

Mechanism of IL-6-related spontaneous atrial fibrillation after coronary artery grafting surgery: *IL-6* knockout mouse study and human observation



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Clinical observation and ex vivo studies have established a strong association between inflammation and postoperative atrial fibrillation (POAF). However, it is unclear whether the inflammatory phenotype is causally linked to this event or is an epiphenomenon, and it is not known which inflammatory mediators may increase susceptibility to POAF. The limitations of available animal models of spontaneous POAF (sPOAF) makes it difficult to select an experimental system. Here, we provide experimental and clinical evidence for mechanistic involvement of interleukin-6 (IL-6) in sPOAF. PHASE I: We established a mouse model of cardiac surgery with nonpaced sPOAF. *IL-6* knockout mice were protected from sPOAF compared with wild-type mice. PHASE II: At 48 hours after surgery, the heart was separated into 6 regions and cultured. IL-6 was expressed in all regions, with highest abundance in the left atrium (LA). In PHASE III, we demonstrated that IL-6 in the LA elicited early profibrotic properties in atria via the pSTAT3/STAT3 signaling pathway and contributed to sPOAF. PHASE IV: In a translational prospective clinical study, we demonstrated that humans with POAF had a higher IL-6 concentration in pericardial drainage (PD). This study provides preliminary evidence of a causal relationship between IL-6 and POAF in a novel nonpaced sPOAF mouse model. IL-6 is a crucial prerequisite for eliciting profibrotic properties in cardiac myocytes via the pSTAT3 pathway during the early postoperative period, leading to an increased susceptibility to POAF. Measuring IL-6 in PD could be a new noninvasive biomarker for the clinical prediction of POAF. (Translational Research 2021; 233:16–31)

Abbreviations: AI = anti-inflammatory; Cx40 = connexin 40; Cx43 = connexin 43; IL-6 = interleukin-6; KO = knockout; LA = left atrium; LV = left ventricle; NAI = non-anti-inflammatory; OPCAB = off-pump coronary bypass grafting surgery; PC = pericardium; PCF = pericardial fluid; PD = pericardial drainage; POAF = postoperative atrial fibrillation; PV = pulmonary vein; RA = right atrium; RV = right ventricle; sPOAF = spontaneous postoperative atrial fibrillation; ssPOAF = sustained spontaneous postoperative atrial fibrillation; STAT3 = signal transducer and activator of transcription 3; WT = wild type

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AT A GLANCE COMMENTARY

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Background

The underlying mechanism of postoperative atrial fibrillation (POAF) is not completely understood, limiting the development of effective predictive and preventive measures. Increasing evidence has established a strong association between inflammation and POAF. However, whether the inflammatory phenotype is causally linked to or its epiphenomenon, and which inflammatory mediators may increase susceptibility to POAF remains unclear.

Translational significance

We revealed a causal relationship between IL-6 and POAF. IL-6 in pericardial drainage holds potential as a new noninvasive biomarker. Given that current strategies for preventing POAF are suboptimal, the IL-6-pSTAT3-fibroblasts axis may be a new promising therapeutic target for this arrhythmia.

INTRODUCTION

Coronary artery bypass grafting (CABG) surgery is the most common surgery performed by cardiac surgeon.¹ The broad use of off-pump CABG (OPCAB) and small incision techniques have largely reduced the incidence of various post-CABG complications, but the incidence of postoperative atrial fibrillation (POAF) remains unchanged (range: 20%–40%) and is a major contributor to morbidity and mortality.² The underlying pathophysiology of POAF is not completely understood, but clinical observation and animal studies have established a strong association between inflammation and POAF.^{2–4}

Atrial fibrosis seems to be a key mechanistic link by which inflammation may contribute to atrial fibrillation (AF); it is thought to alter atrial coupling (eg, connexins), resulting in structural changes that predispose patients to AF.⁵ However, it is still unclear whether inflammation can induce structural atrial changes during early postoperative stage.⁶ In a canine sterile pericarditis model, the percentage of fibrosis in the left atrium (LA) on postoperative day 2 was $30\% \pm 8\%$ in the pericarditis group compared to $6\% \pm 2\%$ in the sham group.⁷ Similar levels of fibrosis were reported in

another rat sterile pericarditis model.⁸ These findings indicated that atrial fibrosis may have occurred at an early stage in relation to atrial inflammation.

Interleukin-6 (IL-6) is a crucial regulatory cytokine that activates signal transducer and activator of transcription 3 (STAT3) signaling, which has been strongly linked to the chronic cardiac fibrosis process.⁹ Recent evidence suggested that IL-6 and fibrinogen levels were elevated 72 hours after CABG surgery, and these increases were associated with POAF.¹⁰ Whether this phenotype is causally linked to or merely an epiphenomenon of POAF and its underlying mechanism is unclear. Current evidence of IL-6 as a biomarker for POAF remains controversial.^{4, 11, 12} Here, we investigated whether IL-6 is causally linked to POAF by activating STAT3 signaling and promoting atrial fibroblast proliferation in the early postoperative stage.

The limitations of available animal models of POAF make it difficult to select a representative experimental system to study the mechanism.¹³ Most prior animal studies exploring the inflammatory mechanism of POAF employed the sterile pericarditis and burst pacing AF models.^{13–15} The pathophysiology induced by sterile pericarditis may be different from that induced by CABG.^{13,16,17} Similarly, AF induced by a burst pacing program also does not represent clinical spontaneous AF.¹⁶

Phase I of the current study established a mouse model of cardiac surgery (according to clinical OPCAB procedures) with nonpaced spontaneous POAF (sPOAF) and determined the causal relationship between IL-6 and sPOAF in wild-type (WT) and *IL-6* knockout (KO) mice. In phase II, IL-6 concentrations in different parts of the heart after cardiac surgery were explored with tissue culture. It is unclear whether IL-6 could be synthesized in atria under OPCAB stimulation, although it could be synthesized in the atria in the setting of cardiac ischemia and reperfusion during cardiopulmonary bypass.¹⁸ In phase III, the region with the highest IL-6 level in phase II was harvested to identify and explore the relevant signaling pathways. To further assess the translational relevance of these findings, IL-6 levels and other inflammatory biomarkers in pericardial drainage (PD) and peripheral blood were determined at different time points postoperatively, and their relationships to POAF were examined in patients undergoing OPCAB in phase IV. We hypothesized that: (1) the atrium produces IL-6 after cardiac surgery and contributes to sPOAF onset by activating the STAT3 signaling pathway and inducing cardiac fibroblast proliferation at the early postoperative stage, and (2) patients with POAF after OPCAB have higher IL-6 concentrations in PD. To our knowledge, this is the first study to explore the causal relationship between IL-6 and POAF in a spontaneous (nonpaced)

POAF animal model, to determine whether it contributes to POAF by eliciting profibrotic atrial properties via the pSTAT3 pathway during the early postoperative period, and to identify pericardial IL-6 as a potential noninvasive biomarker to predict POAF in humans.

METHODS

The animal study procedures were approved by the Capital Medical University Animal Ethics Committee (AEEI-2014-137). All experimental procedures were in compliance with National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Publication, revised 2011).

Phase I: Mouse model of cardiac surgery and telemetry studies. Mouse model of cardiac surgery. The study used age-matched (24–26 weeks) and weight-matched (31.43 ± 4.41 g) C57BL/6 (WT) male mice (Jackson Laboratory, Bar Harbor, ME) and *IL-6* KO mice (B6.129S2-I16tm1Kopf/J, Jackson Laboratory). We used male mice because females tend to have a lower rate of POAF.¹⁹ Mice were randomly divided into 3 groups (10 mice per group): (1) a control group receiving anesthesia only, (2) a non-anti-inflammatory (NAI) group undergoing cardiac surgery, and (3) an anti-inflammatory (AI) group undergoing cardiac surgery with methylprednisolone (2 mg/kg per day) applied to the pericardial surfaces of the heart once daily for 3 days. The first dose was given before chest closure. The other 2 doses were administered via the chest tube on postoperative days 1 and 2. The tube was secured to the rib to ensure that the distal end was above the surface of the heart. Necropsies were performed 48 hours after surgery from the lateral approach to confirm that the distal end of the chest tube was above the heart. Mice in the NAI group were injected with the same volume of vehicle via the chest tube. All surgical procedures were performed by the same team. The operator was blinded to the groups as a research assistant was responsible for methylprednisolone or vehicle preparation.

