Introduction

Dilated cardiomyopathy (DCM), a heart condition characterized by left ventricular dilation and progressive loss of cardiac function, represents the main cause of severe heart failure in younger adults and thus is a challenge for public health [1]. About one-third of DCM cases are genetic in origin, whereas the etiology of the remaining 70% is poorly understood [2]. Autoimmune responses against various myocardial antigens have been proposed to play an important role in the triggering or progression of DCM [3–4]. However, the mechanisms involved in its pathological process have not been elucidated.

Recent studies have reported that the activation of T lymphocytes and an increase in inflammatory cytokines are involved in chronic heart failure due to DCM [5,6]. A role for altered T cell proliferation was indicated by our previous studies which reported elevated ratios of CD4+ /CD8+ T lymphocytes during heart failure in rats [7]. These researches suggest that some certain elements may contribute to T lymphocyte disorder in the pathogenesis of chronic heart failure.

Evidences suggest that antigens newly exposed to the immune system upon cardiac damage trigger a myocardial autoimmune response, leading to ventricular remodeling and further damage to the myocardium [8]. In the 1990s, investigators reported that the

β1-Adrenoceptor Autoantibodies from DCM Patients Enhance the Proliferation of T Lymphocytes through the β1-AR/cAMP/PKA and p38 MAPK Pathways

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Abstract

Background: Autoantibodies against the second extracellular loop of the β1-adrenergic receptor (β1-AA) not only contribute to increased susceptibility to heart failure, but also play a causative role in myocardial remodeling through their sympathomimetic-like effects that are induced upon binding to the β1-adrenergic receptor. However, their role in the function of T lymphocytes has never been previously investigated. Our present study was designed to determine whether β1-AA isolated from the sera of dilated cardiomyopathy (DCM) patients caused the proliferation of T cells and the secretion of cytokines.

Methods: Blood samples were collected from 95 DCM patients as well as 95 healthy subjects, and β1-AA was detected using ELISA. The CD3+ T lymphocytes were selected separately through flow cytometry and the effect of β1-AA on T lymphocyte proliferation was examined by CCK-8 kits and CFSE assay. Western blotting was used to analyze the expressions of phospho-VASP and phospho-p38 MAPK.

Results: β1-AA enhanced the proliferation of T lymphocytes. This effect could be blocked by the selective β1-adrenergic receptor antagonist metoprolol, PKA inhibitor H89, and p38 MAPK inhibitor SB203580. Furthermore, the expression of the phosphorylated forms of phospho-VASP and phospho-p38 MAPK were markedly increased in the presence of β1-AA. β1-AA also inhibited the secretion of interferon-γ (IFN-γ) while promoting an increase in interleukin-4 (IL-4) levels.

Conclusions: These results demonstrate that β1-AA isolated from DCM patients binds to β1-AR on the surface of T cells, causing changes in T-cell proliferation and secretion through the β1-AR/cAMP/PKA and p38 MAPK pathways.

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autoimmune antibody (β1-AA) against the second extracellular loop (β1-AR-EC2) amino acid residues 197–223, 100% sequence identity between humans and rats [9] of the β1-adrenergic receptor is present in the sera of patients with cardiovascular diseases [10–13]. This led to the proposal stating that β1-AA acts similarly to a β1-adrenergic receptor agonist based on findings of an increased beating rate in neonatal rat cardiomyocytes [14]. We have reported that the long-term presence of β1-AA led to myocardial damage with increased ratios of CD4+/CD8+ T lymphocytes [15]. These clinical and experimental results, taken together with the expression of lymphocytes [16], strongly suggest that β1-AA might mediate T cell abnormalities in chronic heart failure patients. However, it is not known if the β1-AA isolated from DCM patients, which mimics the action of catecholamine, could recognize the cognate receptor and interfere with T lymphocytes.

Therefore, the aims of the current study were as follows: (1) to observe the effects of β1-AA on the proliferation and secretion of T cells, and (2) to identify the signaling pathways that mediate T cell responses.

Materials and Methods

Materials

Metoprolol (selective β1-adrenergic receptor antagonist), isoproterenol (β1/β2-adrenergic receptor agonist), SB203580 (selective p38 MAPK inhibitor), and H89 (selective PKA inhibitor) were purchased from Sigma-Aldrich Chemicals Company (USA). Carboxyfluorescein diacetate succinimidyl ester (CFSE) was purchased from Sigma-Aldrich Chemicals Company (USA). Polyclonal antibodies directed against phospho-VASP (Ser157), phospho-p38 MAPK (Thr180/Tyr182), VASP, and p38 MAPK were obtained from Cell Signaling Technologies (USA). All chemicals utilized in this study were of analytical grade.

Animals

Healthy male 8-week-old Sprague–Dawley rats, with normal blood pressure and heart rate, weighing 200 g to 240 g, were selected for this study. The experimental procedures were conducted in adherence to the “Guiding Principles in the Use and Care of Animals” published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996), the Guide for the Care and Use of Laboratory Animals protocol, published by the Ministry of the People’s Republic of China (issued on 3 June 2004), and approved by the Institutional Animal Care and Use Committee of Capital Medical University.

