Plasma levels of TNF-α and IL-6 are associated with diastolic heart failure through downregulation of sarcoplasmic reticulum Ca^{2+} ATPase

Running Title: TNF-α and IL-6 are associated with DHF through SERCA

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ABSTRACT

Objective: The inflammatory process is associated with cardiac diastolic dysfunction, which has been demonstrated to be an independent prognostic marker for the mortality of critically ill patients. We investigated the association between inflammatory cytokines (tumor necrosis factor-α [TNF-α] and interleukin 6 [IL-6]), diastolic heart failure (DHF), and the possible molecular mechanism.

Design: Prospective case-controlled cohort and molecular studies.

Setting: University hospital and research laboratory.

Subjects: Patients with a diagnosis of DHF by echocardiography and matched controls from the general population (study group 1) and also subjects from the intensive care unit (study group 2). Sarcoplasmic reticulum Ca^{2+}-ATPase (*SERCA2*) gene expression and diastolic calcium decay in HL-1 cardiomyocytes were used as molecular phenotypes of DHF.

Interventions: Soluble plasma levels of TNF-α and IL-6 were measured in all subjects. An ~1.75-kb promoter of the *SERCA2* gene was cloned to the pGL3 luciferase reporter. The effect of TNF-α and IL-6 on SERCA2 gene expression and diastolic calcium decay of HL-1 cardiomyocytes were investigated.

Measurements and Main Results: Patients with DHF had significantly higher plasma levels of TNF-α and IL-6 than the controls. Significant correlations ($p < .01$ for each)

were found for TNF-α and E/Em (r = 0.87) and E/A (r = -0.69), and for IL-6 and E/Em (r = 0.80) and E/A (r = -0.65). Cytokine levels were also correlated with diastolic function in critical ill patients (study group 2), and diastolic function improved significantly in association with decrease of cytokines. TNF-α, IL-6 and sera from critically ill patients downregulated the expression of the *SERCA2* gene. TNF-α and IL-6 also delayed the diastolic calcium reuptake and decay in cardiomyocytes.

Conclusions: Through downregulation of *SERCA2* gene expression, inflammatory cytokines may cause cardiac diastolic dysfunction by decreasing diastolic calcium re-uptake. Our study may suggest novel therapeutic strategies for DHF and critically ill patients by modulating inflammatory reactions.

Keywords: cytokines, diastolic heart failure, inflammation, HL-1 cardiomyocytes, sarcoplasmic reticulum Ca^{2+}-ATPase

Immunoinflammatory activation has been demonstrated to play a pivotal role in the development and in the progression of heart failure in some studies (1,2). Specifically, circulating levels of tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6) have been found to be positively correlated to deteriorating heart failure functional class and predictive of a worse outcome (2-4).

A very recent study demonstrated that approximately half of critically ill patients with a normal ejection fraction (EF) and no previous history of heart failure (HF) exhibited evidence of left ventricular (LV) diastolic dysfunction (5). Furthermore, LV diastolic dysfunction was found to be an independent prognostic marker for the mortality of critically ill patients with preserved EF (5). However, the mechanism of diastolic dysfunction in critically ill patients is unknown. A biologically plausible hypothesis is that inflammatory cytokines, such as TNF-α or IL-6 directly induce diastolic dysfunction (6).

Currently, the molecular mechanism of diastolic heart failure (DHF) has not been fully elucidated. Previous experiments with animal models found that active proinflammatory processes were associated with cardiac diastolic dysfunction (7). TNF-α and IL-6 were shown to be associated with DHF in some specific groups, such as patients with coronary artery disease (8) and patients with newly diagnosed systolic HF (9). There have been no studies regarding the most common proinflammatory

markers, TNF-α and interleukin-6 and the development of non-complicated DHF. On the other hand, sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) is one of the most extensively studied calcium handling proteins with respect to diastolic dysfunction. In cardiomyocytes, free calcium increases during systole and induces mechanical contraction by binding to the troponin complex. Subsequently, calcium is removed from the cytosol primarily by the action of SERCA during diastolic relaxation (10). Decreased activity of SERCA slows the removal of calcium from the cytosol, which impairs the diastolic relaxation of contractile proteins (11). SERCA has 3 isoforms which are encoded by three genes and are spliced to produce multiple mRNA transcripts. SERCA2, the major heart isoform, is required for normal cardiac diastolic function. Whether IL-6 or TNF-α regulates SERCA2 expression in cardiomyocytes, and subsequently modulates diastolic function remain unclear.