To establish an experimental system that could represent clinical OPCAB, we developed a mouse model of cardiac surgery according to clinical OPCAB procedures (Table I; Supplementary Fig 1) under sterile surgical conditions. All animals were anesthetized using intraperitoneal injection of pentobarbital sodium (70 mg/kg) prior to the surgery. Tracheal intubation was performed to secure respiration with a tidal volume of 0.2 mL and respiratory rate of 110 breaths/min via a MiniVent 845 mouse ventilator (Harvard Apparatus, Holliston, MA). (1) *Heart exposure.* The chest skin was shaved and sterilized with 75% alcohol and betadine solution. After sterile draping

and preparation, an intercostal incision was performed at the left fourth intercostal space to expose the heart. (2) *Exposure of the target vessel.* After pericardiotomy, the left anterior descending (LAD) artery was targeted. A sterile cotton ball was used to push tissue away for better visualization. (3) *Simulated artery stabilization and clamping.* The clinical OPCAB procedure used a tissue stabilizer to stabilize and temporarily and partially clamp the artery. Forceps were used to depress gently the tissue around the LAD (not on the LAD) for 1 minute to simulate artery stabilization. No myocardial anemia was witnessed in the depressed site, suggesting that ischemia and reperfusion were successfully avoided. Two temporary sutures were placed on the surface of heart, forming a loose snare to simulate temporary artery clamping for 2 minutes, then removed. To avoid myocardial infarction, no major arterial branches were occluded by sutures. (4) *Simulated anastomoses.* Forceps were used to make 10 scratches on the surface of the heart to simulate anastomoses. The heart was irrigated with sterile saline, and suction was performed with a self-made aspirator using a silicone venous catheter (BD, 24G, Franklin Lakes, NJ). (5) *Chest tube placement.* A self-made silicon chest tube (made from a 1.9 Fr NeopICC central venous catheter, Health Line International Corp., Foshan, China) was placed with the distal end positioned above the surface of the heart and the bent and tied proximal end positioned subcutaneously at the abdomen. The chest tube was not attached to an extracorporeal drainage device as it would have required sedation for the entire 1-week observation period, which may have biased the results. (6) *Chest closure.* The chest incision was closed in layers.

Animals were placed on a 37°C heating pad after surgery. After fully recovering from anesthesia, each animal was placed in an individual cage. Postoperative infection was prevented by intramuscular injection of penicillin (20,000 units per day) for 3 days.

Telemetry studies. To observe whether sPOAF occurred in this mouse model of cardiac surgery, a 1.4 cc, 2.2 g wireless telemetry device (HD-X11, Data Sciences International, St. Paul, MN) was implanted within a subcutaneous pocket immediately after chest closure according to manufacturer's instructions. A pair of recording leads were subcutaneously secured to the right pectoral muscle and left caudal rib region. Placement was verified by visualization of electrocardiography (ECG) waveforms on a data acquisition system via Dataquest A.R.T. 4.4 software (Data Sciences International). Data collection and analysis were carried out with Ponemah version 5.2 (Data Sciences International) for 7 consecutive days. ECGs were independently analyzed by 2 researchers with 10 years of clinical practice in AF management. Both were trained in ECG reading. A qualified physician was consulted if

Table I. Mouse model of cardiac surgery

OPCAB procedure	Mouse model of cardiac surgery
•Heart exposure Intercostal incision or median sternotomy. •Harvest arterial or venous conduit for coronary graft. •Target vessel exposure Pericardiotomy performed. Artery is targeted. •Artery stabilization and clamping Tissue stabilizer is placed to stabilize the artery. Aorta is temporarily and partially clamped. •Anastomosis Incision of the target artery. Arterial or venous conduit is anastomosed to the target artery. Irrigation with sterile saline. Suction of blood. •Chest tube placement Place a silicon chest tube. •Chest closure Chest incision closed in layers.	•Heart exposure Intercostal incision performed at the left fourth intercostal space. •— •Target vessel exposure Pericardiotomy performed. Left anterior descending artery (LAD) targeted. •Simulated artery stabilization and clamping Forceps were used to depress the tissue around LAD for 1 min to simulate artery stabilization.[#] Two temporary sutures are placed on the surface of the heart simulating temporary artery clamping for 2 min and then removed. No major arterial branches are occluded by sutures to avoid any myocardial infarction. •Simulate anastomosis Ten scratches are made on the surface of the heart to simulate anastomoses. Irrigation with sterile saline. Suction with a self-made aspirator by a venous catheter (BD, 24G, USA). •Chest tube placement Place a self-made silicon chest tube. •Chest closure Chest incision closed in layers.

Abbreviation: OPCAB, off-pump coronary bypass grafting surgery.

[#]To avoid ischemia and reperfusion, no myocardial anemia was witnessed in the depressed site.

there was disagreement between the 2 examiners about the underlying cardiac rhythm.

The primary endpoint in this phase was the incidence of sPOAF, defined as an episode of a rapid and irregular rhythm lasting for more than 1 second.²⁰ The incidence of sPOAF was defined as the percentage of mice with sPOAF during the 7-day observation period. An sPOAF episode longer than 1 minute was defined as a sustained sPOAF (ssPOAF).²⁰ The number of sPOAF episodes in each mouse was the total number of episodes during the 7-day observation. sPOAF duration was defined as the time between sPOAF onset and return of sinus rhythm. The total duration of sPOAF episodes in each mouse was calculated as the sum of the duration of each POAF episode.

Phase II: Tissue culture studies. We performed tissue culture studies to assess IL-6 productive capacity in specimens collected from 6 heart sites after cardiac surgery and investigated whether it was suppressed by epicardial methylprednisolone therapy. WT and KO mice were randomly divided into 3 groups as described in Phase I ($n=8$ /group). Mice were euthanized 48 hours after surgery, which corresponds to the peak incidence time of POAF. The whole heart was divided into

the pericardium (PC), pulmonary vein (PV), left atrium (LA), right atrium (RA), left ventricle (LV), and right ventricle (RV) and weighed (Sartorius BSA224S, Göttingen, Germany). Tissue samples were flushed with D-Hank's fluid 3 times (10 seconds each) and placed in a plastic 12-well culture plate. Each well contained 200 μ L Roswell Park Memorial Institute 1640 (RPMI 1640 medium), 5% fetal bovine serum, and antibiotics (100 U/mL penicillin, 100 μ g/mL streptomycin, all from Gibco, Thermo Fisher Scientific, Inc., Waltham, MA). Tissue samples were incubated (Thermo Fisher Scientific, Forma 370) for 4 hours at 37°C under 5% CO₂. At 10 minutes and 4 hours after incubation, 100 μ L of supernatants were removed and stored at -80°C, and IL-6 levels were determined using enzyme-linked immunosorbent assays (ELISAs; RD Systems, Minneapolis, MN) in accordance with the manufacturer's instructions. IL-6 concentrations were determined as the ratio of IL-6 level in supernatant to tissue weight.

Phase III: Mechanistic studies. To explore the role IL-6 in early atrial remodeling, 2 additional sets of WT and IL-6 KO mice were randomly divided into 3 groups as described above. One set of mice was monitored for

sPOAF for 48 hours after cardiac surgery and then euthanized. The part of the heart with the most IL-6 after cardiac surgery according to the results of phase II was harvested to analyze the expressions of IL-6, STAT3, phosphorylated STAT3 (pSTAT3), connexin 43 (Cx43), connexin (Cx40), collagen I, and collagen III by western blot and real-time polymerase chain reaction (RT-PCR). Tissues from the other set of mice were subjected to Masson trichrome staining and immunofluorescence tests to analyze collagen deposition and the distributions of IL-6, pSTAT3, and vimentin. Detailed methods are provided in the online supplement.

Phase IV: Animal studies and clinical studies. To evaluate the predictive value of IL-6 in POAF in humans, we measured IL-6 levels in peripheral blood and PD in patients who had undergone OPCAB. Animal experiments were performed prior to the clinical study.

Animal studies. Sixteen WT mice underwent cardiac surgery, and ECGs were monitored for 48 hours with implanted wireless telemetry as described in Phase I. IL-6 levels were measured in peripheral blood and PD. Samples were collected at 0 (the start of surgery), 6-, 12-, and 18-hour postoperatively. Blood samples were collected from orbital veins of mice using microcapillary tubes. PD from chest tube was sampled with insulin syringe (29G, BD). Samples were immediately centrifuged at 1500 g for 15 minutes. Serum or supernatant was stored at -80°C until assay. IL-6 levels were measured using mouse IL-6 Quantikine ELISA Kit (R & D Systems). Assays were conducted in accordance with the protocols provided by the manufacturers. ECG and sPOAF were assessed as described in Phase I. The relationships between IL-6 in PD and blood with sPOAF were examined.

Clinical studies. *Patient population.* This prospective cohort study was conducted in 2 cardiac centers in Beijing, China. The protocols for human research were approved by the ethics committees of Capital Medical University (20119y30). The study was conducted in accordance with The Code of Ethics of The World Medical Association (Declaration of Helsinki). Written informed consent was obtained from each participant. All adult patients with normal sinus rhythm prior to the first time undergoing isolated CABG were consecutively recruited. The exclusion criteria were existing atrial arrhythmia, concurrent procedures, emergent CABG, thoracic bleeding after surgery, implanted pacemaker, evidence of active infection or chronic inflammatory disorders, impaired immune system disease, or AI therapy. Sample size calculation was based on power analysis (2-tailed, $\alpha = 0.05$, power = 0.9) for logistic regression (estimated odds ratio [OR] for IL-6 = 2, estimated incidence of POAF = 30% according to references²) and indicated that 102 patients were

required. Clinical and demographic data were prospectively collected from patient charts by trained researchers. Data quality checks were routinely performed for missing values and incorrect entries.

