Impaired Thymic Export and Apoptosis Contribute to Regulatory T-Cell Defects in Patients with Chronic Heart Failure

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Abstract

Objective: Animal studies suggest that regulatory T (Treg) cells play a beneficial role in ventricular remodeling and our previous data have demonstrated defects of Treg cells in patients with chronic heart failure (CHF). However, the mechanisms behind Treg-cell defects remained unknown. We here sought to elucidate the mechanism of Treg-cell defects in CHF patients.

Methods and Results: We performed flow cytometry analysis and demonstrated reduced numbers of peripheral blood CD4+CD25+FOXP3+CD45RA− naive Treg (nTreg) cells and CD4+CD25+FOXP3−CD45RA+ memory Treg (mTreg) cells in CHF patients as compared with non-CHF controls. Moreover, the nTreg/mTreg ratio (p<0.01), CD4+CD25+FOXP3+CD45RA− CD45RA+ recent thymic emigrant Treg cell (RTE-Treg) frequency (p<0.01), and T-cell receptor excision circle levels in Treg cells from CHF patients than in non-CHF controls. Combined annexin-V and 7-AAD staining showed that peripheral Treg cells from CHF patients exhibited increased spontaneous apoptosis and were more prone to interleukin (IL)-2 deprivation- and CD95 ligand-mediated apoptosis than those from non-CHF individuals. Furthermore, analyses by both flow cytometry and real-time polymerase chain reaction showed that Treg-cell frequency in the mediastinal lymph nodes or Foxp3 expression in hearts of CHF patients was no higher than that of the non-CHF controls.

Conclusion: Our data suggested that the Treg-cell defects of CHF patients were likely caused by decreased thymic output of nascent Treg cells and increased susceptibility to apoptosis in the periphery.

Introduction

Chronic heart failure (CHF) is regarded as a state of chronic inflammation with elevated T-cell activation and inflammatory cytokine production in the circulatory system [1,2]. However, the pathogenic mechanisms responsible for this abnormal immune activation remain unknown. Treg cells represent a unique lineage of T cells that play an essential role in the modulation of immune responses and the control of potentially harmful immune activations because of their immunoregulatory and immunosuppressive characteristics [3]. Among the several types of Treg cells that have been defined, one particular subset that constitutively expresses CD4, CD25 and the transcription factor Foxp3 has received much attention. Alterations in CD4+CD25+Foxp3+ Treg− cell number or function is directly associated with the pathogenesis of several common human diseases, including acute coronary syndrome (ACS) [4,5], multiple sclerosis [6], type 1 diabetes [7], and rheumatoid arthritis [8]. Adoptive transfer of purified Treg cells suppresses immune injury and improves recovery in animal disease models [9–12].
Adverse ventricular remodeling occurs upon acute and chronic injury regardless of etiology, and it is related to poor prognosis of patients with heart failure [13]. There is compelling evidence that inflammatory mechanisms contribute to the process of adverse ventricular remodeling [14]. In animal models of heart failure, previous studies demonstrated that Treg cells could be a target of heart failure therapies because CCR5-mediated Treg-cell recruitment in the infarcted heart [15] and adaptively transferred Treg cells [16] provided protection from adverse cardiac remodeling by preventing expansion of inflammation and fibrosis after adoptive transfer. In a previous publication, we found that circulating Treg cells were reduced and their function was altered in CHF patients, regardless of etiology, suggesting that the defects in Treg cells are responsible for the aberrant chronic immune activation in CHF patients [17]. It is believed that the understanding of mechanisms underlying Treg-cell defects in CHF patients is of great significance, especially with respect to therapy through Treg-cell manipulation. In the present study, we attempt to explore the mechanisms that might account for the Treg-cell defects in CHF patients by studying Treg-cell production, survival, and tissue reallocation in these patients.

Results

1. Reduced nTreg, mTreg, and RTE-Treg-cell frequency in CHF patients

To determine the number of total Treg cells and Treg subsets, PBMCs were obtained from 52 CHF patients and 43 age-matched non-CHF controls followed by 6-color flow cytometric analysis. Basic clinical characteristics of the study population are summarized in Table 1. Within the naive CD4+CD45RA-CD45RO- (R1 in Figure 1A) or memory CD4+CD45RA-CD45RO+ (R2 in Figure 1A) T cells, a small subpopulation of cells with high expression of both CD25 and Foxp3 could be readily detected. mTreg cells were characterized as CD4+CD25-Foxp3+CD45RA-CD45RO+ cells (R3 in Figure 1B, upper panel) and nTreg cells were characterized as CD4+CD25-Foxp3-CD45RA-CD45RO- cells (R4 in Figure 1B, lower panel). nTreg cells exhibited a lower expression of CD25 compared with mTreg cells (mean fluorescent intensity, MFI: nTreg vs. mTreg = 24.1 ± 5.4 vs. 54.6 ± 8.7, P < 0.01). RTE-Treg cells were identified as CD31 co-expressing nTreg cells (Figure 1C). The proportion of Treg cells in total CD4+ T cells was significantly decreased in CHF patients when compared with non-CHF subjects (Figure 1D). The percentages of nTreg and mTreg cells within CD4+ T cells were also significantly lower in CHF patients than in age-matched non-CHF subjects (non-CHF vs. CHF: nTreg: 1.17 ± 0.41% vs. 0.59 ± 0.31%, P < 0.01; mTreg: 3.27 ± 0.92% vs. 2.02 ± 0.65%, P < 0.01; Figure 1D). CHF patients showed a significantly lower nTreg/mTreg ratio (non-CHF vs. CHF: 36 ± 10% vs. 30 ± 12%, P < 0.05; Figure 1E). Furthermore, we observed that proportions of RTE-Treg cells in the total Treg-cell population in CHF patients were significantly reduced when compared to age-matched, non-CHF controls, suggesting that thymic production of Treg cells was impaired in CHF patients (non-CHF vs. CHF: 4.97 ± 2.34% vs. 3.00 ± 0.97%, P < 0.01; Figure 1F). However, no difference in total Treg, nTreg, mTreg, and RTE-Treg cells between IHF or NHF patients was observed (Figure 1D–F). Similar results were obtained when we compared the absolute numbers of total Treg, nTreg, mTreg and RTE-Treg cells between CHF patients and non-CHF controls (Table 2).