Patients and Samples

The study adheres to the principles of the Declaration of Helsinki and its amendments, the Code of Federal Regulations, Part 46, Protection of Human Subjects, Revised 13 November 2001, effective 13 December 2001. Ninety-five chronic heart failure patients were recruited from the Air Force General Hospital of the People’s Liberation Army and General Hospital of Tonghua Mining Group Co., LTD, all of which were suffering from dilated cardiomyopathy (DCM) (New York Heart Association functional class II to IV), with a left ventricular diastolic volume >110 ml/m² and an ejection fraction <45% (by echocardiography). DCM was diagnosed when coronary heart diseases were excluded by angiography and exposures to cardiotoxic substances, myocarditis, or other systemic heart diseases were not evident from clinical history. In ventriculography, all patients exhibited a diffuse reduction in wall motion. At the time of sample acquisition, all patients were stable under therapy with diuretics, ACE inhibitors, digitalis, and nitrates. The control group consisted of 95 healthy subjects randomly selected from the same community with normal clinical, ECG, and echocardiography examinations. On the basis of the resulting measurements of β1-AA, the DCM patients were divided into a β1-AA-positive group (n = 44) and a β1-AA-negative group (n = 51). Clinical characteristics are summarized in Table 1 and Table 2. Venous blood samples were collected without an anticoagulant. After centrifugation at 4 °C, the serum was immediately separated and stored at −80 °C until assay.

The Institutional Committee for the Protection of Human Subjects of Capital Medical University approved this research protocol. All patients were informed of the purpose and protocol of the investigational nature of the study. Both oral informed consent and written consent were obtained.

Peptide Synthesis

The peptide corresponding to the sequence (amino acid residues 197–223) of the second extracellular loop of the human β1-AR [15]: H-W-W-R-A-E-S-D-E-A-R-R-C-Y-N-D-P-K-C-D-C-D-F-V-T-N-R-G was synthesized using an automated peptide synthesizer by solid-phase methods. Peptide purity was judged by high-performance liquid chromatography (HPLC) using an automated amino-acid analyzer. Peptide preparations were 98% pure as judged by analytical HPLC. This work was performed by a contractor (Qiang Yao, Shanghai Bio Scientific Commercial Development Co. Ltd., China).

Enzyme-linked Immunosorbent Assay (ELISA)

The titer of β1-AA was measured by ELISA, and the results are expressed as optical-density (OD) units according to published methods [17]. Briefly, the synthetic peptide described above (5 mg/ml) in 100 mmol/l Na2CO3 (pH 11.0), was coated onto the wells of microtiter plates overnight at 4 °C. The wells were then saturated with 0.1% PMT buffer [0.1% (w/v) albumin bovine V, 1.0% Tween 20, and 0.02% NaN3] until assay.

Table 1. Clinical characteristics of patients with chronic heart failure due to DCM (mean ± SD).

|                | β1-AA-positive group (n = 44) | β1-AA-negative group (n = 51) | Healthy group (n = 95) |
|----------------|-----------------------------|-----------------------------|-----------------------|
| Age (year)     | 62 ± 12                     | 59 ± 6                      | 56 ± 10               |
| Gender (male/female) | 25/19                      | 27/24                       | 53/42                 |
| NYHA           | 36 ± 0.8                    | 32 ± 0.7                    | 43 ± 2.0              |
| LVEF (%)       | 35 ± 6.1***                 | 41 ± 7.0**                 | 64.9 ± 6.6            |
| LVEDD (mm)     | 67.5 ± 6.6**                | 63.8 ± 7.7**               | 46.5 ± 8.3            |
| LVESD (mm)     | 57.6 ± 6.6**                | 55.3 ± 7.9**               | 32.6 ± 7.3            |
| Medications    |                            |                            |                       |
| Diuretics (%)  | 52 (23/21)                  | 59 (30/21)                  |                       |
| Digoxin (%)    | 50 (22/22)                  | 57 (29/22)                  |                       |
| ACE-inhibitors (%) | 100 (44/0)                | 98 (50/1)                   |                       |
| β-Blockers (%) | 45 (20/24)                  | 37 (19/32)                  |                       |
| Anti-arrhythmia agents (%) | 23 (10/34)                | 31 (16/35)                  |                       |

DCM dilated cardiomyopathy; NYHA New York Heart Association; LVEF left ventricular ejection fraction; LVEDD left ventricular end-diastolic diameter; LVESD left ventricular end-systolic diameter. Values are expressed as mean ± SD or number (%). *P<0.01 versus healthy group. doi:10.1371/journal.pone.0052911.t001
Table 2. Holter Electrocardiographic Findings (mean ± SD).

|                      | β₁-AA-positive group (n = 44) | β₁-AA-negative group (n = 51) |
|----------------------|-------------------------------|--------------------------------|
| Atrial fibrillation (%) | 34 (15/29)                   | 27 (14/27)                    |
| PVCs                 | 2,136±3,400                  | 1,564±2,674                   |
| Multiform PVCs (%)   | 82 (35/8)****                | 63 (32/19)                    |
| VT                   | Presence of VT (%)           | 65 (24/20)***                |
|                      | Maximal runs of VT (beats)   | 7±5                           |
|                      | Longest VT duration (s)      | 2.2±1.4                      |
|                      | Fastest VT rate (beats/min)  | 187±32*                      |

PVC: premature ventricular contractions; VT: ventricular tachycardia. Values are expressed as mean ± SD or number (%) of patients. *P<0.05, **P<0.01 β₁-AA-positive group versus β₁-AA-negative group.