Surgical procedures and postoperative management. All surgical interventions were performed in a standard fashion through a median sternotomy. After adequate exposure and stabilization, the target vessel was exposed and snared above the anastomotic site with a 4-0 polypropylene suture and a soft plastic snugger to prevent coronary injury. The coronary artery was then opened to perform anastomosis. Visualization was enhanced using a surgical blower-humidifier. Conduits for bypass included the LAD and/or the internal mammary artery and/or saphenous veins and/or the radial artery. Before closing the sternum, 2 chest tubes were placed in the mediastinal space and connected to a sealed drainage system.

Postoperatively, patients were transferred to the cardiac surgery intensive care unit and weaned from ventilation when they met the following criteria: respiratory and hemodynamic stability, normothermia, and no major bleeding. Pharmacologic therapy with inotropes was used to maintain a cardiac index ≥ 2.0 L/min/m². Mediastinal drainage tubes were removed as soon as the drainage was <10 mL/h for at least 6 hours.

Evaluation of postoperative AF. Heart rate and rhythm were continuously monitored during the first 7 postoperative days with an individual bedside monitor (M8002A, Philips, Amsterdam, the Netherlands) or via telemetry (M3290A, Philips). When clinical evaluation suggested atrial arrhythmia, 12-lead ECG recordings were obtained to confirm the AF episodes. AF in the clinical study was defined as P-wave loss before each QRS complex and irregular ventricular rate for >30 seconds.²¹ Time of onset, duration, and mode of conversion of each AF episode were recorded. Independent POAF diagnoses were made on the basis of 24-hour ECG recordings by 2 physicians who were unaware of patients' clinical status. Disagreements were resolved by joint re-evaluation with a senior physician.

IL-6, CRP, and WBC measurement. A previous study reported that serum IL-6 increased after CABG completion, peaked at 6 hours, and declined thereafter.²² Thus, we collected both peripheral blood samples and PD from the chest tube 6-, 12-, and 18-hour postoperatively. Peripheral blood samples were also collected just before off-pump CABG surgery. Chest tubes were removed on the morning of postoperative day 2 in most patients. PD samples were difficult to obtain at postoperative day 2 and later. A predictor would be relevant if it occurs earlier than POAF, which was at 49.07 ± 24.24 hours

(range from 18 to 151 hours) postoperatively in current study. Therefore, we did not collect PD and blood samples after 18 hours. To collect samples in accordance with the scheduled time points, the drainage bottle was emptied before each collection. Drainage fluid was allowed to accumulate for no more than 1 hour in the drainage tube and sampled from the drainage bottle with a sterile disposable syringe. Samples were centrifuged and stored as described above. IL-6 levels were determined by an ELISA kit (RayBiotech, Atlanta, GA). C-reactive protein (CRP) concentrations were measured using a high-sensitivity CRP Assay Kit with a protein analyzer (Beckman Coulter, Inc, Brea, CA). The sensitivities of IL-6 and CRP were 3 pg/mL and 0.1 mg/L, respectively. White blood cell (WBC) counts were quantified using a Sysmex Automatic Cell Analyzer (Sysmex Shanghai Ltd., Shanghai, China). All assays were conducted in accordance with the manufacturers' protocols and provided by a laboratory technician who was blinded to patient clinical status.

Statistical analysis. Statistical analysis was performed using SPSS version 21.0 (IBM, Armonk, NY). Continuous variables were analyzed with Student's *t* test, one-way analysis of variance, or Mann-Whitney *U* test as appropriate. Categorical variables were analyzed with chi-squared tests or Fisher's exact tests. Correlation coefficients were assessed using Pearson correlations or Spearman's rank tests. Repeated one-way analysis of variance measures were used to compare IL-6, CRP, and WBC levels between patients and mice with and without POAF. Bonferroni correction was applied for multiple comparisons at each time point. Logistic regression was used to test associations between IL-6, CRP, and WBC levels in PD and peripheral blood and POAF. A 2-sided $P < 0.05$ was considered statistically significant.

To further examine the potential causal relationship between IL-6 and POAF, path analysis with the bootstrapping method was performed using IBM SPSS AMOS 21.0. The model fit was judged by χ^2 statistic and standardized root mean square residual (RMR). A small (<3) χ^2 statistic relative to the degrees of freedom and a probability value >0.05 were considered optimal. A standardized RMR < 0.08 was considered adequate. Three other indices were also analyzed to assess model fit: goodness-of-fit index, comparative fit index, and normed fit index. Values >0.90 indicate a good fit.²³ Standardized coefficients were used to measure effect sizes and defined as follows: <0.3 , small; 0.3 – 0.5 , medium; >0.5 , large.²⁴

RESULTS

IL-6 knockout mice were fully protected from ssPOAF. The representative baseline ECGs of sinus rhythm and

spontaneous AF rhythm are shown in Fig 1A, Panels I and II. In the cohort of mice that underwent 7-day observation, ssPOAF and nonsustained sPOAF occurred in 40% and 50% of the mice in the NAI-WT group, respectively. In sharp contrast, almost complete protection from sPOAF was observed in *IL-6* KO mice (NAI-WT vs NAI-KO: 40% vs 0 for ssPOAF, $P = 0.01$; 50% vs 10% for nonsustained sPOAF, $P = 0.044$) and those that received epicardial methylprednisolone (Fig 1A, Panels III–IV). The mean number (Fig 1A, Panel V) and total duration (Fig 1A, Panel VI) of sPOAF episodes in the NAI-WT group were 5- and 16-fold higher than in the NAI-KO mice, respectively. The protective effect of *IL-6* KO against sPOAF was confirmed in a separate set of mice (phase III) that underwent 48-hour observation, with 50% (4/8) having sPOAF in the NAI-WT group and none in the *IL-6* KO and AI groups. This suggested that IL-6 was mechanistically involved in sPOAF onset.

We then compared IL-6 levels in 3 WT groups. In NAI-WT mice, cardiac surgery substantially elevated myocardial IL-6 protein levels (Fig 1B, Panels I and II), but they were profoundly reduced by 62.5% in methylprednisolone-treated mice (AI-WT). The results were confirmed by RT-PCR (Fig 1B, Panel III). We further compared myocardial IL-6 protein levels in NAI-WT mice and found that they were significantly higher in the mice that exhibited sPOAF ($n = 4$) compared to those that did not (0.79 ± 0.09 vs 0.51 ± 0.05 , $P = 0.037$, $n = 4$ for non-POAF).

The LA had the highest IL-6 levels after OPCAB stimulation. The IL-6 protein levels at 6 sites in the supernatant of a 10-minute culture were undetectable in all 6 groups and markedly augmented after culturing for 4 hours in WT mice (still undetectable in *IL-6* KO mice). The LA had the highest IL-6 protein abundance (measured by ELISA, see details in Phase II: Tissue culture studies section), followed by the RA, PV, PC, LV, and RV in the NAI-WT group ($P < 0.001$; post hoc test, $P = 0.016$ for LA vs PV; $P < 0.001$ for LA vs PC; $P < 0.001$ for LA vs LV; $P < 0.001$ for LA vs RV; $P = 0.335$ for LA vs RA, Fig 2). IL-6 concentrations were highest in the NAI-WT group and could be significantly suppressed by epicardial AI therapy (AI-WT group). This finding suggests that IL-6 could be locally synthesized in settings of cardiac surgery without cardiac ischemia and reperfusion, with the highest abundance in the LA.