Consistent with our previous report [17], we observed that total Treg number was negatively correlated with NT-proBNP in CHF patients. Furthermore, the present study also found that NT-proBNP and nTreg or mTreg numbers were negatively correlated (Table 3).

2. Decreased intracellular TREC levels in Treg cells from CHF patients

TREC is a marker for nascent thymic T cells [18]. We studied intracellular levels of TRECs in Treg cells isolated from 25 CHF patients and 15 age-matched non-CHF subjects using quantitative real-time PCR. Flow cytometry was used to determine the purity of Treg cells after cell sorting (Figure 2A, left panel). The TREC content in Treg cells was significantly lower in CHF than that in non-CHF patients (non-CHF vs. CHF patients: 1.38 ± 0.49 × 105/106 cells vs. 0.60 ± 0.32 × 105/106 cells, P < 0.01; Figure 2A, right panel). There was no significant difference in Treg-cell TREC content between IHF and NHF patients. Spearman’s correlation test revealed a positive association between Treg-cell TREC level and RTE-Treg cell proportion in both CHF patients and non-CHF controls (r = 0.75, P < 0.001; Figure 2B).

3. Increased spontaneous and IL-2 deprivation/Fas-mediated apoptosis in Treg cells from CHF patients

Increased apoptosis and decreased survival could be a mechanism of Treg-cell defects in CHF patients. Because of the fixation and permeabilization procedures used for detecting Treg cells using Foxp3 antibodies by FACS, we detected apoptotic Treg cells with antibodies against CD127, a newly identified Treg surface marker that correlates well with Foxp3 [19]. In both CHF patients and non-CHF controls, CD4+CD25+Foxp3+ Treg cells were correlated with CD4+CD25+CD122low/- Treg cells (r = 0.91, P < 0.001, Figure 3). When we gated the CD4+CD25+CD122low/- cells, we found that when this cell population was derived from CHF patients, irrespective of the etiology, there was a significantly higher percentage of apoptotic annexin V-7-AAD+ cells than when derived from non-CHF controls (non-CHF vs. CHF patients: 8.79 ± 3.37% vs. 14.78 ± 4.08%, P < 0.01; Figure 4A).

Enhanced apoptosis often correlates with altered expression of apoptosis-associated genes. We compared the levels of anti-apoptotic gene Bcl-2 and pro-apoptotic gene Bax expression between CD4+CD25+CD122low/- Treg cells isolated from CHF patients and non-CHF controls. Significantly lower Bcl-2 expression (P < 0.01) and higher Bax expression (P < 0.01) were observed in Treg cells from CHF patients when compared with those from non-CHF controls (Figure 4B).

IL-2 is essential for the development, function and homeostasis of Treg cells [20]. However, human Treg cells do not produce this cytokine and therefore are susceptible to IL-2 deprivation, which leads to Treg-cell apoptosis [21]. Treg cells from CHF patients and non-CHF controls might exhibit different susceptibilities to IL-2 deprivation. To test this hypothesis, we incubated Treg cells from the different patient populations with anti-human IL-2 monoclonal antibodies for 3 days. Treg cells from CHF patients were more sensitive to IL-2 deprivation-induced apoptosis when compared with Treg cells from non-CHF subjects (non-CHF vs. CHF: 22.3 ± 4.12% vs. 33.26 ± 5.89%, P < 0.01; Figure 5A). Treg-cell apoptosis could also be induced by the interactions between death receptor CD95 with the CD95 ligand (CD95L) [22]. Human Treg cells constitutively express these death receptors and are thus highly sensitive to CD95L-mediated apoptosis [23]. Increased apoptosis in Treg cells from CHF patients suggests that Treg cells from these patients express high levels of CD95 and/or are more sensitive to CD95L. To test this hypothesis, we compared the expression level of CD95 and sensitivity toward CD95L-triggered apoptosis in Treg cells from CHF patients and non-CHF controls.
Mechanisms for Treg Defects in CHF Patients

Table 1. Clinical characteristics of study population.