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0.1% (v/v) Tween-20 in phosphate-buffered saline (PBS, pH 7.4) for 1 h at 37°C. After washing 3 times with PBS-T, serial dilutions of human sera were added for 1 h at 37°C. After 3 washings, biotinylated goat-antihuman IgG antibodies (Sigma) (1:1000 dilutions in PBT) were added for 1 h at 37°C. After 3 washings, streptavidin-peroxidase conjugate (Sigma) at 1:2000 dilution in the same buffer was added to the wells and incubated under the same conditions. Finally, 2, 2-azino-di-(3-ethylbenzothiazoline) sulfonic acid (ABTS)-H2O2 (Roche, Switzerland) substrate buffer was added and reacted for 30 min in the dark at room temperature. The OD values were measured at 405 nm using a microplate reader (Spectra Max Plus, Molecular Devices, USA). We also calculated antibody titer according to the ratio (P/N) of OD values of positive/negative controls ([specimen OD-blank control OD]/(negative control OD-blank control OD)) [7]. Control samples were prepared as follows: 95 sera samples from healthy humans with an OD value of less than 2.5 times the background OD were pooled and centrifuged at 1,500 rpm for 10 min, and the supernatants were then divided into small aliquots and stored for subsequent use. Samples positive or negative for β₁-AA were defined as P/N ≥2.1 or P/N ≤1.5, respectively.

Preparation of Immunoglobulin G

Immunoglobulin G fractions (IgG) from the sera of 44 β₁-AA-positive or from 51 β₁-AA-negative DCM patients were prepared by MabTrap Kit (Amersham Bioscience, Sweden). The concentrations (µg/ml) and specificities of purified IgGs were determined by the Bicinchoninic Acid (BCA) Protein Assay (Pierce, USA) and ELISA, respectively.

Isolation and Culture of CD3⁺ T Cells

Rats were anesthetized with ether, and a blood sample was taken from the abdominal aorta. Mononuclear cells were prepared from the freshly drawn blood samples by Ficoll-Hypaque (1.077 g/l) density gradients. Whole blood (40 ml) was collected with heparin and centrifuged. After the plasma was discarded, white cells and erythrocytes were taken and suspended in 10 ml of PBS (pH 7.4). These suspensions were added to 5 ml of Ficoll-Hypaque and then centrifuged at 2,000 rpm for 20 min. Mononuclear cells were collected, washed twice with PBS, and centrifuged at 1,500 rpm for 10 min. To eliminate adherent cells (monocytes), cell suspensions were placed into culture flasks with 5 ml of Roswell Park Memorial Institute medium 1640 (RPMI) with gentamicin (100 µg/ml), L-glutamine (2 mmol/l), and 10% fetal calf serum (Invitrogen, USA). Cells were incubated at 37°C in humidified air containing 5% CO2 for 30 min. Nonadherent cells were collected, washed, and isolated by centrifugation at 1,500 rpm for 10 min and suspended in culture medium. CD3⁺ T cells were selected from the mononuclear cells using a flow cytometer (BD Biosciences, USA).

Immunofluorescence Staining

CD3⁺ T cells were gently washed with PBS (pH 7.4) and immediately fixed with 4% paraformaldehyde (w/v) for 20 min. Cells were blocked in PBS containing 5% bovine serum albumin (BSA) (w/v). The cells were then incubated overnight at 4°C with the IgG fractions (25 µg/ml) from β₁-AA-positive DCM patients at a 1:500 dilution. Following three PBS washes, cells were incubated in donkey anti-human IgG tagged with fluorescein isothiocyanate (FITC) as the secondary antibody for 1 hour in the dark at 37°C. After being rinsed with PBS, cover slips with mounting medium containing 4’, 6-diamidino-2-phenylindole (DAPI) stain nuclei were coated. Negative controls were performed by omitting primary antibodies. Images were acquired using a Zeiss 510 Meta Confocal microscope (63 power oil 1.40 NA (Zeiss, Germany), pinhole equals 1.0 Airy Disc) using the Carl Zeiss Imaging software.

Culture of Beating Neonatal Cardiomyocytes

Hearts were removed aseptically from 1 to 2-day-old Sprague-Dawley rats and cultured as described [7]. The number of beats of a selected isolated myocardial cell or a cluster of synchronously contracting cells in each of 10 fields was counted for 15 s each. The IgG fractions from β₁-AA-positive DCM patients and corresponding receptor agonists were added, and the cells were observed for 5 min after each addition. This procedure was repeated three times in different cultures to yield results representing a total of 30 cells or cell clusters. The basal beating rate was 145±15 beats/min.

CD3⁺ T Cell Proliferation Assays

1. CCK-8 assay. CD3⁺ T cells (5×10⁵ cells/ml) were cultured for 48 h with or without mitogens in either the presence or absence of β₁-AA (12.5 µg/ml, 25 µg/ml, and 50 µg/ml), H89 (1 µmol/l), metoprolol (1 µmol/l), isoproterenol (0.1 µmol/l), and SB203580 (1 µmol/l). The mitogens used were 3 µg/ml soluble mouse anti-rat CD3 mAb (Clone 1F4, Biologend, USA) and 1 µg/ml soluble mouse anti-rat CD28 mAb (Clone JI319, Biologend). Agonists were added to cell suspensions together with the mitogens while the antagonists were added 1 h before the agonists. After each treatment, 10 µl CCK-8 solution was added to each well, and the cells were incubated for 4 hours at 37°C. The absorbance at 450 nm was measured using a microplate reader with the wavelength correction set to 630 nm.

