

# Programmed cell death-1 deficiency results in atrial remodeling in C57BL/6 mice

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**Abstract.** Deficiency of the programmed cell death-1 (PD-1) gene enhances T-cell activation and increases inflammation levels. It has been reported that atrial fibrillation (AF) is closely related to inflammation. The aim of the present study was to investigate the role of PD-1 deficiency in the pathogenesis of AF. Two groups of mice were used in our experiment: the C57BL/6 and the C57BL/6-PD-1<sup>-/-</sup> group. The expression of the inflammatory cytokines interleukin (IL)-2, -4, -6, -10, -17, interferon- $\gamma$  and tumor necrosis factor were detected. Furthermore, the levels of atrial myocyte oxidative stress, the atrial effective refractory period (AERP) and the atrial myocardial fibrosis levels were determined. Compared with the C57BL/6 group, we found that the inflammatory cytokines were significantly increased in the PD-1<sup>-/-</sup> group and the levels of atrial myocyte oxidative stress in the PD-1<sup>-/-</sup> group were also higher. The AERP became shorter and the dispersion of AERP was increased in the PD-1<sup>-/-</sup> group. Moreover, the PD-1<sup>-/-</sup> group presented significant atrial myocardial fibrosis but the C57BL/6 group did not. Our findings strongly suggest that the higher levels of inflammatory cytokines and atrial myocyte oxidative stress were present in the PD-1<sup>-/-</sup> mice and resulted in atrial electricity and structural remodeling. Due to the atrial remodeling, the PD-1<sup>-/-</sup> mice were more likely to develop AF.

## Introduction

Atrial fibrillation (AF) is the most common arrhythmia in clinical practice, present in 2% of the general population. The prevalence of AF increases markedly with age, affecting as

many as 9% of individuals 80 years or older (1). AF has several complications such as thromboembolism and heart failure and is regarded as an independent risk factor for cardiovascular mortality (2).

Numerous studies have focused on the pathogenesis of AF. It is generally accepted that AF may be multifactorial and has been linked to, among others, ectopy from the pulmonary veins, catecholamine release and large atrial size. However, the fundamental arrhythmia mechanisms of AF are rapid ectopic firing and reentrant activity. Atrial remodeling may increase the risk of ectopic or reentrant activity through a multitude of potential mechanisms (3) and is considered to be the new pathophysiological mechanism of AF (4).

In the past few years the relationship between inflammation and AF has drawn significant attention. Inflammation may be a cause of AF and this is supported by the high incidence of AF in post-operative cardiac surgery, a state of severe inflammatory process (5-7). However, evidence suggests that inflammation may also play a prominent role in nonoperative onset of AF (8).

Programmed cell death-1 (PD-1, *Pdcd-1*) is a negative immunoreceptor belonging to the CD28/CTLA-4 family. PD-1 deficiency enhances T-cell activation and increases the inflammation level. Previous observations indicated that the PD-1<sup>-/-</sup> mice with different genetic backgrounds presented different types of autoimmune diseases (9,10). Another study found that PD-1<sup>-/-</sup> mice cleared adenovirus infections more rapidly but developed more severe hepatocellular injury compared with the control group (11). Meanwhile, the study of Carter *et al* (12) indicated that the PD-1<sup>-/-</sup> mice produced a significantly higher concentration of inflammatory cytokines [interferon- $\gamma$  (IFN- $\gamma$ ), tumor necrosis factor (TNF), interleukin-6 (IL-6) and IL-17]. In the past few years, as an inflammatory animal model, PD-1<sup>-/-</sup> mice have been used to carry out extensive research, however, none of these studies focused on AF.

In the present study, we compared the expression of inflammatory cytokines, the level of atrial myocyte oxidative stress, the atrial effective refractory period (AERP) and the atrial myocardial fibrosis level of the C57BL/6 PD-1<sup>-/-</sup> with the C57BL/6 mice. We observed the relationship between the inflammation in the C57BL/6 PD-1<sup>-/-</sup> mice and the pathogenesis of AF.

