV, Pandey A, Shrivastava AK, Raizade A, Singh SK, Singh N. Prognostic value of neuron specific enolase and IL-10 in ischemic stroke and its correlation with degree of neurological deficit. *Clin Chim acta*. 2013; 419:136-138.
34. Chang LT, Yuen C, Lian CW, Lu CH, Chang WN, Youssef AA, et al. Link between IL-10 level and outcome after ischemic stroke. *Neuroimmunomodulation*. 2010; 17:223-228.
35. Rodríguez-Yáñez M, Castellanos M, Sobrino T, Brea D, Ramos-Cabrer P, Pedraza S, et al. Interleukin-10 facilitates the selection of patients for systemic thrombolysis. *BMC Neurol*. 2013; 13:62.
36. Chamorro A, Urra X, Planas AM. Infection after acute ischemic stroke: a manifestation of brain-induced immunodepression. *Stroke* 2007; 38:1097-1103.
37. Davenport RJ, Dennis MS, Weelwood L, Warlow CP. Complications after acute stroke. *Stroke*. 1996; 27:415-420.
38. Grau AJ, Buggle F, Schitrler P, Spiel M, Lichy C, Hacke W. Fever and infection early after ischemic stroke. *J Neurol Sci*. 1999; 171:115-120.
39. Langhorne P, Stott DJ, Robertson L, McDonald M, Jones L, McAlpine C, et al. Medical complications after stroke: a multicenter study. *Stroke*. 2000; 31:1223-1229.
40. Katzan IL, Cebul RD, Husak SH, Dawson NV, Baker DW. The effect of pneumonia on mortality among patients hospitalized for acute stroke. *Neurology*. 2003; 60:620-625.
41. Heuschmann PU, Kolominsky-Rabas PL, Misselwitz B, Hermanek P, Leffmann C, Jauzen RW, et al. Predictors of in-hospital mortality and attributable risks of death after ischemic stroke: the German Stroke Registers Study Group. *Arch Intern Med*. 2004; 164:1761-1768.
42. Vargas M, Horcajada J, Obach V, Revilla M, Cervera A, Torres F, et al. Clinical consequences of infection in patients with acute stroke: is it prime time for further antibiotic trials? *Stroke*. 2006; 37:461-465.
43. Wilckens T, De Rijk R. Glucocorticoids and immune function: unknown dimensions and new frontiers. *Immunol Today*. 1997; 18:418-424.
44. Elenkov IJ, Wilder RL, Chrousos GD, Vizi ES. The sympathetic nerve an integrative interface between two supersystems: the brain and the immune system. *Pharmacol Rev*. 2000; 52:595-638.
45. Rodríguez-Yáñez M, Castillo J. Role of inflammatory markers in brain ischemia. *Curr Opin Neurol*. 2008; 21:353-357.
46. O'Garra A, Vieira P. Regulatory T cells and mechanisms of immune system control. *Nat Med*. 2004; 10:801-805.
47. Emsley H, Hopkins SJ. Post-stroke immunodepression and infection: an emerging concept. *Infect Disord Drug Targets*. 2010; 10:91-97.

48. Li P, Mao L, Zhou G, Leak RK, Sun BI, Chen J, et al. Adoptive regulatory T cell therapy preserves systemic immune homeostasis after cerebral ischemia. 203; 44:3509-3515.

Tables

Table 1. Baseline clinical characteristics, vascular risk factors, stroke subtype, biochemical/cellular parameters and neuroimaging findings in patients with good or poor outcome at 3 months.

Variable	Good outcome n = 87	Poor outcome n = 117	P value
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 peripheral 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)	
- Atherothrombotic, n (%)	13 (14.9)	13 (11.1)	
- Lacunar, n (%)	3 (3.4)	4 (3.4)	
- Undetermined, n (%)	44 (56.4)	34 (43.5)	
% Treg cells / total lymphocytes at admission	0.0224±0.0177	0.0197±0.0112	0.962
% Treg cells / total lymphocytes, 48 hours	0.0715±0.0133	0.0231±0.0173	<0.0001
% Treg cells / total lymphocytes), 72 hours	0.0709±0.0064	0.0273±0.0183	<0.0001
% Treg cells / total lymphocytes) 7 days	0.0515±0.0189	0.0209±0.0172	0.001

Figures

Figure 1

Temporal pattern of % Treg-cell (A) and IL-10 levels (pg/mL) (B) at admission, 48 and 72 hours and day 7.

Figure 2

Relationship between mRS score at 3 months and circulating levels of Treg-cells at admission (A), 48 hours (B), 72 hours (C) and day 7 (D).

Figure 3

Relationship between circulating Treg-cells and serum levels of IL-10 at admission (A), 48 hours (B), 72 hours (C) and day 7 (D).