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Electrophysiological Functions of Macrophages are Involved in Atrial Fibrillation

Aim	Inflammation, in which macrophages play an important role as immune cells, is closely related to atrial fibrillation (AF). Recent studies have shown that macrophages participate electrical conduction in the heart, thus indicating that they have electrophysiological characteristics. However, whether the electrophysiology of macrophages is associated with AF or not remained unknown. Thus, we investigated the biological function changes in macrophages using patch-clamping after tachypacing to mimic AF.
Material and methods	Atrial myocytes and macrophages were cultured. RNA sequencing was performed to investigate the expression change of atrial myocytes after tachypacing. Patch-clamping was conducted to measure the change of APD and ICa,L in macrophages after tachypacing. Rapid atrial stimulation was performed to measure the AF incidence in macrophage-specific CX43 knockout mice.
Results	After tachypacing, the time required for 90% repolarization of the action potential and the ICa,L were reduced in macrophages. Furthermore, we found that tachypacing atrial myocytes led to the secretion of Wnt 7a, further inhibiting the expression of CACNA1C in macrophages. Moreover, the knockout of CX43 in macrophages decreased the incidence of AF in a mouse model of chronic inflammation.
Conclusion	The electrophysiology of macrophages is related to the development of AF and might be a potential therapeutic target for AF therapy. Prospects for the transfer of laboratory data to the clinic: 1) Regulation of macrophage electrophysiology might be a potential therapeutic target for atrial fibrillation (AF). 2) A Wnt 7a inhibitor could be used to decrease AF incidence. 3) Blocking the interaction between macrophages and atrial myocytes might be a potential therapeutic target for AF.

Keywords	Atrial fibrillation; macrophages; electrophysiology; CACNA1C; CX43
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Introduction

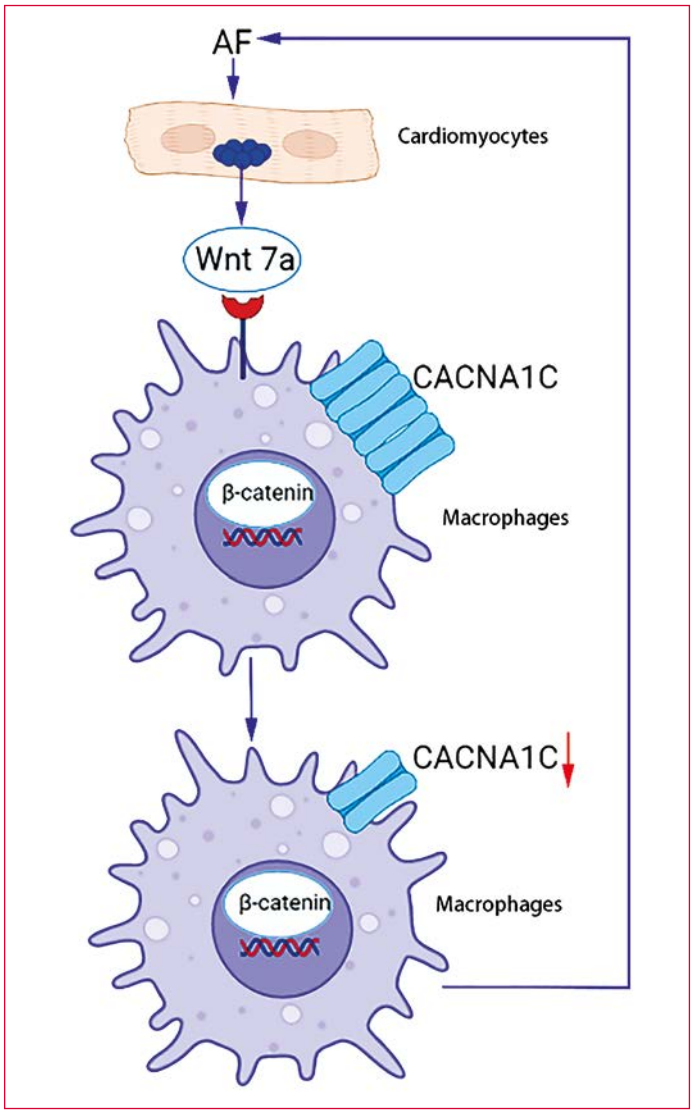
Atrial fibrillation (AF) is the most common type of arrhythmia, and AF can cause stroke and heart failure [1–3]. Hence, AF patients exhibit high mortality and disability rates, leading to a significant burden on their families and on society [4]. The mechanisms of AF remain unclear, and the efficacy of treatments is still unsatisfactory [5]. Thus, investigating the mechanisms of AF is an urgent issue.