IL-6-activated STAT3 promotes fibroblast proliferation and facilitates sPOAF onset. IL-6 promotes fibroblast proliferation. To assess the role of IL-6 in early structural atria remodeling—a known prerequisite of AF—we performed histopathological, RT-PCR, and immunofluorescence studies at 48 hours after cardiac

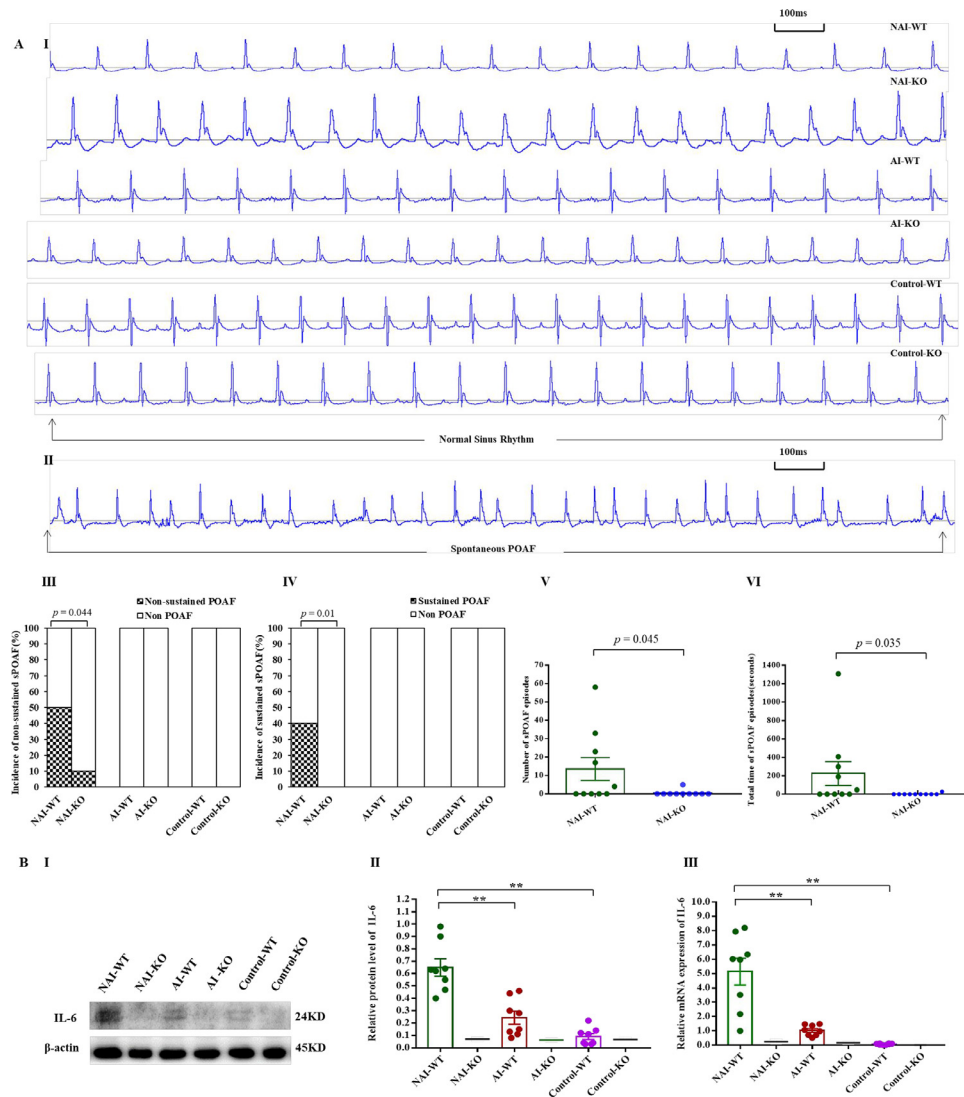


Fig 1. Effects of IL-6 on spontaneous POAF. A. Panel I: Examples of baseline sinus rhythm. Panel II: Examples of sPOAF. Panel III: Incidence of nonsustained sPOAF. Panel IV: Incidence of sustained sPOAF. Panel V: Number of sPOAF episodes. Fivefold higher in the NAI-WT group than that in the NAI-KO group. Panel VI: Total time of sPOAF episodes. Sixteen-fold longer in the NAI-WT group than that in the NAI-KO group. $n = 10$ per group. B. Panel I: Representative western blots for IL-6. Panel II: Quantification of IL-6 relative to β -actin. Panel III: The mRNA expression of IL-6. $n = 8$ per group. Numbers and total time of sPOAF episodes are presented with median and interquartile range. All other values are mean $\pm$ SEM. *: $P < 0.05$; **: $P < 0.001$. WT, wild type; KO, IL-6 knock out; NAI, non-anti-inflammatory; AI, anti-inflammatory; IL-6, interleukin-6; sPOAF, spontaneous postoperative atrial fibrillation.

surgery. In WT mice, cardiac surgery substantially increased myocardial IL-6 mRNA and protein levels, which were suppressed by methylprednisolone (Fig 1B). Masson trichrome staining (Fig 3A, Panels I and II) revealed a 4.9-fold increase in fibroblast proliferation in the cardiac surgery-treated WT mice (NAI-WT group) compared with the control group. Atrial fibroblast proliferation was markedly attenuated in cardiac surgery-treated *IL-6* KO mice (NAI-KO group) and methylprednisolone-treated mice (AI groups)

compared with the NAI-WT group. Results were confirmed by RT-PCR (Fig 3A, Panel III). The mRNA levels of collagen I and III correlated closely with IL-6 levels ($r = 0.899$, $r = 0.891$, respectively, Fig 3B). Consistent with this, dual immunofluorescence (Fig 3C, Panel I) revealed dense fibroblasts and high IL-6 infiltrated in the NAI-WT group, in comparison with scant fibroblasts and attenuated to absent IL-6 infiltration in the *IL-6* KO and AI groups. Confocal laser scanning microscopy (Fig 3C, Panel II) showed that IL-6 and

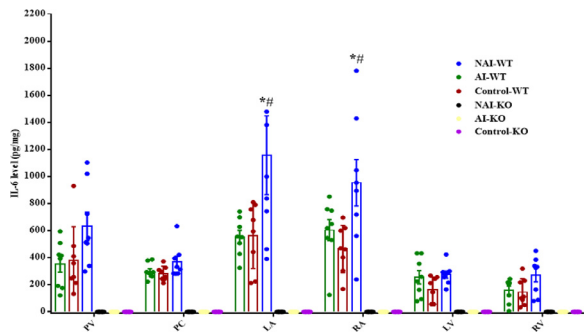


Fig 2. IL-6 levels in the supernatant at 4 hours of culturing from 6 cardiac sites. IL-6 levels were undetectable in *IL-6* KO mice. IL-6 levels were the highest in the NAI-WT group at LA and RA sites when compared with the control-WT and AI-WT groups in WT mice ($P = 0.040, 0.019, 0.064, 0.114, 0.104, 0.069$ for LA, RA, PV, PC, LV, RV, respectively, compared among 3 WT groups). There was no significant difference between the control-WT and AI-WT group at all 6 sites. LA had the highest concentrations of IL-6 protein, followed by RA, PV, PC, LV, and RV in the NAI-WT group ($P < 0.001$; post hoc test, $P = 0.016$ for LA vs PV; $P < 0.001$ for LA vs PC; $P < 0.001$ for LA vs LV; $P < 0.001$ for LA vs RV; $P = 0.335$ for LA vs RA). $n = 8$ per group. *: $P < 0.05$, NAI-WT group vs control-WT group; #: $P < 0.05$, NAI-WT group vs AI-WT group; PV, pulmonary vein; PC, pericardium; LA, left atrium; RA, right atrium; LV, left ventricle; RV, right ventricle; WT, wild type; KO, knock out; NAI, non-anti-inflammatory; AI, anti-inflammatory; IL-6, interleukin-6.

fibroblasts co-localized to a large extent in the cytoplasm. This finding complements the histological and RT-PCR observations of attenuated atrial fibroblast proliferation in *IL-6* KO mice.

IL-6 upregulates pSTAT3 signaling. To further characterize the molecular basis involved in IL-6-dependent atrial remodeling, we measured pSTAT3/STAT3 levels. These levels were increased by 4-fold ($P < 0.001$) in the NAI-WT group compared with the control group (Fig 4A). Western blots showed that they were profoundly reduced in the *IL-6* KO and AI groups compared with the NAI-WT group (Fig 4A). The protein levels of pSTAT3 correlated closely with IL-6 ($r > 0.8$, Fig 4B). Dual immunofluorescence staining (Fig 4C) further clarified the relationship between pSTAT3 and IL-6, demonstrating strong pSTAT3 and IL-6 signals in the NAI-WT group. In contrast, pSTAT3 and IL-6 signals were noticeably attenuated in the *IL-6* KO and methylprednisolone-treated groups (pSTAT3 in NAI-WT and NAI-KO: 2.96 ± 1.10 vs 1.25 ± 0.09 , $P = 0.037$) and were almost undetectable in the control groups (pSTAT3 in Control-WT and Control-KO: $0.07 \pm 0.02, 0.02 \pm 0.02$, respectively), supporting the western blot results.

The activities of pSTAT3/STAT3 were strongly positively correlated with collagen I and III mRNA levels ($r = 0.877, P < 0.001$; $r = 0.896, P < 0.001$, respectively).

IL-6 is inversely correlated with Cx40 and Cx43 expression.

Cx40 and Cx43 protein levels had an inverse relationship with IL-6 levels (Supplementary Fig 2). Additional details can be found in the Online Supplement.