Accordingly, in the present study, we sought to assess plasma levels of IL-6 and TNF-α in patients with DHF, including critically ill patients, and to evaluate the association between these cytokines and left ventricular dysfunction indices. We then explored the effect of IL-6 and TNF-α on the transcriptional regulation and function of SERCA2 in cardiomyocytes.

MATERIAL AND METHODS

Study subjects

Two patient groups were examined in this study. The first group (study group 1) consisted of patients admitted to the cardiovascular ward of National Taiwan University Hospital and its affiliated hospital for coronary angiography or a health examination from July 2007 to March 2009. The second group (study group 2) enrolled 30 consecutive patients that were admitted to the intensive care units (ICUs) of National Taiwan University Hospital. A control population for study group 1 consisted of risk-factor matched controls with no symptoms of HF failure and no objective evidence of diastolic dysfunction selected from the same ward or clinic. Finally, 110 patients with DHF (52 men and 58 women) along with 55 controls were selected in study group 1 and 30 critically ill patients in study group 2.

All participants received echocardiographic examinations as well as blood sampling for the estimation of plasma TNF-α and IL-6 levels. DHF was defined as in our previous reports and as in the recent consensus statement by the European Society of Cardiology (12-15). The subjects admitted to the ICU also received echocardiographic examinations and testing for serum cytokines levels during admission at ICU and after they were transferred to the general ward to compare the change of diastolic function and cytokine levels. The study was approved by the local Institutional Review Board, and all subjects provided their informed consent. The

detailed inclusion and exclusion criteria are listed in the online data supplement in Supplemental Text 1.

Measurements of Plasma TNF-α and IL-6

In all patients, blood samples were collected from the antecubital vein between 08:00 and 10:00 h, in supine position, after 12 h of fasting. Serum IL-6 and TNF-α were measured with high-sensitivity enzyme-linked immunosorbent assays (ELISA). Further detailed methods are provided in Supplemental Text 1.

Cell Culture

HL-1 myocytes were cultured in Claycomb medium (JRH Bioscience, Lenexa, KS, USA) supplemented with 10% fetal bovine serum and maintained in a humid 10% CO2 incubator at 37°C as previously described (16).

Construction of SERCA2 Promoter-Luciferase Fusion Plasmids and Transfection of HL-1 Cardiomyocytes

A 1754 bp promoter fragment of rat *SERCA2* gene was amplified by polymerase chain reaction (PCR) using forward and reverse primers. The ~1.7-kb PCR product was then subcloned into the pGL3Basic Vector at *Bgl*II and *Hind*III sites (16). The constructs were confirmed by deoxyribonucleic acid (DNA) sequencing. Transfection of the HL-1 myocytes was performed using Lipofectamine 2000 reagent. Normalized luciferase activities were measured using the Dual Luciferase Reporter Assay System.

Further detailed methods are provided in Supplemental Text 1.

Fluo-3 Dye Staining and Confocal Laser Scanning Microscopy (CLSM) for Recordings of Intracellular Calcium

Spontaneous calcium transient was recorded in HL-1 cells using the fluorescent dye Fluo 3-AM and CLSM as previously described (16). Cells were loaded with Fluo 3-AM and pluronic F-127, and Fluo-3 fluorescence was monitored using an inverted CLS microscope (LSM 510; Carl Zeiss, Jena, Germany). Further detailed methods are provided in in Supplemental Text 1.

To quantify the rate of recovery of intracellular calcium to diastolic levels, the decay portion of the calcium transient (from 30% to 100% of the decline phase) was measured by the time constant (i.e., Tau) of a single exponential fit, as previously reported (17).