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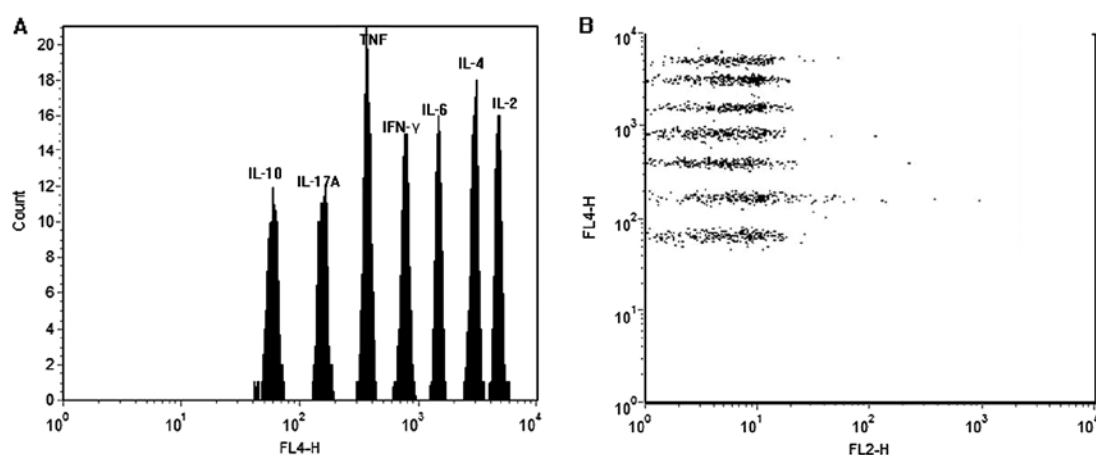


Figure 1. The bead platform for the cytometric bead array system. (A) Particles were internally labeled with different concentrations of a dye that emits discrete fluorescent signals measured in the FL4 channel shown on histograms for the bead populations, (B) while displaying fluorescence measured in the FL2 channel as shown in the dot plot.

## Materials and methods

**Animals.** Two groups of mice were used in our experiment: the C57BL/6 and the C57BL/6-PD-1<sup>-/-</sup> group (15 male mice, 7-8 weeks old, in each group).

The C57BL/6-PD-1<sup>-/-</sup> mice were kindly provided by Professor Tasuku Honjo of Kyoto University, Japan. All experiments conformed to the guidelines for the care and use of laboratory animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996). The protocol was approved by the Animal Care and Use Committee of Tangdu Hospital; all animals were maintained under specific pathogen-free conditions prior to the experiment.

**Cytometric bead array (CBA) for cytokine assessment.** Cytokine assessment was carried out using a mouse Th1/Th2/Th17 cytokine kit (BD Biosciences, San Jose, CA, USA) for simultaneous detection of seven cytokines (IL-2, -4, -6, -10, -17A, TNF and IFN- $\gamma$ ). The kit performance was optimized for analysis of physiologically relevant concentrations (pg/ml level) of specific cytokine proteins in serum samples.

The CBA technique utilizes micro particles or beads dyed with discrete fluorescence intensity. The dye incorporated in the beads fluoresces strongly at 650 nm (measured as FL4 signals in the BD FACSCalibur flow cytometer) when excited with an argon laser. Each particle population of a given intensity represents a discrete population for constructing an immunoassay for a single analyte and each particle is covalently coupled with an antibody directed against a specific analyte. When these capture beads for different analytes are used as a mixture, the level of individual analyte within samples can be measured simultaneously. Detection is mediated by the binding of specific detection antibodies that are directly conjugated with phycoerythrin (PE), thus providing an FL2 fluorescent signal on the appropriate bead. This signal is proportional to the concentration of the analyte (Fig. 1).

Mouse serum (50  $\mu$ l) and PE detection antibody were incubated with capture bead reagent for 2 h in the dark at room temperature. All unbound antibodies were washed (1.0 ml

wash buffer), re-suspended in 250  $\mu$ l before acquiring data on the BD FACSCalibur bio-analyzer (BD Biosciences).