Recent studies have shown that atrial inflammation is closely associated with the development of AF [6], and macrophages play an important role in this process [7]. In a previous study, we found that M1 macrophages increased the incidence of AF by secreting IL1β, which further inhibited the expression of CACNA1C expression as well as the ICa,L in atrial myocytes [8], while M2c macrophages produced an opposite effect. M2c macrophages decreased the incidence of AF by secreting IL10, which led to the inhibition of KCNQ1 in atrial myocytes. These effects were achieved through the secretion of cytokines by macrophages [9].

Besides acting as immune cells, cardiac macrophages express ion channels, and this affects the generation of action potentials and the electrical conduction in the atrioventricular node [10–13]. The electrical conduction between macrophages and cardiomyocytes is achieved by forming gap junctions with cardiomyocytes via connexin 43 (CX43). Additionally, the knockout of CX43 in macrophages leads to complete AV block [10]. This suggests a novel role for macrophages in AV node electrical conduction, even under physiological conditions. However, whether macrophages do, in fact, participate in atrial conduction and the development of AF remained unclear.

Therefore, in the present study, we investigated the relationship between the electrophysiological function

Central illustration. Electrophysiological Functions of Macrophages are Involved in Atrial Fibrillation



Atrial myocytes secret WNT 7a in patients with AF, which further activates the β-catenin pathway and decreases the expression of CACNA1C in macrophages. This change of the electrophysiological functions in macrophages leads to further AF.

of macrophages and AF. First, we co-cultured macrophages and atrial myocytes and performed tachypacing to mimic AF. We then evaluated the electrophysiological changes of macrophages after tachypacing. Furthermore, the incidence of AF was measured in macrophage-specific CX43 knockout mice. As a result, we present here new insights into the mechanisms of AF, and the results suggest a novel target for AF treatment.

Material and methods

Experimental animals

CD11b-DTR, Cx^{3}cr1CreER, and Cx43flox/flox mice (8 wks, 2530 g) were purchased from The Jackson Laboratory (Maine, USA). CD11bDTR mice are transgenic mice that have a diphtheria toxin (DT) inducible system that transiently depletes macrophages in various tissues after DT treatment. The mice were housed in an animal facility maintained at 20–25 °C with 55% relative humidity and an automatic 12 h light/dark cycle. All animals received a standard laboratory diet and tap water ad libitum and were acclimated in the facility for 1 wk before experimentation. Animal studies were performed in compliance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines and the Basel Declaration. All animals received humane care according to the USA National Institutes of Health guidelines.

Generation of the animal model of chronic inflammation

The chronic inflammation animal model was generated as previously described [8]. Briefly, the mice were injected intraperitoneally (i.p.) with saline (control) or with lipopolysaccharides (LPS) from Escherichia coli O111: B4 (Sigma-Aldrich, Maryland, USA, 10 mg/kg) once per week for 2 mos. To deplete macrophages, CD11bDTR mice were injected with diphtheria toxin (DT) (Sigma-Aldrich, Maryland, USA, 25 ng/g body weight) [10]. To block the electrical conduction between macrophages and atrial myocytes, Cx3cr1CreER and Cx43flox/flox mice were crossbred and their offspring were injected with 5 doses of 2 mg tamoxifen (Sigma-Aldrich, Maryland, USA) i.p., with a separation of 24 h between doses.