RNA Extraction, Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (RT–QPCR)

The cells were harvested 0, 2, 6, 12, and 24 hours after treatment with IL-6 (10 ng/mL) or TNF-α (5 ng/mL) for ribonucleic acid (RNA) extraction and quantification. Total RNA was isolated and reverse-transcribed. The single-stranded cDNA was amplified (ABI-Prism 7900; Applied Biosystems, Foster City, CA, USA), using SYBR Green dye. For mouse *SERCA2*, the primers were designed according to the published gene

sequences (18). GAPDH mRNA was used as the internal control. The primer sequences and other detailed methods are provided in Supplemental Text 1.

Protein Extracts, Western Blot Analysis

HL-1 cells were treated with the sera from the critically ill patients or cytokines (IL-6 [10 ng/mL) or TNF-α [5 ng/mL]), and subsequently harvested after 24 h using a Nonide P40 (NP-40) based lysis buffer. SERCA2 protein concentrations were determined by Western blot analysis with a GADPH control. Sera from healthy subjects and stable ICU patients without left ventricular diastolic dysfunction were used as the controls. The detailed methods are provided in Supplemental Text 1.

Statistical Analysis

Data were analyzed using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). Continuous variables are presented as mean values ± standard deviation, while categorical variables are presented as frequencies. Associations between categorical variables were tested by Pearson's chi-squared test. To test whether the data were normally distributed, the Kolmogorov-Smirnov test was applied. The comparisons between the data showing normal distribution were performed using the Student's *t*-test, or otherwise by the Mann-Whitney U test. The associations between cytokines and Doppler parameters were studied with the use of Pearson's correlation coefficient if the data meet the criteria for normal distribution, or otherwise by Spearman's

correlation test. Echocardiographic and serum cytokines levels of the ICU patients were compared by paired-t test for data before and after treatments. A value of $p < .05$ was considered statistically significant.

RESULTS

Baseline Characteristics

The baseline characteristics of the study subjects (study group 1 and group 2) are shown in Table 1 and Table 2. In group 1, all baseline characteristics of the cases were comparable with those of the controls. Echocardiographic parameters for cases and controls are also shown in Table 1. There were no statistically significant differences in left ventricular ejection fraction (LVEF), left atrium size, left ventricular end-diastolic diameter, left ventricular end-systolic diameter between cases and controls. Patients with DHF had longer mitral flow deceleration time, smaller early mitral valve flow E/A ratio, and larger E/Em ratio. Patients admitted to the ICU (study group 2) had markedly higher inflammatory cytokine levels due to their underlying disease. Their Acute Physiology and Chronic Health Evaluation II (APACHE II) scores were 18.9 ± 5.3 (mean ± SD), indicating a critically ill condition during echcardiographic and serum measurement. The time between measurements of echocardiography and serum cytokines levels at critical phase and at stable phase was

15.4 ± 6.1 days (mean ± SD).

Plasma Levels of TNF-α and IL-6 in Patients with DHF

The serum TNF-α levels were significant higher in DHF patients compared to controls (DHF, 5.38 ± 1.68 pg/mL; control, 2.61 ± 1.40 pg/mL; $p < .001$) (Table 1a). Patients with DHF also had significantly higher plasma IL-6 levels than the control group (DHF, 4.99 ± 1.75 pg/mL; control, 2.54 ± 1.74 pg/mL; $p < .001$). Bivariate correlations of LV diastolic function parameters and plasma levels of cytokines are shown in Figures 1A-1F. The correlation test demonstrated that both TNF-α and IL-6 are significantly associated with diastolic parameters.

Cytokine Levels and Diastolic Dysfunction Parameter Changes in ICU Patients

Proinflammatory cytokines, including IL-6 and TNF-α, of critically ill patients in ICU significantly decreased after they were transferred to general ward (Table 2). Accordingly, echocardiographic parameters for diastolic dysfunction (including decelerating time, E/Em, and mitral inflow E/A) improved significantly after their general condition improved (Table 3).

TNF-α and IL-6 Down-regulated *SERCA2* Gene Promoter Activity in Cardiomyocytes

In order to explore whether these cytokines affect diastolic dysfunction through SERCA2, we examined their effect on the *SERCA2* promoter first. The promoter

region of the *SERCA2* gene was cloned to the pGL3 luciferase vector. The luciferase activity expressed in HL-1 cardiomyoctyes increased approximately 20-fold compared to that in the PGL3 basic plasmid, which indicated an efficient transfection (Figure 2A).