By dedicated CBA analysis software, seven individual cytokine standard curves were used to determine the concentration of each analyte in the test sample; detection was calculated from curve estimation for an average of ten assays using power fit and  $R^2 > 0.99$  for all cytokines (Fig. 2).

**Cell isolation.** The isolation of single atrial myocyte from mice was performed as described by Cho *et al.* (13). Mice were sacrificed by cervical dislocation, and the heart was quickly removed. The heart was cannulated by a 24-gauge needle and then retrogradely perfused via the aorta on a Langendorff apparatus. During coronary perfusion, all perfusates were maintained at 37°C and equilibrated with 100% O<sub>2</sub>. Initially the heart was perfused with normal Tyrode solution for 2-3 min to clear the blood. The heart was then perfused with Ca<sup>2+</sup>-free solution for 2 min. Finally, the heart was perfused with enzyme solution for 15 min. The enzyme solution contained 0.15 mg/ml collagenase (Sigma-Aldrich) in Ca<sup>2+</sup>-free solution. Following perfusion with enzyme solution, the atria were separated from the ventricles and chopped into small pieces. Single cells were dissociated in storage medium from these small pieces using a blunt-tip glass pipette.

**Generation of reactive oxygen species (ROS).** Generation of myocardial cell intracellular ROS was measured using the fluorescent dye 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich). DCFH-DA is a non-polar and non-fluorescent compound that can diffuse into the cell where it is deacetylated by cellular esterases into a non-fluorescent polar derivative DCFH that is impermeable to the cell membrane. DCFH is rapidly oxidized to the highly fluorescent dichlorofluorescein (DCF) in the presence of intracellular ROS and can be analyzed with excitation 495 nm/emission 525 nm (measured in the FL1 channel).

Atrial myocytes were seeded in 96-well black tissue culture microplates. The cells received DCFH-DA (2  $\mu$ mol/l) for 20 min at 37°C. Following removal of the DCFH-DA, the micro-plate was incubated at 37°C for 1 h (14), and data was acquired on a BD FACSCalibur bio-analyzer (BD Biosciences).