Enhanced IL-6/pSTAT3/fibroblast levels are correlated with sPOAF onset. To further evaluate the role of IL-6 in sPOAF, we compared the protein levels of IL-6 and pSTAT3 and mRNA levels of collagen I and III within NAI-WT mice. Levels of all 4 were more highly upregulated in mice with sPOAF ($n = 4$) than in those without ($P = 0.037, P = 0.014, P = 0.021$, and $P = 0.020$, respectively, $n = 4$ for non-POAF), whereas no significant differences were observed for Cx40 and Cx43 ($P = 0.281$ and $P = 0.162$, respectively).

To test our hypothesis that enhanced IL-6 promotes sPOAF onset by activating pSTAT3/STAT3 signaling and promoting fibroblast proliferation, we conducted path analysis with AMOS software. Based on the molecular biology studies, an initial hypothetical causal model was formulated (Fig 5A). A revised model (Fig 5B) was generated with good fit (indices all within the suggested criteria: $\chi^2/df = 1.799, P = 0.083$; standardized RMR = 0.052, goodness-of-fit index = 0.923, comparative fit index = 0.983, normed fit index = 0.963).

Each one standard deviation (SD) increases in IL-6 indirectly led to a 0.589-SD increase in ssPOAF (Supplementary Table II). It led to 0.749- and 0.803-SD increases in pSTAT3/STAT3 and collagen I levels, respectively, as well as a 0.507-SD decrease in Cx40 level. Each one-SD increase in pSTAT3/STAT3 was associated with a 0.689- and 0.629-SD increase in collagen III and collagen I, respectively, which directly and indirectly produced a 0.743- and 0.617-SD increase in ssPOAF occurrence. There was no causal effect of Cx40 on POAF onset. The results of path analysis indicated that IL-6 caused POAF through activating pSTAT3 and promoting collagen I and III expression.

Pericardial IL-6 increased the risk of POAF by 2.15 fold.

In phase IV, 6 (37.5%) mice developed sPOAF. Pericardial IL-6 levels were significantly elevated in mice with sPOAF compared to those without, while no differences were observed in serum IL-6 levels measured by ELISA (Fig 6A). Pericardial IL-6 was 127-fold higher compared to serum IL-6 ($P < 0.001$).

A total of 172 patients were assessed for eligibility at 2 cardiac centers. After excluding 48 patients, the remaining 124 patients completed the study (Patient flow chart, Supplementary Fig 3). Forty-four (35.5%) developed POAF at a mean of 49.07 ± 24.24 -hour postoperatively. There were no significant differences in baseline IL-6, WBC, or CRP levels between patients with and without POAF (Table II). Patients with POAF were characterized by older age, history of cerebrovascular disease, higher total drainage volume during the

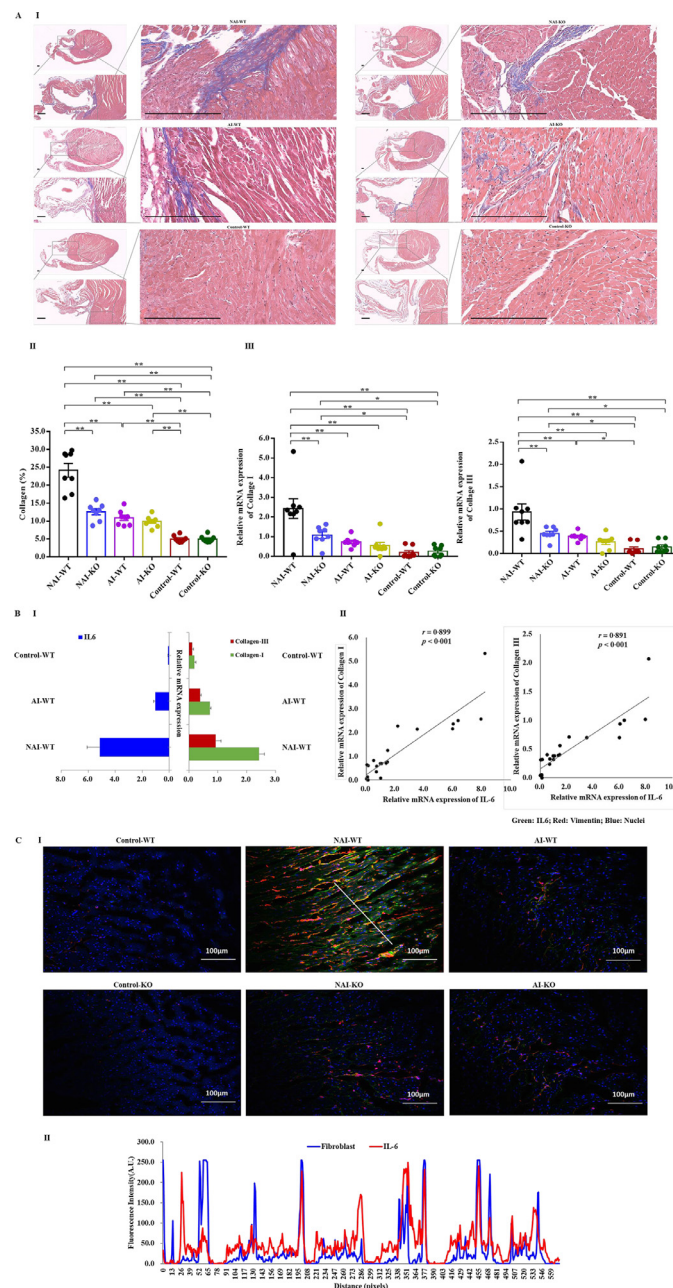


Fig 3. Effects of IL-6 on collagen. **A.** Panel I: Representative histological sections by Masson trichrome stain for visualization of collagen (blue stain) depicting marked collagen deposition in atria of NAI-WT group relative to that in the atria of control, KO, and AI groups. Scale bar = 250 μ m. Panel II: Quantification of collagen. $n = 8$ per group. Panel III: The mRNA expression of collagen I and collagen III. $n = 8$ per group. **B.** Panel I: The mRNA expression of IL-6, collagen I and collagen III in WT mice. Panel II: Relationship between IL-6 and collagen I and collagen III in WT mice. **C.** Panel I: Representative dual immunofluorescence staining of distribution of IL-6 and fibroblasts (vimentin) showing absence to scant signals of IL-6 and fibroblasts in control, KO, and AI groups, with strong IL-6 and fibroblasts signals in the NAI-WT group. IL6 staining is green. Vimentin staining is red. Nuclei was stained with DAPI (blue). Panel II: The overlap of fluorescence intensity peaks of IL-6 and vimentin as indicated in the merge micrograph in NAI-WT group. Confocal micrographs of atria double labeled with IL-6 antibody and vimentin antibody in the NAI-WT group indicating IL-6 complete colocalization with vimentin. All values are the mean $\pm$ SEM. * $P < 0.05$; ** $P < 0.001$; WT, wild type; KO, knock out; NAI, non-anti-inflammatory; AI, anti-inflammatory; IL-6, interleukin-6.