We then evaluated whether TNF-α and IL-6 regulate *SERCA2* gene expression. After treating HL-1 cells with TNF-α (5 ng/mL) and IL-6 (1 0 ng/mL) for 24 h, the luciferase activity of the 1754-bp promoter–reporter construct of the *SERCA2* gene decreased significantly both in the TNF-α and IL-6 group (control, 1.000; TNF-α, 0.837 ± 0.095; $p = .041$) (Fig. 2A); (control, 1.000; IL-6, 0.777 ± 0.366; $p < .001$) (Fig. 2A).

To evaluate dose-response effect, different concentrations of cytokines were administered. The effect on SERCA2 promoter activity was seen for TNF-α ≧ 5 ng/mL and IL-6 ≧ 10 ng/mL (Figure 2B).

TNF-α and IL-6 Attenuated SERCA2 Expression in Cardiomyocytes

We also evaluated whether TNF-α and IL-6 influenced SERCA2 transcription and translation in cardiomyocytes by estimating mRNA levels after treating with cytokines for different time intervals. SERCA2 mRNA levels were decreased as early as 2 hours after TNF-α (5 ng/mL) treatment, and significant attenuation of SERCA2 mRNA was achieved after 24 h of TNF-α treatment (1 for TNF-α at 0 h [Control] vs.

0.83 ± 0.15, 0.69 ± 0.08, 0.75 ± 0.12, and 0.69 ± 0.12 for TNF-α 2 h, TNF-α 6 h, TNF-α 12 h, and TNF-α 24 h, respectively; $p = .01$ for control vs. TNF-α 24 h) (Fig. 2C). On the other hand, SERCA2 mRNA levels declined slightly after treatment with IL-6 (10 ng/mL) for 2 h, and then the most significant changes occurred after 6 h, and although slightly increased, the level of SERCA declined significantly after 24 hours of IL-6 treatment (1 for IL-6 0 hours [Control] vs. 0.99 ± 0.17, 0.94 ± 0.23, 0.99 ± 0.21, and 0.78 ±0.11 for IL-6 2 h, IL-6 6 h, IL-6 12 h, and IL-6 24 h, respectively; p =.03 for control VS IL-6 24 h) (Figure 2D). The fluorescence-cycle number relationship for quantitative real-time RT-PCR was displayed in figure 2F. Similar findings were found in the protein levels (Figure 2E). These results were compatible with the findings of the luciferase-promoter assay.

Serum from Critically ill Patients Attenuated SERCA2 Expression in Cardiomyocytes

We found the association of elevated serum TNF-α and IL-6 cytokines levels and diastolic dysfunction in critically ill patients. We then sought to evaluate whether sera from critically ill patients attenuated SERCA2 expression. The SERCA2 mRNA and protein concentrations decreased significantly after treatment with sera from critically ill patients in cardiomyocytes (Fig. 3A and 3B).

Diastolic Calcium Decay

Figure 4A shows the calcium transients of control HL-1 cells and TNF-α (5 ng/mL; 24 h) and IL-6 (10 ng/mL; 24 h) treated cells. The calcium transients of all cells showed a rapid increase in intracellular calcium followed by a slow decay as intracellular calcium returned to diastolic levels, a molecular phenotype of cardiomyocyte diastolic function (19). When fit to a single exponential, the decay phase of the calcium transient is slower (i.e., Tau is larger) for TNF-α (141.4 ± 4.2 ms) and IL-6 (137.4 ± 5.8 ms) treated cells, as compared to control cells (113.5 ± 6.2 ms; p = .028 and .019 for TNF-α and IL-6, respectively, vs. control; N = 6 preparations for each group). These data suggest that the decay of intracellular calcium to diastolic levels is slower in TNF-α and IL-6 treated cells as compared with control cells, and is compatible with decreased SERCA expression due to TNF-α and IL-6.

