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Role of IL-37 in Preventing AF-Associated Inflammation and Fibrosis in Right Heart Disease

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Mémoire intitulé

Role of IL-37 in Preventing AF-Associated Inflammation and Fibrosis

in Right Heart Disease

Présenté par

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Résumé

Problématique : La fibrillation auriculaire (FA) est la forme d'arythmie cardiaque la plus fréquente. La maladie du cœur droit (MCD) fait partie des facteurs de risque de la FA. L'inflammation, impliquant l'axe NLRP3-IL18-IL1β-IL6, affecterait les fibroblastes (FB) atriaux et jouerait un rôle important dans l'induction de FA. Peu d'études ont évalué le rôle de l'interleukine-(IL)-37, un inhibiteur de la voie IL18, dans la prévention de l'inflammation et la FA dans la MCD.

Hypothèse*:* L'IL37 inhibe l'activation de l'inflammation et prévient le développement du substrat de la FA dans les FB auriculaires de rats MCD.

Méthodes*:* La MCD a été induite chez des rats Wistar en réalisant une ligature du tronc pulmonaire (LTP), et des animaux témoins ont reçu une chirurgie factice (SHAM). Les FB atriaux ont été isolés et cultivés dans 5 conditions de traitement: Milieu normal, Supplémentation pro-inflammatoire [IL-6], Supplémentation anti-inflammatoire [IL-37], Inhibition de voies inflammatoires avec le Tocilizumab [anti-IL6R], Inhibition de voies anti-inflammatoires avec [anti-IL37]. La prolifération et la migration des FB furent évaluées par immunomarquage de (α-SMA). Les niveaux d'expression des molécules liées à la fibrose, les marqueurs inflammatoires et les marqueurs anti-inflammatoires ont été évalués par qPCR (gènes) et western blot (protéines).

Résultats: Trois semaines après la chirurgie, les animaux LTP ont développé de l'hypertrophie et de la dilatation cardiaque associées à de l'inflammation et de la fibrose atriale droite. Comparés aux SHAM, les FB de rats MCD ont exprimé plus de marqueurs d'inflammation et de fibrose et cette surexpression a été exacerbée en présence d'IL-6, et normalisée en présence d'IL-37.

Conclusion: Au niveau des FB isolés, un traitement à l'IL37 semble être une stratégie efficace pour prévenir l'inflammation et la fibrose atriale associées à la MCD.

Mots clés: Arythmies, Insuffisance Cardiaque, Fibrose, Inflammation, Fibrillation Atriale

Abstract

Introduction: Atrial fibrillation (AF) is the most common type of cardiac arrhythmia. Right heart disease (RHD) is one of the risk factors for AF. Inflammation, involving the NLRP3-IL18-IL1β-IL6 axis affects atrial fibroblasts (FB) and plays an important role in AF induction. Few studies have assessed the role of interleukin-(IL)-37, an IL18 pathway inhibitor, in preventing inflammation and AF in RHD.

Hypothesis: IL37 inhibits the activation of inflammation and prevents AF substrate development in atrial FB of RHD rats.

Methods: RHD was induced in Wistar rats by performing pulmonary artery banding (PAB) and control animals received SHAM surgery. Atrial FBs were isolated and cultured under 5 treatment conditions: Normal medium, Pro-inflammatory supplementation [IL-6], Anti-inflammatory supplementation [IL-37], Inhibition of inflammatory pathways with tocilizumab [anti-IL6R], Inhibition of anti-inflammatory pathways with [anti-IL37]. The proliferation and migration of FB were determined with scratch wound healing assay. The expression levels of fibrosis-related molecules, inflammatory markers and anti-inflammatory markers were assessed by qPCR (genes) and western blot (proteins).

Results: Three weeks post-surgery, PAB animals developed right heart enlargement and dilation associated with inflammation and right atrial fibrosis. Compared to SHAM, FB from PAB rats expressed more markers of inflammation and fibrosis, and this overexpression was exacerbated in the presence of IL-6 and normalized in the presence of IL-37.

Conclusion: At the level of isolated FB, treatment with IL37 seems to be an effective strategy to prevent atrial inflammation and fibrosis associated with RHD.

Key words: Arrhythmia, Heart Failure, Fibrosis, Inflammation, Atrial Fibrillation.