|                      | CHF patients (n = 52) | NIHF patients (n = 32) | IHF patients (n = 20) | Non-CHF controls (n = 43) |
|----------------------|-----------------------|------------------------|-----------------------|--------------------------|
| Age (year)           | 44±13                 | 40±14                  | 51±8                  | 42±12                    |
| Gender (Male/Female) | 31/21                 | 17/15                  | 14/6                  | 28/15                    |
| NYHA (I/III/IV)      | 25/21/6               | 12/16/4                | 13/5/2                | —                        |
| LVEF (%)             | 35.38±6.24            | 35.06±6.41             | 35.9±6.09             | —                        |
| LVEDD (cm)           | 6.11±0.47             | 6.16±0.5               | 6.02±0.41             | —                        |
| Hypertension (%)     | 37                    | 16                     | 70                    | 0                        |
| NT-proBNP (pg/ml)    | 2955.52±1971.55       | 2756.33±1628.37        | 3176.8±2442.88        | —                        |
| Medication (%)       | ACEI/ARBs             | 90                     | 91                    | 90                       | 0                        |
|                      | Antisterone           | 42                     | 47                    | 35                       | 0                        |
|                      | Digitalis             | 31                     | 28                    | 35                       | 0                        |
|                      | β-Blocker             | 87                     | 88                    | 85                       | 0                        |
|                      | Diuretics             | 73                     | 50                    | 80                       | 0                        |

Data is presented as mean±SD, or number or percentage of patients or healthy controls (HCs). NIHF: non-ischemic heart failure; IHF: ischemic heart failure; NYHA, New York Heart Association; LVEF, left ventricular ejection fraction; LVEDD, left ventricular end-diastolic dimension; NT-ProBNP, N-terminal Pro B-type natriuretic peptide; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker.

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non-CHF controls. CD95 expression on Treg cells from CHF patients was significantly higher than on Treg cells from non-CHF controls (non-CHF vs. CHF: 73.78±8.12% vs. 84.30±6.67%, p=0.01; Figure 5B). CD95L induced apoptosis of Treg cells from CHF patients in a dose- and time-dependent manner (Figure 5C). CD95L initiated Treg-cell apoptosis in 3 hrs, but apoptosis reached a peak after 12 hrs of induction. When incubated with 100 ng/ml of CD95L for 12 hrs, Treg cells prepared from CHF patients showed higher percentages of cells undergoing CD95L-induced apoptosis than in non-CHF subjects (non-CHF vs. CHF patients: 19.43±6.87% vs. 36.52±12.03%, p<0.01; Figure 5D). These observations could explain the increased Treg-cell apoptosis in CHF patients (Figure 4A). Furthermore, we detected significantly higher plasma levels of soluble CD95L in CHF patients than in non-CHF controls (non-CHF vs. CHF patients: 77.28±5.26% vs. 101.22±5.06%, p<0.01; Figure 5E). Among the CHF subgroups IHH and NIHF, we did not detect any differences in either IL-2 deprivation- or CD95-mediated Treg-cell apoptosis (Figure 5A/5D). Plasma CD95L levels were also similar between CHF, IHH, and NIHF patients (Figure 5E). Taken together, these findings suggest that Treg cells from CHF patients were more prone to apoptosis and that IL-2 and CD95/CD95L might be involved in regulation of Treg-cell survival.

4. Treg cells accumulate neither in mediastinal lymph nodes nor in failing hearts

One possible explanation for reduced Treg-cell number in CHF patients is the reallocation of these cells to the lymph nodes or disease-affected organs. We compared the proportion of CD4+CD25+Foxp3+ Treg cells to total CD4+ T cells in the mediastinal lymph nodes from CHF patients and non-CHF controls. Mediastinal lymph node Treg cells from CHF patients were significantly fewer than from non-CHF controls (Figure 6A/6B). Total lymphocyte Foxp3 mRNA levels were also significantly lower in CHF, IHH and NIHF patients than in non-CHF controls (Figure 6C). To examine whether Treg cell accumulation in the heart was different between CHF and non-CHF controls, Foxp3 RT-PCR was performed on biopsied cardiac samples. No difference was found between failing hearts and hearts from donors, although Foxp3 levels were low in all tested heart samples (Figure 6C).

Discussion

As the final common pathway of many cardiovascular diseases, CHF is a complex multi-step disorder and several mechanisms participate in its pathogenesis. There is compelling evidence that inflammation and autoimmune abnormalities play an important role in the progression of heart failure [1,24,25]. Various autoantibodies, which are directed against different cardiac antigens, such as cardiac myosin, cardiac troponin I, cardiolipin, beta1-adrenergic and M2 muscarinic receptors can be detected in the serum of patients with NIHF or IHF [26–29]. These autoantibodies can lead to cardiac injury, and they correlate with the deterioration of cardiac function. Other autoimmune abnormalities include infiltration of T cells in endomyocardial biopsies from patients with idiopathic dilated cardiomyopathy (DCM). Additionally, the transfer of peripheral blood lymphocytes from DCM patients to severe combined immunodeficiency (SCID) mice leads to ventricular remodeling [30]. In animal models, lymphocytes from rats with IHH can recognize and kill normal neonatal rat cardiac myocytes in vitro [31] and lead to autoimmune myocarditis in vivo after adoptive transfer [32].

Treg cells play a key role in the control of inflammation and autoimmune responses, and altered Treg cells predispose patients for uncontrolled immune activation or autoimmunity [3]. CHF patients were previously reported to have impaired Treg-cell number and function, but the precise mechanism behind this defect remains largely unknown [17]. In this study, we showed that reduced Treg cell number and function in CHF patients might be explained by impaired Treg-cell thymic output and increased apoptosis of these cell populations.

