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ORIGINAL ARTICLE

Activation of T Lymphocytes as a Novel Mechanism in Beta1-Adrenergic Receptor Autoantibody-Induced Cardiac Remodeling

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Abstract

Background Numerous studies have reported significantly elevated titers of serum autoantibody against the second extracellular loop of β_1-adrenoceptor (β_1-AA), a catecholamine-like substance with β_1-adrenergic activity, in patients with heart failure. Although evidence demonstrates that this autoantibody may alter T cell proliferation and secretion, the role of T lymphocytes in heart failure induced by β_1-AA remains unclear. The current study was designed to determine whether T cell disorder contributes to heart failure induced by β_1-AA.

Methods and Results β_1-AA monoclonal antibodies (β_1-AAmAb) produced using the hybridoma technique were administered in wild-type mice or T lymphocyte deficiency nudes for 12 weeks. T lymphocytes from heart failure patients and neonatal cardiomyocytes were utilized in vitro. Mouse protein antibody array analysis was employed to detect the cytokines responsible for β_1-AAmAb-induced heart failure. Compared to wild-type mice, T lymphocyte deficiency mice prevented cardiac function from getting worse, attenuated adverse remodeling, and ameliorated cardiomyocyte apoptosis and fibrosis. As shown by protein array, the serum level of interleukin (IL)-6 was significantly lower in the nude group as compared to wild-type after β_1-AAmAb treatment. Mechanistic studies in vitro demonstrated that T lymphocyte culture supernatants stimulated by β_1-AAmAb caused direct damage in the cardiomyocytes, and β_1-AAmAb promoted proliferation of T lymphocytes isolated from patients with heart failure and increased IL-6 release. IL-6-specific siRNA virtually abolished cardiomyocyte apoptosis, suggesting that IL-6 may be a key cytokine released by T lymphocytes and responsible for β_1-AAmAb-induced cardiac remodeling.

Conclusions Collectively, we demonstrate that β_1-AAmAb-induced cardiac remodeling via mediating T lymphocyte disorder and releasing a variety of IL-6.

Keywords T lymphocytes · Autoantibody · Receptors adrenergic · Beta-1 · Remodeling

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Abbreviations

β_1-AA	Autoantibodies against the second extracellular loop of β_1-adrenergic receptor
β_1-AAmAb	β_1-AA monoclonal antibody
β_1-AR	β_1-adrenergic receptor
β_1-AR-EC_{II}	The second extracellular loop of β_1-adrenergic receptor
CHF	Chronic heart failure
DCM	Dilated cardiomyopathy
LVID (d)	Left ventricular diastolic diameter
Lvmass	Left ventricular mass
EF	Left ventricular ejection fraction
FS	Percent fractional shortening
ELISA	Enzyme-linked immunosorbent assay
IgG	Immunoglobulin fractions G
PBS	Phosphate-buffered saline

Introduction

Chronic heart failure (CHF) is a complex and fatal clinical syndrome with high incidence and mortality rates. More than one-fourth of hospitalized cardiac patients undergoing percutaneous angiography suffer from CHF [1]. Despite the availability of effective clinical treatments, heart failure and its associated risk factors remain uncontrolled in a large number of patients. The specific mechanisms underlying heart failure still remain unclear.

Recent studies have reported that T lymphocyte activation and increased inflammatory cytokines are involved in CHF [2]. Additionally, evidence suggests that recruitment of T lymphocyte to the left ventricular myocardium is associated with heart failure progression [3]. We previously reported an elevated ratio of $CD4^+$ to $CD8^+$ T lymphocytes as well as an increase in the production of autoantibodies against β_1-adrenergic receptors (β_1-AR) in rats during heart failure [4]. Together, the data suggest that T lymphocyte disorder may contribute to the pathogenesis of CHF, a novel relationship that needs further comprehensive elucidation.

The autoimmune antibody against the second extracellular loop (β_1-AR-EC_{II}) of the β_1-adrenergic receptor (β_1-AA) (amino acid residues 197–223, sharing 100% sequence identity between humans and rats [5]) was found in the sera of patients with idiopathic dilated cardiomyopathy [6]. This work led to the hypothesis that β_1-AA may act similarly to β_1-adrenergic receptor agonists, as data also showed based on an increased beating rate of neonatal rat cardiomyocytes [7]. A large number of clinical studies have shown that the positive rate and titer of β_1-AA in patients with heart failure are significantly higher than that in healthy individuals [8–10]. Moreover, studies on clinical heart failure found that complete elimination of β_1-AA by immunoabsorption might improve cardiac function [11, 12]. These clinical findings strongly suggest the existence of a relationship between circulating β_1-AA in the sera of CHF patients and the development of heart failure. In addition, the active immunization and serum-transfer experiment demonstrated that long-term circulating β_1-AA induced cardiac dysfunction in rats [13]. The pathogenic mechanism triggered by β_1-AA involves cardiac electrical dysfunction [14], altered cardiomyocyte contractility [15], cardiomyocyte apoptosis [16], and cardiac fibroblast proliferation [17]. We previously reported that β_1-AA isolated from dilated cardiomyopathy patients binds to β_1-AR on the surface of T cells, altering T cell proliferation and secretion [18]. However, whether the T lymphocyte is the crucial primary target cell of β_1-AA and the relationship between T cell alteration and β_1-AA in heart failure pathogenesis remain unclear and therefore instigated this study. Here, we report the effect of T cell disorder upon cardiac remodeling and dysfunction caused by β_1-AA and propose a novel mechanism responsible for the effect of β_1-AA on cardiac function.

Methods

Animals

All animal experiments were performed in accordance with the guidelines for the care and use of laboratory animals, published by the Ministry of the People's Republic of China (issued June 3, 2004) and were approved by the Institutional Committee of Animal Care at Capital Medical University. β_1-AA-negative male 8-week-old BALB/c nudes and wild-type BALB/c mice ($n = 8$, weighing 20 ± 2 g, exhibiting normal blood pressure and pulse) were used for each group and imaged every 4 weeks for 12 weeks. The animals were sacrificed afterwards and tissues were collected for further analysis. All animals were housed in pathogen-free conditions at 20 °C and were exposed to reverse light: dark conditions with 12: 12 h of light-dark periods daily.

Patients and Samples

The study adheres to the principles of the Declaration of Helsinki and Title 45, U.S. Code of Federal Regulations, Part 46, Protection of Human Subjects, Revised 13 November 2001, effective 13 December 2001. Seven chronic heart failure patients were recruited from General Hospital of Tonghua Mining Group Co., LTD, all of which were suffering from heart failure (HF) (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). At the time of sample acquisition, all patients were β_1-AA-negative and stable under therapy with diuretics, ACE inhibitors, digitalis, and nitrates. Clinical characteristics are summarized in Table 1.

Table 1 Clinical data of patients and healthy subjects (mean ± SD)

	HF group ($n = 7$)
Age (year)	71 ± 9
Gender (male/female)	6/1
NYHA	2.9 ± 0.3
CAD, *n* (%)	100(7/7)
Cardiomyopathy, *n* (%)	42.9(3/7)
EF (%)	38.5 ± 1.8
ACE-inhibitor, *n* (%)	100(7/7)
β-blocker, *n* (%)	0(0/7)

HF heart failure, *NYHA* New York Heart Association classification, *CAD* coronary artery disease, *EF* left ventricular ejection fraction

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.

