Peripheral Frequency of CD4+ CD28− Cells in Acute Ischemic Stroke

Relationship With Stroke Subtype and Severity Markers

Antonino Tuttolomondo, MD, PhD, Rosaria Pecoraro, MD, Alessandra Casuccio, MD, Domenico Di Raimondo, MD, PhD, Carmelo Buttà, MD, Giuseppe Clemente, MD, Vittoriano della Corte, MD, Giuliana Guggino, MD, Valentina Arnau, MD, Carlo Maida, MD, Irene Simonetta, MD, Rosario Maugeri, MD, Rosario Squatrito, MD, and Antonio Pinto, MD

Abstract: CD4+ CD28− T cells also called CD28 null null cells have been reported as increased in the clinical setting of acute coronary syndrome. Only 2 studies previously analyzed peripheral frequency of CD28 null cells in subjects with acute ischemic stroke but, to our knowledge, peripheral frequency of CD28 null cells in each TOAST subtype of ischemic stroke has never been evaluated. We hypothesized that CD4+ cells and, in particular, the CD28 null cell subset could show a different degree of peripheral percentage in subjects with acute ischemic stroke in relation to clinical subtype and severity of ischemic stroke.

The aim of our study was to analyze peripheral frequency of CD28 null cells in subjects with acute ischemic stroke in relation to TOAST diagnostic subtype, and to evaluate their relationship with scores of clinical severity of acute ischemic stroke, and their predictive role in the diagnosis of acute ischemic stroke and diagnostic subtype.

We enrolled 98 consecutive subjects admitted to our recruitment wards with a diagnosis of ischemic stroke. As controls we enrolled 66 hospitalized patients without a diagnosis of acute ischemic stroke. Peripheral frequency of CD4+ and CD28 null cells has been evaluated with a FACS Calibur flow cytometer.

Subjects with acute ischemic stroke had a significantly higher peripheral frequency of CD4+ cells and CD28 null cells compared to control subjects without acute ischemic stroke. Subjects with cardioenbolic stroke had a significantly higher peripheral frequency of CD4+ cells and CD28 null cells compared to subjects with other TOAST subtypes. We observed a significant relationship between CD28 null cells peripheral percentage and Scandinavian Stroke Scale and NIHSS scores. ROC curve analysis showed that CD28 null cell percentage may be useful to differentiate between stroke subtypes.

INTRODUCTION

In the late 1980s, Hansen and Martin described a subset of T CD4+ cells also called CD28 null cells that are increased in the clinical setting of acute coronary syndrome (ACS). These findings suggest a possible role for a T-cell component in acute ischemic stroke clinical setting showing a different peripheral frequency of CD28 null cells in relation of each TOAST subtype of stroke.

These findings seem suggest a possible role for a T-cell component also in acute ischemic stroke clinical setting showing a different peripheral frequency of CD28 null cells in relation of each TOAST subtype of stroke.

(Medicine 94(20):e813)

DOI: 10.1097/MD.0000000000000813

ISSN: 0025-7974

Volume 94, Number 20, May 2015 www.md-journal.com

**Abbreviations**: ACS = acute coronary syndrome, AMI = acute myocardial infarction, ANOVA = analysis of variance, AUP = Azienda Ospedaliero Universitaria Policlinico, BBB = blood brain barrier, Bcl-2 = B-cell lymphoma, brain CT = brain computed tomography, CD4+CD28− = cluster differentiation (CD), CEI = cardioembolic infarct, ECG = electrocardiogram, EDTA = ethylenediaminetetraacetic acid, IFN-γ = interferon gamma, IgG1-FITC = IgG1 antibodies conjugated with fluorescein isothiocyanate, IgG2a-PE = IgG2a conjugated with phycoerythrine, IL-1β = interleukin 1β, IL-2R (IL-2Ra) = interleukin 2 receptor (IL2RA), IL-6 = interleukin 6, LAAS = large artery atherosclerosis, LAAS = large artery atherosclerotic stroke, LAC = lacunar, LACunar, LACunar infarct, md-rankin = modified rankin, MRI = magnetic resonance imaging, NIHSS = National Institutes of Health Stroke Scale, ODE = other determined etiology, PBMCs = peripheral blood mononuclear cells, ROC = receiver operating characteristic, SD = standard deviation, TIA = transient ischemic attack, TNF-α = tumor necrosis alfa, TOAST = trial Org 10172 in acute stroke treatment, Treg CD4+ CD25 null cells = T-regulator CD4+ CD25 null cells, UDE = undetermined etiology.

**ACRONYMS**: AT = acute thromboembolic stroke, APTT = activated partial thromboplastin time, AUC = area under the curve, BAV = brain vascular, BBB = blood brain barrier, Bcl-2 = B-cell lymphoma, brain CT = brain computed tomography, CD4+CD28− = cluster differentiation (CD), CEI = cardioembolic infarct, ECG = electrocardiogram, EDTA = ethylenediaminetetraacetic acid, IFN-γ = interferon gamma, IgG1-FITC = IgG1 antibodies conjugated with fluorescein isothiocyanate, IgG2a-PE = IgG2a conjugated with phycoerythrine, IL-1β = interleukin 1β, IL-2R (IL-2Ra) = interleukin 2 receptor (IL2RA), IL-6 = interleukin 6, LAAS = large artery atherosclerosis, LAAS = large artery atherosclerotic stroke, LAC = lacunar, LACunar, LACunar infarct, md-rankin = modified rankin, MRI = magnetic resonance imaging, NIHSS = National Institutes of Health Stroke Scale, ODE = other determined etiology, PBMCs = peripheral blood mononuclear cells, ROC = receiver operating characteristic, SD = standard deviation, TIA = transient ischemic attack, TNF-α = tumor necrosis alfa, TOAST = trial Org 10172 in acute stroke treatment, Treg CD4+ CD25 null cells = T-regulator CD4+ CD25 null cells, UDE = undetermined etiology.