Like other T cells, Treg cells develop in the thymus [33]. A small fraction of Treg cells with a naive CD45RA+CD150+ surface profile (nTreg) has recently been detected in the circulation. However, this nTreg subset declines with age, as does thymic...
output and other naïve T cells [34]. By contrast, the majority of circulating Treg cells appear as a mature population with a memory CD45RA CD45RO phenotype; these mTreg cells are stable throughout the life span, and the levels of mTreg cells increase during aging [35,36]. nTreg cells could represent the de novo generation of thymic lymphocytes, so the assessment of nTreg.

Table 2. Absolute number of Treg, nTreg, mTreg and RTE-Treg in the study population.

|                  | CD4⁺ T cells (10⁶/L) | Treg (10⁶/L) | nTreg (10⁶/L) | mTreg (10⁶/L) | RTE-Treg (10⁶/L) |
|------------------|----------------------|--------------|---------------|---------------|------------------|
| CHF patients (n = 52) | 341.95±206.28 | 9.92±5.78  | 1.92±1.24* | 6.84±4.30* | 0.31±0.24* |
| NIHF patients (n = 32) | 346.55±205.81 | 10.52±6.26* | 2.06±1.37* | 7.38±4.81* | 0.35±0.29* |
| IHF patients (n = 20) | 334.60±212.16 | 8.96±4.92* | 1.69±0.97* | 5.97±3.27* | 0.25±0.13* |
| Non-CHF controls (n = 43) | 289.99±155.36 | 13.96±7.6  | 3.33±2.07  | 9.51±5.49  | 0.70±0.43 |

Data is presented as mean±SD. Treg regulatory T cells; nTreg naïve Treg; mTreg memory Treg; RTE-Treg recent thymic emigrant Treg.*p<0.05 vs. non-CHF controls.
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Mechanisms for Treg Defects in CHF Patients

Table 3. Correlation analysis between Treg or its subset frequency and NT-proBNP in CHF patients.

|         | Treg | nTreg | mTreg |
|---------|------|-------|-------|
| Coefficients | -0.589 | -0.557 | -0.446 |
| p values | <0.01 | <0.01 | <0.01 |

NT-proBNP, N-terminal Pro B-type natriuretic peptide; Treg, regulatory T cells; nTreg, naive Treg; mTreg, memory Treg.

For example, intrathyroidal CD4+ Treg cells from patients with autoimmune thyroid diseases were prone to apoptosis, which led to a local Treg-cell reduction [40]. In contrast, patients with metastatic epithelial cancer demonstrated a significantly elevated proportion of peripheral Treg cells, and these cells were protected from apoptosis [41]. Apoptosis not only reduces the number of Treg cells, but also reduces their functions. By using T-effector cell suppression assays, Treg-cell apoptosis was closely associated with their capacity to inhibit T-effector cell proliferation [42]. In patients with type 1 diabetes, an increase in apoptosis was correlated with a decline in the suppressive potential of Treg cells [43]. As suggested by these studies, high sensitivity to IL-2 deprivation or FasL-induced apoptosis may contribute in part to the defect of Treg cells in CHF patients. Treg cells from CHF patients were more susceptible to apoptosis following IL-2 deprivation. Upon antigen activation, T cells induce the expression of CD95, a member of the tumor necrosis factor receptor/nerve growth factor receptor superfamily that induces apoptosis by binding to CD95L and subsequently activating.

The homeostasis of Treg cells is maintained by a balance between Treg-cell generation and depletion. Apoptosis-induced alteration of Treg-cell levels has been associated with several diseases. For example, intrathyroidal CD4+CD25+ Treg cells from patients with autoimmune thyroid diseases were prone to apoptosis, which led to a local Treg-cell reduction [40]. In contrast, patients with metastatic epithelial cancer demonstrated a significantly elevated proportion of peripheral Treg cells, and these cells were protected from apoptosis [41]. Apoptosis not only reduces the number of Treg cells, but also reduces their functions. By using T-effector cell suppression assays, Treg-cell apoptosis was closely associated with their capacity to inhibit T-effector cell proliferation [42]. In patients with type 1 diabetes, an increase in apoptosis was correlated with a decline in the suppressive potential of Treg cells [43]. As suggested by these studies, high sensitivity to IL-2 deprivation or FasL-induced apoptosis may contribute in part to the defect of Treg cells in CHF patients. Treg cells from CHF patients were more susceptible to apoptosis following IL-2 deprivation. Upon antigen activation, T cells induce the expression of CD95, a member of the tumor necrosis factor receptor/nerve growth factor receptor superfamily that induces apoptosis by binding to CD95L and subsequently activating.
In the present study, we demonstrated that T<sub>reg</sub> cells in CHF patients had higher CD95 expression levels and were more sensitive to CD95/CD95L-mediated apoptosis than those in non-CHF subjects. Indeed, we also detected concurrent increases in serum soluble CD95L levels in CHF patients, consistent with prior observations [45]. These findings strongly suggest that the CD95/CD95L pathway is an important regulator of T<sub>reg</sub>-cell apoptosis in CHF patients.