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List of Abbreviations

AF : Atrial Fibrillation

AFL : Atrial Flutter

A-SMA : Alpha Smooth Muscle Actin

AT : Acceleration Time

BMI : Body Mass Index

BW : Body Weight

CL : Cycle Length

CM : Cardiomyocyte

DAMPs : Damage-associated Molecular Pattern

DMEM : Dulbecco's Modified Medium

ECM : Extracellular Matrix

ECG : Electrocardiogram

FB : Fibroblast

FBS : Fetal Bovine Serum

HF : Heart Failure

HRP : Horseraddish peroxidase

LADd : Left Atrial Dimension at end Diastole

LADs : Left Atrial Dimension at end Systole

LA : Left atrium

MDB : Membrane desalting buffer

MI : Myocardial Infarction

NDS : Normal donkey serum

PAB : Pulmonary artery banding

PAH : Pulmonary Arterial Hypertension

RAD : Right Atrial Dimension

RAA : Right Atrial Area

RAAd : Right Atrial Area at end Diastole

RHD : Right Heart Disease

RVAWd : Right Ventricular thickness

RV : Right Ventricle

SR : Systolic Contractility

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I´m in all humbleness, and gratefulness to acknowledge my deep, and sincere gratitude to all the individuals who have contributed to the success of my project, and the realization of this mémoire.

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Eventually, I'm extremely grateful for my beloved Jad, my backbone, for his ultimate support, his constant care and love, for encouraging me and lifting me when I felt so down. I'm so lucky to have you in my life.

Chapter 1- Introduction

1.1. ***AF Definition and Risk Factors***

Atrial fibrillation (AF) is the most common type of cardiac arrhythmia. It is characterized by abnormal and uncoordinated rapid electrical activity within the atria. AF is associated with increased morbidity and mortality [1]. Worldwide, it was recently estimated that up to 33,5 million patients are affected by AF, and its prevalence is expected to become more than double over the next 40 years [2,3]. The main risk factors of AF include cardiovascular diseases: Heart failure (HF), myocardial infarction (MI), or non-modifiable risks: genetics, race, age, sex, or modifiable risks: decreased physical activity, smoking, diabetes, increased body mass index (BMI), increase blood pressure [4]. AF can provoke severe complications and cardiovascular events, such as heart failure, stroke, myocardial infarction, and sudden death [3].

1.2. ***Structural and Electrical Alterations Initiate AF***

Evidence suggests that AF is directly correlated with the presence of pre-existent pathological triggers, perpetuators, and substrates generated from conditions that cause structural and electrical deleterious remodeling [5].

AF has been shown to be associated with the development of atrial fibrosis, which can occur in response to cardiac cell death and cardiac injuries [6]. Atrial fibrosis is a physical barrier that can generate re-entrant circuits by altering the normal conduction of electrical signals across the atria. Various ion channels and gap-junctions promoting excitation-contraction influx between cardiomyocytes can be affected by the development of atrial fibrosis. The Loss of inter-cellular gap-junctions affects action potential duration, promoting atrial conduction heterogeneity and arrhythmogenicity [7].

Electrical alteration that occurs due to pathological stimuli involves changes in the expression of ion channels (KCNQ1, CACN1Ac, SCNA5), gap-junctions (connexion-40, connexion-43), exchangers (Na/K-ATPase, SERCA), and calcium (Ca^{2+}) handling proteins (l-type Ca^{2+} channels, RyR2, Phospholamban), promoting ectopic activity [8]. Such remodeling results in slowed electrical conduction, prolonged refractoriness, and loss of myocardial voltage [5]. As AF evolves,

electrical remodelling will encompass the changes in the properties of ion channels to prevent conduction alteration. In addition, other structural changes in the cardiac tissue size, thickness, and properties will be developed to preserve normal sinus rhythm [9]. The persistence of electrical disturbance can promote chronic conduction abnormalities, leading to more cardiac remodelling and functional impairment associated with atrial arrhythmias [10].

Persistent AF negatively affects the healthcare system. In fact, abnormal atrial contraction during AF can lead to the formation of blood clots, which increases the risk of stroke. Hence, AF patients are often exposed to an increased risk of life-threatening complications, including sudden death. Then, it is understandable that more substantial efforts should be made in terms of fundamental research, and clinical innovation to prevent and cure AF and its burdens [11].

