Defective Circulating CD4⁺CD25⁺Foxp3⁺CD127^{low} Regulatory T-cells in Patients with Chronic Heart Failure

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Key Words

Chronic heart failure • Immunity • Regulatory T cells

Abstract

Aims: Increasing evidences confirm the role of immune activation in the pathogenesis of chronic heart failure (CHF). Regulatory T cells appear central to the control of immune homeostasis. We assessed the hypothesis that the circulating frequency and function of CD4⁺CD25⁺ Foxp3⁺CD127^{low} T regulatory cells (Tregs) would be deranged in patients with CHF. **Methods:** Ninety-nine CHF patients due to non-ischemic (NIHF) or ischemic etiology (IHF) and 24 control donors were enrolled in the study. Frequency of circulating Tregs was evaluated by flow cytometry. Foxp3 in peripheral blood mononuclear cells (PBMCs) was assayed at the mRNA level by real-time PCR. Functional properties of Tregs to suppress proliferation and pro-inflammatory cytokines secretion of activated CD4⁺CD25⁺ T cells were measured by proliferation assay and ELISA. **Results:** The results demonstrated that CHF patients had significantly lower frequency of circulating Tregs and reduced Foxp3 expression in PBMCs compared with control

donors. Moreover, Tregs from CHF patients showed compromised function to suppress CD4⁺CD25⁺ T cells proliferation and pro-inflammatory cytokines secretion. A similar pattern with reduced Tregs frequency and compromised function was found in both NIHF and IHF patients. Correlation analysis suggested that Tregs frequency and function positively correlated with LVEF, whereas negatively correlated with LVEDD and NT-proBNP in patients with CHF. **Conclusions:** Our data are the first to demonstrate that frequencies of circulating Tregs in patients with CHF are reduced and their suppressive function compromised independently of the etiology. Defective Tregs may be an underlying mechanism of immune activation in CHF patients.

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Introduction

Tregs are a subgroup of T cells with suppressive effect on immune response. In addition to other subpopulation of Tregs, including interleukin-10 producing Tr1, transforming growth factor β producing Th3 cells,

CD8⁺ T suppressor cells, natural killer T cells, CD4-CD8- T cells and $\gamma\delta$ T cells [1], CD4⁺CD25⁺ Foxp3⁺ Tregs are the most well-documented. Recently, it was reported that the absence or low expression of CD127 can be used as a new and reliable marker of human Tregs [2]. Previous studies have demonstrated altered status of Tregs in several autoimmune diseases, infectious diseases and tumors [3-8]. These studies suggest that status of circulating Tregs may be used as a marker for immune status in these patients. While immune activation and persistent inflammation are believed to contribute to the progression of chronic heart failure (CHF) [9], mechanisms for the observed immune activation remain to be elucidated. Yndestad et al [10] have reported that T cells in patients with CHF exhibited an enhanced expression of inflammatory cytokines in CHF. We and others have demonstrated a systemic T-helper (Th) 1 cell immune response in CHF [11-12]. Since previous study has reported that deficiency of Tregs could result in increased T cells activity in normal subjects [13], one possibility to explain the T cells activation in CHF could be deficient of Tregs. So far, there was no previous report concerning Tregs in CHF. We therefore attempt to investigate any changes in the frequency and/or function of circulating Tregs in patients with CHF.

Materials and Methods

Subjects

This study consisted of 99 CHF patients due to non-ischemic or ischemic etiology. Diagnosis of CHF was based on clinical history, physical examination and results of echocardiography, chest X-ray, electrocardiography and N-terminal pro-brain natriuretic peptide (NT-proBNP). Patients were classified as NIHF (n=57, 33 men and 24 women, mean $\pm$ SD age, 51 $\pm$ 12 years) if they had no history of myocardial infarction and absence of significant coronary artery stenosis on coronary angiography, including 40 patients with dilated cardiomyopathy and 17 patients with hypertensive heart disease. Patients were classified as IHF (n=42, 28 men and 14 women, mean $\pm$ SD age, 54 $\pm$ 7 years) if the coronary angiography presented significant coronary artery disease (> 50% stenosis in more than one major epicardial coronary artery) or the patients have history of myocardial infarction or previous revascularization. All patients were stable on optimal treatment for at least 3 months. Patients were excluded from the analysis if they were treated with anti-inflammatory drugs such as non-steroidal anti-inflammatory drugs and steroids or had collagen diseases, thromboembolism, disseminated intravascular coagulation, advanced liver disease, renal failure, malignant disease, other inflammatory disease (such as septicemia, pneumonia, etc.), valvular heart disease, atrial fibrillation or were using a pacemaker. Patients who

received statin therapy within three months were also excluded. For comparison, we also included 24 age- and sex- matched controls (14 men and 10 women, mean $\pm$ SD age, 50 $\pm$ 11 years). The investigation conforms with the principles outlined in the Declaration of Helsinki. The trial was approved by the country's ethics committee and patients or controls provided written informed consent.