Electrophysiology and programmed stimulation in vivo

The electrophysiological studies and in vivo programmed stimulation were conducted as previously described [8]. Briefly, mice were anesthetized with isoflurane (1.5% v/v) and then subcutaneously injected with 0.03 mg/kg buprenorphine hydrochloride. Subdermal needle electrodes were placed in the four legs to record the electrocardiogram (ECG). A 1.1F electrophysiology catheter (Scisense Inc., Ontario, Canada) was inserted through the jugular vein. AF inducibility was determined using right atrial burst pacing. Three trains of 2 s burst pacing were given as follows: the first 2 s burst was applied at a cycle length of 40 ms with a pulse duration of 5 ms. After 3 min of stabilization, the second 2 s burst was set at a cycle length of 20 ms with a pulse duration of 5 ms. After another 3 min of stabilization, the final 2 s burst was given at a cycle length of 20 ms with a pulse duration of 10 ms. AF was defined as a rapid and irregular atrial rhythm with irregular R–R intervals for at least 1 s on the surface ECG.

Cell culture

HL1 atrial myocytes were cultured as previously described [8]. Briefly, cells were cultured with Claycomb Medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin/streptomycin, 0.1 mM norepinephrine,

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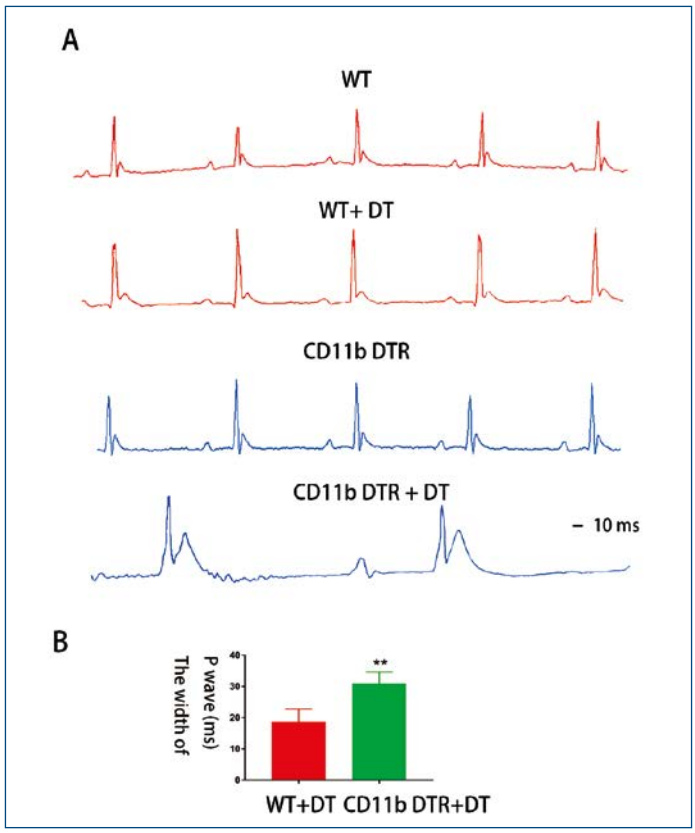
and 2 mM Lglutamine. RAW264.7 macrophages were obtained from the American Type Culture Collection (ATCC, Virginia, USA) and cultured with Dulbecco's Modified Eagle's Medium supplemented with 10% FBS. Then, HL1 atrial myocytes and RAW 264.7 cells were co-cultured in the same dish with a 10:1 ratio. Cell viability was detected using a CCK8 kit (Sangon, Shanghai, China). After co-cultured for 72 hrs, cells tachypacing was achieved by rapid stimulation for 24 h at 5 Hz (18 V, 4 ms) using a cell pacing system (Ionoptix, Massachusetts, USA).

Wholecell patchclamp

The wholecell patchclamp was performed as previously described [8]. The bath solution contained 140 mM TEACl, 2 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM HEPES, and 5 mM glucose at pH 7.4 (tetraethylammonium hydroxide). The internal solution contained 120 mM CsCl, 1 mM $MgCl_2$, 10 mM HEPES, 4 mM MgATP, 10 mM EGTA, and 0.3 mM Na_2GTP at pH 7.2 (CsOH). The Cav IVcurve was generated using the following protocol: a single cell was clamped at a holding potential of −60 mV and the Cav current was measured using a stimulus voltage pattern consisting of a 500 ms test pulses from −60 to +60 mV, each separated by a 1 s test interval. For steady-state channel inactivation, the cell was clamped at a holding potential of −80 mV and stepped to a voltage between −60 and 80 mV for 2,000 ms to inactivate the Cav current. The cell was then clamped to 10 mV for 250 ms to elicit the Cav current.