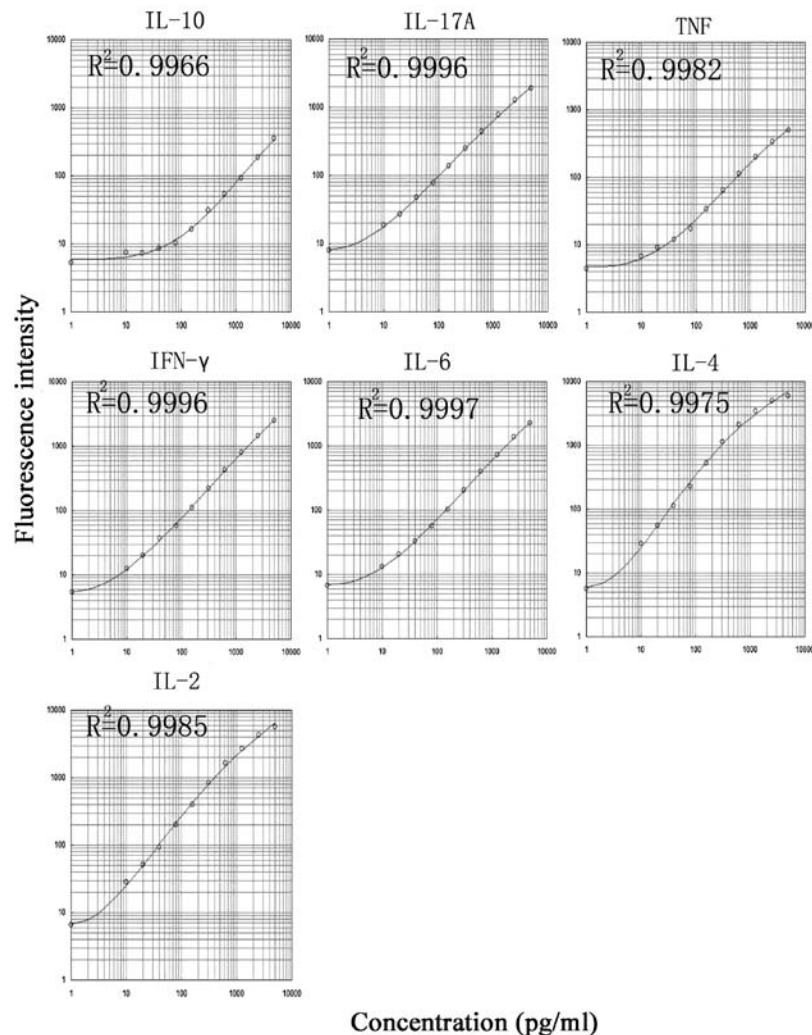


Figure 2. Cytokine calibration curve estimation. Power fit was used to determine the linearity derived from an average of 10 individual assays for each cytokine. The limit of detection was 1-5 pg/ml.

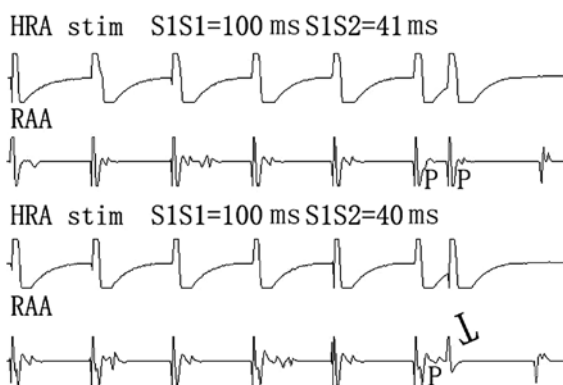


Figure 3. Recordings from a PD-1<sup>-/-</sup> mouse during S1S2 protocol. Note successful AV conduction at S1S2 of 41 ms and block at S1S2 of 40 ms. HRA, high lateral right atrium; RAA, right atrial appendage.

**Atrial effective refractory periods (AERPs).** The electrophysiology research was performed according to Etzion *et al* (15). All mice were anesthetized with intraperitoneal injection of sodium pentobarbital (50 mg/kg). Following intubation and

mechanical ventilation, the chest was opened through the right fourth intercostal space, and two bipolar screws-in pacing lead were fixed in proper sequence to four sites in the right atrial as follows: appendage, high lateral, low lateral and anterior walls. During ventilation, the arterial blood gases were adjusted to between pH 7.35 and 7.45.

Electrograms were recorded in bipolar mode at a filter setting of 30-500 Hz and stored digitally on a custom acquisition system (Quinton Electrophysiology, Canada). A programmed stimulator (Fukuda Denshi BC02A, Japan) was used to deliver square-wave impulses of 1-ms duration. The AERPs were measured at each of two sites at basic cycle lengths (100 ms) after 30 sec of continuous pacing to achieve a steady state; an atrial extra-stimulus was introduced after every six drive beat, and all stimuli were twice the diastolic threshold. The initial extra-stimulus coupling interval was set at 30 ms, and the coupling interval was set in steps of 5 ms increasing after every six beat until the extra-stimulus resulted in atrial capture. The coupling interval was then reduced by 5 ms and increased in steps of 1 ms until atria captured again by extra-stimulus. AERP was defined as the longest S1S2 coupling interval that failed to lead to atrial capture (Fig. 3).