Sample preparation and peripheral blood mononuclear cells (PBMCs) isolation

Blood samples were collected and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation and then were used for flow cytometric analysis, real-time polymerase chain reaction (PCR) or cell culture. Serum was obtained after centrifugation and stored at -80 °C for the measurement of NT-proBNP.

Flow cytometric analysis

Cells were stained with surface markers as anti-CD4-PerCP, anti-CD25-FITC, anti-CD127-Alexa Fluor® 647 (eBioscience), for 20 min at 4°C. After the surface staining, the cells were stained with anti-Foxp3-PE (eBioscience) after fixation and permeabilization according to the manufacturer's instructions. Isotype controls were given to enable correct compensation and confirm antibody specificity. Stained cells were analyzed by flow cytometry with FACS Calibur (BD).

Foxp3 expression by real-time PCR

Total cell RNA was extracted using Trizol lysis buffer according to manufacturer's instruction. cDNA was synthesized using random hexamer primers and RNase H-reverse transcriptase (Invitrogen) and mRNA expression of Foxp3 was assayed by real-time PCR using SYBR Green Master Mix (Takara). Primer pairs were as follows:

Foxp3: F, 5'-GAAACAGCACATTCCCAGAGTTC-3'

R, 5'-ATGGCCCAGCGGATGAG-3'

GAPDH: F, 5'-CCACATCGCTCAGACACCAT-3'

R, 5'-GGCAACAATATCCACTTTACCAGAGT-3'

Data were analyzed on an ABI Prism 7900 Sequence Detection system (Applied Biosystems). For each sample, mRNA expression level was normalized to the level of GAPDH housekeeping genes. Means of duplicate measurements were normalized and expressed as a ratio of Foxp3 mRNA copies/GAPDH mRNA copies.

Electrochemiluminescence immunoassay of serum NT-proBNP

Serum NT-proBNP was measured using the Roche Elecsys Electrochemiluminescence immunoassay (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions.

Cell separation

In some experiments, CD4⁺CD25⁻ T cells and CD4⁺CD25⁺CD127^{low} Tregs were MACS-sorted using a CD4⁺CD25⁺CD127^{dim/-} Regulatory T Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions. Briefly, PBMCs were first incubated with biotin labeled cocktail antibodies and CD4⁺CD127^{low} T cells were isolated by a step

Characteristics	Control group (n=24)	CHF group (n= 99)	NIHF group (n=57)	IHF group (n=42)
Age, y	50±11	52±9	51±11	54±7
Male/Female, %	14/10, 58/42	61/38, 62/38	33/24, 58/42	28/14, 67/34
Hypertension, No. (%)	5 (21)	38 (37)	17 (27)	21 (50) [#]
NHYA classification II/III/IV		29/51/19	17/29/11	12/22/8
LVEF (%)	62.08±4.85	37.30±6.95*	37.23±7.13*	37.40±6.78*
LVEDD, mm	47.37±3.82	64.00±5.86*	64.86±6.22*	62.83±5.18*
TC, mmol/L	4.14±0.46	4.22±0.48	4.20±0.49	4.27±0.46
HDL cholesterol, mmol/L	1.10±0.24	1.19±0.26	1.15±0.25	1.23±0.26
LDL cholesterol, mmol/L	2.42±0.41	2.49±0.47	2.52±0.53	2.45±0.38
Triglycerides, mmol/L	1.26±0.36	1.23±0.26	1.20±0.25	1.25±0.28
NT-proBNP, pg/ml	184.92±143.92	3776.49±2469.15*	3812.53±2529.37*	3727.57±2414.55*
Medications				
ACEI/ ARBs	4 (17)	88 (88) *	53 (93) *	35 (83) *
Antisterone	0 (0)	56 (57) *	32 (56) *	24 (57) *
Digitalis	0 (0)	39 (39) *	23 (40) *	16 (38) *
β-Blocker	3 (13)	75 (76) *	44 (77) *	31 (74) *
Diuretics	0 (0)	82 (83) *	46 (81) *	36(86) *

Table 1. Clinical characteristics of study population. Values are presented as mean ± SD, **p*<0.05 vs. control group, [#]*p*<0.05 vs. NIHF group. NYHA, New York Heart Association; LVEF, left ventricular ejection fraction; LVEDD, left ventricular end-diastolic dimension; TC, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NT-ProBNP, N-terminal Pro B-type natriuretic peptide; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker.

of negative selection, then CD25⁺ T cells were removed by positive selection after incubation with streptavidin labeled anti-CD25. Purified CD4⁺CD25⁺ T cells and CD4⁺CD25⁺CD127^{low} Tregs were used for cell culture, proliferation and suppression assay.