*1.3. **Right Heart Disease Can Cause AF***

Among AF risk factors, Right Heart Disease (RHD) has been found to increase AF vulnerability [12]. RHD is a clinical syndrome caused by structural and functional myocardial remodeling that impairs the right side of the heart, promoting atrial arrhythmogenesis, favouring right atrial fibrosis, and maintaining atrial fibrillation, in addition to conduction abnormalities [2]. For instance, RHD could be induced by Pulmonary Arterial Hypertension (PAH). PAH is mainly caused by a decrease in the diameter of the pulmonary artery due to vasoconstriction, remodeling of the pulmonary vessel wall, or thrombosis [13]. These disorders lead to haemodynamic changes, characterized by increased pressure and volume overload, causing myocardial dilation and death [14]. RHD is diagnosed by the presence of mean pulmonary arterial pressure >25mmHg at rest [15], and studies have shown that it is directly correlated with AF and atrial flutter (AFL), since up to 25.1% of patients with PAH are experiencing atrial electrical disturbance [16].

In detail, due to a narrowed pulmonary artery, the pressure in the blood vessels leading to the lungs becomes too high, resulting in excessive afterload in PAH patients. Therefore, this chronic atrial stress appeared to be one of the most prominent trigger mechanisms for signaling changes involved in the pathogenesis of AF [6]. Consequently, long-standing disturbances will eventually increase cavity stiffness, raise end-diastolic pressure, and decrease right ventricular (RV) lateral wall systolic contractility. These structural perturbations can increase RV workload and cause

systolic and diastolic dysfunction [7, 17]. Nevertheless, the process underlying the transition from myocardial adaptation to heart failure remains unclear.

*1.4. **Fibroblasts and Atrial Remodeling***

One of the key features of right ventricular adaptation to chronic pressure is hypertrophy [17]. It starts from the augmented blood volume in the RV that exposes the cardiac muscle to high filling pressure and mechanical stress, diastolic, systolic, and contractile impairment. Therefore, changes in the density and structural organization of sarcomeres will develop to increase the size of cardiomyocytes, myocardial thickness, and RV mass [7, 11]. Under prolonged pressure/volume overload, physiological adaptation becomes insufficient, so cardiomyocytes will exhibit increased rates of apoptosis, which results in chamber dilation, reduced ventricular mass, and abnormal contractile capability due to hypertensive insult [18].

Chronic mechanical stretch and degenerated cardiomyocytes (CM) constitute a pro-fibrotic stimulus for fibroblast (FB) activation [19]. The pathway starts from damage-associated molecular patterns (DAMPs) released from injured CM that activate a cascade of intracellular proteins in cardiac cells (macrophages, CM, FB), ending at the transcription factor protein (NF-kB) [20], which can upregulate the expression of several key pro-inflammatory stimuli genes, such as cytokines (IL-6, IL18, IL-1b), and chemokines (CXCL-1, CXCL-2). These secreted molecules are key elements for the transition from the pro-inflammatory to the reparative phase, through the stimulation of migration, proliferation, and differentiation of FB into myo-FB, which in turn upregulate the expression of many fibrosis-related genes. Consequently, these events provoke the over-production of specific proteins, including collagen, fibronectin, or laminin, involved in thickening the extracellular matrix (ECM) [21, 22]. In addition, the overexpression of alpha-smooth muscle actin (α-SMA) by the myo-FB contributes to rescuing the contractile activity [23] *(Figure 1)*.