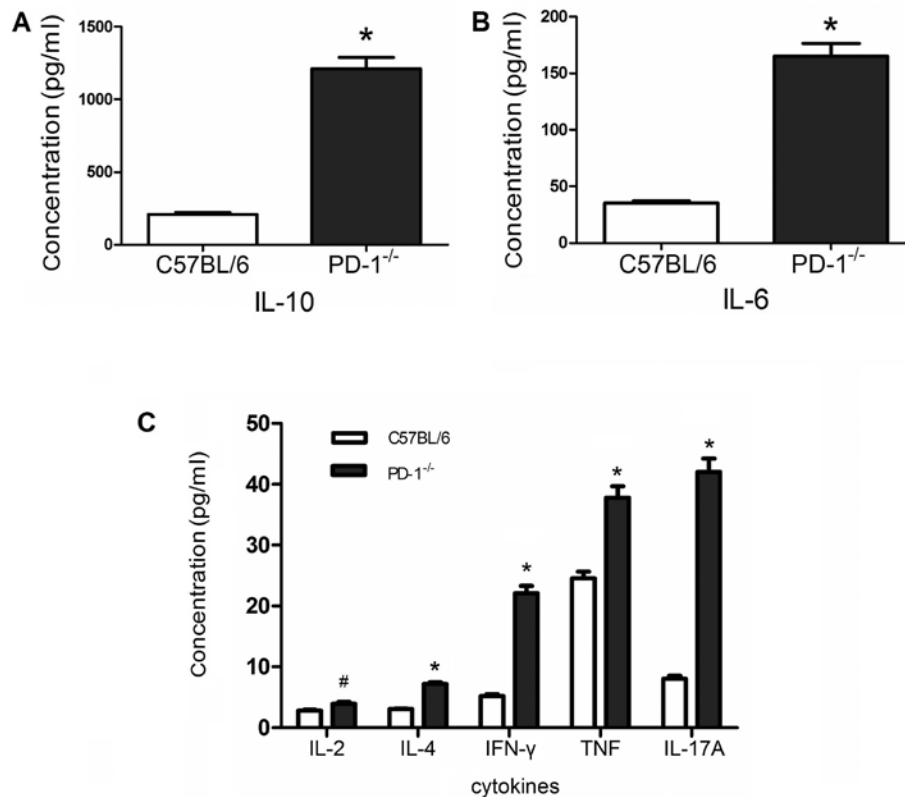


Figure 4. Comparison of inflammatory cytokine levels between the PD-1<sup>-/-</sup> and the C57BL/6 mouse groups. Concentrations of cytokines (pg/ml) in serum were measured by cytometric bead array. Inflammatory cytokines were all significantly increased in the PD-1<sup>-/-</sup> mice. Comparison of (A) IL-10, (B) IL-6, (C) IL-2, -4, -17A, TNF and INF-γ. \*P<0.001 and #P<0.01 compared with the C57BL/6 mice.

Dispersion of AERP (dAERP) equaled to maximum AERP minus minimum AERP among the four sides.

**Histological examination.** Atrial cardiac muscle was dehydrated by storing in 70% ethanol, cleared and embedded in paraffin. Routine 4 μm serial section was performed, and slides were dried in a 60°C oven. For Masson staining, slides were submerged in Masson trichrome solution (the Fourth Military Medical University Pathology Laboratory, Xi'an, China) for 5 min, washed with 0.2% acetic acid for 10 sec, followed by 5% phosphotungstic acid for 10 min, then washed twice with 0.2% acetic acid solution, stained by 2% aniline blue solution for 5 min, washed twice by 0.2% acetic acid, dehydrated using gradient ethanol, cleared in xylene and sealed using neutral Balsam.

**Statistical analysis.** All statistical analyses were performed using SPSS software (version 18; SPSS Inc., Chicago, IL, USA). Data are expressed as the means ± SD and compared by the two-sample Student's t-test. Differences were considered to be statistically significant when P<0.05.

## Results

**Inflammatory cytokines.** There were significant differences in inflammatory cytokine levels between the two groups. The inflammatory cytokine levels of PD-1<sup>-/-</sup> mice were significantly higher than those of the C57BL/6 mice. IL-10, 1212.35±300.42 vs. 208.8±57.25 pg/ml, P<0.001 (Fig. 4A); IL-6, 165.21±42.49

Table I. Comparison of AERP between the C57BL/6 and the PD-1 deficient mice.