**OPEN**
former conducted by Nadareishvili et al. prospectively evaluated subjects with acute ischemic stroke analyzing the relationship between CD28 null cells and recurrence of a cerebrovascular event showing that high peripheral levels of CD28 null cells were significantly associated with event recurrence risk at 1-year follow-up. In a second study by Nowik et al., authors reported that CD4+ CD28 null cells were involved in mechanisms that increase stroke risk.

Thus, we hypothesized that CD4+ cells and, in particular, the CD4+ CD28− subset could show a different degree of increased peripheral percentage in subjects with acute ischemic stroke in relation to clinical subtype and severity of ischemic stroke.

The aim of this study was as follows:

1. To analyze peripheral frequency of CD4+CD28− cells in subjects with acute ischemic stroke in relation to TOAST diagnostic subtype;
2. To evaluate relationship of peripheral frequency of CD4+CD28− cells with scores of clinical severity of acute ischemic stroke;
3. To evaluate the ability of peripheral frequency of CD4+CD28− cells to predict stroke and its subtypes classified according TOAST classification.

MATERIALS AND METHODS

We enrolled consecutive subjects admitted to our recruitment wards (ward of Internal Medicine, AOU “P.Giaccone” Palermo; ward of Vascular Medicine AOU “P.Giaccone”, Pronto Soccorso Unit, Fondazione Istituto S. Raffaele/Giglio of Cefalù) with a diagnosis of acute ischemic stroke, in a recruitment period from June 2011 to December 2013. As controls we enrolled hospitalized patients without a diagnosis of acute ischemic stroke, admitted in the same period to our Internal Medicine Ward for any cause other than acute cardiovascular and cerebrovascular events. All enrolled patients underwent a general and neurological evaluation and an instrumental evaluation (ECG, ECG-holter 24 hours, epicranial vessel echography, brain CT or MRI, transesophageal echocardiography, and, in some cases, transesophageal).

Ischemic stroke has been defined as “a clinical syndrome of rapidly developing symptoms or signs of focal loss of cerebral function with symptoms lasting more than 24 hours and no apparent cause other than the vascular origin.” Patients and controls were excluded if they had 1 of the exclusion criteria: rheumatologic disorders, chronic inflammatory disease, acute systemic infections, recent venous thrombosis, recent acute myocardial infarction (AMI) (within 3 months), and recent cerebrovascular event (TIA or stroke within 6 months) (all these conditions may influence inflammatory cytokine and cell levels).

CRITERIA FOR EVALUATION OF CARDIOVASCULAR RISK FACTORS FOR CASES AND CONTROLS

Type 2 diabetes mellitus was determined using a clinically based algorithm that considered age at onset, presenting weight and symptoms, family history, onset of insulin treatment, and history of ketoacidosis. Hypertension was defined according to the 2007 European Society of Hypertension and the European Society of Cardiology Guidelines (2007 ESH/ESC 2007) as follows: (i) systolic blood pressure (SBP) >140 mmHg, and/or diastolic blood pressure (DBP) >90 mmHg in patients not receiving antihypertensive medication; (ii) previously documented diagnosis of hypertension in patients with the concurrent use of diet or antihypertensive medication regardless of current SBP and DBP values. Hypercholesterolemia was defined as total serum cholesterol ≥200 mg/dL and hypertriglyceridemia as total serum triglyceride ≥150 mg/dL on the basis of the National Cholesterol Education Program—Adult Treatment Panel III reports that define this cutoff for optimal total serum cholesterol and triglyceride levels. All patients had blood pressure, serum glucose, creatinine, serum uric acid, serum cholesterol levels, serum triglyceride levels, and urinary albumin excretion values measured on admission to the hospital. Coronary artery disease was identified on the basis of a history of physician-diagnosed angina, myocardial infarction, or any previous revascularization procedure determined by a questionnaire. Cerebrovascular disease (TIA/ischemic stroke) was identified by patient history, specific neurologic examination performed by specialists, and hospital or radiological records (brain computed tomography or brain magnetic resonance) of definite TIA or stroke. The protocol study was approved by Ethics Committee of the Policlinico P Giaccone Hospital and of Fondazione Istituto S. Raffaele/Giglio of Cefalu and all the patients gave their written informed consent to participate in the study, as well as for sampling and banking of the biological material. Study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

BLOOD SAMPLE COLLECTION TIMING

Blood samples has been drawn after 48 hours after symptom onset owing to the fact that several reports from experimental models to humans can sustain a possible “peripheral blood translation” of a neuroinflammatory cascade, either in terms of inflammatory cytokines or in terms of “cellular trafficking,” so it is plausible to hypothesize a peripheral increase of some cell subset of the T-cell population within 48 hours after an acute ischemic cerebral event. Activated T-cells on the periphery of the immune compartment once recruitment by means of cytokines has been fulfilled, enter the cerebral level through blood brain barrier (BBB) disruption and thus it appears plausible to expect a higher frequency of some T-cell subsets on peripheral blood in parallel with their course of intracerebral inflow.