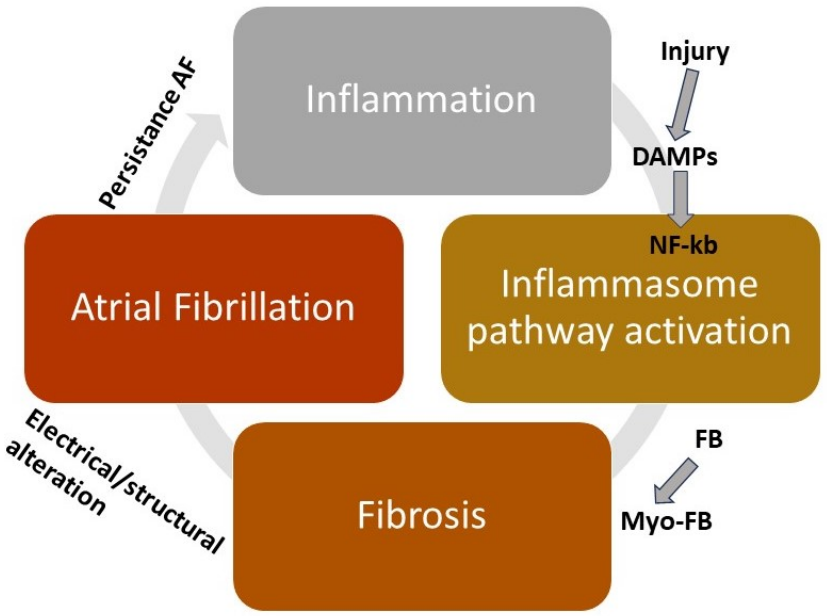

Figure 1: Pathway of Fibrosis Leading to AF During Inflammation. *Cardiac injury causes the release of Damage-Associated Molecular Patterns (DAMPs), which activate the inflammasome pathway in certain cells (example: Fibroblasts). The FBs differentiate into myo-FBs and start to build fibrotic tissue to replace the damaged area. New tissue is non-contractile and non-excitable, it causes atrial fibrillation (AF) and exacerbates the inflammation. (Original schematic realized by Rim Younes)*

In normal physiological conditions, FBs act as scaffolds for CM by maintaining the shape, structure, and mechanical force of myocardial tissue. On the other hand, electrical action potential spreads through CM due to direct coupling between the cells via gap junctions [8].

In RHD, when FBs start bridging the gaps between surviving CMs, and because neither FBs nor myo-FBs are electrically excitable, the heterogeneity of electrical conduction is disrupted [8]. Persistent stimulation and failure to resolve acute atrial inflammatory response have been shown to be involved in AF pathogenesis [23].

Accumulated evidence supporting the link between inflammation and AF generates the hypothesis that pharmacological intervention might be efficacious in preventing AF. This could be done by looking for a suitable target in the inflammatory pathway [14]. However, the mechanisms underlying the passage from inflammation to atrial remodeling associated with AF are not very well understood. Among the experimental strategies adopted to clarify the association between

RHD, inflammation, and AF incidence, Pulmonary artery banding (PAB) has been shown to successfully increase pressure overload in rats, in the understanding of the molecular and cellular pathways involved in right heart remodeling [24]. Hence, PAB appears to be an ideal method to detect possible targets for therapeutic treatments that can prevent or cure AF in an experimentally provoked atrial inflammation status associated with RHD.

1.5. Hypothesis

We hypothesized that pro-inflammatory cytokine IL-6 activates the development of AF substrates, whereas IL-37 counteracts the inflammatory signals, thereby preventing inflammatory-induced atrial remodeling and fibrillation.

1.6. Aims of this Study

In this context, our study aims to:

(1) Evaluate the effect of RHD-induced atrial remodeling on AF susceptibility in rats.

(2) Determine the expression level of inflammatory markers, inflammasome, and fibrosis-related molecules in response to pro-inflammatory and anti-inflammatory treatments.

(3) Characterize the implication of FBs differentiation, proliferation, and migration in atrial remodeling in response to pro-arrhythmogenic stimuli.

Chapter 2 - Materials and Methods

2.1. ***Pressure Overload Model in Rat***

Right heart disease (RHD) was induced through Pulmonary artery banding surgery (PAB) [25]. Male Wistar rats (~250 g) were subjected to intraperitoneal injection with pre-operative analgesic (buprenorphine 0.03 mg/kg) and anaesthetized with 2-3٪ isoflurane enriched with oxygen (2L/min). The body temperature (37°C) was maintained throughout the surgery using an electrical heating pad. An endotracheal tube was inserted and connected to a respirator at 70-80 respiration/min. The surgery was initiated by creating a 2 cm incision between the last rib and the base of the left anterior membrane. Muscles were separated to expose the thoracic cage, and a deep incision was made in the intercostal muscle between the 3rd and 4th rib. At this stage, the upper part of the heart became visible, allowing to pass a surgical suture beneath the pulmonary artery. 3 knots were quickly tied to prevent the interruption of blood circulation (*Figure 2*).

Upon completing the surgery, the respirator was blocked, and lung function was monitored. The opening between the ribs was closed using surgical silk, and the rat received a fluid injection. Subsequently, isoflurane was closed, the oxygen was left open until we observed some reflections, and then the rat was returned to its cage. A dose of Buprenorphine was administrated between 6-8 h post-surgery. For the control rats (SHAM), the same steps were followed, except the knots around the pulmonary artery were omitted.