AERPs BCL (ms)	C57BL/6 (n=15)	PD-1 deficient (n=15)
RAA 100	42.47±0.74	41.73±1.03 <sup>a</sup>
LRA 100	42.87±0.99	41.47±2.17 <sup>a</sup>
HRA 100	42.80±0.94	41.87±1.19 <sup>a</sup>
ARA 100	42.60±1.45	41.53±1.30 <sup>a</sup>

AERP, atrial effective refractory period; RAA, right atrial appendage; LRA, low lateral right atrium; HRA, high lateral right atrium; ARA, anterior right atrium. <sup>a</sup>P<0.05 compared with the control group.

vs. 35.47±6.04 pg/ml, P<0.001 (Fig. 4B); IL-2, 3.95±1.06 vs. 2.8±0.63 pg/ml, P<0.01; IL-4, 7.19±1.07 vs. 3.11±0.35 pg/ml, P<0.001; IFN-γ, 22.1±4.72 vs. 5.17±1.27 pg/ml, P<0.001; TNF, 36.49±8.21 vs. 24.55±4.29 pg/ml, P<0.001; IL-17A, 42.03±8.53 vs. 8.05±1.76 pg/ml, P<0.001 (Fig. 4C).

**Oxidative stress.** Intracellular ROS levels were measured by fluorescent probe DCFH-DA (Fig. 5). There were significant differences in the mean DCF fluorescence intensity between two groups (30.21±1.66 vs. 24.35±1.82, P<0.001).

**AERPs.** AERPs at each site were significantly shortened in PD-1<sup>-/-</sup> mice compared with the C57BL/6 mice (Table I). We

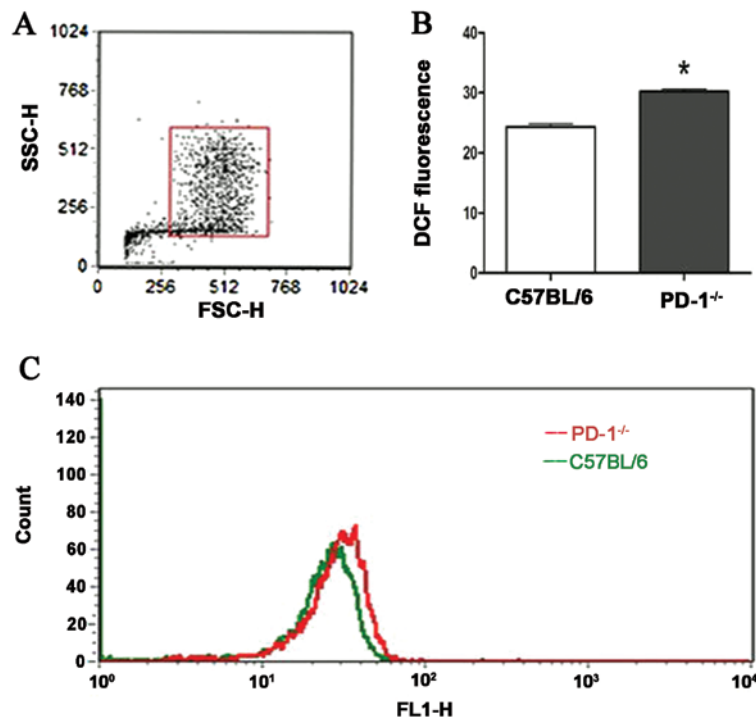


Figure 5. DCF fluorescence of 10,000 cells in the two groups was measured by flow cytometry. (A) The DCF positive cells were selected as shown in gate. (B) The comparison of mean DCF fluorescence intensity (\* $P < 0.001$  compared with C57BL/6 mice). (C) Comparison of DCF fluorescence histograms of single analyte.