Figure 1. Macrophages participated in the electrical conduction in the whole heart



Panel A. The depletion of macrophages led to prolonged P wave, PR interval, and QRS complex. **Panel B.** Statistical analysis of the width of the P wave between WT+DT and CD11b DTR+DT groups. See text for description of mice types, WT and CD11b DTR. DT, diphtheria toxin.

RNA sequencing (RNA-seq)

Total RNA was extracted using the RNeasy Micro kit (Qiagen, Maryland, USA). Then, the cDNA libraries were prepared using the NEBNext Ultra RNA Directional Library Prep kit for Illumina following the manufacturer's protocol (NEB, Massachusetts, USA). Libraries were quantified by real-time qPCR using the KAPA Library Quantification kit (Roche, California, USA) and sequenced as single-end 50 base reads on an Illumina HiSeq 2000 in the high-output mode (Illumina, California, USA).

Cell transfection

The DNA transfection was performed as previously described [8]. For cardiomyocytes, 2 μg DNA was added into a 200 μl jetPRIME buffer, followed by the addition of 4 μl jetPRIME. After 10 min of incubation, the transfection mix was added to each well. For macrophages, 6 μg DNA was added to 50 μL of 150 mM NaCl followed by the addition of 50 μL jetPEI–Macrophage solution. After 30 min of incubation, the transfection mix was added to each well.

Cells sorting

GFP positive HL1 cells and RFP positive Raw264.7 were co-cultured. Then, the cells were harvested and washed. Next, RFP positive GFP negative cells were isolated using FACS sorting with the FITC and PE channels.

Statistical analyses

Data are expressed as mean±standard deviation (SDs). Unpaired Student's t-tests were used to compare two groups after demonstrating the homogeneity of the variance by the F test. Fisher's exact test was used to evaluate the incidence of AF. A $p<0.05$ was considered statistically significant.

Ethics approval

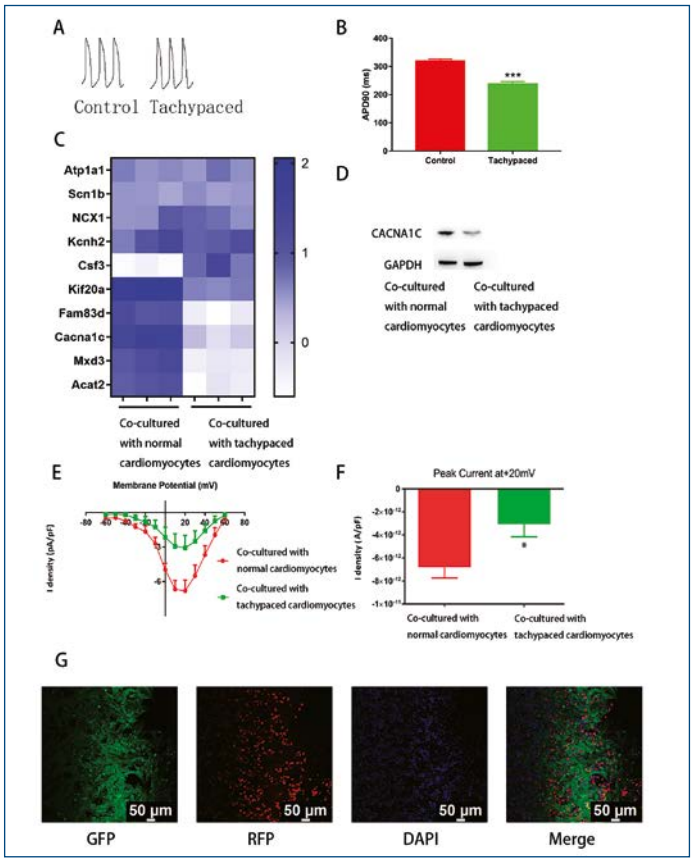
The present study was approved by the research ethics committee of the First Affiliated Hospital, College of Medicine, Zhejiang University (Reference number: 201972). This work does not contain any studies involving human subjects.