Generation and Characterization of β_1-AA Monoclonal Antibody

Two peptides corresponding to the sequence (amino acid residues 197–223) of the second extracellular loop of the human β_1-AR [12] (peptide1, H-W-W-R-A-E-S-D-E-A-R-R-C-Y-N-D-P-K-C-C-D-F-V-T-N-R-C; Peptide2, C-H-W-W-R-A-E-S-D-E-A-R-R; 98% purity, Qiang Yao, Shanghai Bio Scientific Commercial Development Co. Ltd., China) were coupled to keyhole limpet hemocyanin. Three wild-type mice were immunized with two fusion peptides at the ratio of 2:1 (peptide1: peptide2). Splenocytes of three immunized wild-type mice were fused with the SP2/0 myeloma cell line at a ratio of 1:10 [13]. Hybridoma clones were tested by ELISA for the production of antibodies against the two coupling peptides. Positive clones were cultivated in Iscove's medium supplemented with 10% fetal calf serum, 2 mM glutathione, 1 mM sodium pyruvate, 100 IU mL^{-1} penicillin-streptomycin, and 50 mM β-mercaptoethanol. Two wild-type mice were vaccinated with hybridoma cells secreting anti-β_1-AR monoclonal antibody (β_1-AAmAb). After 10–14 days, ascitic fluid from immunized mice was collected. IgGs were purified via protein G antibody affinity chromatography. Bio-layer interferometry (BLI) technique, co-immunoprecipitation analysis, and confocal staining were performed to detect the binding and affinity of β_1-AAmAb for β_1-AR on rat neonatal cardiomyocytes. Beating frequency and intracellular cAMP accumulation in rat neonatal cardiomyocytes served as reporters of β_1-AAmAb functions.

In Vivo Immunization by β_1-AA Monoclonal Antibody

Eight-week-old β_1-AA-negative wild-type mice and nudes were intraperitoneally immunized on day 0 with either β_1-AA monoclonal antibody or saline (5 μg g^{-1} body weight). Every 2 weeks thereafter until week 12, a booster injection of β_1-AA monoclonal antibody (5 μg g^{-1} of body weight) was administered. Blood samples were taken prior to intraperitoneal injections; sera were collected and stored at − 80 °C for subsequent analysis.

Echocardiography

Transthoracic echo Doppler examinations were performed in a blinded fashion by an experienced echocardiographer. The mice were lightly anesthetized with 2% isoflurane, shaved (chest only), and placed on a specially designed apparatus. Echocardiography was performed using a High-Resolution Imaging System Vevo 770™ (Visual Sonics Inc., Canada) equipped with a 10-MHz phased array transducer. M-mode tracings were recorded at baseline (before immunization) and every subsequent 4 weeks, in the parasternal long- and short-axis views through the aortic valve at the base of the aortic leaflets and through the anterior and posterior left ventricle (LV) wall at the papillary muscle level. Left ventricular ejection fraction (EF) and percent fractional shortening (FS) were automatically determined in M-mode by averaging the results from three consecutive heartbeats. Wall thickness and LV internal dimensions were measured on the screen (online). Pulsed-wave Doppler spectra of mitral inflow and LV outflow were recorded from the apical four- and five-chamber views, respectively. LV mass was assessed via a modified cube formula equation.

To determine the reproducibility of mice and nude echocardiographies, all baseline examinations were repeated within 24 h. To evaluate the accuracy of M-mode and Doppler (online) measurements, a second set of unmarked M-mode and Doppler images per animal was stored digitally throughout the study.

Blood Pressure and Pulse Measurements

Blood pressure and pulse were measured every 4 weeks, on conscious, restrained mice using the Visitech tail-cuff system (Apex, NC, USA). To avoid procedure-induced anxiety, mice were initially accustomed to the instrument for 5 consecutive days before the actual recorded measurements. Moreover, the first 10 of 30 blood pressure values recorded at each session were disregarded, and the remaining 20 values were averaged and used for analysis.

Histology

The heart tissues from immunized mice were fixed with 10% neutral formaldehyde and dehydrated in graded ethanol (85%, 95%, and 100%). After xylene permeation, tissues were embedded in paraffin. The paraffin blocks were sectioned 3–5 μm thick and mounted onto glass slides. Cardiac collagen content was assessed via Masson's trichrome staining.

Determination of $CD4^+$ and $CD8^+$T Cells

For measurement of $CD4^+$ and $CD8^+$ T lymphocytes in the mouse's spleen, preparation of samples and determination by flow cytometry (BD Biosciences, USA). The following monoclonal antibodies were used for combining T lymphocyte: fluorescein isothiocyanate (FITC)-labeled anti-mouse CD4 and phycoerythrin-labeled mouse CD8 (Biolegend, USA).

CCK-8 Proliferation Assay

Blood samples were collected from seven β_1-AA-negative patients with heart failure. Specific clinical data were summarized in Table 1. Human T cells were isolated as previously described [11] and were cultured for 48 h with or without mitogens in the presence or absence of β_1-AAmAb (25 μg mL^{-1}). The mitogens utilized were 3 μg mL^{-1} soluble anti-human CD3 mAb (Biolegend, USA) and 1 μg mL^{-1} soluble anti-human CD28 mAb (Biolegend, USA). Antagonists were added 1 h before the addition of β_1-AAmAb. After each treatment, 10 μl CCK-8 solution was added to each well. Cells were incubated for 4 h at 37 °C. The absorbance at 450 nm was measured on a microplate reader (wavelength correction at 630 nm).

Assessment of Cardiomyocyte Apoptosis

Cardiomyocyte apoptosis was assessed with Annexin V/propidium iodide (Invitrogen, USA) [15]. Briefly, cardiomyocytes from a neonatal rat were seeded (2.6×10^5 cells $well^{-1}$) in a six-well microtiter plate, followed by overnight incubation. After the appropriate treatment, the cells were trypsinized, washed, and incubated with Annexin V-FITC solution for 15 min in the dark. After washing, a propidium iodide solution was added and the cells were immediately analyzed by flow cytometry (BD Biosciences, USA).

Co-immunoprecipitation and Immunoblotting

Cells were lysed with cold lysis buffer, homogenized, and analyzed. Briefly, cell lysates were pre-cleared with the corresponding nonimmune IgG and incubated together with protein A/G plus-Sepharose for 30 min at 4 °C after which they were incubated with 2 μg of anti-β_1-AAmAb. Cell lysates were then incubated with protein A/G plus-Sepharose overnight at 4 °C. Nonimmune mouse IgG served as negative control. Protein A/G beads were then extensively washed with lysis buffer after incubation. Proteins were eluted from the beads with elution buffer and analyzed by Western blot.

Samples with 2 × SDS loading buffer were heated and separated by electrophoresis. After transfer to PVDF membranes, proteins were immunoblotted with anti-β_1-AR, anti-MMP2, anti-MMP9, anti-TGF-beta1, anti-IL-6, or anti-GAPDH antibodies (Cell Signaling Technology, USA). Protein abundance was quantified by densitometry.