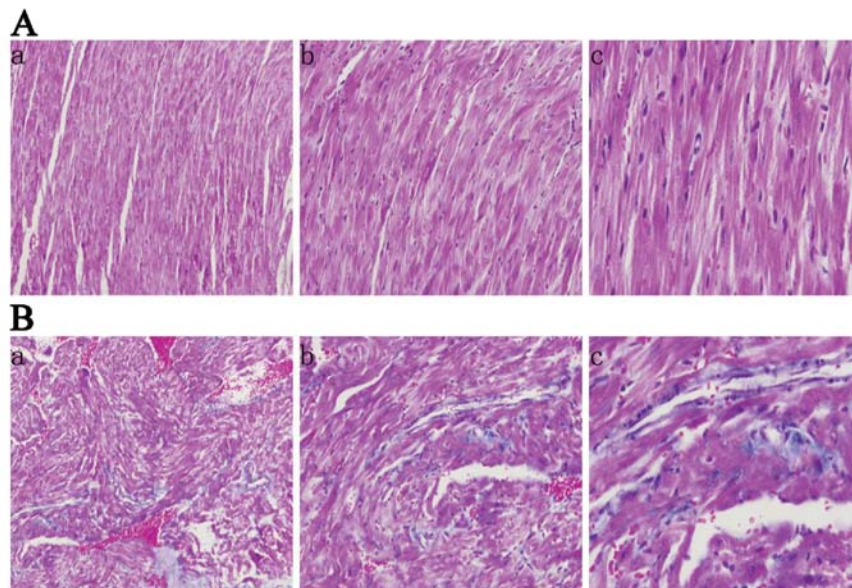


Figure 6. Representative histological sections of the right atrial from the two groups. (A) C57BL/6 mice and (B) PD-1<sup>-/-</sup> mice: magnification, (a) x100, (b) x200, (c) x400, stained with Masson's trichrome. Collagen fiber (dye blue) were detected in the PD-1<sup>-/-</sup>, but not in the C57BL/6 group.

also found that the dAERP in PD-1<sup>-/-</sup> mice was significantly increased, compared with the C57BL/6 mice,  $2.73 \pm 1.44$  vs.  $1.53 \pm 0.92$  ms,  $P < 0.05$ .

**Pathological examination.** Histological studies were performed to verify the potential pathological substrate difference between the two groups. Atrial myocardial fibrosis was detected in the PD-1<sup>-/-</sup> group, but not in the C57BL/6 group (Fig. 6).

## Discussion

**PD-1<sup>-/-</sup> mice and cardiovascular diseases.** Programmed cell death-1 (PD-1) is an inhibitory receptor in the CD28/CTLA-4 family and it can be inducibly expressed on CD8 and CD4 T-cells, natural killer T-cells, B-cells and activated monocytes (9,10,16). The ligands for PD-1 (PD-Ls) are PD-L1 and PD-L2. PD-Ls pathways control the induction and main-



tenance of peripheral T-cell tolerance (10). PD-1 deficiency enhances T-cell activation and increases the inflammation level.

PD-1<sup>-/-</sup> mice were first reported by Nishimura *et al* (17) in 1998. Experiments showed that the PD-1<sup>-/-</sup> mice with different genetic backgrounds presented different types of diseases and several were cardiovascular diseases. Nishimura *et al* (18) found that some 2C-PD-1<sup>-/-</sup>H-2<sup>b/d</sup> mice died of a Graft-Versus-Host-like (GVH-like) disease; the survivor mouse myocardium presented inflammatory cell infiltration. Studies also indicated that the heart of BALB/c-PD-1<sup>-/-</sup> mice showed various degrees of inflammation with marked deposition of immune complex on the surface of cardiomyocytes, and their sera contained high titer auto-antibodies against cardiac troponin I (cTnI) (19-21). In addition, MRL-PD-1<sup>-/-</sup> mice developed fatal myocarditis (22), massive infiltration of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells and myeloid cells were found in their hearts concomitant with the production of high-titer auto-antibodies against cardiac myosin (22).

These studies indicated that the PD-1<sup>-/-</sup> mice are closely associated with cardiovascular diseases; the basic mechanism is the high system inflammation level resulting in the inflammatory cell infiltration and/or the generation of auto-antibodies against cardiac tissue.

In the present study, we found that the PD-1<sup>-/-</sup> group presented atrial electricity remodeling (shorter AERP and increased dAERP) and structural remodeling (atrial myocardial fibrosis). It is generally accepted that AF results from the presence of multiple reentrant circuits originating in the atria (23), and atrial remodeling increases the probability of generating multiple atrial wavelets by dispersion of refractoriness and rapid atrial activation (4). Our findings strongly suggest that the PD-1<sup>-/-</sup> mice are more likely to develop AF.

*Inflammatory cytokines, reactive oxygen species (ROS) and atrial remodeling.* Previous studies indicated that inflammation may lead to 'atrial myocarditis'; atrial electrical and structural remodeling resulting from it subsequently led to the initiation and maintenance of AF (24,25).