After release from the thymus, T<sub>reg</sub> cells circulate continuously from blood to lymphoid tissues. In disease conditions, the expression of chemokine receptors, such as CCR4 and CCR8, on T<sub>reg</sub> cells allows their migration and recruitment to the site of inflammation [46]. In several human diseases, T<sub>reg</sub> cells preferentially accumulate at lymphoid tissues or sites of affected organs [47,48]. Therefore, it is possible that decreases in peripheral T<sub>reg</sub> cells in CHF patients are caused by T<sub>reg</sub>-cell reallocation rather than an overall decrease. To investigate this possibility, we compared the T<sub>reg</sub>-cell numbers in the mediastinal lymph nodes or Foxp3 expression in cardiac biopsies between CHF patients and non-CHF controls. The results revealed that T<sub>reg</sub>-cell frequency in the mediastinal lymph nodes or Foxp3 expression in hearts of CHF patients was no higher than that of the non-CHF controls. However, this possibility could not be excluded due to the very small sample number. In addition to generation in the thymus, T<sub>reg</sub> cells can also be converted from activated effector or memory CD4<sup>+</sup>CD25<sup>+</sup> T cells in the periphery [49]. Peripherally converted T<sub>reg</sub> cells and thymus-generated T<sub>reg</sub> cells demonstrate a similar phenotype and suppressive functions. It is possible that such peripheral T-cell phenotype conversion was altered in CHF patients. This hypothesis merits further investigation.

TNF-α is central in the inflammatory cytokines response in CHF and play a role in the pathogenesis and clinical progression of the disease [50]. IL-10, an anti-inflammatory cytokine, may offer protection against TNF-α and an improvement in cardiac function in CHF has been associated with an increase in IL-10 [51] or a decrease in TNF-α/IL-10 ratio [52]. Our data indicated that T<sub>reg</sub> frequency was negatively correlated with serum level of TNF-α or the TNF-α/IL-10 ratio (Figure S1). In both our previous study [17] and the present study, we observed that total T<sub>reg</sub> number was significantly negatively correlated NT-proBNP which is considered as the most sensitive index of cardiac dysfunction in CHF patients. Based on these observations, we may speculate that T<sub>reg</sub> cells provided protection for the failing heart.

**Figure 4. Spontaneous apoptosis of T<sub>reg</sub> cells from CHF patients and non-CHF controls.** PBMCs of 47 CHF patients and 38 non-CHF controls were stained with anti-CD4, anti-CD25, anti-CD127, annexin-V and 7-AAD and analyzed by flow cytometry. a. Representative FACS analyses from one non-CHF control and one CHF patient are shown. A small subpopulation of CD25<sup>+</sup>CD127<sup>low</sup>/2 cells were gated and identified as T<sub>reg</sub> cells (left panels). The staining of annexin-V and 7-AAD was further analyzed on gated T<sub>reg</sub> cells (middle), and apoptosis levels of the T<sub>reg</sub> cells are calculated as percentage of annexin-V<sup>-</sup>7-AAD<sup>-</sup> cells among 7-AAD<sup>-</sup> cells (right; *p<0.01 vs. non-CHF controls). b. CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low</sup>/2 T<sub>reg</sub> cells from CHF patients (n = 25, 14 NIHF and 11 IHF) and non-CHF controls (n = 15) were isolated by magnetic selection (left), and the expression of both the anti-apoptotic gene Bcl-2 (top panel) and the pro-apoptotic gene Bak (bottom panel) was measured. *p<0.05 vs. non-CHF controls.

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heart and defects in T_{reg} cells is involved in the deterioration of cardiac function in CHF patients. However, the direct effect of T_{reg} cells on cardiac dysfunction still needs to be studied in animal model.

IL-10 and TGF-β1 have been identified as the main effector cytokines of T_{reg} cells [53]. We investigated the hypothesis that impaired T_{reg}-cell function was associated with the decreased expression of these two cytokines. Disappointedly, we failed to observe a decrease in the expression of either IL-10 or TGF-β1 in CHF patients (Figure S2).

To conclude, our study revealed that both impaired export from the thymus and enhanced apoptosis can account for impaired T_{reg}-cell number and function in CHF patients, offering a mechanistic explanation for the phenotypes and providing possible novel targets for CHF therapy through T_{reg}-cell manipulation.

**Materials and Methods**

1. **Subjects**

   samples were collected from 52 CHF patients (31 men and 21 women, 44±13 years old) and 43 non-CHF controls (28 men and 15 women, 42±12 years old). Peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll density gradient centrifugation (Sigma, USA). Plasma was obtained after centrifugation and stored at ~80°C. CHF diagnoses were based on clinical history, physical examination, echocardiography, chest X-ray, electrocardiography and levels of N-terminal pro-brain natriuretic peptide (NT-proBNP), according to available guidelines pertaining to CHF. Patients were classified as having non-ischemic heart failure (NIHF) (n = 32, 17 men and 15 women) if they had no history of myocardial infarction and did not have significant coronary artery stenosis upon coronary angiography. Patients were considered to have ischemic heart failure (IHF) (n = 20, 14 men and 6 women) if the coronary angiography presented significant coronary artery disease (>50% stenosis in more than one major epicardial coronary artery) or the patients had a history of myocardial infarction or previous revascularization. Patients were excluded (1) if they were currently treated with anti-inflammatory drugs, such as non-steroidal anti-inflammatory drugs and steroids, (2) if they had collagen disease, thromboembolism, disseminated intravascular coagulation, advanced liver disease, renal failure, malignant disease, other inflammatory disease (such as septicemia, pneumonia), valvular heart disease, or atrial fibrillation, or (3) if they had pacemakers. Patients with higher serum cholesterol than the target values after risk stratification [54], who received statin therapy within 3 months, were also excluded. Mediastinal lymph nodes