2. CFSE-labeling of lymphocytes. CFSE (10 mol/l in DMSO [Invitrogen]) was diluted in PBS. CD3⁺ T lymphocytes were suspended in PBS supplemented with 0.05% BSA and 4 mol/l CFSE. (2×10⁵ cells/ml) for 10 min at 37°C in a 5% CO2 atmosphere. Cells were washed, diluted in 0.5 ml culture medium and incubated for 30 min at 37°C in 5% CO2 to stabilize the CFSE-labeling. The efficiency of labeling untreated cells was >95%.
β1-AA Enhanced the Proliferation of T Lymphocyte

β1-AA Bound to β1-ARs on the Surface of CD3+T Cells

To determine the purity of the CD3+ T cells preparation, multi-color flow cytometry was used. As illustrated in Fig. 2A, after sorting, the CD3+ T cells represented 92.2% of the cell population. We next employed immunofluorescence staining to determine whether the IgG fraction isolated from the β1-AR-positive sera of DCM patients could bind to β1-ARs. We found that β1-AA (25 μg/ml) showed that the β1-AR staining was localized mainly to the membrane, while DAPI staining was only observed in the nucleus (Fig. 2C). Therefore, we concluded that the IgG fraction isolated from β1-AA-positive sera of DCM patients exhibited a pattern of β1-AR specific binding virtually identical to commercially available β1-AR-specific antibodies.

β1-AA Increased the Beat Frequency of Cultured Cardiomyocytes

The IgG fractions (25 μg/ml) isolated from β1-AR-positive sera of DCM patients increased cardiomyocyte beat frequency, similar to the effects of the β1-adrenergic receptor agonist isoproterenol (0.1 μmol/l). This effect of β1-AA was abolished by the addition of the β1-adrenergic receptor antagonist metoprolol (1 μmol/l) (Fig. 3).

β1-AA Promoted the Proliferation of T Lymphocytes

We found that β1-AA enhanced T cell proliferation in a concentration-dependent manner (Fig. 4A). Therefore, we chose 25 μg/ml for further study because it is comparable to the concentration in the sera of heart failure patients [18–19]. Freshly isolated CD3+ T cells were stimulated with anti-CD3/CD28 mAb in either the presence or absence of β1-AA for 48 h. As summarized in Fig. 4, the presence of β1-AA increased CD3+ T cell proliferation (0.127±0.028 vs. 0.0745±0.016, p<0.05) (Fig. 4B). However, administration of β1-AA-negative IgG purified from 51 DCM patients did not detectably affect proliferation (0.004±0.0059 vs. 0.0745±0.016, p>0.05) (Fig. 4B). Similar results were obtained when we used the CFSE assay (Figs. 4C and 4D). Taken together, the results presented in Fig. 4 demonstrate that β1-AA present in DCM patients induced CD3+ T cells proliferation.

β1-AA Enhanced T Lymphocyte Proliferation through the β1-AR/cAMP/PKA Pathway

The most common signaling mechanism initiated by β1-AR stimulation is the β1-AR/cAMP/PKA pathway [18,20]. To determine whether β1-AA-stimulated T cell proliferation resulted from the triggering of this pathway, the selective β1-AR antagonist metoprolol (1 μmol/l) and the PKA inhibitor H89 (1 μmol/l) were used to block the pathway before β1-AA administration, and then the activity of PKA was determined. The results demonstrated that T cell proliferation mediated by β1-AA was partially inhibited by metoprolol (0.094±0.0044 vs. 0.127±0.028) and H89 (0.094±0.0044 vs. 0.0745±0.016).
β1-AA Enhanced the Proliferation of T Lymphocyte

Involvement of p38 MAPK in β1-AA-mediated T Lymphocyte Proliferation

In immune cells, p38 MAPK plays a role in regulating the production of mature T cells [21–22]. To investigate the role of activation of p38 MAPK in β1-AA-stimulated T cell proliferation, the selective p38 MAPK antagonist SB203580 (1 μmol/l) was used to block the pathway before stimulation with β1-AA (25 μg/ml), and then the activity of p38 MAPK was determined. As depicted in Fig. 6A, T cell proliferation stimulated by β1-AA was partially inhibited by SB203580 (0.121 ± 0.00415 vs. β1-AA group 0.137 ± 0.0086, p < 0.05; 0.121 ± 0.00415 vs. vehicle group 0.109 ± 0.0052, p < 0.05). Although H89 and SB203580 were used together before β1-AA administration, the proliferation of T cells induced by β1-AA was still partially blocked (0.12 ± 0.0043 vs. β1-AA group 0.137 ± 0.0086, p < 0.05; 0.12 ± 0.0043 vs. vehicle group 0.109 ± 0.0052, p < 0.05) (Fig. 6A). In immunoblot analysis, β1-AA stimulated VASP phosphorylation, which was inhibited by metoprolol and H89. However, it had no effect on total VASP (Fig. 5C, 5D). Collectively, these results suggest that the β1-AR/cAMP/PKA pathway was involved in T cell proliferation stimulated by β1-AA.