Cell Isolation, Staining, and Flow Cytometry

Peripheral blood has been drawn at 48 hours after symptom onset and after informed consent had been obtained from the patient or his/her authorized representative. Peripheral blood mononuclear cells (PBMCs) have been obtained by density gradient centrifugation using the lymphocyte separation medium (ICN Pharmaceutical, Costa Mesa, CA). This protocol yields an average PBMC composition of 60% T cells, 15% monocytes/macrophages, 10% B cells, and 15% natural killer cells. White cells were obtained from 2 mL of peripheral EDTA-anticoagulated venous blood. Cells were labeled with human monoclonal anti-CD4 antibodies conjugated with fluorescein isothiocyanate and anti-CD28 antibodies conjugated with phycoerythrine (Becton, Dickinson and Company 1 Becton Drive Franklin Lakes, New Jersey). Mouse IgG1 antibodies conjugated with fluorescein isothiocyanate (IgG1-FITC) and IgG2a conjugated with phycoerythrine IgG2a-PE (Becton Dickinson) were used as the isotype controls. The samples were incubated for 30 minutes in the dark at...
STROKE SUBTYPE EVALUATION

The type of acute ischemic stroke was classified according to the TOAST classification: large artery atherosclerosis (LAAS); cardioembolic infarct (CEI); LACunar infarct (LAC); stroke of other determined etiology (ODE); and stroke of undetermined etiology (UDE).

FUNCTIONAL EVALUATION

National Institute of Health Stroke Scale (NIHSS) and Scandinavian Stroke Scale (SSS) were used to evaluate acute neurological deficit grade at 48 hours after admission in all enrolled patients with acute ischemic stroke. Modified Rankin score (mRankin) was used to assess disability grade at discharge.

Statistical Analysis

Statistical analysis of quantitative and qualitative data, including descriptive statistics, was performed for all items. Continuous data are expressed as mean ± standard deviation, unless otherwise specified. Baseline differences between groups were assessed by the χ² test or Fisher exact test as needed for categorical variables, and by the independent Student t test for continuous parameters. The univariate analysis of variance (ANOVA) was performed for parametric variables, and post hoc analysis with the Bonferroni test was used to determine whether there were pairwise differences. Linear regression analysis examined the correlation between various patient characteristics (independent variables), and CD4+ cells or CD4+ CD28− cells peripheral percentages (dependent variable) in simple and multiple regression models; at multivariate analysis we analyzed relationship between prognostic indexes (SSS, NIHSS, and rankin scores and death) and CD4+ and CD28 null cell peripheral percentage after adjustment for

| Variables                  | Patients With Ischemic Stroke (n: 98) | Controls (n: 67) | P     |
|----------------------------|--------------------------------------|-----------------|-------|
| Age, y (mean ± SD)         | 74.3 ± 12.1                          | 73.7 ± 10.7     | 0.702 |
| Diabetes (n/%)             | 39 (40.2)                            | 30 (44.7)       | 0.506 |
| Hypertension (%)           | 85 (87.0)                            | 53 (79.1)       | 0.203 |
| Hypercholesterolemia (n/%) | 33 (34.0)                            | 27 (27.8)       | 0.397 |
| Atrial fibrillation (n/%)  | 43 (44.3)                            | 28 (28.9)       | 0.854 |
| Previous stroke (n/%)      | 47 (48.5)                            | 12 (17.9)       | 0.0001|
| Previous TIA (n/%)         | 12 (12.4)                            | 4 (5.9)         | 0.207 |
| SAP, mm Hg (mean ± SD)     | 150.7 ± 28.6                         | 132.1 ± 11.0    | 0.0001|
| DAP, mm Hg (mean ± SD)     | 80.8 ± 14.5                          | 78.9 ± 6.6      | 0.310 |
| Glucose blood levels, mmol/L (mean ± SD) | 4.36 ± 1.13          | 4.12 ± 0.57    | 0.110 |
| Total cholesterol levels, mmol/L (mean ± SD) | 2.45 ± 0.65          | 2.47 ± 0.64    | 0.820 |
| Triglycerides, mmol/L (mean ± SD) | 1.65 ± 0.58          | 1.39 ± 0.24    | 0.310 |
| WBC, ×10⁹/L                | 9.35 ± 4.96                         | 7.21 ± 2.14     | 0.001 |
| Neutrophil (%)             | 67.2 ± 13.1                         | 51.8 ± 9.1      | 0.005 |
| TOAST subtype              |                                      |                 |       |
| LAAS                       | 38 (39.2)                            | 38.8            |       |
| Lacunar                    | 25 (25.8)                            | 25.5            |       |
| CEI                        | 32 (32.9)                            | 32.6            |       |
| ODE                        | 3 (30.9)                             | 3.1             |       |
| NIHSS (mean ± SD)          | 18.4 ± 15.1                          |                 |       |
| SSS (mean ± SD)            | 30.5 ± 13.8                          |                 |       |
| mRS (mean ± SD)            | 3.4 ± 1.7                            |                 |       |
| ESR, mm/h                  | 26.6 ± 16.7                          | 14.8 ± 9.1      | 0.0001|
| CRP, mg/dL                 | 5.4 ± 1.7                            | 3.6 ± 1.8       | 0.011 |
| CD4+ cells (%)             | 51.4 ± 6.8                           | 34.4 ± 6.4      | 0.0001|
| CD4+ CD28− (%)             | 5.7 ± 2.4                            | 2.8 ± 0.9       | 0.0001|
| TNF-α, pg/mL               | 28.2 ± 29.3                          | 12.3 ± 4.5      | 0.0001|
| IL-1β, pg/mL               | 7.9 ± 2.6                            | 4.7 ± 1.6       | 0.0001|
| IL-6, pg/mL                | 8.2 ± 2.4                            | 4.2 ± 1.5       | 0.0001|