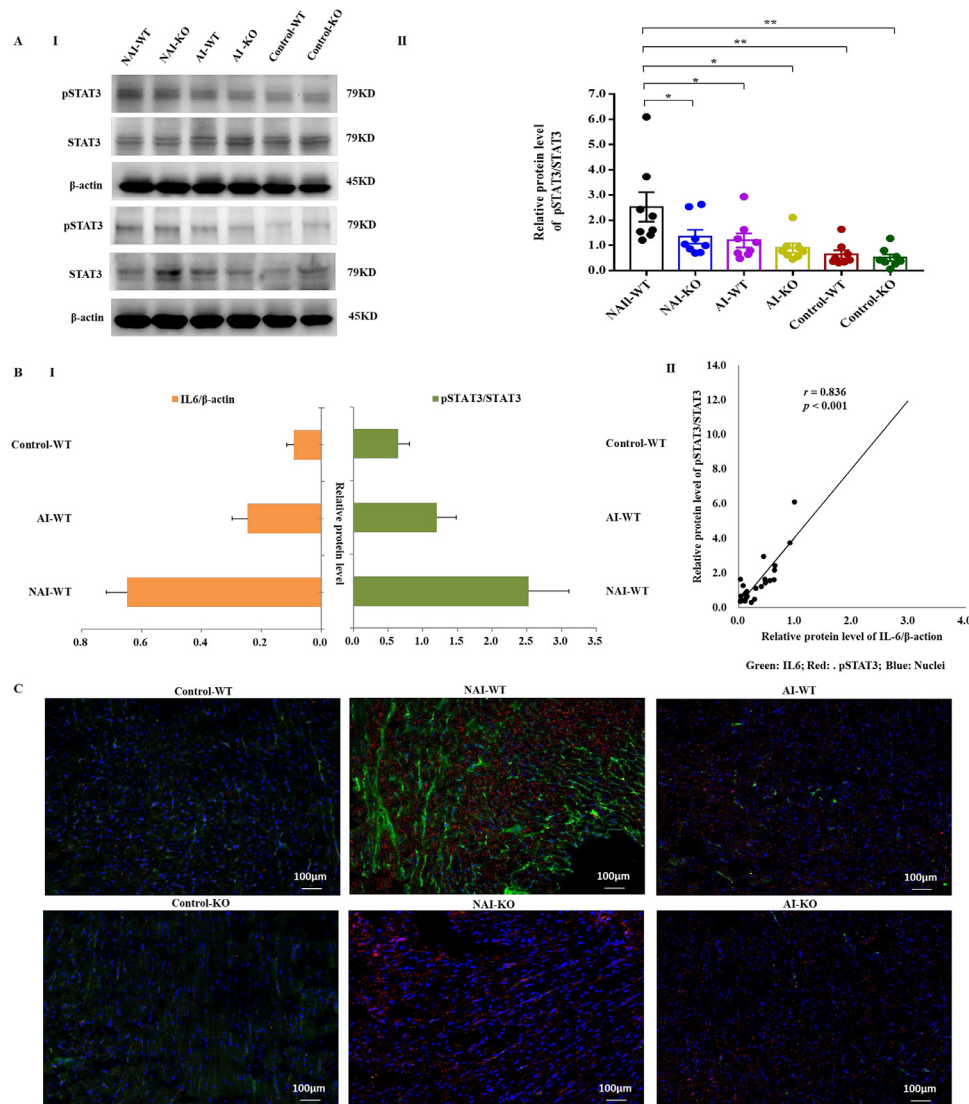


Fig 4. Effects of IL-6 on pSTAT3. **A.** Panel I: Representative western blots for pSTAT3 and STAT3. Panel II: Quantification of pSTAT3/STAT3 relative to β -actin. $n = 8$ per group. **B.** Panel I: Quantification of IL-6, pSTAT3/STAT3 relative to β -actin in WT mice. Panel II: Relationship between IL-6 and pSTAT3/STAT3 in WT mice. **C.** Representative dual immunofluorescence staining of distribution of IL-6 and pSTAT3 showing absence to scant IL-6 and pSTAT3 immunoreactivity in control, KO, AI groups, and strong IL-6 and pSTAT3 immunoreactivity in the NAI-WT group. IL-6 staining is green. pSTAT3 staining is red. Nuclei was stained with DAPI (blue). All values are mean $\pm$ SEM. *: $P < 0.05$; **: $P < 0.001$. WT, wild type; KO, knockout; NAI, non-anti-inflammatory; AI, anti-inflammatory; IL-6, interleukin-6; pSTAT3, phosphorylated-signal transducer and activator of transcription 3; STAT3, signal transducer and activator of transcription 3.

first 18 postoperative hours, and higher pericardial IL-6 at 18-hour postoperatively (Table II, Fig 6B). Univariate logistic regression showed that PD IL-6 level at 18 hours was the only inflammatory biomarker significantly associated with POAF (unadjusted OR = 1.493, 95% confidence interval, 1.036–2.152, $P = 0.032$). After adjusting for the confounding effects of age, cerebrovascular disease, hypertension, angiotensin-converting enzyme inhibitor or angiotensin receptor blocker use, and drainage volume with multivariable

logistic regression (area under the curve = 0.822), the OR increased to 2.154 with a 95% confidence interval of 1.218–3.811 ($P = 0.008$, Table III). No significant difference in serum IL-6 levels was observed between the 2 groups at the 3 time points (interactive effect $P = 0.509$; Fig 6B). Neither CRP levels in PD or blood nor WBCs differed significantly between patients with and without POAF (Supplementary Fig 4).

IL-6 in PD might originate from intrapericardial production or the systemic circulation. Therefore, we

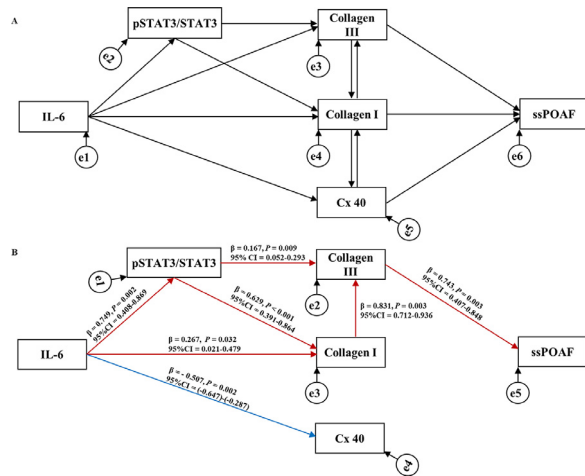


Fig 5. Path analysis of the direct and indirect contributions of cytokines to POAF. A. Initial model. B. Revised model. Black lines: hypothetical path; red lines: significant ($P < 0.05$) excitatory path; blue lines: significant ($P < 0.05$) inhibitory path. Each one standard deviation (SD) increases in IL-6 indirectly led to a 0.589 SD increase in ssPOAF. It led to a 0.749 and 0.803 SD increase (total effects) in pSTAT3/STAT3 and collagen I levels, as well as a 0.507 SD decrease in Cx40 level. Each one SD increase in pSTAT3/STAT3 was associated with a 0.689 and 0.629 SD increase in collagen III and collagen I, respectively, which directly and indirectly produced a 0.743 and 0.617 SD increase in the occurrence of ssPOAF. There was no causal effect of Cx40 on the onset of POAF. Numbers are standardized coefficients for each path. IL-6, interleukin-6; Cx40, connexin 40; pSTAT3, phosphorylated-signal transducer and activator of transcription 3; STAT3, signal transducer and activator of transcription 3; ssPOAF, sustained spontaneous postoperative atrial fibrillation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

compared pericardial and serum levels of IL-6 to determine whether local pericardial production is the main source of IL-6. We found that pericardial IL-6 was 26-fold higher compared to serum IL-6 ($P < 0.001$), and no correlations were found between serum and pericardial IL-6 levels at the 6, 12, or 18-hour postoperatively ($r = 0.124, P = 0.171$; $r = 0.041, P = 0.655$; and $r = -0.042, P = 0.642$, respectively). Serum CRP levels at 6 h, 12 h, 18 h and WBC counts in blood at 18 h postoperatively were significantly higher than corresponding pericardial levels. (Supplementary Fig 4). These results suggest that local production of IL-6 in PD plays an essential role in sPOAF, and raise the possibility that pericardial IL-6 may be used as a noninvasive predictive biomarker.

DISCUSSION

This study provides new evidence for the “atrial inflammation theory” in POAF onset. Our findings are summarized schematically in Fig 7. For the first time,

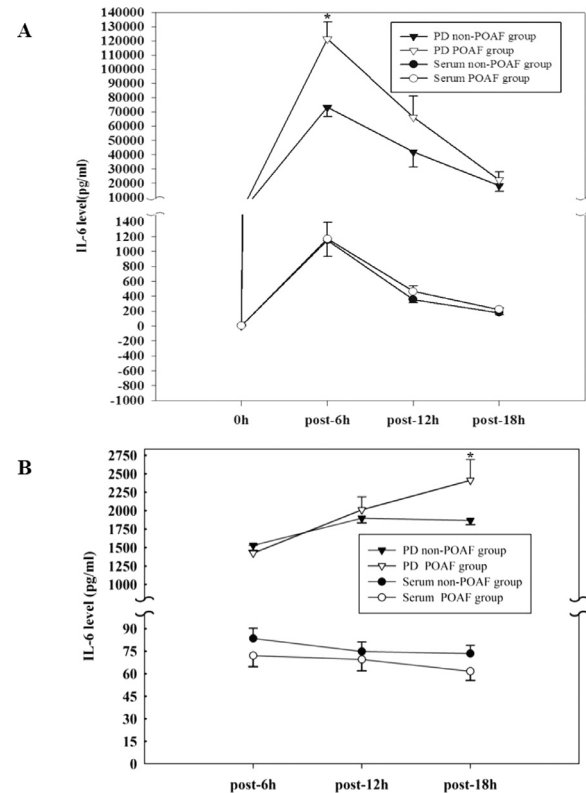


Fig 6. IL-6 levels in pericardial drainage and peripheral serum at different time points in mice and patients with POAF and non-POAF. A. Mice with POAF had a significantly higher level of IL-6 in PD at 6-hour postoperatively ($P_{6h} = 0.002$; $P_{\text{interactive effect}} = 0.014$). No significant differences were observed in serum IL-6 levels between the 2 groups ($P_{\text{interactive effect}} = 0.770$). $n = 16$. B. Patients with POAF had a significantly higher level of IL-6 in PD at 18-hour postoperatively ($P_{18h} = 0.015$; $P_{\text{interactive effect}} = 0.002$). No significant differences in serum IL-6 levels at 3 time points were observed between the 2 groups ($P_{\text{interactive effect}} = 0.509$). $n = 124$. Data are presented as the mean $\pm$ SEM. * $P < 0.05$ after Bonferroni correction for multiple comparisons at each time point. IL-6, interleukin-6; POAF, postoperative atrial fibrillation; PD, pericardial drainage. $P_{\text{interactive effect}}$ values were calculated by analysis of variance for repeated measures. P_{18h} and P_{6h} values were calculated by independent-samples t test.