Mouse Protein Antibody Array

Mouse protein antibody arrays, containing 308 antibodies (RayBiotech, Georgia, USA) were used following the manufacturer's instructions. Sera obtained from BALB/c mice or nude mice in the presence or absence of β_1-AAmAb were dialyzed and labeled using biotin. Each of the antibodies had two replicates that were printed on a coated glass microscope slide, along with multiple positive and negative controls. Fluorescent detection was scanned with a GenePix × 4000 scanner (GenePix 4000B, Axon Instruments, USA) until the glass chips were completely dry; images were analyzed with GenePix Pro 6.0 (Axon Instruments, USA). After subtracting background signals and normalizing to positive controls, the signal intensities between and among array images were compared to determine relative differences in the expression levels of each protein between groups. Any ≥ 2.0-fold increase or ≤ 0.5-fold decrease in the signal intensity for a single analyst between samples or groups was considered a significant difference in expression, provided that both sets of signals were well above the background signals.

Small Interfering RNA Transfection

T lymphocytes were transfected with a siIMPORTER siRNA transfection kit (Qiagen Science Inc. Benelux, Venlo, Netherlands) according to the manufacturer's protocol using siRNA duplexes against IL-6 and universal control oligonucleotides (Santa Cruz Biotechnology, Californian, USA). Briefly, T lymphocytes were plated onto six-well plates before transfection. After reaching 80% confluence, siRNA was applied to each well (final concentration 50 nM).

Bio-layer Interferometry Binding Assay

Real-time binding assays between β_1-AAmAb/β_1-AA IgGs from heart failure patients and β_1-AR were performed using BLI with an Octet Red 96 instrument (Fortebio). In the BLI experiments, β_1-AR protein was biotinylated by the EZ-Link

Sulfo-NHS-LC-Biotinylation kit (catalog no. 21435) from Thermo Scientific. Sulfo-NHS-LC-biotin was conjugated onto target proteins via the reaction between the NHS ester and the primary amino groups (including the N terminus of the protein and primary amines on the sidechain of lysine) on proteins. Briefly, we incubated β_1-AR protein and Sulfo-NHS-LC-biotin at 1:1 M ratio in ddH_2O (600 μL volume), at 25 °C for 40 min. After that, the excess free Sulfo-NHS-LC-biotin was removed by applying the protein sample to a desalting column (Zeba Spin Desalting Columns, 5 mL, for 500- to 2000-μL samples, 7000 molecular weight cutoff (MWCO)). After centrifugation of the column at 1000×*g* for 2 min, the collected flow-through solution is the biotin-labeled β_1-AR protein for subsequent BLI experiments.

Biotinylated β_1-AR (in 20 mM Hepes, pH 7.4, 150 mM NaCl, and 1 mM DTT) were first immobilized onto streptavidin biosensors (ForteBio) at a speed of 1000 rpm for 4 min. The immobilized sensors were equilibrated in reaction buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM DTT, and 0.02% Tween20) at a speed of 200 rpm for 3 min. Association curves were obtained by incubating β_1-AR-coated biosensor with β_1-AAmAb or β_1-AA IgG solutions (1 μM protein in reaction buffer), the biosensor was rotated at a speed of 200 rpm for 8 min, and dissociations were detected by incubating in reaction buffer without β_1-AAmAb or β_1-AA IgG proteins in the same condition. Data were acquired using an Octet Data Acquisition 7.0.1.17 according to the manufacturers' instructions. The assays were analyzed with the Octet Data Analysis Software 7.0.1.3.

Statistical Analysis

Values are expressed as mean ± standard deviation (SD) or mean ± standard error of the mean (SEM). Statistical analysis was performed using SPSS 18.0 software [16]. Student's *t* test was used to compare two independent sample means, whereas one-way ANOVA was used to compare the means of more than two samples. *P* values <0.05 were considered statistically significant.

Results

T Lymphocyte Deficiency Prevented Left Ventricular Cardiac Function from Getting Worse β_1-AAmAbs produced by the hybridoma technique bind with native β_1-ARs and exert the same biological effects as β_1-AAs circulating in patients with heart failure (Supplementary Fig. 1). β_1-AA-negative T lymphocyte deficiency BALB/c nudes and wild-type BALB/c mice ($n = 8$) were used for each group. Serum β_1-AAmAb titers were markedly increased in both β_1-AA-positive mice and nudes throughout the experiment, suggesting the establishment of successful passive immunization models (Fig. 1a, b). The effect of β_1-AAmAb upon cardiac function was determined 12 weeks after β_1-AAmAb passive immunization. Compared to the vehicle group, the β_1-AAmAb-positive wild-type mice exhibited significantly decreased EF and FS (Fig. 1c, e) while there was no significant change in β_1-AAmAb-positive nudes (Fig. 1d, f). However, there is no difference in heart rate (Supplementary Fig. 2) and blood pressure (Supplementary Fig. 3) in both β_1-AAmAb-positive wild-type mice and nudes. As shown in Fig. 1g, the ratios of $CD4^+$/ $CD8^+$ T cells increased remarkably after 4 weeks in β_1-AAmAb-positive wild-type mice in comparison to the vehicle group; suggesting that the long-term existence of β_1-AAmAb induced the T lymphocyte disorder. In order to further detect whether the change in $CD4^+/CD8^+$ T cell ratio is a specific effect of β_1-AAmAb, a non-specific IgG control group was added. Freshly isolated $CD3^+$ T cells were stimulated with non-specific IgGs for 48 h, and it was found that non-specific IgGs had no effect on the $CD4^+/CD8^+$ T cell ratio (Supplementary Fig. 4), suggesting that the change in $CD4^+$/ $CD8^+$ T cell ratio is indeed a specific effect of β_1-AAmAb. Taken together, these data demonstrate that T lymphocyte deficiency protected both the LV systolic and diastolic function.

T Lymphocyte Deficiency Attenuates Left Ventricular Enlargement and Dilation Induced by β_1-AAmAb Having demonstrated that T lymphocyte deficiency protects cardiac function, we further investigated whether T lymphocyte deficiency may prevent pathological remodeling, the primary cause of β_1-AA-induced cardiac dysfunction and heart failure. Over the course of 12 weeks, β_1-AAmAb administration increased cardiac size and elevated the heart to body weight ratio, but had no significant effect on nudes when compared to the vehicle group (Fig. 2a). Moreover, β_1-AAmAb treatment significantly increased the left ventricular end-diastolic diameter (LVEDD) and left ventricular mass (LV mass) in wild-type mice (Fig. 2c, e), but no changes were observed for either of these parameters in β_1-AAmAb-positive nudes (Fig. 2d, f). Taken together, these data present the first evidence that T lymphocyte deficiency attenuates adverse cardiac heart failure remodeling.