CD4+ CD28− cells = cluster of differentiation 4 positive and cluster of differentiation 28 negative cells, CD4+ cells = cluster of differentiation 4 positive cells, CRP = C-reactive protein, DAP = diastolic blood pressure, ESR = Erytrocyte sedimentation rate, HDL = high density lipoprotein, IL-1β = Interleukin-1β, IL-6 = Interleukin-6, LDL = low-density lipoprotein, mRS = modified Rankin Scale score, NIHSS = National Institutes of Health Stroke Scale, SAP = systolic blood pressure, SSS = Scandinavian Stroke Scale score, TNF-α = tumor necrosis factor alfa, TOAST = Trial of ORG 10172 in Acute Stroke Treatment, WBC = white blood count.
other variable resulted significant at univariate analysis. To assess the predictive rate of different cutoff values of CD4 or CD4CD28 peripheral percentages with regard of stroke and TOAST subtype, a receiver operating characteristic (ROC) curve with calculations of area under the curve and 95% CIs was constructed and sensitivity and specificity values were calculated. Data were analyzed by the Epi Info software (version 6.0, Centers for Disease Control and Prevention, Atlanta, GA) and IBM SPSS Software 21.0 version (SPSS, Inc., Chicago, IL). All P values were 2-sided and P values <0.05 were considered statistically significant.

RESULTS

We enrolled 98 subjects with acute ischemic stroke and 66 control subjects. Demographic, laboratory, and clinical variables of subjects with acute ischemic stroke and control subjects are reported in Table 1.

Subjects with acute ischemic stroke had a significantly higher peripheral frequency of CD4+ cells compared to controls without acute ischemic stroke (51.4 ± 6.8% vs 34.4 ± 6.4%; P = 0.0001); similarly, ischemic stroke subjects had a significantly higher peripheral frequency of CD4+CD28− cells compared to controls without acute ischemic stroke (5.7 ± 2.4% vs 2.8 ± 0.9%; P = 0.0001). Stroke subjects also showed higher cytokine plasma levels such as TNF-α (28.2 ± 29.3 vs 12.3 ± 4.5 pg/mL; P = 0.0001), IL-1β (7.9 ± 2.6 vs 4.7 ± 1.6 pg/mL; P = 0.0001) and IL-6 (8.2 ± 2.4 vs 4.2 ± 1.5 pg/mL; P = 0.0001) compared to controls (see Table 1).

Demographic, clinical and laboratory variables of subjects with acute ischemic stroke in relation to TOAST subtype are listed in Table 2.

Subjects with cardioembolic stroke showed a significantly higher peripheral frequency of CD4+ cells compared to subjects with LAAS and lacunar subtype (51.7 ± 6.1% vs 45.3 ± 6.9% vs 43.6 ± 7.5%; P = 0.012) and of CD28 null cells compared to subjects with LAAS and lacunar subtypes (7.5 ± 2.0% vs 5.4 ± 1.8% vs 3.7 ± 1.5%; P = 0.0001). Subjects with cardioembolic subtype had significantly higher blood levels of TNF-α (39.6 ± 6.2 vs 32.2 ± 15.3 vs 18.1 ± 10.7 pg/mL); IL-6 (9.03 ± 1.90 vs 8.44 ± 1.73 vs 7.04 pg/mL; P = 0.006) and IL-1β (7.7 ± 2.0 vs 6.1 ± 2.3 vs 9.3 ± 2.4 pg/mL) compared to subjects with LAAS and lacunar subtypes (see Table 2).

Regarding the relationship between immune-inflammatory variables and severity markers (see Table 3), we have observed no significant relationship between peripheral frequency of CD4+ cells and chosen stroke severity indicators, whereas

### TABLE 2. General, Clinical, and Laboratory Variables of Patients With Acute Ischemic Stroke in Relation to TOAST Subtype

