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The ratio of circulating regulatory cluster of differentiation 4 T cells to endothelial progenitor cells predicts clinically significant acute rejection after heart transplantation

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KEYWORDS:
Treg/EPC ratio;
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acute rejection

BACKGROUND: The aim of this study was to determine the value of the ratio of the percentage of circulating regulatory cluster of differentiation 4 T cells (%Tregs) to the percentage of endothelial progenitor cells (%EPCs; Treg/EPC ratio) for predicting clinically significant acute rejection.

METHODS: Peripheral blood %Tregs and %EPCs were quantified in 91 cardiac transplant recipients using flow cytometry at a mean of 42 ± 13 days after transplant. The primary end point was clinically significant acute rejection, defined as an event that led to an acute augmentation of immunosuppression in conjunction with an International Society for Heart and Lung Transplantation grade ≥ 2R in a right ventricular endomyocardial biopsy specimen or non-cellular rejection (specimen-negative rejection) with hemodynamic compromise (decrease in left ventricular ejection fraction by > 25%).

RESULTS: Significant rejection occurred in 27 recipients (29.7%) during a median of 49.4 months (interquartile range, 37.0–62.0 months). The mean %Tregs and %EPCs were not significantly different between those with and without an episode of significant rejection, but the mean Treg/EPC ratio was significantly lower in recipients with significant rejection (44.9 vs 106.7, $p = 0.001$). Receiver operating characteristic curve analysis showed an area under the curve value for significant rejection for a Treg/EPC ratio of 0.712. The best cutoff value of the Treg/EPC ratio that distinguished between those with or without significant rejection was ≤ 18 by receiver operating characteristic curve analysis. Kaplan-Meier analysis revealed that patients with a Treg/EPC ratio of ≤ 18 had a significantly higher rate of rejection than those with a Treg/EPC ratio > 18 (61.5% vs 16.9%, log-rank $p < 0.0001$). A low Treg/EPC ratio was an independent predictor of significant rejection.

CONCLUSIONS: A low Treg/EPC ratio measured soon after heart transplantation is an independent predictor of acute rejection. The Treg/EPC ratio has potential as an early biomarker after heart transplantation for predicting acute rejection.

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A considerable body of animal research suggests that regulatory cluster of differentiation (CD) 4 T cells (Tregs), by inhibiting the activity of alloreactive T cells, can create a

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tolerant state that reduces the requirement for immunosuppressive drugs.[1–9] Several studies have shown that the number of circulating endothelial progenitor cells (EPCs) is directly related to a reduced risk of cardiac death and other coronary events.[10–14] However, EPCs may be pathologic after heart transplantation because of allograft antigenicity and contribute to vasculopathy. Furthermore, cardiac allograft vasculopathy (CAV) and rejection may be a result of defective EPC repair mechanisms.[15] Several studies have demonstrated an unfavorable role of EPCs after heart transplantation by causing allograft rejection or CAV.[15–19] Therefore, higher levels of Tregs and lower levels of EPCs may be beneficial after heart transplantation.

This study investigated longitudinal clinical outcome data in cardiac transplant recipients and evaluated the prognostic value of the ratio of the percentage of Tregs (% Tregs) to the percentage of EPC (%EPCs), referred to as the Treg/EPC ratio. Therefore, the aim of this study was to determine the prognostic value of the Treg/EPC ratio after heart transplantation.

Methods

Design

This was a retrospective analysis of prospectively collected data from a randomized, controlled, multicenter study investigating the role of ramipril, an angiotensin-converting enzyme (ACE) inhibitor, in preventing the development of CAV early after heart transplantation.[20] This study was conducted at Stanford University, the Palo Alto VA Health Care System, and the Cedars-Sinai Medical Center between 2009 and 2014 (NCT01078363). This study was approved by an Institutional Review Committee from each participating site.

Subjects underwent coronary angiography with measurement of fractional flow reserve, coronary flow reserve, and the index of microcirculatory resistance and performance of intravascular ultrasound (IVUS) in the left anterior descending coronary artery within 8 weeks of heart transplantation. Subjects were then randomized in a double-blind fashion to ramipril or placebo. This assessment was repeated after 1 year. The %Tregs and %EPCs in the circulation were measured within 8 weeks after heart transplantation.