DISCUSSION

Main Findings

In the present study, we first demonstrated that the levels of pro-inflammatory cytokines IL-6 and TNF-α were higher in non-complicated DHF patients. We also explored the possible mechanism, and found that inflammatory cytokines had a direct effect on SERCA2 at the transcription level. Due to the strong correlation between

heart diastolic function and SERCA2 expression, we hypothesized that plasma levels of TNF-α and IL-6 are associated with DHF through downregulation of SERCA2. Accordingly, we also demonstrated that high plasma levels of TNF-α and IL-6 were associated with diastolic dysfunction in critically ill patients.

Implications in Critically ill Patients

Recently, it has been demonstrated that inflammatory processes and release of inflammatory mediators in critically ill patients may cause LV diastolic dysfunction, which is also an important prognostic factor and is associated with mortality and worse outcomes (5). Some studies have addressed the impact of inflammation in heart failure with preserved left ventricular systolic function in patients with coronary artery disease (CAD) (8,20). In our study group 1 without critically ill condition, we excluded patients with coronary artery or peripheral artery disease. Therefore, we demonstrated the direct association between inflammatory cytokines and DHF without the confounding effect of medications or CAD.

Subsequently, in our study group 2 with critically ill patients, we further demonstrated the direct association between inflammatory cytokines and LV diastolic dysfunction. Importantly, the evidence is even stronger because we directly show that sera from critically ill patients downregulate SERCA2 expression, which is the most important protein regarding LV diastolic dysfunction. Moreover, the cytokines

concentrations remained higher in group 2 than that in the DHF cohort and the controls. The echo parameters improved after treatment but did not normalize. Along with the correlation we discovered between cytokines and measures of LV diastolic dysfunction, the results further support the association and potential mechanistic relationship between these inflammatory cytokines in diastolic dysfunction especially in the critically illness patients.

Mechanism of DHF, a Distinct Form other than Systolic Heart Failure (SHF)

Heart failure is a commonly encountered hemodynamic and neurohormonal syndrome with increasing prevalence as the population ages (21). Abnormalities of left ventricular diastolic function are postulated as the earliest, and at times, the sole manifestation of myocardial ischemia before changes in systolic function (22,23). However, in recent years, more structural, functional, and molecular biological evidence support that DHF and SHF present and evolve not as a single, but two different syndromes.

Structurally, patients with SHF have eccentric LV hypertrophy, whereas those with DHF have concentric LV hypertrophy (24). With respect to the molecular aspects of the conditions, the cytoskeletal protein titin, which functions as a bidirectional spring is responsible for early diastolic LV recoil and late diastolic resistance to stretch. Borbély et al. (25) concluded that a shift in titin isoform phosphorylation, with

relative hypopophosphorylation of the stiff N2B titin isoform, is a novel mechanism in the formation of DHF. Patients with SHF have a more compliant isoform of titin than those with DHF (26,27). Apart from titin, patients with DHF tend to have decreased matrix degradation because of downregulation of matrix metalloproteinases (MMPs) and upregulation of tissue inhibitors of MMPs, whereas in dilated cardiomyopathy, there is increased matrix degradation (28). These studies emphasize the importance of myofilament or cytoskeletal protein alterations in the development of cardiac diastolic dysfunction. Moreover, in patients with type 1 diabetes mellitus, a positive correlation was found between serum levels of advanced glycation end products (AGE) and echocardiographic diastolic parameters (29). Berg et al detected that the cross-linking properties of AGEs might have an influence on diastolic function.

Cytokines, SERCA, and DHF

In addition to myofilament or cytoskeletal protein alterations, defective calcium dynamics is even more important in the mechanism of DHF. In apparently normal hearts, SERCA2 is essential to the regulation of intracellular calcium levels in cardiomyocytes, and remove most of the calcium in the cytosol during diastolic relaxation (10). He et al. (30) demonstrated that the downregulation of SERCA2

contributed to both diastolic and systolic dysfunction in hypertrophied and failing myocardium. Recent studies suggested that proinflammatory markers, including TNF-α and IL-6, are associated with myocardial dysfunction (31,32). Impaired calcium homeostasis of cardiomyocytes is one of the pathophysiological hallmarks of HF. Of numerous calcium-regulatory proteins, decreased SERCA2 expression and altered regulation of Na^{+}/Ca^{2+}exchanger (NCX) or phospholamban levels have been reported and appear to correlate with diastolic function (33). In the present study, we show that TNF-α and IL-6 directly downregulate the expression of SERCA2 and decrease the function of SERCA2, which may provide the molecular mechanism of cytokine-induced diastolic dysfunction.