T Lymphocyte Deficiency Inhibits Cardiomyocyte Apoptosis and Fibrosis Induced by β_1-AAmAb To determine the cellular mechanisms responsible for β_1-AAmAb-mediating remodeling effect, the extent of apoptosis and interstitial fibrosis were assessed. T lymphocyte deficiency dramatically attenuated cleaved caspase-3 expression (Fig. 3a). Western blot analysis demonstrated that mouse T cells expressed β_1-AR, and the binding of β_1-AAmAb and β_1-AR was confirmed by confocal microscopy (Supplementary Fig. 5). Freshly isolated $CD3^+$ T cells were stimulated with anti-CD3/CD28 mAb in the presence or absence of β_1-AAmAb (25 μg mL^{-1}) for 48 h

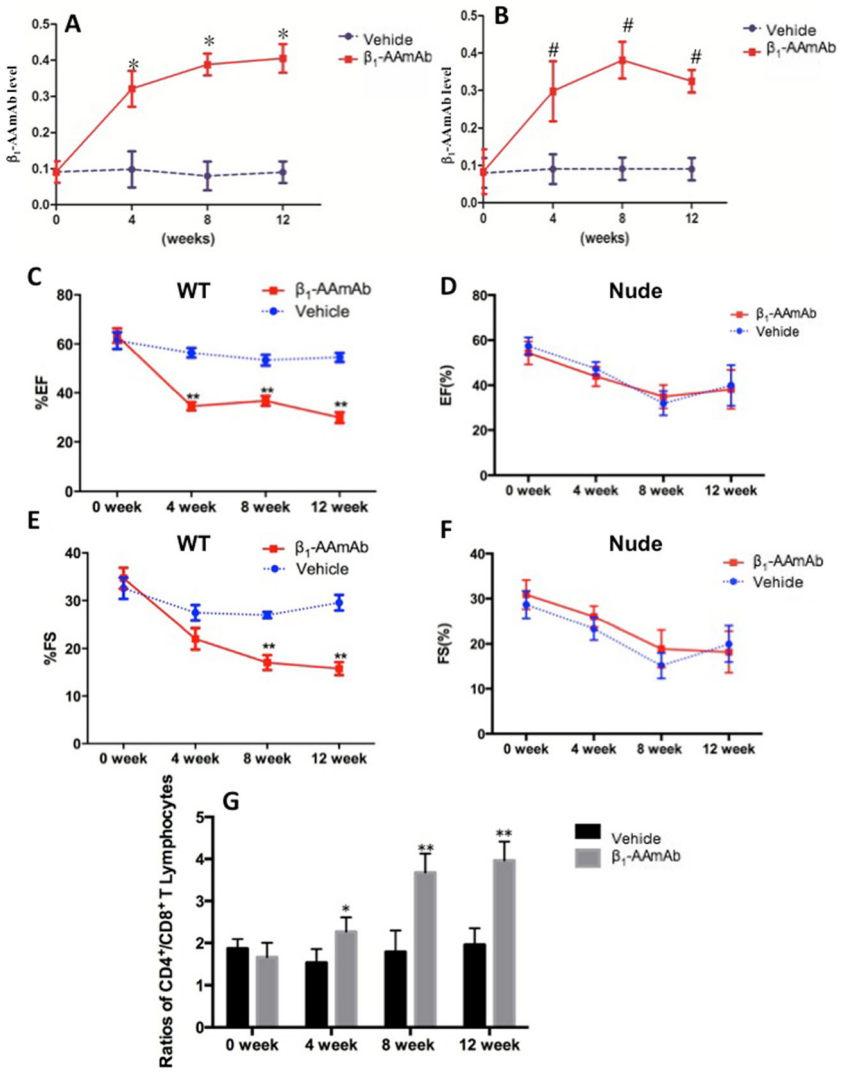

Fig. 1 T lymphocyte deficiency protects left ventricular cardiac function. **a** Changes in the level of β_1-AAmAb in BALB/c mice during passive immunization at different time points. **b** Changes in the level of β_1-AA in BALB/c nudes during passive immunization at different time points. The effects of β_1-AAmAb treatment on LV function were determined up to 12 weeks. **c**, **d** Ejection fraction (EF). **e**, **f** Fractional shortening (FS). **g** The ratios of $CD4^+/CD8^+$ T lymphocytes in BALB/c mice. Data presented as mean ± SEM. WT: wild-type mice; Nude: T lymphocyte deficiency mice; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ β_1-AA group vs. Vehicle group, $n=8$/group

before collecting the T lymphocyte supernatant. We evaluated the induction of cardiomyocyte apoptosis in β_1-AAmAb-stimulated T lymphocyte supernatant via flow cytometric analysis. We observed that the rate of cardiomyocyte apoptosis increased to 40.9% when cardiomyocytes were stimulated for 12 h (Figs. 3b). T lymphocyte deficiency significantly suppressed interstitial fibrosis (Fig. 4a) and decreased MMP2/MMP9 activity and TGF-β1 production, the two most significant mechanisms contributing to cardiac fibrosis (Fig. 4b). Collectively, these results demonstrate that T lymphocyte deficiency may prevent β_1-AAmAb-induced LV remodeling by reducing apoptosis and fibrosis.

IL-6 Secreted by T Lymphocyte Is the Key Cytokine Responsible for β_1-AAmAb-Induced Remodeling To further determine the mechanisms responsible for β_1-AAmAb-induced remodeling, we performed protein array experiments and compared the levels of more than 300 mouse cytokines secreted by wild-type mice and nudes in the presence or absence of β_1-AAmAb. Interestingly, we found that only nine of 308 labeled proteins were associated with both T lymphocyte deficiency and β_1-AAmAb compared with the vehicle group; eight cytokines were upregulated, and only IL-6 was downregulated in T lymphocyte deficiency mice (Fig. 5a, b and Table 2). These results were further confirmed by ELISA (Fig. 5c). siRNA was employed to specifically downregulate endogenous IL-6 production in freshly isolated mouse T lymphocytes. After 48 h of transfection with siRNA, T lymphocytes were incubated with either vehicle or β_1-AAmAb (25 μg mL^{-1}) for 6 h. Western blot analysis showed that the siRNA successfully suppressed IL-6 by 70–80% (Fig. 5d). The supernatant of the IL-6 siRNA-transfected T cells was also used to treat cardiomyocytes and resulted in a reduction of cardiomyocyte apoptosis, as observed by a decrease in Annexin staining and apoptosis rate (Fig. 5e). Taken together, the results shown in Fig. 5 suggest that IL-6 may be the key cytokine released by T lymphocytes and responsible for β_1-AAmAb-induced remodeling.