Patients were included if they were aged $\geq$ 12 years, had a serum creatinine of $<$ 2.0 mg/dl, had not previously received a cardiac transplant, and were willing and able to provide informed written consent. Parent(s) of pediatric patients provided consent, and the child signed assent. Patients were excluded if they had more than 1 solid organ transplant at the time of heart transplant, had serum creatinine $>$ 2.0 mg/dl, or were pregnant. Patients were further excluded from the present sub-study if the %Tregs and % EPC data were not available.

Sample collection and peripheral blood mononuclear cell isolation

Whole blood was collected in heparinized collection tubes. Most samples were processed the same day. Other samples were shipped overnight to Stanford University from Cedars-Sinai (Los Angeles) at ambient temperature and then processed immediately (all within 48 hours of being drawn). Peripheral blood mononuclear cells (PBMCs) were separated via centrifugation over Ficoll-Paque Plus (GE Healthcare), following the manufacturer's directions, and preserved in 10% dimethyl sulfoxide/90% fetal calf serum medium in liquid nitrogen vapor.

Flow cytometry

Samples were rapidly thawed and rested for 2 hours in complete media (Roswell Park Memorial Institute–10% fetal calf serum) at 37°C and humidified 5% CO_2 atmosphere before surface staining with the following anti-human monoclonal antibodies [clones]: CD3 [SK7], CD14 [MfP9], CD16 [3G8], CD19 [SJ25C1], CD20 [L27], CD56 [NCAM16.2], CD31 [WM59], CD34 [581], and CD45 [HI30], all from BD Biosciences; and CD127 [eBioRDR5] and CD4 [RPA-T4] from Ebiosciences.[21] Samples were then stained with Live/Dead Blue (Thermo Fisher), fixed, permeabilized, and stained with anti-human forkhead box P3 (FOXP3) [PC101] using FOXP3 staining buffer (eBioscience). After staining, all samples were fixed with 2% paraformaldehyde in phosphate buffered saline (Electron Microscopy Sciences) and stored at 4°C until acquisition.

Data were acquired on an LSR II instrument (BD Biosciences) running FACSDIVA software in the Stanford Shared FACS Facility. Post-acquisition analysis was done using FlowJo (Tree Star; Figure 1). We determined the frequency of CD4 Tregs within total CD4 lymphocytes (%Tregs) in recipient PBMCs by surface and intracellular staining at a mean of 42 $\pm$ 13 days after transplant. In conjunction with %Treg analyses, EPCs were also enumerated in the same recipient PBMC sample. We determined the frequency of EPCs as a function of total live PBMCs (%EPCs).[22]

Clinical follow-up

The main outcome measure was development of significant rejection. Significant acute rejection was defined as an event that led to an acute augmentation of immunosuppression in conjunction with an International Society for Heart and Lung Transplantation grade $\geq$ 2R right ventricular endomyocardial result on the biopsy specimen or non-cellular rejection (specimen negative for rejection) with hemodynamic compromise (decrease in left ventricular ejection fraction by $>$ 25%). Patients were monitored for acute rejection by routine surveillance endomyocardial biopsy and echocardiography to evaluate allograft function at 2 weeks and at 1, 2, 3, 4, 6, 9, and 12 months in the first year after heart transplantation. During the second year, routine surveillance biopsy was performed every 3 to 4 months through 24 months. After 2 years, biopsies were performed every 6 months until 3 years, and afterwards only based on clinical suspicion. The main outcome was obtained from review of the electronic medical record when not available in the study database. If patients had multiple episodes of rejection, the time to the first event was counted as the censored outcome.

Statistical analysis

Values are expressed as mean $\pm$ standard deviation or as numbers (percentages). The Mann-Whitney U test was used to compare continuous variables. Categoric variables were summed as percentages and compared using chi-square statistics. Receiver operating characteristic curve analysis was done to establish the best cutoff value of the Treg/EPC ratio to differentiate those with and without significant rejection. The area under the curve was calculated with 95% confidence intervals (CIs). The freedom from