However, we could not rule out the possibility that TNF-α and IL-6 may also affect calcium dynamics through Na^{+}/Ca^{2+}exchanger (NCX) or phospholamban. Furthermore, the effects of TNF-α and IL-6 on the myocardium are complex. For example, TNF-α could modulate β adrenergic receptor function and thus lead to delayed contractile dysfunction (34). TNF-α and IL-6 could inhibit contractility of papillary muscles through mediation of myocardial nitric oxide synthase (35). Both cytokines can also impact myocardial function via effects on the extracellular matrix (6). Therefore, TNF-α and IL-6 may induce DHF through non-calcium dependent mechanism, which have not been addressed in the present study. Nevertheless, the

present investigation successfully demonstrated that proinflammatory cytokines downregulate SERCA2 at the transcription level, and may therefore be associated with pure DHF. Our current finding showed that inflammatory markers had a more prominent effect than angiotensin II over SERCA2 in cardiomyocyte (36), which implies that blockage of these cytokines may lead to better results than blockage of angiotensin II in DHF patients. These findings support further investigation in the use of anti-inflammatory medications in patients with DHF.

SERCA2 expression and changes had been found to be correlated to clinical presentation of cardiac dysfunction. Hasenfuss et al proposed the functional relevance of SERCA2 in falling human myocardium (37). SERCA2 levels reduced by around 30% in failing compared with non-failing myocardium. In another analysis about cardiac systolic and diastolic dysfunction after a cholesterol-rich diet (38), SERCA2 mRNA levels decreased around 17% after 4 days of diet intervention and was sufficient to correlate to impaired cardiac diastolic dysfunction. There is also evidence that around 17~40% of changes of SERCA2 could have substantial influence for cardiac diastolic function (39,40). As to the changes of Ca^{2+} transient, Halow et al demonstrated that slowed Ca^{2+} transient decline well represented slowed relaxation in a rat heart model (41). Therefore, changes in Ca^{2+} transient decay can be linearly translated into changes of ventricular relaxation. According to these references, we

believed that our findings over cellular models might be large enough for substantial physiological implications.

Limitations

This study had several limitations. First, we did not directly measure the diastolic calcium transient decay rate and SERCA2 expression in patients with DHF. Theoretically, this is not feasible in human studies. Second, this is a hypothesis generating study. The mechanism proposed in the study is still speculative. Third, we did not observe any differences in SR calcium content or release, but only decreases in calcium reuptake. During heart failure, decreased SERCA2 function is a long-lasting process, which eventually results in decrease SR content. In our experiment, we treated myocytes with cytokine for 24 hours. This scale is relatively short, and can only represent an acute or sub-acute course. Therefore, we postulated after longer time of stimulation, decreased SERCA2 function may result in decreased SR content and slower calcium release.

CONCLUSIONS

This study revealed a significant correlation between the immunoinflammatory system and the development of DHF. We also demonstrated that cytokines have a significant impact on SERCA2, one of the most essential calcium handling proteins,

pertinent to diastolic dysfunction at the cellular level. While there is little information regarding the medical management of DHF, a better understanding of the nature of inflammatory activation in patients with DHF may lead to improvement of outcomes by applying novel therapies aimed at limiting inflammatory reactions, particularly in the setting of patients in intensive care units.

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Figure Legends

Figure 1.

Title: Correlation between interleukin-6 (IL-6), tissue necrosis factor-alpha (TNF-α) and echocardiographic diastolic function parameters

Caption: A) TNF-α and mitral valve ejection flow deceleration time (DT) (r = 0.72, p < .001). B) TNF-α and the ratio of mitral valve ejection flow (E) divided by mitral valve atrium flow (A) (r = -0.69, p < .001). C) TNF-α and the ratio of E divided by early diastolic lengthening velocities in tissue Doppler imaging (Em) (r = 0.78, p < .001). D) IL-6 and DT (r = 0.72, p < .001). E) IL-6 and E/A (r = -0.65, p < .001). F) IL-6 and E/Em (r = 0.80, p < .001).