the role of atrial inflammation in POAF was assessed in a cardiac surgery mouse model with nonpaced sPOAF. We uncovered preliminary evidence of a causal relationship between IL-6 and POAF, as mice with IL-6 KO or inhibition by methylprednisolone were completely protected from ssPOAF. We found that IL-6 could be synthesized in the atria in a cardiac surgery setting without cardiac ischemia and reperfusion. IL-6 elicited early profibrotic properties in the atria via the pSTAT3/STAT3 signaling pathway and contributed to POAF onset. The translational relevance of these findings is supported by our prospective clinical study. We investigated for the first time the

Table II. Clinical and surgical variables in patients with and without atrial fibrillation after coronary artery bypass grafting

Characteristic	POAF (n = 44)	Non-POAF (n = 80)	P value
Age (yrs)	65.91 ± 8.27	58.26 ± 9.22	<0.001
Male, n (%)	35 (79.5)	58 (72.5)	0.386
Body mass index, kg/m ²	25.41 ± 3.45	25.48 ± 2.95	0.909
Diabetes, n (%)	16 (36.4)	38 (47.5)	0.231
Left atrium diameter, mm	37.30 ± 6.50	36.30 ± 5.00	0.574
Hypertension, n (%)	37 (84.1)	55 (68.8)	0.062
COPD, n (%)	1 (2.3)	1 (1.2)	1.000
Cerebrovascular disease, n (%)	13 (29.5)	11 (13.8)	0.033
Myocardial infarction, n (%)	24 (54.5)	38 (47.5)	0.453
NYHA classification, n (%)			0.218
I	23 (52.3)	50 (62.5)	
II	17 (38.6)	21 (26.2)	
III–IV	4 (9.1)	9 (11.3)	
Left ventricle ejection fraction, %	59.43 ± 14.34	61.93 ± 11.92	0.302
Calcium channel blockers, n (%)	18 (40.9)	30 (37.5)	0.709
β-Blockers, n (%)	36 (81.8)	64 (80.0)	0.806
ACEIs or ARBs, n (%)	30 (68.2)	44 (55.0)	0.152
Statins, n (%)	38 (86.4)	69 (86.2)	0.986
Perioperative data			
Grafts number	3.09 ± 0.64	3.00 ± 0.72	0.486
Operative time (h)	4.41 ± 0.74	4.33 ± 0.86	0.613
Blood loss (mL)	622.73 ± 249.52	621.25 ± 262.15	0.975
Drainage volume (mL)	573.30 ± 203.11	462.69 ± 188.21	0.003
IABP, n (%)	9 (20.5)	11 (13.8)	0.331
Use vasoactive drugs, n (%)	40 (90.9)	75 (93.8)	0.825
Postoperative β-blockers, n (%)	38 (86.4)	72 (90.0)	0.752
Preoperative inflammatory markers			
IL-6 (pg/mL)	7.43 ± 7.90	6.55 ± 11.58	0.619
CRP (mg/L)	5.06 ± 4.29	4.30 ± 4.14	0.346
WBC (× 10 ⁹)	6.68 ± 1.47	7.16 ± 1.90	0.150

Abbreviations: ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; COPD, chronic obstructive pulmonary disease; IABP, intra-aortic balloon pump; NYHA, New York Heart Association; POAF, postoperative atrial fibrillation. Drainage volume, the total drainage volume during the first 18 hours. Data are presented as mean ± SD or frequencies (percentages).

Table III. IL-6 levels in pericardial drainage at 18-hour postop as a predictor of new-onset atrial fibrillation with (multivariate models) and without (univariate model) adjustments

Models	Variables	Odds ratio	95% CI	P value
Univariate	IL-6 (unadjusted, + 1 ng/mL)	1.493	1.036–2.152	0.032
Multivariate	IL-6 (covariate-adjusted*, + 1 ng/mL)	2.154	1.218–3.811	0.008
	Age	1.115	1.053–1.181	<0.001
	Drainage volume	1.004	1.001–1.006	0.002
	Hypertension	1.925	0.571–6.496	0.291
	Cerebrovascular disease	1.500	0.456–4.935	0.505
	ACEIs or ARBs	1.030	0.384–2.763	0.953

*Clinical covariates (risk factors and operative variables) used in adjustment were age, hypertension, cerebrovascular disease, preoperative medications (ACEIs or ARBs), and drainage volume. Drainage volume: the total drainage volume during the first 18 hours. Abbreviations: ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; CI, confidence interval; IL-6, interleukin-6.

pericardial and serum IL-6 and CRP concentrations and WBCs and demonstrated that local (PD but not serum) IL-6 levels were more strongly elevated in patients that developed POAF. Pericardial IL-6 holds potential as a novel, noninvasive biomarker to predict POAF in patients undergoing OPCAB.

Sterile pericarditis with burst pacing is a commonly used animal model of POAF.^{14–16} However, it is argued that the pathophysiology induced by a sterile talc may be different from that by OPCAB,^{13, 17} and the model ignores the complex effects of OPCAB in patients.¹³ An atria pacing model may not represent clinically

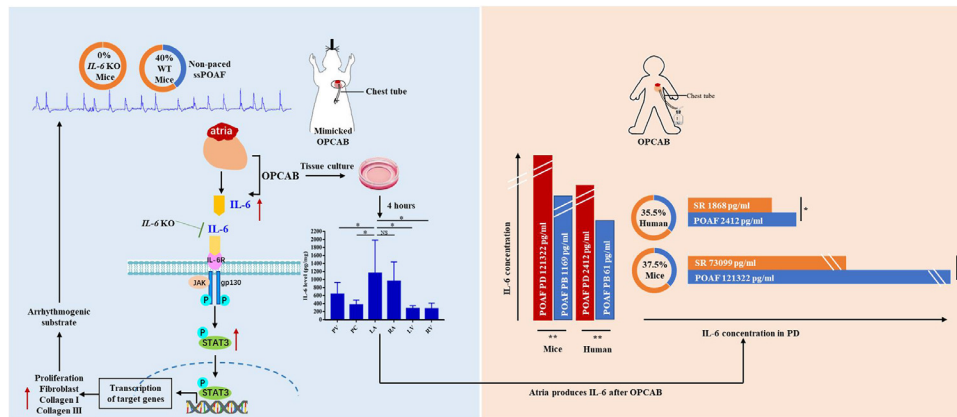


Fig 7. Schematic illustration of our findings. We established a mouse model of cardiac surgery (according to clinical OPCAB procedures) with nonpaced, spontaneous POAF (sPOAF). 40% WT mice had sustained sPOAF (ssPOAF) while mice with genetic depletion of *IL-6* were completely protected from ssPOAF. Atria could produce *IL-6* after stimulation of OPCAB, with LA the highest site. Atrial *IL-6* elicited early profibrotic properties in the atria via the pSTAT3/STAT3 signaling pathway and contributed to the onset of sPOAF. Atrial *IL-6* might be the source of *IL-6* in PD. Pericardial *IL-6* holds potential as a new noninvasive biomarker for the prediction of POAF for patients with OPCAB. OPCAB, off-pump coronary bypass grafting surgery; PD, pericardial drainage; PB, peripheral blood; PV, pulmonary vein; PC, pericardium; LA, left atrium; RA, right atrium; LV, left ventricle; RV, right ventricle. Red arrows indicate excitatory effects; “⊥” indicates inhibitory effects. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

sPOAF.¹⁶ Our mouse model of cardiac surgery followed the clinical OPCAB procedure step by step, which may be more representative of pathophysiology observed in clinical settings. We successfully observed sPOAF in this model at an incidence of 40.0%, which is consistent with the incidence rates reported in literature.¹³ This suggests that our mouse model may be suitable to study the mechanism of “clinical” sPOAF.