Proliferation and suppression assays

In proliferation assay, CD4⁺CD25⁺ cells (1×10⁵/well) were cultured with plate-bound (5 µg/ml) anti-CD3 and soluble anti-CD28 (2 µg/ml) in complete RPMI1640 at 37 °C in 5% CO₂ for 72h triplicately in U-bottom 96-well plates. One µCi of [³H]-thymidine (Amersham Biosciences, Piscataway, NJ) was added to each well 16 h before harvesting and the incorporation of [³H]-thymidine was assayed by scintillation counting.

Suppression assays using human Tregs were performed essentially as previously described [14]. Briefly, T cell proliferation was induced by stimulation with a combination of plate-bound anti-CD3 and soluble anti-CD28 antibodies in U-bottom 96-well plates in duplicate at a CD4⁺CD25⁺ T cells (responder T cells)/ CD4⁺CD25⁺CD127^{low} T cells (Tregs) ratio of 1:1, 2:1 and 4:1 in a final volume of 200 µl. After 72h, one µCi of [³H]-thymidine was added to each well 16h before harvesting and the incorporation of [³H]-thymidine was assayed by scintillation counting.

Cytokines detection

1×10⁵ MACS sorted CD4⁺CD25⁺CD127^{low} T cells (Tregs) and 1×10⁵ CD4⁺CD25⁺ T cells were co-cultured with plate-bound

anti-CD3 (5 µg/ml) and soluble anti-CD28 (2 µg/ml) antibodies in U-bottom 96-well plates in a final volume of 200 µl. Three days later, culture supernatants were collected and tumor necrosis factor-α (TNF- α) and interferon- γ (IFN- γ) were quantified using enzyme-linked immunosorbent assay (ELISA) kits (ebioscience) according to the manufacturer's instructions.

Statistical Analyses

Values are expressed as means ± standard deviation (SD) in the text and figures. One-way ANOVA (n>2 groups) or student's *t* test was used for statistical analysis of group comparisons. Spearman's correlation analysis was performed to test for the correlation between the variables. In all cases, two-tailed *p*<0.05 was considered significant.

Results

Basic clinical characteristics

Basic clinical characteristics of study population are summarized in Table 1. CHF patients and controls were matched for age, sex and blood lipids. Compared with controls, CHF patients showed significantly lower left ventricular ejection fraction (LVEF), larger left ventricular end-diastolic dimension (LVEDD) and higher NT-proBNP.

Fig. 1. Decreased frequency of circulating CD4⁺CD25⁺Foxp3⁺CD127^{low} Tregs and reduced Foxp3 expression in PBMCs in patients with CHF. a. Representative FACS pictures from a single person in CHF group and control group. b. Frequency of circulating Tregs decreased significantly in CHF patients, but it was not the case with the proportion of CD4⁺CD25⁺ T cells. c. Reduced Foxp3 mRNA expression in PBMCs in CHF patients detected by real-time PCR (**p*<0.05 vs. controls).

The frequency of circulating Tregs significantly decreased in patients with CHF

Cell surface phenotype analyses of PBMCs isolated from CHF patients and controls to encompass human Tregs (CD4⁺CD25⁺Foxp3⁺CD127^{low}) in a 4-color assay clearly showed a reduction in the number of such cells in CHF patients compared with controls (CHF vs. control: 1.57±0.83% vs. 5.59±2.47% of CD4⁺ T cells, *p*<0.05). However, no significant difference in the number of CD4⁺CD25⁺ T cells between CHF patients and controls was observed (CHF vs. control: 10.16±3.47% vs. 11.17±4.22% of CD4⁺ T cells, *p*>0.05). We confirmed our data by real-time PCR, which suggested that Foxp3 expression in PBMCs was significantly lower in the CHF patients than that in the controls (Fig. 1a-c).

In CHF patients, the proportion of Tregs was not significantly different between NIHF group and IHF (NIHF vs. IHF: 1.51±0.81% vs. 1.66±0.86%, *p*>0.05), and so was the proportion of CD4⁺CD25⁺ T cells (NIHF vs. IHF: 10.33±3.51% vs. 9.92±3.45%, *p*>0.05) (Fig. 2).