Results

Macrophages participated in the atrial electrical conduction

Previously, Hulsmans et al. found that macrophages participated in the electrical conduction of the atrioventricular node [10]. However, whether macrophages also

Figure 2. Tachypacing led to electrophysiological changes



Panel A. Tachypacing of macrophages shortened the action potential. **Panel B.** The APD 90 decreased after tachypacing in macrophages. **Panel C.** The RNA-seq showed that the expression of CACNA1C was inhibited in macrophages after tachypacing. **Panel D.** Western blotting confirmed the CACNA1C inhibition after tachypacing in macrophages. **Panel E.** ICa,L changes after tachypacing. **Panel F.** Statistical analysis of the peak current at 20 mV between control and tachypacing groups showed an inhibition after tachypacing. **Panel G.** Green and red fluorescence in HL1 cells and Raw264.7 cells.

contributed to the atrial electrical conduction remained unclear. In the present study, we depleted macrophages by injecting DT into CD11b-DTR mice and the ECG was continuous recorded using implantable ECG telemetry before DT injection. The depletion of macrophages caused prolonged P and QRS waves within 24 hs after DT injection (Figure 1). The P wave width was 18.7±4.2 ms in wild type (WT) +DT mice, while 31.0±3.6 ms in CD11b DTR+ DT mice (n=10, p=0.0179). These results indicated that macrophages participated in the electrical conduction in the whole heart, including the atria, atrioventricular node, and ventricles.

The tachypacing of atrial cardiomyocytes modified macrophages' electrophysiological properties

In our previous studies, we found that different types of macrophages have different roles in the development of AF [8, 9]. Specifically, M1 macrophages increased the incidence of AF, whereas M2 macrophages inhibited AF. However, these effects were achieved through the immunologic functions of macrophages, and whether the electrophysiological functions of macrophages were involved in the development of AF remained undetermined.

Hence, in the present study, we transfected HL1 atrial myocytes with a GFP plasmid, while an RFP plasmid was used to label RAW 264.7 macrophages. Then, HL1 and RAW 264.7 cells were co-cultured and tachypaced. The electrophysiological functions of RFP + RAW 264.7 cells were analyzed using patch-clamping. Co-culture with tachypaced HL1 cells led to the shortening of the time required for 90% repolarization of the action potential (APD90) (Figure 2A and B). The APD90 was 319.02±7.78 and 237.98±8.71 ms, respectively, (n=10, p=009) in macrophages which were co-cultured with either normal or tachypaced cardiomyocytes. To investigate the underlying mechanisms, we separated RFP + RAW 264.7 cells using fluorescence-activated cell sorting. Then, RNA-seq was performed. Co-culture with tachypaced HL1 cells led to the inhibition of CACNA1C (Figure 2C). This phenomenon was further verified by measuring the L-type calcium current. Co-culture with tachypaced HL1 cells led to the decrease of ICa,L (Figure 2 E and F). The peak currents at +20 mV were –6.8075 x 10–12±1.8580 x 10–12 A/pF, and –3.0562 x 10–12±2.6897 x 10–12 A/pF in macrophages which were co-cultured with normal or tachypaced cardiomyocytes (n=10, p=0.0425). The fluorescence in HL1 cells and Raw264.7 cells is shown in Figure 2G.

Tachypacing of HL1 cells inhibited the expression of CACNA1C in macrophages through Wnt-β catenin signaling

To investigate the mechanisms involved in the electrophysiological changes in macrophages after tachypacing, we performed RNA-seq with separated HL1 cells using FACS. The tachypacing of HL1 cells increased the concentrations of Wnt 7a (Figure 3A and 3B). Thus, we hypothesized that the inhibition of CACNA1C was mediated by Wntβ catenin signaling.