β1-AA Inhibited IFN-γ Secretion, but Promoted IL-4 Production

As illustrated in Fig. 7A, the addition of IgG isolated from β1-AA-positive sera of DCM patients caused a reduction in IFN-γ production (6.788 ± 1.46 pg/ml vs. vehicle group 56.22 ± 2.29 pg/ml, p < 0.01; 6.788 ± 1.46 pg/ml vs. Negative IgG group 22.96 ± 0.903 pg/ml, p < 0.01) (Fig. 7A). The effect was completely blocked by the addition of the selective β1-AR antagonist metoprolol (1 μmol/l) (p < 0.05) (Fig. 7A). We next examined the effects of β1-AA on the production of IL-4 in T cells. The results suggest that β1-AA promoted the secretion of IL-4 (95.37 ± 61.79 pg/ml vs. 413.19 ± 32.495 pg/ml, p < 0.01) (Fig. 7B), while the increase in IL-4 was antagonized by metoprolol (1 μmol/l) (p < 0.05). Collectively, these results suggest that β1-AA regulated the secretion of T cells.

Discussion

The concentration of circulating autoantibodies directed against the second extracellular loop of β1-AR is known to be increased in patients with heart failure [17] when compared with healthy subjects. Our present results confirm these findings, and we report here that autoantibodies directed against the second extracellular loop of β1-AR were present in 46.3% of sera collected from 95 patients with heart failure due to DCM, which was significantly higher than in the samples from 95 healthy subjects. Moreover, we noted that their overall prevalence was 8.42% in healthy subjects, which is clearly more than has been reported to date [19,20,23]. We believe that these differences are essentially due to different methods used to detect the autoantibodies as well as the discriminant criteria. In the present study, the P/N ratio was used to represent the positive rate of β1-AA detection, which can effectively reduce the risk for false negative results [6]. Furthermore, during the myocardial remodeling process in rats, the generation of β1-AA showed a characteristic self-growth and time-course decline, and the existence of β1-AA in rats lasted for a short period, with the titers gradually tapering after about two to three months [2]. Additionally, in concert with our animal experiments, it was reported, clinically, that autoantibodies against the β1-AR existing in the sera of DCM patients also present a time-course decrease and cardiac autoantibodies in patients with DCM become undetectable with disease progression [24–25]. Therefore,
the presence of $\beta_1$-AA in the sera of DCM patients showed a time-course decrease, and the different pathological processes of heart failure patients could lead to the changes of incidence of $\beta_1$-AA. Moreover, using the immunofluorescence and radioligand-binding techniques, we could demonstrate that $\beta_1$-AA isolated from DCM patients recognized $\beta_1$-ARs expressed either on CD3$^+$T cells or on H9c2 rat cardiomyoblast cells transiently transfected with $\beta_1$-AR (Fig. S1C, Fig. 2), whereas after $\beta_2$-ARs expressed on CD3$^+$T cells

Figure 2. $\beta_1$-AA from DCM patients bound to $\beta_1$-ARs on the surface of CD3$^+$T cells. A. After FACS, the purity of selected rat CD3$^+$T lymphocytes by immunomagnetic separation was 92.2%. B, C. The binding of $\beta_1$-AA (25 $\mu$g/ml) with the $\beta_1$-ARs on the CD3$^+$T cells was determined by confocal microscopy, and $\beta_1$-AR was identified using an anti-$\beta_1$-AR antibody (green). Nuclei were labeled with DAPI (blue). The negative control was performed by omitting primary antibodies during the incubation. Bar, 30 $\mu$m.

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were blocked by the specific β2-AR antagonist ICI118551, CD3^+ T cells can still be stained (Fig. S1B). Additionally, in order to enhance specificity, the monoclonal antibody, which was obtained by immunizing Balb/C mice with free peptide H26R corresponding to the second extracellular loop of the human β1-AR, and had the same biological effect with β1-AA IgGs isolated from heart failure patients (Fig. S3), was used to serve as positive control (Fig. S1A). These experiments revealed that β1-AA isolated from DCM patients can bind to β1-ARs on CD3^+ T cells, and did not cross-react with the very closely related β2-AR expressed on CD3^+ T cells. All of these antibodies were directed against the second extracellular domain, which is known to affect ligand binding [26] and may induce immune responses [11]. They all increased the beat frequency and cAMP level of cultured cardiomyocytes (Fig. S4), in the same way as the effect of IgG fractions isolated from individual β1-AA-positive sera of DCM patient (Fig. S5). Our results indicated that β1-AA may be one of the abnormal immune phenomena in heart failure and suggests their involvement in the pathophysiology of essential heart failure.