![Figure 5. IL-2 deprivation and FasL-mediated T_{reg}-cell apoptosis.](image)
**Mechanisms for Treg Defects in CHF Patients**

4. T\(_{\text{reg}}\) cell isolation

A two-step selection using a CD4\(^+\)CD25\(^+\)CD127\(^{dim-}\) Regulatory T cell Isolation Kit (Miltenyi Biotech, Germany) was used to isolate T\(_{\text{reg}}\) cells according to the manufacturer’s instructions. Briefly, non-CD4\(^+\) and CD127\(^{high}\) cells were magnetically labeled with a cocktail of biotin-conjugated antibodies and anti-biotin microbeads and subsequently depleted by negative selection. Pre-enriched CD4\(^+\) T cells were then labeled with anti-CD25 microbeads, and CD4\(^+\)CD25\(^+\)CD127\(^{dim-}\)/T\(_{\text{reg}}\) cells were isolated by positive selection. FACS was used to confirm the purity (>90\%) of the isolated T\(_{\text{reg}}\) cells.

5. Quantification of T-cell receptor excision circles (TRECs)

The Wizard\textsuperscript{®} Genomic DNA Purification Kit (Promega, USA) was used to extract genomic DNA (gDNA) from purified T\(_{\text{reg}}\) cells. Quantitative real-time PCR on an ABI Prism 7900 sequence detection system (Applied Biosystems, USA) was used to determine the number of TRECs. Primer pairs and probes were as follows:

- TREC: F: 5’-aacagctttgggacaatagt-3’, R: 5’-gctgtaattgccacctcpgag-3’, probe: 5’-6FAM-cacaccttcacacagtgcagcttctg- TAMAR-3’;
- RAG2: F: 5’-ggaacatgttagaagtctg-3’, R: 5’-ggctgtaatcatctcacc-3’, probe: 5’-6FAM-cctctttctgtgctgtgtgttgga-TRAMIRA-3’. Data were expressed as TRECs/10\(^6\) cells.

6. Apoptosis assays

Freshly isolated PBMCs were first stained with surface antibodies APC/Cy7 anti-human CD4, PE anti-human CD25, PerCp/Cy5.5 anti-human CD95 (Fas) (Biolegend, USA) and Alexa Fluor\textsuperscript{®} 647 anti-human CD127 (eBioscience, USA). Cells with the phenotype CD4\(^+\)CD25\(^+\)CD127\(^{dim-}\) were identified as T\(_{\text{reg}}\) cells. Apoptosis was measured using annexin V and 7-aminoactinomycin D (7-AAD) co-staining (Bender MedSystems, USA). The proportion of annexin V\(^+\)7-AAD\(^-\) apoptotic cells in 7-AAD\(^-\) viable T\(_{\text{reg}}\) cells and the surface expression of CD95 on T\(_{\text{reg}}\) cells were analyzed using FACS Aria (BD Biosciences, USA).

For IL-2 deprivation-mediated apoptosis, cells were stimulated with 2 \(\mu\)g/ml plate-bound anti-CD3 (eBioscience, USA) and anti-human IL-2 monoclonal antibodies (Peprotech, 2 \(\mu\)g/ml) for 3 days. For Fas ligand (FasL)-induced apoptosis, cells were stimulated with gradient concentrations of soluble recombinant FasL (Peprotech, USA) in complete RPMI1640 containing IL-2 (100 IU/ml) for 12 hrs [56]. CD4\(^+\)CD25\(^+\)CD127\(^{dim-}\)/T\(_{\text{reg}}\) cells were also gated for annexin V\(^+\)7-AAD\(^-\) to determine apoptotic cell populations. Cell death was presented as (Percent of IL-2 deprivation- or FasL-mediated apoptosis - percent of apoptosis in the absence of anti-human IL-2 or FasL) / (100% - percent of cells in the absence of anti-human IL-2 or FasL)\times100.

7. Soluble CD95 ligand (sCD95L) ELISA detection

Human FasL/TNFSF6 Quantikine ELISA Kit (R&D Systems, USA) was used to determine the plasma sCD95L levels. The minimal detectable concentration was 2.66 pg/ml, and intra-assay and inter-assay coefficients of variation were <10\%. All samples were measured in duplicate.

8. T\(_{\text{reg}}\)-cell detection in mediastinal lymph node

Mediastinal lymph nodes were minced and filtered through a cell strainer to create a single cell suspension preparation. Lymphocytes were isolated using Ficoll-Hypaque, stained with specific antibodies for CD4, CD25 and Foxp3, and subjected to FACS analysis. The number of T\(_{\text{reg}}\) cells in the lymph nodes was quantified by flow cytometry.
9. Real-time PCR

Total RNA was extracted using Trizol lysis buffer (Invitrogen, USA), and cDNA was prepared using the RevertAAce kit (Toyobo, Japan). Expression of target genes (Bcl-2 and Bak in purified Treg and Foxp3 cells) in heart tissues and lymphocytes isolated from mediastinal lymph nodes was quantified using the SYBR Green Master Mix (Takara, Japan) on an ABI Prism 7900 Sequence Detection system (Applied Biosystems, USA). Primer pairs were as follows:

Bcl-2: F, 5′-tacctgcaagggcacctg-3; R, 5′-ggcgtacagttcacaaggg-3;
Bak: F, 5′-ctggccgctgtgatggcggtaaaa-3; R, 5′-atggcccagcggat-3′
GAPDH: F, 5′-cccacagctgacacactc-3; R, 5′-ggcacaactacactcagcag-3′

For each sample, the mRNA expression level was normalized to that of GAPDH. The mean of duplicate measurements was normalized and expressed as a ratio of target mRNA copies to GAPDH mRNA copies.