| Variables                  | LAAS (n: 38)          | Lacunar (n: 25)       | Cardioembolic (n: 32) | P  |
|----------------------------|-----------------------|-----------------------|-----------------------|----|
| Age, y (mean ± SD)         | 72.3 ± 11.9           | 73.1 ± 13.7           | 77.4 ± 11.0           | 0.302 |
| F/M (n%)                  | 14/24 (36.8/63.1)     | 14/11 (35.6/44)       | 22/10 (68.7/31.2)     | 0.058 |
| Diabetes (n%)             | 16 (42.1)             | 11 (45.8)             | 12 (37.5)             | 0.477 |
| Hypertension (%) (n%)     | 32 (84.2)             | 22 (88)               | 28 (87.5)             | 0.754 |
| Atrial fibrillation (n%)  | 18 (47.4)             | 5 (20.8)              | 9 (28.1)              | 0.119 |
| Previous stroke (n%)      | 18 (47.4)             | 11 (44)               | 17 (53.1)             | 0.888 |
| Previous TIA (n%)         | 6 (15.8)              | 2 (8)                 | 4 (12.5)              | 0.755 |
| SAP, mm Hg (mean ± SD)    | 148.9 ± 31.5          | 157.0 ± 29.6          | 149.3 ± 24.9          | 0.528 |
| DAP, mm Hg (mean ± SD)    | 80.9 ± 13.8           | 83.1 ± 14.1           | 78.9 ± 16.3           | 0.769 |
| Glucose blood levels, mmol/L (mean ± SD) | 8.66 ± 2.46          | 7.65 ± 2.72           | 7.70 ± 3.08           | 0.605 |
| Total cholesterol levels, mmol/L (mean ± SD) | 4.37 ± 1.09          | 4.29 ± 1.11           | 4.31 ± 1.21           | 0.571 |
| LDL cholesterol levels, mmol/L (mean ± SD) | 2.63 ± 0.82          | 2.40 ± 0.87           | 2.73 ± 1.08           | 0.850 |
| HDL cholesterol levels, mmol/L (mean ± SD) | 1.15 ± 0.33          | 1.23 ± 0.49           | 39.7 ± 0.32           | 0.240 |
| Triglycerides, mmol/L (mean ± SD) | 1.31 ± 0.64          | 1.45 ± 0.62           | 1.20 ± 0.42           | 0.476 |
| WBC, ×10⁹/L               | 10.25 ± 4.14          | 9.47 ± 3.34           | 8.35 ± 6.71           | 0.340 |
| Neutrophil (%)            | 67.9 ± 12.9           | 70.4 ± 10.9           | 63.7 ± 13.3           | 0.273 |
| SSSS (mean ± SD)          | 31.6 ± 13.0           | 40.5 ± 7.3            | 21.6 ± 13.1           | 0.0001 |
| NIHSS (mean ± SD)         | 14.9 ± 12.1           | 10.0 ± 10.2           | 29.9 ± 15.3           | 0.0001 |
| mRS (mean ± SD)           | 3.6 ± 1.8             | 2.1 ± 1.3             | 4.1 ± 1.1             | 0.0001 |
| ESR, mm/h                 | 23.8 ± 15.7           | 29.6 ± 14.1           | 27.9 ± 20.8           | 0.505 |
| CRP, mg/dL                | 3.471 ± 1.98          | 3.9 ± 1.7             | 3.4 ± 2.6             | 0.987 |
| CD4+ cells (%)            | 45.3 ± 6.9            | 43.6 ± 7.5            | 51.7 ± 6.1            | 0.012 |
| CD4+ CD28− (%)            | 5.4 ± 1.8             | 3.7 ± 1.5             | 7.5 ± 2.0             | 0.0001 |
| TNF-α, pg/mL              | 32.2 ± 15.3           | 18.0 ± 10.7           | 39.6 ± 6.2            | 0.025 |
| IL-1β, pg/mL              | 7.7 ± 2.0             | 6.1 ± 2.3             | 9.3 ± 2.4             | 0.0001 |
| IL-6, pg/mL               | 8.4 ± 1.7             | 7.0 ± 3.2             | 9.0 ± 1.9             | 0.006 |

CD4+ CD28− cells = cluster of differentiation 4 positive and cluster of differentiation 28 negative cells, CD4+ cells = cluster of differentiation 4 positive cells, CRP = C-reactive protein, DAP = diastolic blood pressure, ESR = erythrocyte sedimentation rate, HDL = high-density lipoprotein, IL-1β = interleukin-1β, IL-6 = interleukin-6, LDL = low-density lipoprotein, mRS = modified Rankin Scale score, NIHSS = National Institutes of Health Stroke Scale, SAP = systolic blood pressure, SSS = Scandinavian Stroke Scale score, TNF-α = tumor necrosis factor alpha, WBC = white blood count.
we observed a significant relationship between peripheral frequency of CD4+/CD28− cells and SSS (β = 0.049; P = 0.046) and NIHSS (β = −0.460; P = 0.042) scores.

At multivariate analysis, we reported a significant relationship between TNF-α (P = 0.039 and P = 0.041), IL-1β (P = 0.044 and P = 0.042), IL-6 plasma levels (P = 0.029 and P = 0.032), SSS and NIHSS scores, whereas CD28 null cell peripheral frequency was significantly associated with inflammatory cytokine blood levels at multivariate analysis, such as IL-6, IL-1β, and TNF-α (see Tables 4 and 5).

### DISCUSSION

This study found a significantly higher peripheral frequency of CD4+ and CD28− cells in subjects with acute ischemic stroke compared to controls. Consistent with findings already reported by previous studies in the clinical setting of ACSs4,7,8 our results seem to suggest a possible role for a T-cell component also in ischemic stroke setting.

The acute phase of ischemic stroke is characterized by a high degree of immune-inflammatory activation in terms of increased plasma levels of cytokines, adhesion molecules, and selectins12,13,14,15,23–26 and our study has yet addressed this issue.

Several reports from experimental models to humans16,27 can sustain a possible “translation” of a neuroinflammatory cascade, either in terms of inflammatory cytokines or in terms of “cellular trafficking,” so it is plausible to hypothesize a peripheral increase of some cell subset of the T-cell population within 48 hours after an acute ischemic cerebral event. Activated T-cells on the periphery of the immune compartment once recruitment by means of cytokines has been fulfilled, enter the cerebral level through BBB disruption and thus it appears plausible to expect a higher frequency of some T-cell subsets on peripheral blood16,27 in parallel with their course of intracerebral inflow.

Among these T-cells, CD28 null cells that show several characteristics of pathogenic cells and they are less susceptible to regulation by Treg CD4+ cells, cluster of differentiation 28 negative cells, CD28 null cells = cluster of differentiation 28 positive cells, IL-1β = interleukin-1β, IL-6 = interleukin-6, TNF-α = tumor necrosis factor-α. After adjustment for atrial fibrillation prevalence.