Fig. 2. Numbers of circulating CD4⁺CD25⁺ T cells and Tregs were not significantly different between NIHF group and IHF group.

Tregs display compromised function in patients with CHF

Quantitative analysis of the suppressive function of Tregs was performed by co-culturing MACS-sorted CD4⁺CD25⁺CD127^{low} Tregs with autologous CD4⁺CD25⁺ T responder cells stimulated by anti-CD3/28 at different ratios (suppressor cells: responder cells: 1:1, 1:2 and 1:4).

Fig. 3. Tregs from CHF patients exhibited reduced capacity to suppress proliferation. CD4⁺CD25⁺CD127^{low} cells and CD4⁺CD25⁻ responder cells from CHF patients (15 patients from NIHF group and 10 from IHF group) and controls (n=15) were purified by MACS-sorting for suppression and proliferation assay. a. Reduced suppressive function of CD4⁺CD25⁺CD127^{low} Tregs from CHF patients suggested by suppression assay. b. Similar proliferative capacity of CD4⁺CD25⁻ responder T cells between CHF group and control group. c. Tregs from NIHF group and IHF group showed similar suppressive capacity on proliferation.

Fig. 4. Tregs from CHF patients were less effective to inhibit cytokines production. Sorted CD4⁺CD25⁻ T cells from controls were cultured alone (1:0) or with CD4⁺CD25⁺CD127^{low} Tregs (1:1) from CHF patients (7 patients from NIHF group and 8 from IHF group) and controls (n=15). a. Comparison of cytokines production by activated CD4⁺CD25⁻ T cells from CHF patients and controls. b. c. Percentage of inhibition of the cytokines production relative to CD4⁺CD25⁻ T cells stimulated alone (**p*<0.05 vs. controls).

Tregs from CHF patients were less effective suppressor with reduced ability to suppress proliferation of CD4⁺CD25⁻ T cells. The reduced regulatory function of Tregs to suppress proliferation in CHF patients could be ascribe to an reduced proliferation of CD4⁺CD25⁻ T responder cells to polyclonal anti-CD3/28. We next assessed the proliferation of CD4⁺CD25⁻ T cell activated by anti-CD3/28, and no significant difference was found between the two groups. There was no significant difference in the ability of Tregs to suppress proliferation between the NIHF group and IHF group (Fig. 3).

Pro-inflammatory cytokines such as TNF-α and IFN-γ play an essential role in the pathogenesis of CHF, we also investigated whether Tregs from CHF patients were effective to suppress TNF-α and IFN-γ production

by CD4⁺CD25⁻ T effectors. As CD4⁺CD25⁻ T effectors from CHF patients and controls may differ in the ability to produce TNF-α and IFN-γ upon activation, we firstly measured the production of cytokines by activated T effectors from the two groups. The result suggested an increased production of TNF-α and IFN-γ in activated T effectors from CHF patients compared with controls. Therefore, Tregs from the two groups were co-cultured with CD4⁺CD25⁻ T effectors from CHF patients. In agreement with the results of suppression assay, Tregs from the CHF patients were less effective to inhibit the production of the two cytokines compared with controls. There was no significant difference in the ability of Tregs to suppress cytokines production between the NIHF group and IHF group (Fig. 4).

Fig. 5. Spearman correlation of Tregs and indexes of cardiac function in CHF patients. a-c. Percentage of Tregs in CD4⁺ T cells was negatively correlated with LVEDD ($r = -0.506$, $p < 0.05$) and NT-proBNP ($r = -0.399$, $p < 0.05$), whereas it was positively correlated with LVEF ($r = 0.450$, $p < 0.05$); c-e. Suppressive function of Tregs on proliferation when they were cultured with T responders at the ratio of 1:1 was negatively correlated with LVEDD ($r = -0.447$, $p < 0.05$) and NT-proBNP ($r = -0.609$, $p < 0.05$), whereas it was positively correlated with LVEF ($r = 0.475$, $p < 0.05$).

Tregs correlated with cardiac function in CHF patients

Next, we performed correlation analysis of Tregs frequency, suppressive function on proliferation (Tregs: T responders: 1:1) and the indexes of cardiac function in patients with CHF. Correlation analysis showed that both the Tregs frequency and function were positively correlated with LVEF, whereas they were negatively correlated with LVEDD and NT-proBNP in patients with CHF (Fig. 5).

Discussion

The present work demonstrated, for the first time, that circulating Tregs in patients with CHF decreased in number and their suppressive capacity on proliferation and pro-inflammatory cytokines production compromised. CHF patients in the NIHF and IHF groups showed a similar pattern of Tregs deficiency which seemed to be independent of the etiology. In addition, we observed a significant inverse correlation for the proportion and suppressive function of Tregs with cardiac function. Our findings suggest that deficiency of Tregs may be a sign of disturbed immune homeostasis in CHF, possibly playing a potential role in the immune activation and the progression of this disorder.