10. Quantification of transforming growth factor (TGF-α) and interleukin (IL)-10 expression in Treg cells

PBMCs were cultured in RPMI1640 containing 10% FBS and stimulated with PMA (50ng/ml; Sigma-Aldrich, USA), ionomycin (1 μg/ml, Sigma-Aldrich, USA) and monensin (1 μM, Becton & Dickenh, USA) for 4 h. Cells were stained with surface antibodies for APC/Cy7 anti-human CD4, PE anti-human eBioscience, USA) for 30 min at 4°C. After surface staining, cells were fixed, permeabilized, and stained with PE-cy7 anti-human TNF-α or PE-cy7 anti-human IL-10 (Biolgend, USA). Stained cells were analyzed by flow cytometry with FACS Aria (BD Biosciences, USA).

11. Tumor necrosis factor (TNF-α) and IL-10 ELISA detection

Commercial ELISA Kits (Invitrogen, USA) were used to determine the plasma TNF-α and IL-10 levels. The minimal detectable concentrations were 0.5 pg/ml and 0.78 pg/ml for TNF-α and IL-10 respectively. All samples were measured in duplicate.

12. Statistical analysis

Values are expressed as means ± standard deviation (SD) or percentage in the text and figures. For variables with normal distribution and homogeneity of variance, independent t-test or one-way analysis of variance (ANOVA) was used to test differences among two or more groups. For skewed variables, either non-parametric Kruskal-Wallis H test or Mann-Whitney U test were used for analyses. For the ranked data, Pearson’s chi-square test or Fisher’s exact test were used for the comparison between multiple groups. Spearman’s correlation analysis was performed to determine correlation between the variables. In all cases, two-tailed, p<0.05 was considered significant.

Supporting information

Figure S1 Correlation analysis between Treg frequency and plasma levels of cytokines in CHF patients (n = 20).

Figure S2 Comparison of intracellular IL-10 and TGF-β1 in CD4+CD25+CD127low Treg between CHF patients (n = 10) and healthy controls (n = 10).