### TABLE 3. Multivariate Analysis of Relationship Between Immunoinflammatory and Prognostic Variables

|            | B      | β     | B 95% CI   | Sig (P) |
|------------|--------|-------|------------|---------|
| Relationship between CD4+ cells and prognostic variables |        |       |            |         |
| SSS        | 0.068  | 0.137 | 0.108–0.244| 0.443   |
| NIHSS      | 0.115  | 0.790 | 0.043–0.272| 0.151   |
| RS         | −0.611 | 0.183 | −1.514–0.293| 0.183   |
| Death      | 0.997  | 0.062 | −0.043–0.272| 0.563   |
| Relationship between CD4+ / CD28− cells and prognostic variables |        |       |            |         |
| SSS        | −0.049 | 0.026 | −0.100–0.001| 0.046   |
| NIHSS      | 0.460  | 0.023 | 0.0001–0.091| 0.042   |
| mRS        | −0.935 | 0.054 | −4.913–3.042| 0.642   |
| Death      | −6.385 | −0.091| 21.403–8.632| 0.401   |

CD4+ / CD28− cells = cluster of differentiation 28 positive and cluster of differentiation 28 negative cells, CD4+ cells = cluster of differentiation 28 positive cells, IL-1β = interleukin-1β, IL-6 = interleukin-6, TNF-α = tumor necrosis factor-α. After adjustment for atrial fibrillation prevalence.

By means of ROC curve analysis we showed a good sensitivity and specificity of CD4+ peripheral frequency to predict ischemic stroke (AUC = 0.964, P = 0.0001; cutoff value > 41.2%, sensitivity = 90.9, specificity = 95.5; see Figure 1); in regard to the peripheral frequency of CD28− cells ROC curve analysis demonstrated good sensitivity and specificity to predict stroke (AUC = 0.880, P = 0.0001; sensitivity = 70.4, specificity = 98.5, cutoff value > 4.1% (see Figure 1).

The sensitivity and specificity of CD4+ cells to predict TOAST subtype of ischemic stroke at ROC curve analysis did not show a significant association with LAAS (AUC = 0.638, P = 0.06; sensitivity = 68.4, specificity = 60.0 cutoff ≥53.1%), and cardioembolic subtype (AUC = 0.792, P = 0.227; sensitivity = 65.6, specificity = 60.0, cutoff value ≥53). ROC curve analysis showed good sensitivity and specificity values of CD28 null cell peripheral frequency to predict cardioembolic (AUC = 0.932, P = 0.0001; specificity = 75.0, sensitivity = 96.0, cutoff value ≥6.1%), and LAAS TOAST subtypes (AUC = 0.787, P = 0.0001; sensitivity = 78.9, specificity = 72.0, cutoff value ≥3.9%) (see Figures 2 and 3).

### TABLE 4. Multivariate Analysis of Relationship Between CD4+ / CD28− Cells and Immunoinflammatory Variables

|            | B      | β     | B 95% CI   | Sig (P) |
|------------|--------|-------|------------|---------|
| TNF-α      | 0.321  | 0.312 | 0.120–0.407| 0.0001  *
| IL-1β      | 0.297  | 0.341 | 0.160–0.435| 0.0001  *
| IL-6       | 0.261  | 0.323 | 0.140–0.397| 0.0001  *

CD4+ / CD28− cells = cluster of differentiation 28 positive and cluster of differentiation 28 negative cells, IL-1β = interleukin-1β, IL-6 = interleukin-6, TNF-α = tumor necrosis factor-α. After adjustment for atrial fibrillation prevalence.

Copyright © 2015 Wolters Kluwer Health, Inc. All rights reserved.
evaluate the relationship between neurological deficit grade and CD4CD28 null cell count, whereas Nowik et al\textsuperscript{10} reported that the severity of neurological deficits assessed on admission did not correlate with percentage of CD4\textsuperscript{þ}CD28\textsuperscript{−} lymphocytes. It explains how our observed relationship between CD4\textsuperscript{þ}CD28\textsuperscript{−} peripheral percentage and NIHSS and SSS scores appear a novel finding.

Ischemic stroke induces a profound local inflammatory response. Within hours, various types of immune cells transmigrate over the activated endothelium to invade the damaged brain in a timed fashion. Although previous studies mostly focused on neutrophils and monocytes,\textsuperscript{16,28,29} the role of lymphocytes, especially T cells in ischemic stroke, has long been neglected, although T cells are localized in close vicinity to blood vessels in the infarct boundary as early as 24 hours after experimental focal cerebral ischemia in rodents.\textsuperscript{30,31} T cells have been identified in the brain as early as 24 hours after ischemia.\textsuperscript{32,33} Involvement of adaptive immunity comes from studies on the role of lymphocytes in models of focal cerebral ischemia reporting how ischemia leads to infiltration of the major lymphocytes subtypes into the ischemic brain.\textsuperscript{33}

Lymphocytes invade the ischemic brain and contribute to tissue damage, but the rapidity of their deleterious effect is not consistent with an adaptive immune response targeted to the brain. Nevertheless, this lymphocyte role in brain ischemia pathogenesis could offer biological plausibility to our findings owing to the proinflammatory properties of CD28 null cells.\textsuperscript{33}

We previously reported that immune-inflammatory activation of the acute phase of ischemic stroke is associated with stroke volume and severity degree in terms of acute neurologic deficit grade evaluated by NIHSS,\textsuperscript{14} thus the role of CD4\textsuperscript{+}CD28\textsuperscript{−} subset could represent a natural extension of cytokine, selectins, and adhesion molecule activation. The severity of neurological symptoms, assumed to reflect the size of the