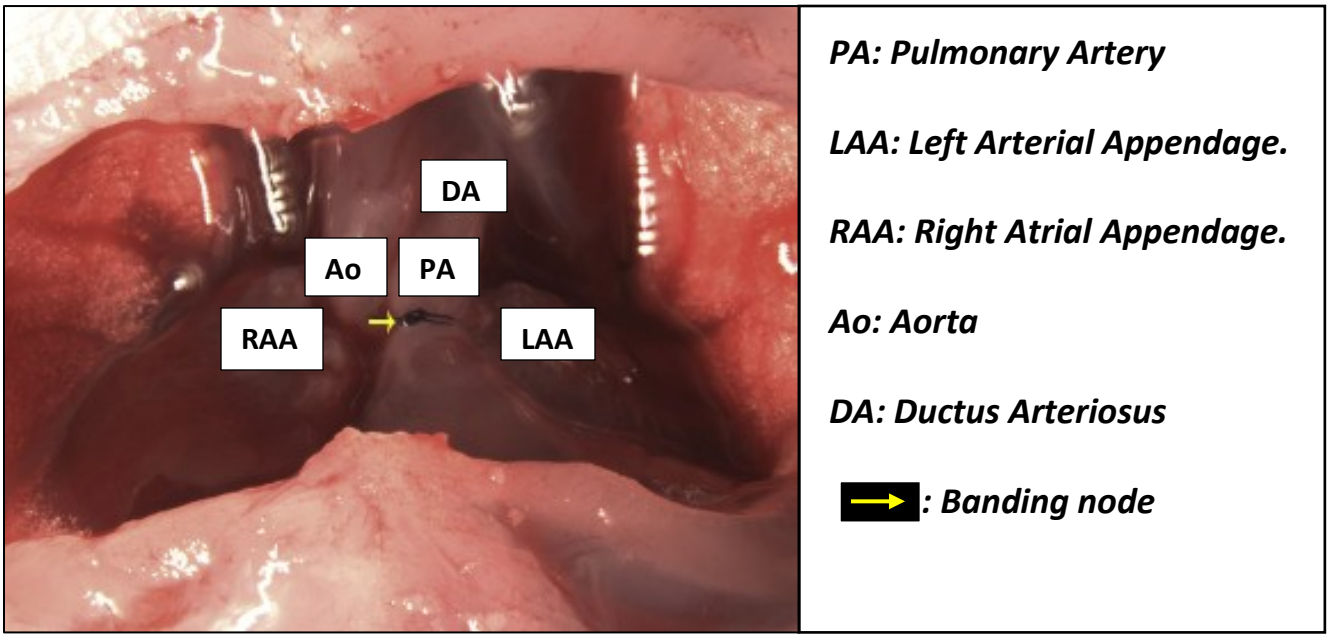

Figure 2: Pulmonary Artery Banding Node *[30]. The picture represents a surgical node around the pulmonary artery (PA) indicated by the yellow arrow.*

2.2. ***Echocardiography***

Twenty-one days post-surgery, rats were shaved in the thoracic region, and subjected to anesthesia using inhaled isoflurane enriched with (2L/min) oxygen throughout the process. Each measurement was done at an average of 3-6 consecutive cardiac cycles. A phased array probe ultrasound (4.5 – 11.5 MHz) connected to a vivid 7-dimension system was used to check the banding width (BW) of pulmonary artery. Subsequently, the Continuous Wave Doppler was employed to determine the peak velocity and mean gradient at the site of banding. Additionally, Pulse Wave Doppler was utilized to measure trans-mitral and trans-tricuspid flow.

In-depth 2D images in the long axis view were performed to facilitate the examination of cardiovascular parameters, such as right atrial dimension (RAD), area at end-diastole (RAAd), and trans-tricuspid regurgitation jet area. Furthermore, the M-mode technique was used to determine the right ventricular anterior wall thickness, as well as the right ventricular dimension, enabling an assessment of right ventricular systolic function. Concurrently, the M-mode was also used to assess the left atrial dimension at end-systole (LADs) and diastole (LADd). Additionally, LV anterior wall thickness was gauged, thereby facilitating the evaluation of structural remodeling.

*2.3. **Electrophysiological Measurements***

On day 21 post-surgery, rats were anesthetized with inhaled isoflurane as previously described [12]. A 4F quadripolar catheter (St. Jude Medical, Saint Paul, Minnesota) was inserted in the esophagus *(Figure 3),* and then we delivered electrical signals in the form of 3 series of 12 bursts pacing (50 Hz, 4x threshold), 3 seconds each, via transesophageal stimulation to trigger AF in susceptible rats. AF was defined as a rapid, irregular atrial rhythm (>800 bpm), and atrial flutter (AFL) as a regular atrial tachyarrhythmia at a cycle length (CL) between 600 and 800 bpm. AF-duration was calculated as the mean duration of all induced AF-episodes. Acquisition and analysis of ECG and catheter signals were performed with Iox2 software (version 2.8.0.13, EMKA technologies, Paris, France).