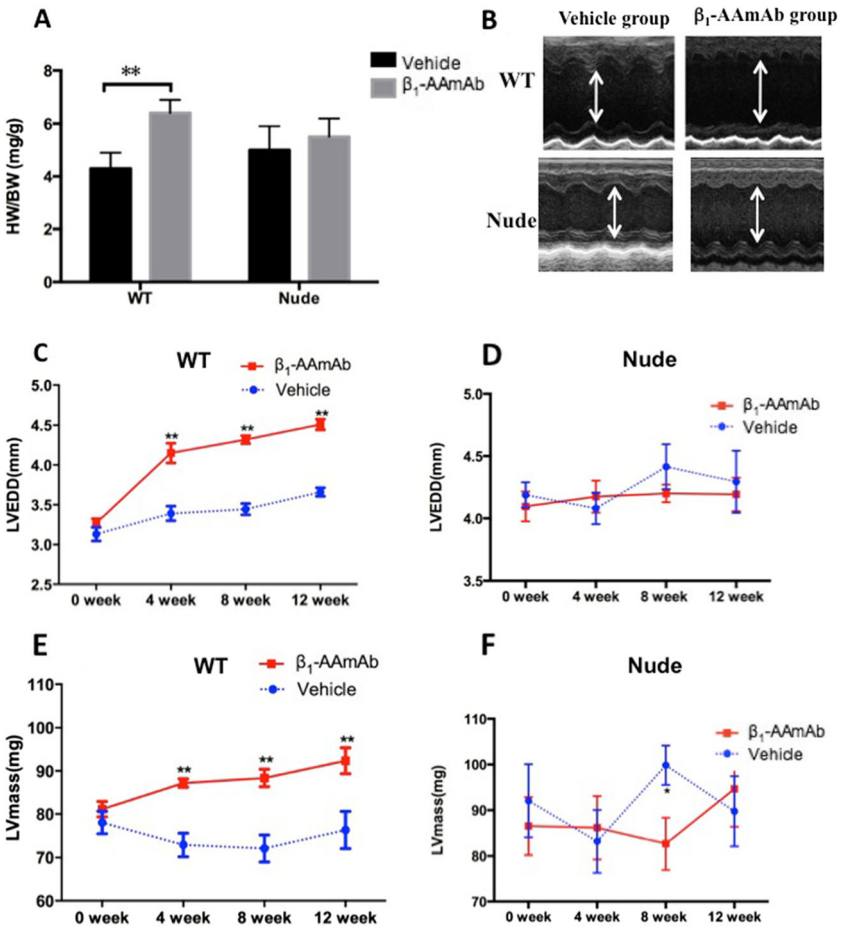

Fig. 2 T lymphocyte deficiency prevents left ventricular cardiac remodeling induced by β_1-AAmAb. **a** Statistics of heart weight/body weight (HW/BW). **b** Representative echocardiogram images. **c**, **d** Left ventricular end-diastolic diameter (LVEDD, mm). **e**, **f** left ventricular mass (LV mass, mg). Data presented as mean ± SEM. WT: wild-type mice; Nude: T lymphocyte deficiency mice; $^{**}P < 0.01$, $^{***}P < 0.001$ β_1-AA group vs. Vehicle group, $n = 8$/group

β_1-AAmAb Increased the Production of IL-6 in T Lymphocyte from Patients with Heart Failure To further confirm whether IL-6 secreted by T lymphocytes is also a significant cytokine responsible for β_1-AAmAb-induced remodeling under pathological conditions, fresh T lymphocytes from heart failure patients were isolated. CCK-8 assay showed that β_1-AAmAb promoted T lymphocyte proliferation (Fig. 6a). Additionally, β_1-AAmAb enhanced IL-6 secretion from T lymphocytes in patients with heart failure (Fig. 6b). Taken together, these data demonstrate IL-6 is responsible for β_1-AAmAb-induced remodeling under both normal and pathological conditions.

Discussion

Our study presents several important observations: (1) β_1-AA monoclonal antibody-induced pathological remodeling; (2) T lymphocyte deficiency prevented cardiac function from getting worse and attenuated the pathological remodeling induced by β_1-AAmAb, as evidenced by preserved LV chamber dimensions, decreased interstitial fibrosis, and reduced apoptosis; (3) IL-6 is the key cytokine responsible for β_1-AAmAb-induced remodeling (Fig. 6c).

β_1-AAs utilized in previous studies were IgG fractions isolated from the sera of β_1-AA-positive animals [19] or patients [20]. Since β_1-AAs act in concert with other GPCR autoantibodies in heart failure patients, IgG fractions also included non-specific IgGs and were not specific for the second extracellular loop of β_1-AR. Moreover, we also tried to purify specific β_1-AA from heart failure patients, but the concentration of β_1-AA was so low that it is impossible to conduct in vitro experiments. Therefore, in this study, the hybridoma technique was employed to produce monoclonal antibodies against the second extracellular loop of β_1-AR (β_1-AAmAb) without the presence of other antibodies. In line with other studies, our data demonstrate that β_1-AAmAbs may combine with native β_1-ARs to yield identical biological effects (beating rate of neonatal rat cardiomyocytes and cAMP level) as β_1-AAs circulating in patients with heart failure. Bio-layer interferometry (BLI) technique identified that β_1-AAmAb has a much higher affinity than β_1-AA IgGs from β_1-AA-positive patients (Supplementary Fig. 1). Co-immunoprecipitation analysis showed that β_1-AAmAb bonded with β_1-ARs, but not β_2-ARs, on T lymphocytes (Supplementary Fig. 6A). Moreover, β_1-AR selective blocker inhibited T lymphocyte proliferation induced by β_1-AAmAb, but specific β_2-AR antagonist had no effect on this

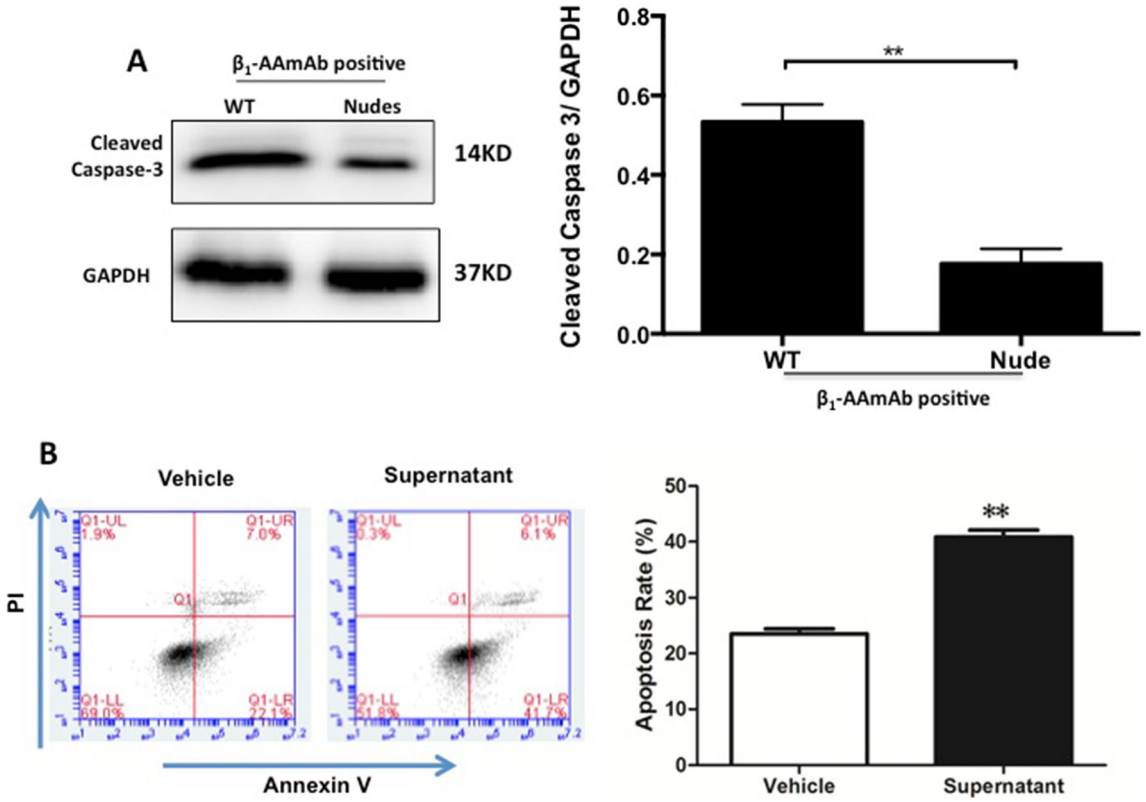

Fig. 3 Effect of β_1-AAmAb on apoptosis. Cardiomyocyte apoptosis was determined by cleaved caspase-3 expression (**a**). β_1-AAmAb-stimulated T lymphocyte supernatant-induced apoptotic cell death in cardiomyocytes was determined by flow cytometry with Annexin V/PI assay (**b**). Representative scatter plots of PI (*y*-axis) vs. Annexin V (*x*-axis) are shown. The lower right quadrants represent Annexin V-positive and PI-negative apoptotic cells; quantitative data are shown. $^{**}P < 0.01$, $^{*}P < 0.05$ vs. Vehicle group; $^{\#\#}P < 0.01$ vs. β_1-AA-positive wild-type group. Data are presented as mean ± SD of three independent experiments