To verify this hypothesis, we performed immunofluorescence analyses. First, HL1 cells were tachypaced, then the cell culture supernatant was used for RAW 264.7 culturing. Co-culture with tachypaced HL1 cells increased the concentrations of β-catenin in the nuclei of macrophages (Figure 3D). Moreover, we treated macrophages with recombinant Wnt 7a (100 ng/ml) which also led to the downregulation of CACNA1C (Figure 3C). The knockdown of Wnt 7a in atrial myocytes abolished the inhibition of CACNA1C in macrophages after tachypacing (Figure 3E). Altogether, these results demonstrated that the tachypacing of HL1 cells led to the inhibition of CACNA1C in macrophages via Wntβ catenin signaling.

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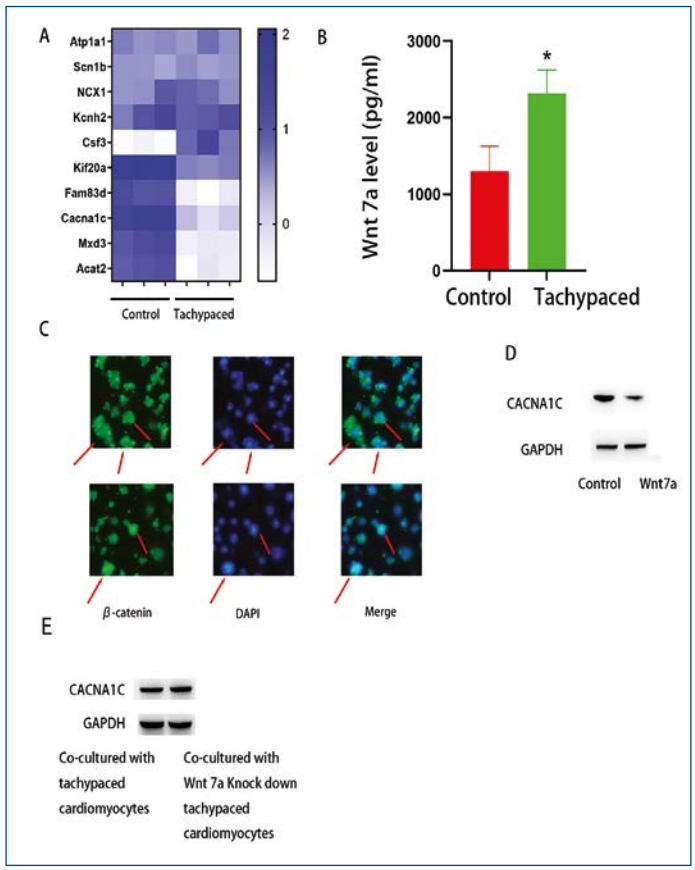
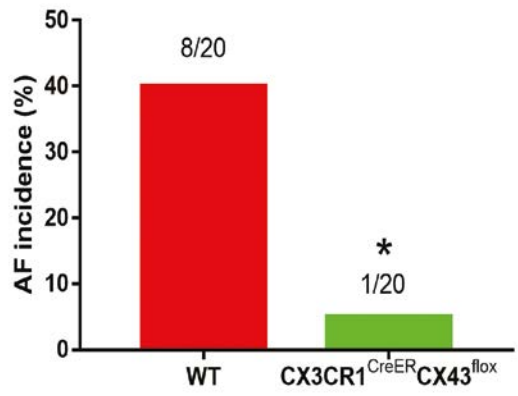
Figure 3. The inhibition of CACNA1C was mediated by the secretion of Wnt 7a in atrial myocytes



Panels A and B. The RNA-seq and Elisa assay showed that tachypacing of atrial myocytes led to the secretion of Wnt 7a. **Panel C.** The nuclear localization of Wnt 7a also increased in macrophages co-cultured with tachypaced cardiomyocytes. **Panel D.** The treatment of macrophages with Wnt 7a led to the inhibition of CACNA1C. **Panel E.** The knockdown of Wnt 7a in cardiomyocytes restored the CACNA1C inhibition in macrophages after tachypacing.

Figure 4. Blocking the electrical conduction between macrophages and atrial myocytes led to the inhibition of AF in a mouse model of chronic inflammation



Blocking the electrical conduction between macrophages and atrial myocytes was achieved by crossbreeding CX3CR1CreER and CX43flox/flox mice. After a chronic inflammation model was constructed by LPS injection, the incidence of AF was measured using rapid atrial stimulation.