Recent clinical reports showed that the selective β1-AR antagonist, metoprolol, decreased the frequency and the geometric mean titer of β1-AA [23,27]. Moreover, Wallukat [28] demonstrated that the β1-adrenergic receptor antagonists were able to block the effect of the antibodies and displace the anti-β1-adrenergic receptor antibodies from their binding sites on the receptor, leading to a decrease of β1-AA titers in patients with

Figure 3. β1-AA from DCM patients increased the beat frequency of cultured cardiomyocytes. The bar graph shows the increase in beat frequency of isolated myocardial cells stimulated by β1-AA (25 μg/ml) or isoproterenol (0.1 μmol/l). **p<0.01 versus vehicle group. Data were presented as means ± SD of three independent experiments. ISO: isoproterenol, MET: metoprolol. doi:10.1371/journal.pone.0052911.g003

Figure 4. β1-AA from DCM patients significantly promoted the proliferation of CD3^+ T cells. A. β1-AA promoted CD3^+ T cell proliferation in a concentration-dependent manner. B. CD3^+ T cells (5×10^5 cells/ml) were incubated for 48 h at 37°C and 5% CO₂ in the presence of β1-AA (25 μg/ml) or isoproterenol (0.1 μmol/l). Cell proliferation was measured at 450 nm by CCK-8 uptake assay. ***p<0.01 versus vehicle group; **p<0.01 versus negative IgG group. n=9 per group. C. CD3^+ T cells were labeled with 4 μmol/l CFSE, and cell proliferation was measured by flow cytometry. Data shown here are representative of one of three different experiments with similar results. D. The bar graph shows the percentage of proliferating (CFSE^-) T cells among the total CD3^+ T cell population. n=3, *p<0.05 versus vehicle group. **p<0.01 versus vehicle group; ***p<0.01 versus Negative IgG group, ISO: isoproterenol. doi:10.1371/journal.pone.0052911.g004
Figure 5. β1-AA-mediated T cells proliferation through the β1-AR/cAMP/PKA pathway. A. T cells were stimulated with the selective β1-AR antagonist metoprolol (1 μmol/l) and the selective PKA inhibitor H89 (1 μmol/l) for 1 h at 37°C in 5% CO2 before the addition of β1-AA (25 μg/ml). ∗∗p<0.05, ∗∗∗p<0.01 versus vehicle group; ∗p<0.05, ∗∗∗p<0.01 versus β1-AA group. n=9 per group. B. The effect of β1-AA or isoproterenol on the production of cAMP (expressed as pg/ml) in T lymphocytes was examined by ELISA. ∗p<0.01 versus vehicle group, #p<0.01 versus negative IgG group, ##p<0.01 versus β1-AA group. Data were presented as means ± SD of 6 independent experiments. C. Immunoblot detection of phosphorylated VASP (p-VASP) and total VASP from CD3^+ T cells stimulated with β1-AA for 30 min. Images are representative of 3 independent experiments. D. The bar graph shows the ratio of p-VASP to total VASP. n=3, **p<0.01 versus vehicle group, ∗∗∗p<0.01 versus β1-AA group, MET: metoprolol.

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According to published paper [14], the patients with DCM also develop functionally active antibodies against the first extracellular loop of the β1-AR (β1-AR-EC1). Therefore, to detect whether there were anti-β1-AR-EC1 antibodies in DCM patients involved in this study, we added peptide corresponding to the sequence of the 1st (β1-AR-EC1) as negative control, and found that the supernatant produced by incubating β1-AA-positive IgG with β1-AR-EC1 still promoted CD3^+ T lymphocytes proliferation (Fig. S7A). However, the supernatant produced by incubating β1-AA-positive IgG with peptide corresponding to the sequence of the 2nd (β1-AR-EC2) had no effect on CD3^+ T lymphocytes (Fig. S7B). These results suggest that β1-AA-positive IgG isolated from DCM patients promoted the proliferation of CD3^+ T lymphocytes by binding to β1-AR-EC1.

The most common signaling mechanism initiated by β1-AR stimulation is the β1-AR/cAMP/PKA pathway [18,20]. In order to explore whether β1-AA promoted T lymphocyte proliferation through this pathway, the β1-AR selective antagonist metoprolol was added to T lymphocytes before β1-AA administration. As a result, the proliferative effect could be blocked, suggesting that β1-ARs on the surface of CD3^+ T cells could be activated by the β1-AA from DCM patients. Furthermore, we also found that each β1-AA sample increased mitogen-stimulated cAMP production in a receptor-mediated fashion.
Previous studies have demonstrated the PKA-dependent effects in immune cells by either assessing agonist-stimulated PKA activity through in vitro assays or demonstrating the actions of pharmacologic PKA inhibitors and activators [30–31]. Here we used a more direct approach in which we analyzed PKA activity by assaying for the phosphorylation of VASP at Ser157, which is mediated directly and selectively by PKA [32–33]. We found that β1-AR activation by β1-AA rapidly led to VASP phosphorylation.