Figure 3: Rat During EPS in vivo. *(Original picture realized by Rim Younes)*

*2.4. **Fibroblasts Isolation***

FBs were isolated from SHAM and PAB rats using the Langendorff system *(Figure 4 A)*. The procedure was initiated with the intraperitoneal administration of 1000 UI Heparin (0.5 ml/rat) to the rat, 5 minutes later, the rat was anesthetized with inhaled 2-3% isoflurane mixed with oxygen (2L/min) throughout the procedure. Once the rat was completely anesthetized, the chest was opened to expose and extract the heart. The excised heart was cannulated through the aorta (*Figure 4 B*) and perfused with 200 µM Ca^{2+} containing Tyrode solution for 5 minutes, followed by an additional 5-minute perfusion with Ca^{2+}-free Tyrode solution. Subsequently, the heart was

perfused with Tyrode solution enriched with collagenase enzyme for 25 to 30 minutes, depending on the mass of the rat.

After confirming the digestion, right atria were collected in Dulbecco's Modified Medium (DMEM) supplemented with 10٪ Fetal Bovine Serum (FBS) and 1٪ penicillin/streptomycin. Physical dissociation of tissue was done using tweezers until large tissue structures were fully decomposed. The next step involves the separation of the FBs from CMs. We first centrifuged at 800 rpm for 5 minutes, to facilitate the floating of FBs within the supernatant, followed by a secondary centrifugation at 1750 rpm for 8 minutes, resulting in the precipitate of the FBs within the pellet.