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Author Contributions

Conceived and designed the experiments: XC TTT ZFZ JW WCZ. Performed the experiments: XC TTT ZFZ JW WCZ HG XLD JHX NGD WS NX XXY SFZ RX JX HY MYL. Analyzed the data: XC TTT ZFZ JW WCZ HG XLD JHX NGD WS NX XXY SFZ RX JX HY MYL. Contributed reagents/materials/analysis tools: XC TTT ZFZ JW WCZ HG XLD JHX NGD WS NX XXY SFZ RX JX HY MYL. Wrote the paper: XC TTT ZFZ JW WCZ.
20. Nelson BH (2004) IL-2, regulatory T cells, and tolerance. J Immunol 172: 3963–3968.
21. Taams LS, Smith J, Rustin MH, Salmon M, Poulter LW, et al. (2001) Human aortic suppressive CD4(+CD25(+) T cells: a highly differentiated and apoptosis-prone population. Eur J Immunol 31: 1122–1131.
22. Krammer PH (2000) CD95's deadly mission in the immune system. Nature 407: 795–795.
23. Fritzsching B, Obereiner E, Eberfaedt N, Quick S, Haass J, et al. (2005) In contrast to effector T cells, CD4(+CD25(+)Foxp3(+) regulatory T cells are highly susceptible to CD95 lipid- but not to TCR-mediated cell death. J Immunol 175: 32–36.
24. Frangogiannis NG, Smith CW, Enman ML (2002) The inflammatory response in myocardial infarction. Cardiovasc Res 53: 31–47.
25. Mason JW (2003) Myocarditis and dilated cardiomyopathy: an inflammatory link. Cardiovasc Res 60: 5–10.
26. Caforio AL, Tona F, Bottaro S, Vinci A, Dequidt G, et al. (2008) Clinical implications of anti-heart autoantibodies in myocarditis and dilated cardiomyopathy. Autoimmunity 41: 35–45.
27. Teplokudov AT, Bolotskaia LA, Dibirov MM, Stepacheva TA, et al. (2008) Clinicoinmunological disorders in patients with postinfarction left ventricular remodeling and chronic cardiac failure. Ter Arkh 80: 52–57.
28. Düngeoen HD, Plattecek M, Vollert J, Scalf J, Müller C, et al. (2010) Autoantibodies against cardiac troponin I in patients with congestive heart failure. Eur J Heart Fail 12: 668–675.
29. Deubner N, Berliner D, Schlipp A, Gelbrich G, Caforio AL, et al. (2010) Etiology, Type-Course, and Survival-Study Group. Cardiac beta1-adrenoceptor autoantibodies in human heart disease: rationale and design of the Etiology, Type-Course, and Survival (ETiCS) Study. Eur J Heart Fail 12: 733–762.
30. Omelovic E, Bellino E, Andresson B, Kujacic V, Schlae W, et al. (2006) Induction of cardiomyopathy in severe combined immunodeficiency mice by transfer of lymphocytes from patients with idiopathic dilated cardiomyopathy. Autoimmunity 32: 271–280.
31. Vardas-Bloom L, Leor J, Olah DG, Hasin Y, Amar M, et al. (2000) Cytotoxic T lymphocytes are activated following myocardial infarction and can recognize and kill healthy myocytes in vitro. J Mol Cell Cardiol 32: 2141–2149.
32. Maisel A, Cesario D, Baird S, Rehman J, Haghighi P, et al. (1998) Experimental implications of anti-heart autoantibodies in myocarditis and dilated cardiomyopathy. Autoimmunity 31: 1122–1131.
33. Josefowicz SZ, Rudensky A (2009) Control of regulatory T cell lineage commitment and maintenance. Immunity 30: 616–625.
34. Fritzsching B, Korporal M, Haas J, Krammer PH, Suri-Payer E, et al. (2006) Apoptosis-induced decrease of intrathymic CD4(+)CD25(+) regulatory T cells in autoimmune thyroid diseases. Thyroid 17: 25–31.
35. Venken K, Hellinga N, Broekmans T, Hensen K, Rummens JL, et al. (2008) Natural naive CD4(+)CD25(+)CD127(lo) regulatory T cell (Treg) development and function are disturbed in multiple sclerosis patients: recovery of memory Treg homeostasis during disease progression. J Immunol 180: 6411–6420.
36. Nakano A, Watanabe M, Isla T, Kuroda S, Matsuzaka F, et al. (1997) Apoptosis-induced decrease of intrathymic CD4(+)CD25(+) regulatory T cells in autoimmune thyroid diseases. Thyroid 17: 25–31.
37. Stanzener D, Dandachi N, Balic M, Reseli M, Samonigg H, et al. (2008) Resistance to apoptosis and expansion of regulatory T cells in relation to the detection of circulating tumor cells in patients with metastatic epithelial cancer. J Clin Immunol 28: 107–114.
38. Fritzsching B, Korpormal M, Haas J, Krammer PH, Suri-Payer E, et al. (2006) Similar sensitivity of regulatory T cells towards CD95L-mediated apoptosis in patients with multiple sclerosis and healthy individuals. J Neurol Sci 251: 91–97.
39. Glicic-Milosavljevic S, Waukau J, Jablana P, Jana S, Khoo HJ, et al. (2007) At-risk and recent-onset type 1 diabetic subjects have increased apoptosis in the CD4(+)CD25(+) T-cell fraction. PLoS One 2: e184.
40. Xu G, Shi Y (2007) Apoptosis signaling pathways and lymphocyte homeostasis. Cell Res 17: 759–771.
41. Yamaguchi S, Yamaoka M, Okuyama M, Niohbe J, Fukui A, et al. (1999) Elevated circulating levels and cardiac secretion of soluble Fas ligand in patients with congestive heart failure. Am J Cardiol 83: 1508–1503.
42. Weir S, Keyzeck I, Zusi W (2006) Regulatory T-cell compartmentalization and trafficking. Blood 108: 426–431.
43. Hesse M, Picciello CA, Belkaid Y, Pruffer J, Mentink-Kane M, et al. (2004) The pathogenesis of schistosomiasis is controlled by cooperating IL-10-producing regulatory T cells. J Immunol 172: 3157–3166.
44. Suvas S, Azkar AK, Kim BS, Kumaraguru U, Rouse BT (2004) CD4(+)CD25(+) regulatory T cells control the severity of viral immunoinflammatory lesions. J Immunol 172: 4123–4132.
45. Liaoq N, Alard P, Zhao Y, Farrall S, Clark SL, et al. (2005) Conversion of CD4(+)CD25(+) cells into CD4(+)CD25(+) regulatory T cells in vivo requires B7 costimulation, but not the thymus. J Exp Med 201: 127–137.
46. Kleinberger P, Schulte R, Heusch G (2011) TNFα in myocardial ischemia/reperfusion, remodeling and heart failure. Heart Fail Rev 16(1): 49–69.
47. Adamopoulos S, Parissis JT, Paraskevaidis I, et al. (2003) Effects of growth hormone on circulating cytokine network, and left ventricular contractile performance and geometry in patients with idiopathic dilated cardiomyopathy. Eur Heart J 24(24): 2186–96.
48. Stumpf C, Lehner G, Yilmaz A, Daniel WG, Garfichs CD (2005) Decrease of serum levels of the anti-inflammatory cytokine interleukin-10 in patients with advanced chronic heart failure. Clin Sci (London) 105(1): 45–50.
49. Askenasy N, Kamnitz A, Yarkoni S (2008) Mechanisms of T regulatory cell function. Autoimmun Rev 7(7): 370–5.
50. Joint committee for developing Chinese guidelines on prevention and treatment of dyslipidemia in adults (2007) Chinese guidelines on prevention and treatment of dyslipidemia in adults. Chin J Cardiol 35: 390–419.
51. Lippi P, Rudert WA, Zanone MM, Stassi G, Trucco G, et al. (1998) Idiopathic dilated cardiomyopathy: a superantigen-driven autoimmune disease. Circulation 98: 777–785.
52. Miyara M, Amoura Z, Parizot C, Badousal C, Dorgamian K, et al. (2005) Global natural regulatory T cell depletion in active systemic lupus erythematosus. J Immunol 175: 8392–8400.