Figure 6. β1-AA-mediated T cell proliferation mediated by activation of p38 MAPK. A. T cells were pretreated with the selective PKA inhibitor H89 (1 μmol/l) and the selective p38 MAPK inhibitor SB203580 (1 μmol/l) for 1 h at 37°C in 5% CO2, before stimulation by β1-AA (25 μg/ml). *p<0.05, **p<0.01 versus vehicle group; §p<0.05 versus β1-AA group. n=9 per group. B. Immunoblot detection of phosphorylated p38 MAPK (p-p38 MAPK) and total p38 MAPK from CD3+ T cells stimulated with β1-AA for 30 min. Images are representative of three independent experiments. C. The bar graph shows the ratio of p-p38 MAPK to total p38 MAPK. n=3, **p<0.01 versus Vehicle group, ***p<0.01 versus β1-AA group, SB: SB203580.
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Figure 7. β1-AA inhibited IFN-γ secretion and promoted IL-4 production in T cells. A. T cells (5×10^5 cells/ml) were pretreated with 1 μmol/l metoprolol in the presence of β1-AA (25 μg/ml) for 48 h, and then IFN-γ levels were analyzed by ELISA. **p<0.01 versus vehicle group, ##p<0.01 versus negative IgG group, n=9/group. B. The effect of β1-AA (25 μg/ml) on IL-4 levels was examined by ELISA, **p<0.01 versus vehicle group, ###p<0.01 versus negative IgG group, ***p<0.01 versus β1-AA group, n=9 per group. Data are presented as means ± SD of 6 independent experiments.
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at Ser157. The selective β1-AR antagonist metoprolol decreased the level of VASP phosphorylation stimulated by β1-AR. In addition, inhibition of PKA by compound H89 abrogated β1-AA-induced phosphorylation of VASP at Ser157. However, β1-AR had no effect on total VASP. Taken together, all of these results strongly implicate the β1-AR/cAMP/PKA pathway as the principal signaling system modulating the β1-AA-induced phosphorylation of VASP at Ser157.

In T cells, one physiological effect of p38 MAPK activity is the regulation of cell growth and cell death, which are especially important in the thymus during T cell development [34]. Dysregulation of p38 MAPK can result in negative selection-induced cell death and the subsequent absence of T cell populations in the peripheral immune system [35]. Our present results suggest that the proliferation of T cells induced by β1-AA was partially blocked by the p38 MAPK-selective inhibitor SB203580. Moreover, immunoblot assays revealed an apparent increase in phosphorylation of p38 MAPK following treatments with β1-AR, in the absence of an effect on total p38 MAPK levels. These results indicated that activation of p38 MAPK was correlated with the production of mature T cells. Furthermore, when H89 and SB203580 were used together before stimulation with β1-AR, the proliferation of T cells was also partially inhibited. These results suggest that both β1-AR/cAMP/PKA and p38 MAPK pathways contributed to β1-AA-mediated proliferation of T cells. However, other pathways may also participate in this process. The study by Antonio et al. [18] showed that the effect of β1-AR on cardiomyocytes could be blocked by tyrosine kinase inhibitor PP2. Other studies have also reported that the role of β1-AR could be mediated by PI3-kinase, PKC, or PKA in the trigger phase of ischemic preconditioning [36]. Based on the studies mentioned, further researches are necessary to investigate the other possible pathways stimulated by β1-AR.

The main form of T cell activation is to secrete cytokines. Therefore, in the current study, the levels of IFN-γ and IL-4, the characteristic cytokines secreted by T cells, were chosen to detect the effect of β1-AA on T lymphocytes secretion. IFN-γ is a major proinflammatory effector and regulatory cytokine produced by activated T lymphocytes, which can inhibit humoral immunity by suppressing the production of Th2 cells, but promotes cell-mediated immunity [37]. Our results suggest that both β1-AA-positive and -negative IgGs might inhibit the secretion of IFN-γ, though the effect of β1-AA-positive IgGs was more pronounced than that of β1-AA-negative IgGs. However, the IgGs isolated from healthy subjects did not enhance IFN-γ secretion (Fig. S8A). Moreover, in order to analyze whether the reduction of IFN-γ production caused by the β1-AA-negative IgG preparation of DCM patients might have an effect of anti-β1-AR-EG1-antibodies, we added peptide corresponding to the sequence of the 1st extracellular loop of the receptor (β1-AR-EG1) as a negative control. We found unexpectedly that the supernatant produced by incubating β1-AA-negative IgGs with β1-AR-EG1 also decreased IFN-γ production, however, compared with β1-AA-negative IgGs, the inhibition of IFN-γ level was alleviated (Fig. S9). These results suggest that there may be anti-β1-AR-EG1-antibodies in β1-AA-negative DCM patients, which inhibited IFN-γ production. Besides that, heart failure itself may be a risk factor in inhibiting cell-mediated immunity, and the effect may be magnified due to the presence of β1-AA. However, the mechanism for the β1-AA-mediated decrease in IFN-γ production is unknown, and further investigations are needed to explain this phenomenon.

IL-4 is the characteristic cytokine secreted by Th2 cells, which decreases the production of Th1 cells and promotes humoral immunity [38–39]. It has been reported that the β-AR agonist isoproterenol may activate Th2 cells and promote IL-4 production mainly via binding to β1-ARs expressed on T lymphocytes [40]. In the current study, we found that β1-AA from DCM patients can enhance IL-4 release through combining with β1-ARs also expressed on T lymphocytes, but the IgGs isolated from healthy subjects did not enhance IL-4 secretion (Fig. S8B). These results suggest that although β1-AA mediates β1-AR agonist-like actions; it is different from the β1-AR agonist isoproterenol and might activate Th2 cells through the β1-AR pathway. Additionally, β1-AA serves as a kind of antibody that is produced by B cells and Th2 cells, and the present study shows that β1-AA may enhance humoral immunity, while inhibiting cell-mediated immunity. This suggests that there is positive feedback between β1-AA and Th2 cells. However, further studies are required to support this conclusion.