(Supplementary Fig. 6B). It suggested that β_1-AAmAb specifically bonded with β_1-AR, and no cross-reactivity with β_2-AR. β_1-AAmAb is indeed a valid model for autoantibodies. As a result, we decided to further expand our research by also using the β_1-AAmAbs to determine the role of T lymphocytes in heart failure induced by β_1-AA.

Previous studies have frequently utilized active immunization models with β_1-AR-EC_{II} [21, 22]. However, active immunity carries the uncertainty of other unknown antigens contributing to myocardial injury. Furthermore, the mean titer of β_1-AA produced in the active immunization model is significantly greater than that observed in the sera of heart failure patients [23], and therefore, the clinical relevance is questionable. In the current study, we therefore established passive immunization models with β_1-AAmAb in wild-type mice or T lymphocyte deficiency nudes. In fact, during the course of this study, we discovered that the initial cardiac function of nude mice is normal. However, with the prolongation of immunization, the heart function of nude mice decreased significantly in both the immunized group and the vehicle group, reaching the lowest point at the 8th week after passive immunization, and recovered at the 12th week of immunization. This result is not what we expected. The explanations are as follows: Firstly, due to T lymphocyte deficiency, resulting in imperfect immune system in nude mice, the basal cardiac function of nude mice is lower than that of wild-type mice. Therefore, regardless of which agent is exogenously administered, it may rapidly trigger the inflammatory response, release a large number of cytokines (such as IL-6), and eventually lead to decreased cardiac function. Secondly, during the process of echocardiography, nudes are more sensitive to anesthetics, leading to an additional reduction in cardiac function. However, the β_1-AAmAb did not induce a further decrease in nude as it was seen with the wild-type mice, suggesting that T lymphocyte disorders are more significant in the process of β_1-AAmAb-induced cardiac insufficiency. Furthermore, according to our previous published paper [22], active immunization with β_1-AR-EC_{II} peptide took 18 months to induce heart failure in rats. Therefore, the 12-week model of β_1-AAmAb passive immunization used in this study was only in the early stages of β_1-AA-induced heart failure. In the early stages, T lymphocytes may be the first target cell of β_1-AA. β_1-AA may first bind with β_1-AR on the surface of T cells, mediate T lymphocytes disorder, and then release a large number of cytokines, inducing inflammatory reactions and heart damage. Therefore, T lymphocyte

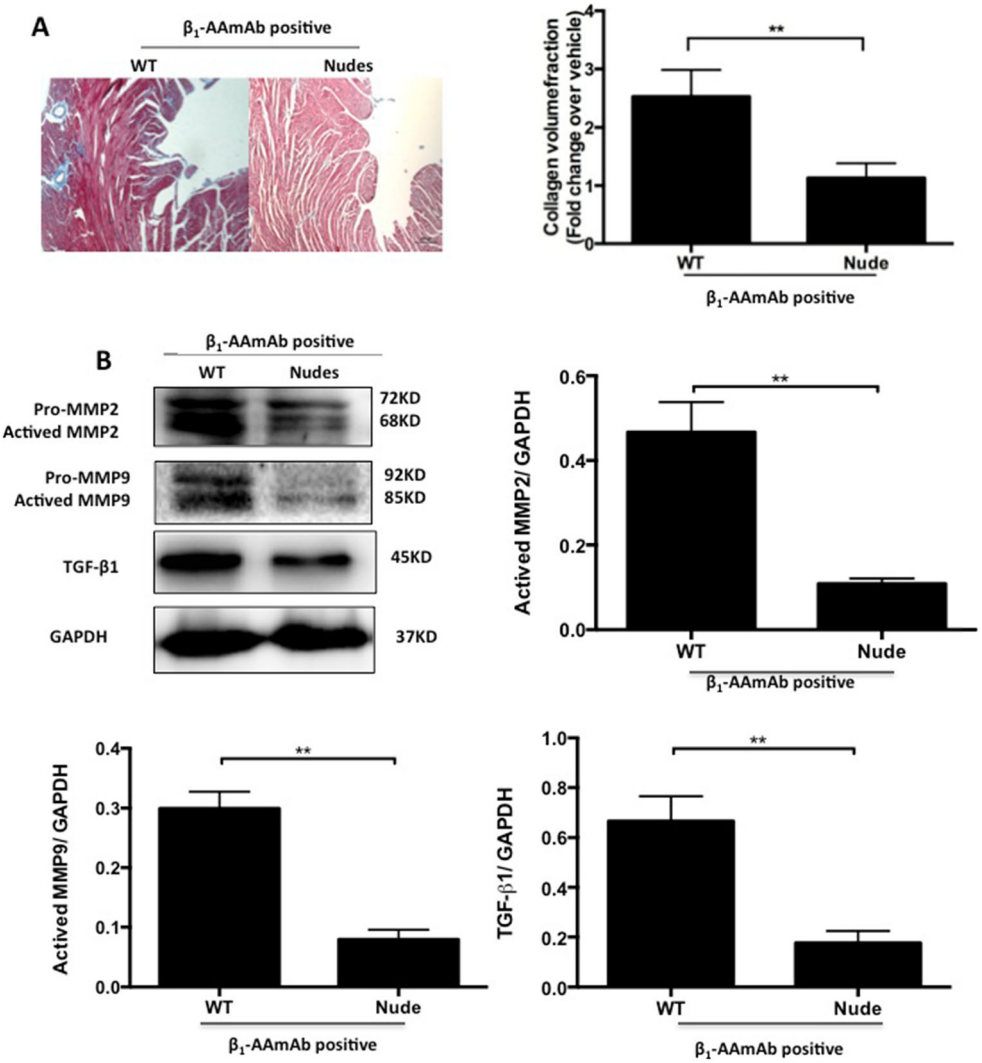

Fig. 4 Effect of β_1-AAmAb on fibrosis. **a** Interstitial fibrosis. Scale bar = 200 μm. **b** Western blot analysis of MMP2, MMP9, and TGF-β1. $^{**}P < 0.01$ vs. Vehicle group; $^{\#\#}P < 0.01$ vs. β_1-AA-positive wild-type group. Data are presented as mean ± SD of three independent experiments

deficiency has a protective effect in the early stages of β_1-AA-induced heart failure.