Other studies indicated that T lymphocytes participated in the cardiac remodeling (26,27) and Th1 and Th2 responses are involved in the most fiber proliferative disorders. Th1 cytokines (IFN- $\gamma$ , TNF and IL-2) are considered to be involved in the initiation phase of fibrosis and the Th2 cytokines (IL-4, -6 and -10) during the latter stages (28). Th17 cells were characterized by the production of IL-17 as signature cytokines and also participate in the process of cardiac remodeling. Studies have shown that IL-17 could promote the production of collagen in cardiac fibroblasts (29,30).

Inflammation is also involved in the process of electrical remodeling. Atrial inflammation following cardiac surgery could influence the electrical properties of atrial tissue, and the level of atrial inflammation was associated with a proportional increase in the inhomogeneity of atrial conduction and AF duration (31). In this process, inflammation cytokines may play an important role. Previous studies found that overexpression of TNF could downregulate connexin 40 (32), Kv4.2, Kv4.3 and Kv1.5 and KChIP-2 (33,34). Downregulation of connexin 40 slows down the conduction and increases the

susceptibility of atrial arrhythmias, decreases potassium channel-interacting protein, reduces  $I_{to}$  (transient outward K current), *IK*, *slow1*, and *IK*, *slow2*, which attenuate the effective refractory period (ERP).

In the present study, although two groups of mice lived in the same environment and were fed the same food and water, the pathogen existing in the environment including microbe, parasites and virus aggravated the inflammation process in PD-1<sup>-/-</sup> mice. Therefore, the PD-1<sup>-/-</sup> group presented higher levels of inflammatory cytokines.

Oxidative stress refers to the total burden of potentially harmful ROS formed during cellular metabolism. High oxidative stress levels may be closely related to atrial electrical remodeling. Carnes *et al* (35) found that atrial tachy-pacing provoked increased protein nitration indicating enhanced oxidative stress in a dog model and the process was accompanied by a decrease in the ERP. It also showed that pretreatment of the animals with oral vitamin C (a water-soluble antioxidant) attenuated the ERP shortening (36). In addition, another study indicated that ROS also played an important role in cardiac remodeling by angiotensin II (37).

In several pathophysiological conditions, inflammation can augment oxidative stress and vice versa. Therefore, some people speculate that inflammation and oxidative stress cooperate at some level facilitating atrial remodeling (25,38).

In the present study, we found that the PD-1<sup>-/-</sup> group presented higher levels of inflammatory cytokines compared with the C57BL/6 group. At the same time, intracellular ROS of cardiac atrial myocytes were also higher. They may be interrelated and result in shorter AERPs, increased dAERP and myocardial fibrosis.

Our study is the first to prove that PD-1 deficiency results in atrial electrical and structural remodeling, raising the vulnerability of C57BL/6 mice to AF. However, it should be noted that this was an animal study with a special gene background and should not be over-interpreted. A clinical trial is required to confirm that the systemic inflammation causes the onset of AF in nonoperative settings. Sata *et al* (39) observed 15 patients with paroxysmal AF and 11 patients with normal sinus rhythm. CRP, IL-6 and TNF- $\alpha$  were measured at baseline, 24 h, and two weeks after cardioversion, and they found that CRP, IL-6 and TNF- $\alpha$  were greater in the AF group and did not normalize two weeks after cardioversion. The study suggested that inflammation may be an independent risk factor for AF, however their sample size was limited.

Tang *et al* (40) hypothesized that *Chlamydia pneumoniae* infection was a possible cause of AF by initiating inflammation response. They found that there was a close epidemiological and pathological relationship between both sides, but the hypothesis remains to be confirmed by clinical experiments. Bigger sample clinical trials need to be performed to validate the role of systemic inflammation in the onset of nonoperative AF.

In conclusion, PD-1 deficiency resulted in a significant increase in inflammatory cytokines and intracellular ROS levels in C57BL/6 mice. Higher inflammatory cytokines and intracellular ROS levels led to atrial remodeling, and, due to atrial remodeling, the C57BL/6 PD-1<sup>-/-</sup> mice may be more susceptible to AF.

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