Inhibition of the electrical conduction between macrophages and atrial myocytes decreased the incidence of AF

Furthermore, to investigate the electrophysiological effects of macrophages in AF, we crossbreed CX3CR1CreERT2 mice with CX43flox/flox mice to achieve a macrophage-specific CX43 knockout. The macrophage-specific CX43 knockout and WT mice were then injected with LPS once per week for two months to generate a model with chronic inflammation and the accompanying increased tendency for AF. Next, programmed stimulation was performed and the incidence of AF was measured. Overall, the incidence of AF was markedly reduced in the CX43 knockout mice with inhibited macrophages (Figure 4).

Discussion

The main findings of the present study are: 1) Macrophages participated in electrical conduction in the atrium. 2) The tachypacing of atrial myocytes to mimic AF modified macrophages' electrophysiological characteristics, as manifested by the decrease of CACNA1C concentrations and ICa,L. 3) The changes in CACNA1C concentrations in macrophages were mediated by the secretion of Wnt 7a from atrial myocytes. 4) Blocking the electrical conduction between macrophages and atrial myocytes decreased the incidence of AF.

Although AF has been investigated for decades, it remains a challenge in clinical practice. Treatments, including drugs and catheter ablation, have limited efficacy and can lead to frequent recurrence. Thus, it is urgent to determine the underlying mechanisms of AF, so that more effective therapy can be developed. In recent years, inflammation has been reported to be closely related to AF, and many immune cells are involved in the development of AF. Among these cells, macrophages have different roles in AF. For example, in our previous study, we found that M1 macrophages increased the incidence of AF while M2c macrophages inhibited it [8, 9].

Besides acting as immune cells, macrophages have action potentials, and they participate in AV nodal conduction. Hence, we hypothesized that macrophages might have similar functions in the atrium. In the current study, we found that macrophages participated in the electrical conduction of the whole heart, not just in the AV node. This was manifested by prolonged atrial P waves and ventricular QRS complexes (Figure 2A). These results were consistent with previous studies that showed that macrophages are present in the whole heart, including the atrium, AV node, and ventricle [8, 10].

Since macrophages participated in the heart conduction, we hypothesized that the unusual electrophysiology of macrophages would be involved in the development of

arrhythmia. In the present study, we demonstrated that the tachypacing of atrial myocytes led to electrophysiological changes in macrophages, manifested by APD 90 shortening. Then, we showed that this change might be caused by CACNA1C downregulation. This was consistent with the results in which atrial myocytes also presented inhibition of CACNA1C after tachypacing. Thus, the tachypacing led to electrophysiological changes in both atrial myocytes and macrophages. We also showed that the Wnt/β catenin signaling was involved in the downregulation of CACNA1C. Since inhibitors of this signaling pathway are currently available [14], the current finding provide a novel target for AF treatment.

In our previous study, we showed that the depletion of macrophages prevented the incidence of AF in a mouse model of chronic inflammation [8]. However, we did not evaluate whether this effect was caused by an immune or an electrophysiological function. Here, we knocked down CX43 in macrophages, while the expression of CX43 was not affected in cardiomyocytes. The immune function of macrophages remained active since they were still alive and could secret cytokines. Thus, we showed that the electrophysiology of macrophages was also involved in the development of AF. Therefore, AF was closely associated with the macrophages in the atrium, and both their immune and electrophysiological functions were involved. Thus, the results provide novel insight in to the mechanisms of AF, and they suggest a potential, new target for AF therapy.

In conclusion, we demonstrated that tachypacing, which was performed to mimic AF, increased the wnt7a secretion in atrial myocytes, which further activates the β-catenin pathway and decreases the expression of CACNA1C in macrophages. This change led to the APD 90 shortening and ICa,L decrease in macrophages, which further increased the AF incidence. Thus, there seems to be a positive feedback in atrial myocytes and macrophages interaction in the development of AF and could be a potential target.

Author contributions

Zewei Sun designed the study and analyzed the data. Jieqiong Wang performed the patch-clamping and cell culture. Wenjing Chen performed the animal study.

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No conflict of interest is reported.

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