β_1-AA was first discovered in Chagas disease, and then, it was detected in the serum of patients with cardiovascular diseases [10]. Since circulating β_1-AA has a positive correlation with the degree of heart failure [24–26], neutralizing β_1-AA may be an effective therapeutic strategy to restore cardiac function. Immunoadsorption is a more selective method to remove β_1-AA, but it is not popular because of its high price and complicated operation. In recent years, it has been reported that the aptamers (short single-stranded or double-stranded RNA or DNA sequences) are a new class of molecules that bind to a specific target molecule (e.g., autoantibodies) with high specificity, modulating the target's function [27]. A trial studying the safety and dosing of BC 007, an aptamer that functions as a broad-spectrum neutralizer of pathogenic autoantibodies bound with β_1-AAs isolated from patients with DCM, Chagas' cardiomyopathy, or peripartum cardiomyopathy and neutralized their pathogenic functions by reducing β_1-AA-induced positive chronotropic effect and cardiomyocyte apoptosis [28, 29]. Moreover, in animal studies, the long-term exposure of β_1-AAs caused adverse cardiac remodeling such as ventricular hypertrophy, chamber walls weakening, cardiomyocytes apoptosis and fibrosis [17]. Consistent with previous reports we demonstrated that β_1-AAmAb caused an increase of myocardial apoptosis and fibrosis in vivo, while T lymphocyte deficiency attenuated β_1-AAmAb-induced cardiac remodeling. Therefore, T lymphocytes were speculated to be involved in β_1-AAmAb-induced cardiac remodeling. It is known that T lymphocytes, the main effector cells, play a central role in cell-mediated immunity. Once T lymphocytes are activated, they divide rapidly and secrete a variety of cytokines that regulate or assist in the active immune response. Excessively activated T lymphocytes may induce myocardial damage, including cell-mediated immune response and inflammatory cytokines [2]. Previous studies indicated that β_1-adrenergic receptor agonists might inhibit the secretion of cytokines from Th1/Th2 cells [30], thereby inducing myocardial injury by invoking T lymphocytes infiltration [31]. Likewise, we have demonstrated that β_1-AA from heart failure patients

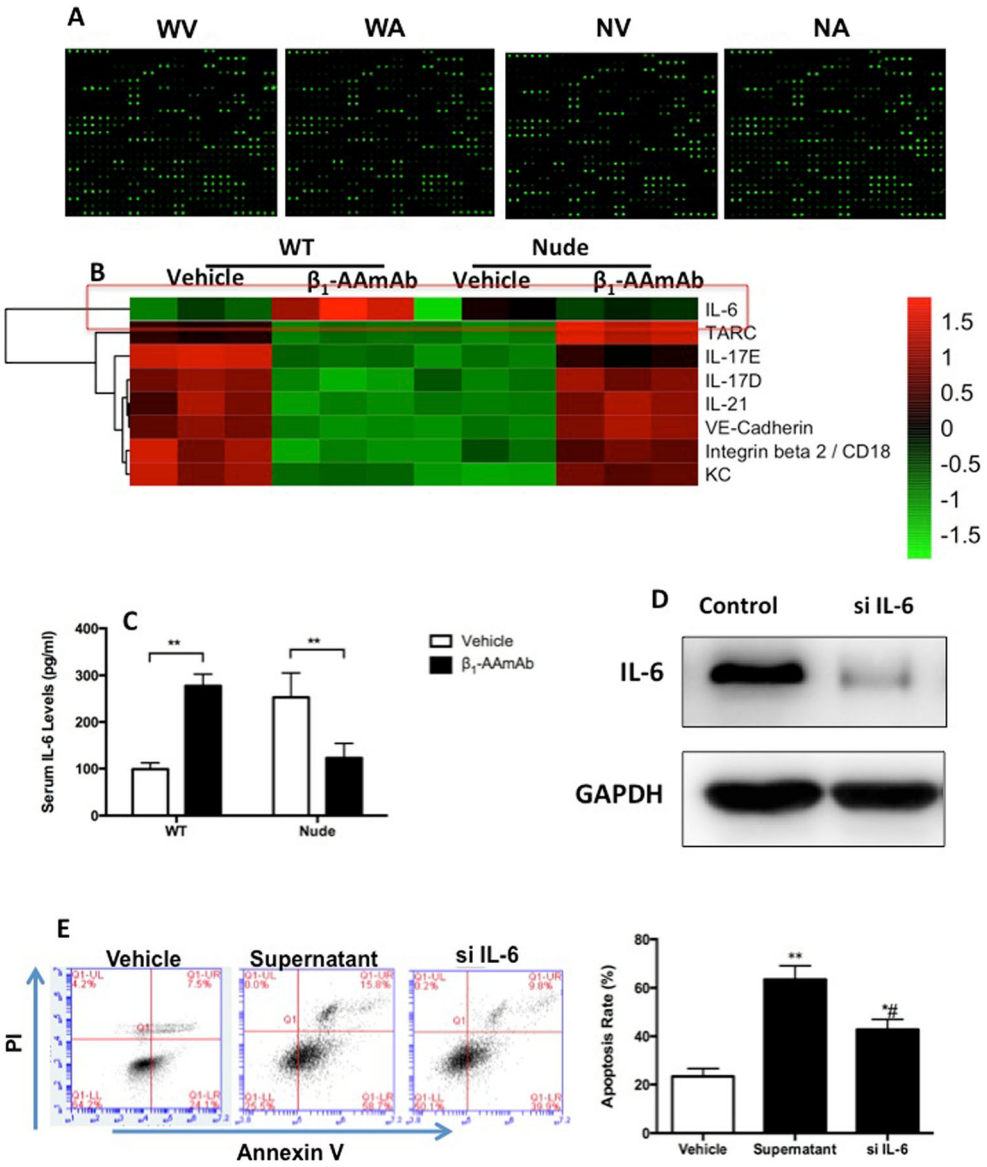

Fig. 5 IL-6 secreted by T lymphocyte is the key cytokine responsible for β_1-AAmAb-induced remodeling. **a** Representative images of arrays performed with the sera from wild-type mice and nude mice are displayed here. WV: wild-type vehicle group; WA: wild-type β_1-AAmAb group; NV: nude vehicle group; NA: nude β_1-AAmAb group. **b** A partial heat map of upregulated and downregulated cytokines. **c** Serum IL-6 levels. **d** Western blot analysis confirmed that siRNA caused a significant reduction in IL-6 protein concentrations. **e** β_1-AAmAb-stimulated T lymphocyte supernatant-induced apoptotic cell death in cardiomyocytes was determined by flow cytometry with Annexin V/PI assay. Representative scatter plots of PI (*y*-axis) vs. Annexin V (*x*-axis) are shown. The lower right quadrants represent Annexin V-positive and PI-negative apoptotic cells. Data are presented as mean ± SD of three independent experiments. $^{**}P < 0.01$ β_1-AA-positive group vs. vehicle group

Table 2 Cytokines significantly changed in mouse and nude sera

Cytokines	WV	WA	NV	NA	Fold change (WA/WV)	Fold change (NA/NV)
Upregulated						
IL-17E	484	217	171	342	0.4489	2.002
Integrin beta2/CD18	358	122	137	275	0.341	2.005
KC	265	88	61	217	0.331	3.5596
TARC	429	207	229	653	0.483	2.853
VE-Cadherin	253	116	99	271	0.458	2.745
IL-13	231	106	124	262	0.457	2.11
IL-17D	255	99	128	286	0.3895	2.243
IL-21	227	78	105	261	0.344	2.488
Downregulated						
IL-6	151	297	266	184	2.016	0.498