Bruins et al²⁵ first reported an association between *IL-6* and POAF in 1997, but that study only included 19 patients, and the conclusion needs further validation. In last 2 decades, increasing evidence has emerged supporting the association between *IL-6* and POAF, although the results are conflicting.^{4, 12, 26, 27} These inconsistent clinical findings might be due to the fact that most studies collected preoperative blood samples³ or intraoperative atrial tissue,²⁸ representing systemic inflammation or atrial inflammatory status before surgery. However, POAF typically occurs the second postoperative day and decreases to 2% at discharge.² Patients undergoing surgery with more frequent heart manipulation are more likely to have POAF, with a rate of 3% after noncardiac surgeries²⁹ vs 11%–16% after heart transplant surgeries^{30, 31} and 30%–50% after CABG or valve surgeries.³² It is argued that a local intracardiac inflammatory milieu induced by surgery might be more critical in determining temporary POAF onset.^{3, 33}

In current study, the role of atrial inflammation was assessed in mouse and clinical studies. The results are

the first preliminary evidence for a causal relationship between *IL-6* and POAF, as *IL-6* KO mice were completely protected from ssPOAF. The role of *IL-6* in POAF was further assessed by human observation. Among 3 markers of inflammation, *IL-6* in PD was the only one associated with POAF and was found to increase the risk by 2.15-fold. Our finding that *IL-6* in PD, but not in peripheral blood, was independently associated with POAF supports the “atrial inflammation theory” in POAF onset. In a study of 100 patients undergoing CABG, intracardiac *IL-6* levels in the LA appendage (but not peripheral levels) were associated with POAF.³⁴ CABG surgery causes direct trauma to cardiac tissue, induces a local inflammatory response, and disrupts the normal composition of pericardial fluid (PCF). As a result, the heart bathes in PCF containing inflammatory cytokines.³⁵ An increase of *IL-6* in PCF can potentially have adverse effects on the thin atrial wall, eliciting profibrotic properties in cardiac myocytes during the early postoperative period (as evidenced in our mouse study), and increasing susceptibility to POAF.

Similar to the study of Gilicze et al,³⁶ we observed persistent 26-fold (human) and 127-fold (mice) elevations in *IL-6* in PD compared with that in blood. That the *IL-6* level in PD is independent of that in serum suggests that *IL-6* present in the PD might be produced by the heart following CABG. This hypothesis was supported by our tissue culture results. We observed remarkably increased *IL-6* expression in the atria after 4-hour tissue culture following cardiac surgery without

ischemia and reperfusion, with the highest abundance in the LA. This is in line with previous reports that IL-6 can be synthesized in cardiac myocytes in settings of ischemia and reperfusion and hypoxia in vitro.¹⁸ The IL-6 and IL-6R genes are relatively abundantly expressed in the atria. Thus, the stimulation of cardiac surgery may increase local IL-6 levels.

Any cardiac arrhythmia requires a susceptible electrophysiological substrate.³⁷ Atrial fibrosis can be activated by the inflammation pathway and plays a pivotal role as a susceptible AF substrate because it separates myocyte bundles and disturbs cell-to-cell pulse propagation.³⁷ Controversy exists regarding the role of fibrosis in POAF.³⁸ However, in a canine pericarditis model, the degree of fibrosis in the LA was as high as 30% on postoperative day 2.⁷ A similar atrial fibrosis finding was recently reported in a rat pericarditis model.⁸ In the current study, NAI-WT mice showed increased mRNA levels of collagen I and collagen III, extensive fibroblasts (Masson's trichrome staining) and vimentin expression (immunofluorescence studies), IL-6 upregulation, and a significantly higher incidence of POAF at 48-hour postoperatively. In contrast, *IL-6* KO mice were fully protected from POAF and had markedly fewer atrial fibroblasts and less collagen deposition. These findings indicate that IL-6 can elicit early profibrotic properties and contribute to POAF onset. Our results confirm previous observations in a rat sterile pericarditis model suggesting that IL-6 and collagen I and III were highly upregulated 3 days after pericardiotomy and may disturb atrial conduction, contributing to POAF pathogenesis.¹⁴ Notably, significant fibrosis was still evident in *IL-6* KO mice although it was remarkably attenuated compared with WT mice. These findings suggest that other inflammatory and oxidative cytokines (eg, myeloperoxidase)²⁰ might also be involved in early postoperative atrial fibrotic remodeling.

To identify molecular mechanisms underlying IL-6-induced fibroblast proliferation, we focused on active form (phosphorylation) of STAT3 levels. Abnormal STAT3 phosphorylation reportedly promotes fibroblast proliferation and differentiation in ventricular hypertrophy and remodeling.⁹ STAT3 is activated by numerous cytokines, and IL-6 is a major regulator via the IL-6/sIL-6R/GP130 complex.³⁹ In the current study, pSTAT3 levels were strongly associated with a proportional increase in atrial fibroblast numbers and IL-6 levels. IL-6/pSTAT3/fibroblast axis promotion of POAF was further supported by our path analysis. Reduced atrial pSTAT3 levels in *IL-6* KO mice was accompanied by substantially less collagen deposition, consistent with the idea that IL-6 regulation of pSTAT3 is a key contributor to increased fibroblast proliferation in the atrium. Our observations are consistent with a previous report

suggesting that culturing cardiac fibroblasts in the presence of IL-6 for 24 hours progressively augmented pSTAT3 levels and cell proliferation.¹⁴

We also demonstrated that higher IL-6 levels directly disturbed Cx40 and Cx43. However, a direct link between connexins and POAF was not been established in this study. Li⁴⁰ analyzed atrial samples taken from 45 patients before and during CABG surgery and found that both Cx40 and Cx43 significantly decreased during CABG surgery; however, the changes were not related to POAF. Evidence regarding the relationship between connexins and AF is conflicting.^{40, 41} It is possible that during the early postoperative stage, acute atrial inflammation coupled with cardiac surgery could cause direct myocardial injury, resulting in widespread and massive reductions in connexin levels.⁴² A uniform and homogeneous decrease in connexin may not be sufficient to cause heterogeneity in electrical conduction.⁴²

Our data may also suggest evidence of a damage-associated molecular pattern (DAMP), also known as a danger signal, in the ventricles and atria. IL-6 can issue a danger signal in the event of tissue damage.⁴³ DAMP is known to promote priming and activation of the nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3) inflammasome and cause myocardial inflammation through Toll-like receptor-mediated pathways.⁴⁴ This triggers transformation of procaspase-1 to caspase-1, secretion of mature IL-1 β and IL-18,⁴⁴ and IL-6 production.⁴⁵ The net-effect of these mechanisms and the potential IL-6/DAMP/NLRP3 feedback loop may set up an intensive proinflammatory and profibrotic environment that leads to an AF substrate.

Another finding of the current study is that topical delivery of methylprednisolone significantly reduced atrial inflammation and POAF incidence. Steroids can inhibit both inflammation and inflammation-induced myocardial fibroblast proliferation.⁴⁶ However, evidence regarding their effect on POAF is inconsistent.⁴⁷ Recent reviews suggested that multiple steroid doses at various perioperative intervals had protective effects against POAF, while single doses often failed to produce similar results.⁴⁸ In our model, steroids were directly delivered to the heart via a chest tube for 3 consecutive days after cardiac surgery. The steroid dosage used was calculated to completely suppress the inflammatory response and was localized to the atria to maximize the ratio of myocardial-to-systemic drug concentration and minimize potential side effects.⁴⁹ It is still unclear whether this dosage and delivery method is clinically acceptable. Nevertheless, in contrast to systemic administration, the direct introduction of AI medication into the pericardial space can provide a higher local tissue concentration for a defined period time, which might be beneficial in the prevention of POAF.

Our findings need to be interpreted in the context of several limitations. First, the preliminary evidence of causal relationship between IL-6 and POAF in mice needs to be further verified by administering recombinant IL-6 to *IL-6* KO mice to evaluate if it can induce POAF. Second, the causative involvement of STAT3 needs further experimental validation. Third, atrial conduction is a complex process that depends on several factors beyond fibrosis. There may be other pathways in addition to the IL-6/pSTAT3/fibroblast axis that are important in POAF pathogenesis. Fourth, the efficacies of *IL-6* KO and AI therapy on POAF need further verification because the definition of AF in mice was based on ECG recordings. Future studies should further evaluate its efficacy on clinically significant POAF (AF requiring treatment). Furthermore, we did not examine patients' preoperative PC fluid. We could not avoid this limitation due to previous local operation room regulations. In another ongoing study, almost undetectable baseline pericardial IL-6 (35, 50, 55 times lower than IL-6 at 6, 12, and 18-hour postoperatively, respectively) suggested that the absence of those baseline data may not have a large impact on our conclusions. In addition, although we found a causal relationship between IL-6 and POAF in mice, our clinical investigation could only assess association. Confounding factors affect prospective cohort study findings, so future randomized control trials are warranted to evaluate IL-6 in PD as a POAF biomarker. Although we could not rule out interindividual heterogeneity in clinical studies, our animal studies employed the same strain of mice, and the results supported our hypothesis.

Collectively, our results suggest that IL-6 is intimately involved in POAF pathogenesis. The detection of IL-6 in PD raises the possibility that it may be a new noninvasive biomarker. Given that the current strategies for preventing POAF are suboptimal, the IL-6-pSTAT3-fibroblast axis may be a promising therapeutic target for this arrhythmia.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:[10.1016/j.trsl.2021.01.007](https://doi.org/10.1016/j.trsl.2021.01.007).

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