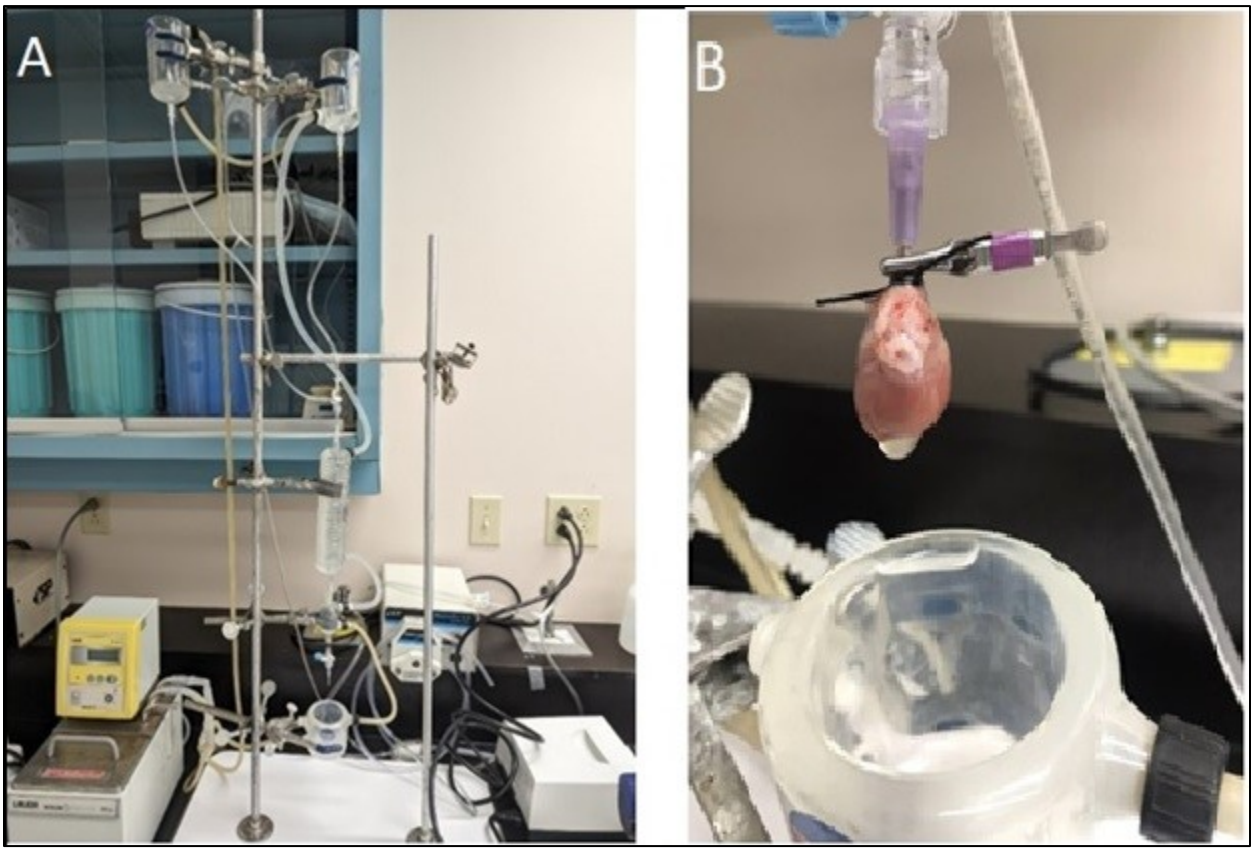

Figure 4: Fibroblast Isolation Using Langendorff System. *(A) the 200 µM Ca^{2+} Tyrode solution in the top left cylinder, and the Ca^{2+} -free Tyrode solution in the top right cylinder. (B) The heart is canulated through the aorta and perfused with Tyrode solution. (Original pictures realized by Rim Younes).*

*2.5. **FB Culture***

Following the isolation procedure, FBs were resuspended in 1 ml of culture medium, then the cells were seeded in an individual well of a 12 -well culture plate. After 3 hours in the incubator, the FBs were attached to the surface of the plate, then washed and incubated at 37°C 3-4 days to become confluent. Once they reached 75٪ confluency, the medium was removed, and the cells were detached from the plate by adding 300 µl trypsin-EDTA enzyme for 5 minutes, the enzymatic activity was inhibited by the addition of 5 ml culture medium to the FBs.

Subsequently, these detached FBs were seeded into 5 wells and allowed to reattach to the plate's surface. After 24 h, the cells were incubated in FBS free-DMEM medium to stop the proliferation of the cells prior to adding the treatments, 24 h later, different treatments were administrated in each well: 50 ng/ml IL6, 100 ng/ml anti-IL6, 100 ng/ml IL-37, 200 ng/ml anti-IL37, control: no treatment is added (*Figure 5*). Following a 48 h of incubation, the culture medium was stored at -80°C for protein analysis (Western Blot), and 350 µl (RA1 buffer + ß-mercaptoethanol) was added in each well, then the cells were stored at -20°C for genetic analysis (qPCR).

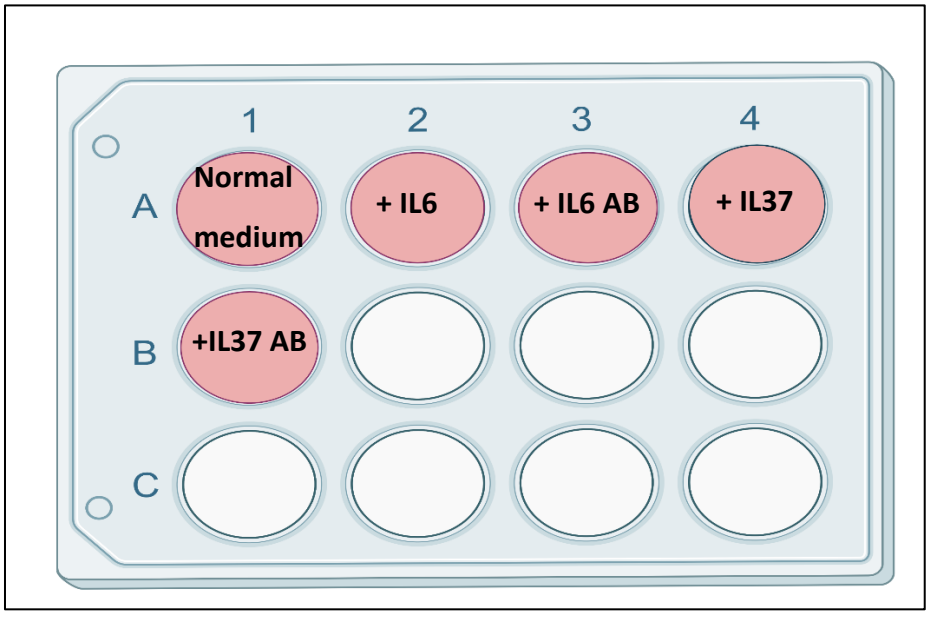

***Figure 5: Fibroblasts in Culture With 5 Different Conditions**. Right atrial fibroblasts from either a SHAM or PAB rat are divided and seeded in 5 wells in the normal medium or with a specific treatment. (Original schematic realized by Rim Younes).*