Figure 2.

Title: TNF-α and IL-6 downregulated *SERCA2* gene promoter activity and effect of TNF-α or IL-6 on mRNA expression and protein level of SERCA2 in HL-1 cardiomyocytes

Caption: HL-1 cardiomyocytes were transiently transfected with the SERCA2 gene 1754-bp promoter-PGL3Basic luciferase reporter fusion plasmids and the pRL-TK vector for an internal control, and luciferase activities were measured after stimulation with vehicle (control) or TNF-α 5 ng/mL or IL-6 10 ng/mL for 24 hours. Data

represent mean ± SE of 3 experiments (Panel A and B). Serum-depleted cardiomyocytes were treated with either vehicle (control) or TNF-α or IL-6 at the indicated concentrations for indicated times (C and D). The level of SERCA2 mRNA was analyzed by quantitative real-time RT-PCR and GAPDH was used as an internal control. Serum-depleted cardiomyocytes were treated with either vehicle (control) or TNF-α 5 ng/mL (C) or IL-6 10 ng/mL (D) for the indicated times. (E). The level of SERCA2 protein was analyzed by Western blot (WB) and GAPDH was used as an internal control. Serum-depleted cardiomyocytes were treated with either vehicle (control) or TNF-α 5 ng/mL or IL-6 10 ng/mL for the indicated times. Data represent mean ± SE of 3 experiments. (F). The fluorescence-cycle number relationship for the quantitative real-time RT-PCR. For any given fluorescence level, more PCR cycles were needed for TNF-α and IL-6 treated cells compared to control cells, indicating lower mRNA levels. *P<0.05 vs control, **P<0.01 vs control.

Figure 3

Title: Effect of sera from critically ill patients on protein expression of SERCA2 in cardiomyocytes

Caption: The levels of SERCA2 mRNA (A) and protein (B) were analyzed by quantitative real-time RT-PCR and Western blotting, respectively, after treating HL-1

cells with either sera from critically ill patients (P; PAT), healthy subjects (C; CTL1) or for 24 h with GAPDH as an internal control. The control subjects included healthy subjects (CTL1) and stable ICU patients (CTL2). *P<0.05 and **P<0.01 compared to controls.

Figure 4

Title: Calcium transients recorded in control, TNF (5 ng/mL; 24 h) and IL-6 (10 ng/mL; 24 h) treated cells

Caption: Calcium transients are expressed as fluorescence levels (F) normalized to diastolic fluorescence levels (F/F0). Return of intracellular calcium to diastolic levels is slower for TNF-α and IL-6 treated cells, as indicated by exponential fit (thick red line) to the decay phase of the calcium transient. The calcium transients are representative of 6 independent preparations. Inset, two-dimensional image to show the possible scanning lines for calcium transient recordings (red arrows).

Table 1. Baseline Characteristics and Cytokines Levels of Patients and Controls (group 1)

	DHF (n = 110)	Control (n = 50)	p
Age (years)	72.44 ± 9.86	72.16 ± 9.62	0.88
Sex (M/F)	52/58	26/29	1.00
BMI (kg/m2)	25.1 ± 4.7	25.1 ± 2.8	0.97
Diabetes mellitus (%)	32 (29)	18 (36)	0.72
Hypertension (%)	76 (69)	39 (78)	0.65
Creatinine (mg/dL)	1.35 ± 0.71	1.37 ± 0.76	0.91
Smoking (%)	31 (28)	20 (40)	0.29
WBC (/mm^3)	7.3 ± 2.0	7.6 ± 2.0	0.47
Cholesterol (mg/dL)	196.2 ± 38.2	202.6 ± 38.6	0.38
Triglyceride (mg/dL)	137.8 ± 89.7	128.8 ± 101.3	0.62
Low density cholesterol (mg/dL)	126.9 ± 39.8	128.2 ± 35.8	0.86
Anti-hypertension therapy			
ACEI + ARB (%)	30 (27)	12 (22)	0.28
β-blocker (%)	24 (22)	16 (29)	0.44
Calcium channel blocker (%)	38 (35)	20 (36)	0.79
Echocardiographic data			