### TABLE 5. Area Under ROC Curve, Sensitivity, and Specificity of CD4\textsuperscript{+} and CD28\textsuperscript{−} Cells Cutoff Values Diagnosis of Ischemic Stroke and TOAST Subtype

| Variable            | Cutoff (%) | Area Under ROC Curve | Sensitivity (95% CI) | Specificity (95% CI) | P     |
|---------------------|------------|-----------------------|----------------------|----------------------|-------|
| CD4\textsuperscript{+} cells | >41.2      | 0.964                 | 90.8 (83.3–95.7)     | 95.5 (87.3–99.0)     | 0.0001|
| CD28\textsuperscript{−} cells | >4.1       | 0.880                 | 70.4 (60.3–79.2)     | 98.5 (91.8–99.7)     | 0.0001|
| LAAS                |            |                       |                      |                      |       |
| CD4\textsuperscript{+} cells | ≥53.1      | 0.638                 | 68.4 (51.3–82.5)     | 60.0 (38.7–78.8)     | 0.057 |
| CD28\textsuperscript{−} cells | >3.9       | 0.787                 | 78.9 (62.7–90.4)     | 72.0 (50.6–87.9)     | 0.0001|
| Cardioembolic       |            |                       |                      |                      |       |
| CD4\textsuperscript{+} cells | ≥53        | 0.592                 | 65.6 (46.8–81.4)     | 60.0 (38.7–78.8)     | 0.227 |
| CD28\textsuperscript{−} cells | >6.1       | 0.932                 | 75.0 (56.6–88.5)     | 96.0 (79.6–99.3)     | 0.0001|

CD4\textsuperscript{+}CD28\textsuperscript{−} cells = cluster of differentiation 4 positive and cluster of differentiation 28 negative cells, CD4\textsuperscript{+} cells = cluster of differentiation 4 positive cells, LAAS = Large Artery Atherosclerotic Stroke, ROC = receiving operator curve, TOAST = Trial of ORG 10172 in Acute Stroke Treatment.
ischemic lesion, correlated with the percentage of CD4+CD28− lymphocytes. According to the hypothesis that stroke is followed by an increase in the number of these cells as a response to antigens released from injured brain tissue, a higher percentage of peripheral CD4CD28 null cells could be related to a more profound brain injury.

The possible neuroinflammatory equivalence between CD4+CD28− cell components and cytokine activation just reported by our group14,23 and other groups21,22 is further confirmed by our finding concerning the relationship between frequency of peripheral CD28 null cells and some severity markers such as the SSS score and the NIHSS, used as indicators of the degree and type of neurological deficit of the acute phase. The significant association between levels of CD28− T cells and serum levels of inflammatory cytokines assessed in our current study (IL-6, TNF-α, IL-1β) may explain this relationship.

Finally, at ROC curve analysis our findings showed that CD28 null cell peripheral percentage may be useful to differentiate between stroke subtypes. The greater frequency of CD4+CD28− in subjects with ischemic stroke compared to controls and the significant association with the degree and type of neurological deficit and with the cardioembolic subtype offers the possibility to analyze a possible application of the peripheral percentage of some T-cell subsets on peripheral blood14,15 after an acute ischemic stroke underlying the role of CD4CD28 null cells that are only a component of lymphocyte cell family. The profound damage to the CNS caused by ischemic lesions has been well documented.

Yet, relatively little is known about the contribution to and effects on the immune system during stroke. Some authors have focused on both early and late events in the peripheral immune system during stroke in mice and have observed an early activation of splenocytes that conceivably could result in immune-mediated damage in the developing CNS lesion,37 followed by global immunosuppression that affects the spleen, thymus, lymph nodes, and circulation that has been reported as mediated by a stroke-induced apoptosis of CD4+CD28+ cells in lymphoid organs23,38.

Nevertheless, it is conceivable that CD4+D28 null subset of T-cells could be resistant to stroke-induced apoptosis. CD4+T-cells deficient in CD28 expression and compared with their CD28+ counterparts, they produce significantly higher levels of IFN-γ giving them the ability to function as proinflammatory cells. Moreover CD4+D28null T cell are highly olygoclonal and clones persist for years in circulation.36 Longevity of these cells appears to be related to their relative resistance to spontaneous cell death even in the absence of IL-2.39 This phenomenon is associated with low levels of expression of the α-chain of the IL-2R (IL-2Ra), despite their ability to produce large amounts of IL-2, and an increased expression of the anti-apoptotic molecule Bcl-2.40–43

In conclusion, we provided evidence of a higher peripheral percentage of some subsets of T-lymphocyte cells in subjects with acute ischemic stroke, a significant association with neurological deficit degree, and a predictive role of CD28 null cell peripheral percentage toward stroke diagnosis and TOAST subtype.

REFERENCES

1. Morishita Y, Martin PJ, Bean MA, et al. Antigen-specific functions of a CD4+ subset of human T lymphocytes with granular morphology. J Immunol. 1986;136:2095–2102.

2. Vallejo AN, Nestel AR, Schirmer M, et al. Aging-related deficiency of CD28 expression in CD4+ T cells is associated with the loss of gene-specific nuclear factor binding activity. J Biol Chem. 1998;273:8119–8122.

3. Vallejo AN, Brandes JC, Weyand CM, et al. Modulation of CD28 expression: distinct regulatory pathways during activation and replicative senescence. J Immunol. 1999;162:6572.