Nonetheless, our work leaves some unanswered questions and paths for future work. The β1-AA used in this study was not specific for the second extracellular loop of β1-AR, and some nonspecific IgGs were involved. Additionally, because of clinical and ethical reasons, DCM patients involved in the current study only stopped β-blocker therapy for one week, which can not completely preclude effects of β-blocker on β1-AA synthesis. Therefore, further studies using monoclonal antibodies specific for the second extracellular loop of β1-AR would be carried out to yield more conclusive results. Moreover, whether there are any differences in T lymphocytes in β1-AA positive and negative patients has not been elucidated. In addition, in the present study, ELISA and binding rate of isolated neonatal cardiomyocytes were employed to detect the titer and function of β1-AA in the patients with DCM. However, recent research reported that a novel molecular and/or fluorescence-based diagnostic method of detecting β1-AA in patients with heart failure was proved to be fast and highly sensitive [41]. Therefore, further investigations using new diagnostic methods would be conducted to provide functional and conclusive diagnostic data.

Supporting Information

**Figure S1** Colocalization experiments. A. Anti-β1-AR monoclonal antibody was used as a positive control. B. CD3+T cells were pretreated with IC1118351 for 1 h in the presence of β1-AA, and then the binding of β1-AA with the β1-ARs on CD3+T cells was determined by confocal microscopy respectively. C. Colocalization experiments with H9c2 cells transiently expressing β1-AR. (TIF)

**Figure S2** Inhibitory effect of β1-AA from DCM patients with different concentrations on [125I]-PIN binding to β1-AR. Results are expressed as percentage of binding in the absence of β1-AA. (TIF)

**Figure S3** Anti-β1-AR monoclonal antibody has been synthesized successfully. A. The level of β1-AA in the supernatant of hybridoma cell was detected using ELISA. **p < 0.01 vs. Vehicle group; n = 3/group. B. Western blot method was used to analyze the combination between anti-β1-AR monoclonal
antibody and β1-AR on the surface of H9C2 cell. n = 3/group. Supernatant group: the supernatant of hybridoma cell. Positive control antibody group: commercial anti-β1-AR polyclonal antibody. (G) Radioiodinating experiment was employed to investigate the co-localization of anti-β1-AR monoclonal antibody to the β1-ARs on the surface of H9C2 cell. (D) Anti-β1-AR monoclonal antibody increased the beat frequency of cultured cardiomyocytes. The bar graph shows the increase in beat frequency of isolated myocardial cells stimulated by anti-β1-AR monoclonal antibody (25 μg/ml) or β1-AR isolated from DCM patients (25 μg/ml). Data were presented as means ± SD of 3 independent experiments.

Figure S4 Increases in basal cAMP levels in cultured neonatal rat cardiomyocytes expressing β1-AR upon incubation with β1-AA. **p<0.01 versus vehicle group. n = 6 per group. Data are presented as means ± SD of 3 independent experiments. (TIF)

Figure S5 Functional assays with β1-AA purified from individual DCM patient. A. β1-AA from individual DCM patient increased the beat frequency of cultured cardiomyocytes. B. Increases in basal cAMP levels in cultured neonatal rat cardiomyocytes incubation with β1-AA from individual DCM patient. **p<0.01 versus vehicle group. n = 6 per group. Data are presented as means ± SD of 3 independent experiments. (TIF)

Figure S6 The effects of β1-AA on CD3+ T cells proliferation. A. β1-AA had no effect on resting rat CD3+ T cells. B, C. D. β1-AA enhanced the proliferation of activated CD3+ T cells isolated from β1-AA-positive/− negative DCM patients and healthy subjects, respectively. **p<0.01 versus vehicle group; **p<0.01 versus negative IgG group. n = 6 per group. Data are presented as means ± SD of 3 independent experiments. (TIF)

Figure S7 The proliferation of CD3+T lymphocytes induced by β1-AA was blocked by β1-AR-ECI. The supernatant produced by incubating β1-AA with β1-AR-EC1 promoted CD3+T lymphocytes proliferation. The proliferation of CD3+T lymphocytes induced by β1-AA was blocked by β1-AR-ECI. **p<0.01, *p<0.05 versus vehicle group. n = 6 per group. Data are presented as means ± SD of 3 independent experiments. (TIF)

Figure S8 The IgGs isolated from healthy subjects revealed no effect on the levels of IFN-γ and IL-4 in CD3+T cells. n = 6 per group. Data are presented as means ± SD of 3 independent experiments. (TIF)

Figure S9 The reduce of IFN-γ induced by β1-AA negative IgG was partially blocked by β1-AR-EC1. β1-AA negative IgG (25 μg/ml) and the peptide corresponding to the sequence of the first extracellular loop of the human β1-AR (β1-AR-EC1 1 μmol/l) co-incubated for 1 h at 37°C. Supernatants were then collected to treat CD3+T lymphocytes, and finally IFN-γ level was analyzed by ELISA. **p<0.01, *p<0.05 versus vehicle group; ΔΔp<0.01 versus negative IgG group. n = 6 per group. Data are presented as means ± SD of 3 independent experiments. (TIF)

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Author Contributions

Conceived and designed the experiments: YD HL. Performed the experiments: YD LY JW HX LX. Analyzed the data: YD LY XL. Contributed reagents/materials/analysis tools: WZ JC KS. Wrote the paper: YD HL.

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