LA diameter (mm)	37.3 ± 6.0	36.6 ± 4.8	0.51
LVEF (%)	67.6 ± 8.0	68.6 ± 5.2	0.35
LVEDD (mm)	45.9 ± 5.9	47.0 ± 4.8	0.24
LVESD (mm)	28.6 ± 5.7	29.6 ± 4.4	0.26
DT (cm/s)	304.9 ± 43.9	189.1 ± 64.2	<0.001
E/A	0.53 ± 0.17	1.17 ± 0.44	<0.001
E/Em	11.15 ± 3.65	6.88 ± 2.57	<0.001
Cytokine levels			
Interleukin 6 (pg/mL)	4.99 ± 1.75	2.54 ± 1.74	<0.001
	5.38 ± 1.68	2.61 ± 1.40	<0.00
Tissue necrosis factor-α (pg/mL)			1

Continues variables are presented as mean ± SD while categorical variables are presented as frequencies.

DHF, diastolic heart failure; BMI, body mass index; BP, blood pressure; ACEI, angiotensin-converting enzyme blocker; ARB, angiotensin Ⅱ type I receptor blocker; LA, left atrium; LVEF, left ventricular ejection fraction; LVEDD, left ventricular end diastolic dimension; LVESD, left ventricular end systolic dimension; DT, mitral flow deceleration time; E/A, early mitral valve flow velocity (E) divided by late mitral flow velocity (A); E/Em, E divided by early diastolic (Em) lengthening velocities in tissue

Doppler imaging.

Table 2. Baseline Characteristics and Cytokines Levels in Critically Ill Patients (n = 30)

Age (years)	65.6 ± 12.3
Sex (M/F)	17/13
BMI (kg/m2)	24.1 ± 5.5
Diabetes mellitus (%)	16 (53)
Hypertension (%)	18 (60)
Creatinine (mg/dL)	2.5 ± 1.8
Smoking (%)	10 (33)
WBC (/mm^3)	19.6 ± 7.7
Cholesterol (mg/dL)	187.2 ± 45.1
Triglyceride (mg/dL)	135.9 ± 86.2
Low density cholesterol (mg/dL)	134.7 ± 35.1
Cause of critical illness	
Pneumonia with respiratory failure (%)	10 (33)
Sepsis (%)	16 (53)
Myocardial infarction (%)	4 (13)
Echocardiographic Data	
LA diameter (mm)	36.9 ± 6.6

LVEF (%)	56.9 ± 7.1
LVEDD (mm)	49.3 ± 8.7
LVESD (mm)	30.2 ± 7.5
Cytokine levels	
Interleukin-6 (pg/mL)	83.3 ± 49.2
Tissue necrosis factor-α (pg/mL)	38.9 ± 20.4

Continues variables are presented as means ± SD while categorical variables are presented as frequencies.

BMI, body mass index; BP, blood pressure; LA, left atrium; LVEF, left ventricular ejection fraction; LVEDD, left ventricular end diastolic dimension; LVESD, left ventricular end systolic dimension.

Table 3. Interleukin-6, tissue necrosis factor-alpha, and echocardiographic parameters in critically ill patients while in the intensive care unit and after transfer to the general ward

	In Intensive Care Unit	After Transfer to General Ward	*p*
Interleukin-6 (pg/mL)	83.3 ± 49.2	17.6 ± 9.6	<.0001
Tissue necrosis factor-α (pg/mL)	38.9 ± 20.4	20.9 ± 12.6	.0002
DT	284.7 ± 48.3	263.2 ± 31.8	.019
E/A	0.48 ± 0.16	0.6 ± 0.27	.045
E/Em	17.0 ± 4.4	13.1 ± 6.5	.003

DT, mitral flow deceleration time; E/A, early mitral valve flow velocity (E) divided by late mitral flow velocity (A); E/Em, E divided by early diastolic (Em) lengthening velocities in tissue Doppler imaging.