As the final common pathway of most cardiovascular diseases, CHF is a complex multi-step disorder in which several physiologic systems participate in its pathogenesis. Systemic inflammation plays an important role in the development and progression of this disorder [15]. Many lines of evidence have indicated that elevated circulating inflammatory cytokines play potential role in the progression of CHF [16, 17]. T cells, which play a central role in the adaptive immunity, were identified as a critical cellular source for the persistent inflammation in CHF by clinical and experimental evidences. Circulating T cells in CHF patients are markedly activated as evidenced by enhanced gene expression of both chemokines and inflammatory cytokines as well as by surface expression of activated markers [10]. Endomyocardial biopsies from patients with idiopathic dilated cardiomyopathy (DCM) showed infiltration of T cells and transfer of peripheral blood lymphocytes from DCM patients to severe combined immunodeficiency (SCID) mice lead to ventricular remodeling [18]. Lymphocytes from rats with IHF can recognize and kill normal neonatal rat cardiac myocytes *in vitro* [19] and lead to autoimmune myocarditis *in vivo* after adoptive transfer [20]. TNF- α is the best studied inflammatory cytokine and closely correlates with the severity of CHF [21]. Satoh et al. have reported that T cells from CHF patients showed increased production of TNF- α [22]. We and others have demonstrated an increased

proportion of IFN- γ ⁺ Th1 cells in CHF patients which correlated with the severity of the disorder [11, 12, 23]. Corresponding to the previous studies, we observed that T cells from CHF patients had increased production of TNF- α and IFN- γ during activation compared with controls in the present work.

Recently, Tregs have been showed to play a critical role in the control of systemic inflammation and pathogenic T cells activation [24]. Several studies have addressed the role of Tregs in modulate pathogenic T cells responses. Selective depletion of Tregs prevents animals from cerebral malaria during a second parasitic challenge induced by pathogenic T helper 1 cells [25]. Adoptive transfer of Tregs inhibits the differentiation of autoantigen-specific T cells to T effectors [26]. In the present work, we observed a reduced number of Tregs with compromised function in CHF patients. Moreover, correlation analysis showed that both the Tregs frequency and function were positively correlated with LVEF, whereas they were negatively correlated with LVEDD and NT-proBNP in patients with CHF, suggesting that defective Tregs correlated with the severity of heart failure. Based on our data, one may speculate that defective Tregs may be involved in the disturbed immune regulation and responsible for the uncontrolled T cells activation in CHF, which further lead to myocardial injury and aggravation of cardiac function. Our study demonstrated a defective circulating Tregs in both non-ischemic and ischemic heart failure. One explanation for this may be that immune activation in CHF is independently of the etiology and represents a common pathogenic feature and potentially also a common pathogenic pathway. T helper 17 cells (Th17) are a newly discovered subset of T helper cells with opposite actions of Tregs [27]. The imbalance between protective Treg and pathogenic Th17 may contribute the pathogenesis of inflammation [28]. Our previous work has demonstrated a Th17/Treg imbalance in patients with acute coronary syndrome [29]. However, there were no

significant differences in the frequency of circulating Th17, serum level of Th17 related cytokine (interleukin-17) and expression of its key transcriptional factor (retinoic acid-related orphan receptor γ t) in PBMCs between CHF patients and controls (data not shown).

Tregs are firstly found within the CD4⁺CD25⁺ population by Sakaguchi et al [30]. As CD25 is also expressed on activated T cells, the additional measurement of Foxp3 expression allowed a more specific analysis of Tregs [31]. CD127, a new tool to identify Tregs, correlates well with Foxp3 and their regulatory function [2]. Corresponding to the previous study, our data demonstrated that CD127 were low expressed on nearly all CD4⁺CD25⁺Foxp3⁺ T cells and sorted CD4⁺CD25⁺CD127^{low} T cells could suppress proliferation of T responders in vitro. It should be addressed that patients receiving statins therapy within three months were excluded from our study as statins have been demonstrated to increase human naturally occurring Tregs by a recent research [32].

In conclusion, this study is the first to demonstrate that frequencies of circulating Tregs in patients with CHF are reduced and their suppressive function compromised, which may underlie the systemic inflammation and T cell activation in this disorder and contribute to its pathogenesis. Further studies are necessary to establish the mechanism of deficit of Tregs in CHF patients and its correlation with systemic inflammation and immune activation.

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