WV wild-type vehicle group, *WA* wild-type β_1-AAmAb group, *NV* nude vehicle group, *NA* nude β_1-AAmAb group

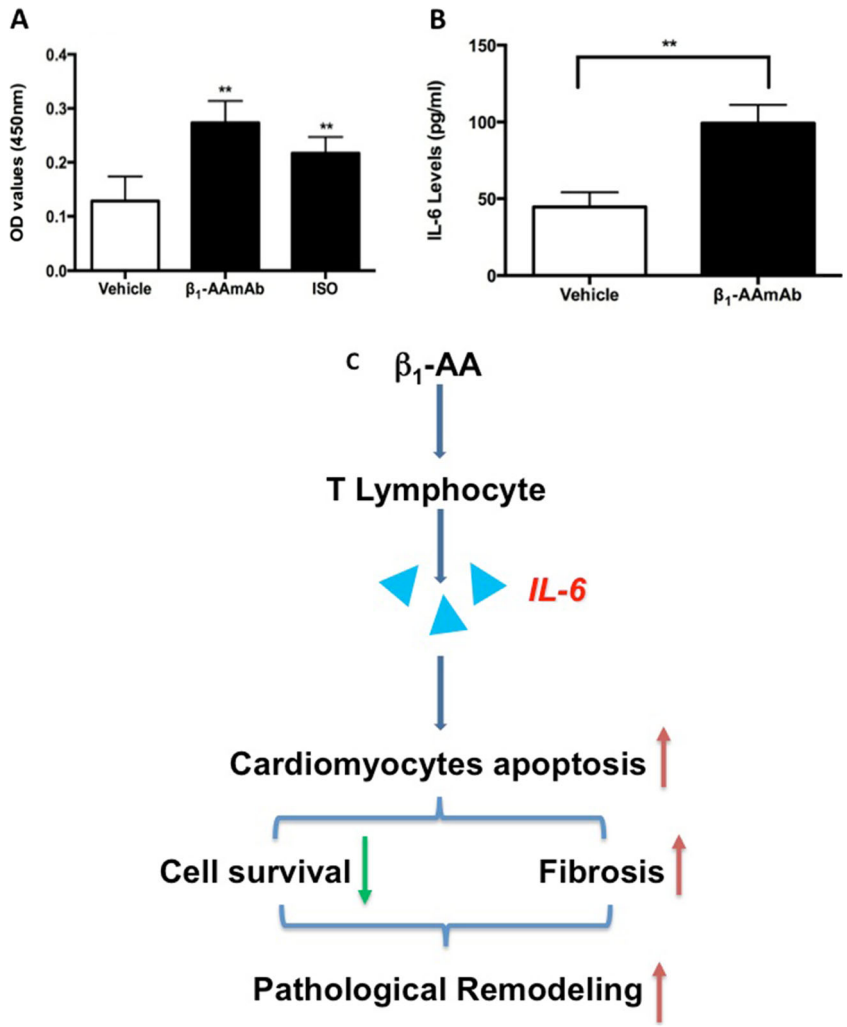

Fig. 6 β_1-AAmAb increased the production of IL-6 in T lymphocyte from patients with heart failure. **a** T cells (5×10^5 cells mL^{-1}) from patients with heart failure were incubated for 48 h at 37 °C and 5% CO_2 in the presence of β_1-AAmAb (25 μg mL^{-1}). Cell proliferation was measured at 450 nm by CCK-8 uptake assay. **b** The effect of β_1-AAmAb on IL-6 levels was examined by ELISA. Data are presented as means ± SD of six independent experiments. $N = 6$/group, Vehicle group: anti-CD3/CD28 antibody group. $^{**}P < 0.01$ vs. vehicle group. ISO: isoproterenol; MET: metoprolol. **c** Schematic of the mechanisms underlying β_1-AAmAb-induced remodeling

promoted rat T lymphocyte activation in vitro and led to T lymphocytes secretion disorder [18]. In the current study, we did observe that not only β_1-AAmAb-induced proliferation of T lymphocytes but also β_1-AAmAb-stimulated T lymphocyte supernatant directly injured the cardiomyocytes, and increased cardiomyocyte apoptosis. Therefore, both in vivo and in vitro studies have demonstrated that β_1-AAmAb-mediated T lymphocyte disorder plays a critical role in cardiac remodeling induced by β_1-AAmAb.

Many cytokines have been reported to either mediate adverse remodeling induced by heart failure or prevent cardiac remodeling and dysfunction. Cytokine secretion balance in the immune system is completely broken down when T lymphocytes disorder occurs. IL-6, a pro-inflammatory cytokine, is secreted by T cells and macrophages to stimulate an immune response [32]. Clinical and translational studies have demonstrated an association between increased serum levels of IL-6 and cardiovascular diseases [33, 34]. Elevated IL-6 levels lead to chronic inflammation and fibrotic disorders [35]. In the current study, protein array analysis showed that long-term circulating β_1-AAmAb elevated IL-6 serum levels in wild-type mice, while it significantly decreased serum IL-6 in T lymphocyte deficiency nudes. Furthermore, results from the in vitro experiments suggest that β_1-AAmAb promoted IL-6 secretion from T lymphocytes in patients with heart failure. siRNA-mediated suppression of IL-6 secretion significantly attenuated the cardiomyocyte injury caused by β_1-AAmAb-stimulated T lymphocyte supernatant. Our results were in-line with another study that immunization with β_1-AR-EC_{II} was unable to induce an early stage phenotype of cardiomyopathy in IL-6 knockout mice when compared to wild-type mice [20]. These results suggest that T lymphocytes secreting IL-6 may play a pivotal role in myocardial remodeling induced by β_1-AAmAb.

Since the distribution of β_1-AR is different between normal and pathological conditions [36], the role of β_1-AA in normal T lymphocytes may not be same with T cells from pathological states. Therefore, in the current study, we investigated the effects of β_1-AAmAb on T lymphocytes from patients with heart failure proliferation and IL-6 secretion. We found that although under pathological conditions, β_1-AAmAb still enhanced T lymphocytes proliferation and IL-6 secretion

(Fig. 6a, b), indicating that T cell disorder is indeed responsible for β_1-AAmAb-induced remodeling.

In conclusion, we have demonstrated that β_1-AA-induced apoptotic death of cardiomyocytes and myocardial fibrosis is mediated by T lymphocyte disorder causing increased IL-6 secretion. Thus, neutralizing β_1-AA or preventing T lymphocyte dysfunction may be a promising therapeutic avenue to restore cardiac function and mitigate the heart failure phenotype in β_1-AA-positive patients.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All animal experiments were performed in accordance with the guidelines for the care and use of laboratory animals, published by the Ministry of the People's Republic of China (issued June 3, 2004), and were approved by the Institutional Committee of Animal Care at Capital Medical University.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The Institutional Committee for the Protection of Human Subjects of Capital Medical University approved this research protocol.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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