4. Liuzzo G, Kopecky SL, Frye RL, et al. Perturbation of the T-cell repertoire in patients with unstable angina. Circulation. 1999;100:2135–2139.
17. Adams HP Jr, Bendixen BH, Kappelle LJ, et al. Classification of acute ischemic stroke: putative role for cytokines, adhesion molecules and i-NOS in inflammatory markers and arterial stiffness indexes in subjects with acute ischemic stroke. J Cereb Blood Flow Metab. 1999;19:639–644.

18. Brott T, Adams HP Jr, Olinger CP, et al. Measurements of acute coronary syndromes. Circulation. 2000;101:2883–2888.

19. Del Zoppo G, Ginis I, Hallenbeck JM, et al. Inflammation and thrombotic/fibrinolytic genes in patients with acute ischemic stroke: association with time of onset and diabetic state. J Immunopathol Pharmacol. 2006;19:639–644.

20. Nowik M, Nowacki P, Grabarek J, et al. Can we talk about CD4+ CD28null T lymphocytes and stroke recurrence and death. Neurology. 2004;63:1446–1451.

21. Del Zoppo G, Ginis I, Hallenbeck JM, et al. Inflammation and plaque instability in acute coronary syndromes. Circulation. 1993;87:964–975.

22. Ferrarone C, Mascarucci P, Zoaia C, et al. Increased cytokine release from peripheral blood cells after acute stroke. J Cereb Blood Flow Metab. 1999;19:1004–1009.

23. Tuttolomondo A, Di Raimondo D, Pecoraro R, et al. Immune-inflammatory markers and arterial stiffness indexes in subjects with acute ischemic stroke. Atherosclerosis. 2010;213:311–318.

24. Tuttolomondo A, Di Raimondo D, Pecoraro R, et al. Inflammation in ischemic stroke subtypes. Curr Pharm Des. 2012;18:4289–4310.

25. Pinto A, Tuttolomondo A, Casuccio A, et al. Immunoinflammatory predictors of stroke at follow-up in patients with chronic non-valvular atrial fibrillation. Clin Sci. 2009;116:781–789.

26. Tuttolomondo A, Di Sciaccia R, Di Raimondo D, et al. Plasma levels of inflammatory and thrombotic-fibrinolytic markers in acute ischemic strokes: relationship with TOAST subtype, outcome and infarct size. J Neurol Immunol. 2009;2015:84–89.

27. Arumugam TV, Granger DN, Mattson MP. Stroke and T-cells. Neurol Med. 2005;7:229–242.

28. Schroeter M, Jander S, Witte OW, et al. Local immune responses in the rat cerebral cortex after middle cerebral artery occlusion. J Neurol Neurosurg Psychiatry. 1994;55:195–203.

29. Kochanek PM, Hallenbeck JM. Polymorphonuclear leucocytes and monocytes/macrophages in the pathogenesis of cerebral ischemia and stroke. Stroke. 1992;23:1367–1379.

30. Crocker SJ, Pagenstecher A, Campbell IL. The TIMPs tango with MMPs and more in the central nervous system. J Neurosci Res. 2004;75:1–11.

31. Liu J, Hars M, Richter M, et al. Stroke-induced immunodepression and pressor-stroke infections: lessons from the preventive antibacterial therapy in stroke trial. Neuroscience. 2009;158:1184–1193.

32. Offner H, Vandenbark AA. Hurn PDEffect of experimental stroke on peripheral immunity: CNS induced suppresses profound immunosuppression. Neuroscience. 2009;158:1098–1111.

33. Gendron A, Teitelbaum J, Ossette C, et al. Temporal effects of left versus right middle cerebral artery occlusion on spleen lymphocyte subsets and mitogenic response in Wistar rats. Brain Res. 2002;955:85–97.

34. Assar P, Keiser C, Haller BJ, et al. Oligoclonal T cell proliferation in patients with rheumatoid arthritis and their unaffected siblings. Arthritis Rheum. 1996;39:904.

35. Park W, Weyand CM, Gwaltney JM, et al. Co-stimulatory pathways controlling activation and peripheral tolerance of human brain response to ischemia. Brain Pathol. 2000;10:95.

36. Ferrarone C, Mascarucci P, Zoaia C, et al. Increased cytokine release from peripheral blood cells after acute stroke. J Cereb Blood Flow Metab. 1999;19:1004–1009.

37. Gendron A, Teitelbaum J, Ossette C, et al. Temporal effects of left versus right middle cerebral artery occlusion on spleen lymphocyte subsets and mitogenic response in Wistar rats. Brain Res. 2002;955:85–97.

38. Assar P, Keiser C, Haller BJ, et al. Oligoclonal T cell proliferation in patients with rheumatoid arthritis and their unaffected siblings. Arthritis Rheum. 1996;39:904.

39. Park W, Weyand CM, Gwaltney JM, et al. Co-stimulatory pathways controlling activation and peripheral tolerance of human brain response to ischemia. Brain Pathol. 2000;10:95.

40. Gendron A, Teitelbaum J, Ossette C, et al. Temporal effects of left versus right middle cerebral artery occlusion on spleen lymphocyte subsets and mitogenic response in Wistar rats. Brain Res. 2002;955:85–97.

41. Assar P, Keiser C, Haller BJ, et al. Oligoclonal T cell proliferation in patients with rheumatoid arthritis and their unaffected siblings. Arthritis Rheum. 1996;39:904.