*2.6. **Quantitative Polymerase Chain Reaction (qPCR)***

RNA extraction was performed on isolated cells. Cardiac cells were contained in a 2ml tube with filer and centrifuged at 11,000 x g for 1 min to clear the lysate cells, then 350 µl of 70% ethanol was added to each tube. For total isolation and purification of RNA from cultured cells, the lysate was loaded in the NucleoSpin RNA column, then spined for 1min. To increase the effectiveness of rDNase later, 350 µl membrane desalting buffer (MDB) was added in each tube and centrifuged for 1min, then 95 µl DNase reaction mixture (10 µL reconstituted rDNase + 90 µL Reaction Buffer for rDNase) was added to each tube, and the tubes were incubated 15 min at room temperature. To deactivate DNase, 200 µL Buffer RAW2 was added, followed by a 1-minute spin at 11,000 x g. The buffer was washed away by adding 600 µL RA3 to each tube, followed by the addition of 250 µL to dry the membrane. Ultimately, RNA was eluted by adding 60 µL RNase-free H_2O.

Subsequently, cDNA synthesis was performed using the cDNA Reverse Transcription Kit. In each tube, 10 µL of 2X RT master mix was combined with 10 µL RNA, and the resultant samples were diluted to obtain a concentration of 20 ng/L. 5 ml of the diluted cDNA was seeded into a well of a 96-well plate, with each well containing one pair of primers specific to a single gene each. Lastly, the Step One Plus qPCR machine was used for PCR amplification.

*2.7. **Immunohistochemistry***

Formalin-fixed paraffin-embedded right atrial tissue was deparaffinized and sectioned into 6 µm slices. After rehydration through xylene (5min), 100% alcohol (3min), 95% alcohol (3 min), 70% alcohol (3min), and water (2min), tissue-bound epitopes were retrieved by high temperature (100◦C) for 1h to unmask the desired antigen, then peroxidase enzyme was added, and the slides were rinsed with water, followed by Phosphate Buffer Saline (PBS). Primary antibody diluted in 1% NGS was applied on the slides, then they were stored at 4°C overnight.

24 h later, the slides were thoroughly washed with PBS, and the secondary antibody was added for 1 hour. After washing the slides, tissue sections were covered with chromogenic substrate (DAP kit). The substrate underwent oxidation in the presence of hydrogen peroxide catalyzed by horseradish peroxidase (HRP) bound to the secondary antibody. The progression of stain development was monitored under a light microscope. Once the colors were clearly observed,

the development of stains was arrested by immersing the slides in PBS for 3 minutes. Finally, the slides were dehydrated through alcohol and xylene.

*2.8. **Immunofluorescence***

The protocol starts as described above, the tissue underwent the sequential rehydrated, and antigen retrieval, then incubated for 1 h with 2% NDS (Normal Donkey Solution) and 1.5 Triton in a PBS solution to obstruct non-target antigens. The tissue was then incubated with the primary antibody in a solution of 1% NDS and triton solution. The concentration and the time incubation were determined according to the properties of this antibody.

After washing the slides with PBS to eliminate unbound antibody, the secondary antibody was introduced in 1% NDS + Triton + DAPI, upon its addition, the secondary antibody bound to the primary antibody, and because it's conjugated to a fluorophore, it emitted light when excited with a specific wavelength, so the samples are washed with PBS for 5 min three times to avoid background fluorescence. To get optimal results, the microscopic analysis was done directly after the mounting of the samples.

*2.9. **Masson's Trichrome Staining***

After the rehydration of right atrial tissue sections through xylene and alcohol, they were initially incubated in Bouin solution at 56◦C for 1 h and set to reach room temperature for (10 min). Thereafter, the slides underwent thorough washing with tap water until becoming clear. Subsequently, Weigert´s iron hematoxylin was added for 10 min, then washed with tap water. Scarlet-Fuchisine is added for 15 min, followed by 4 successive rinses with distilled water, then Phosphomolybdic-Phosphotungstic acid was administrated for (8 min) followed by aniline blue for another 8 min. Afterwards, the slides were rinsed four times with distilled water, and treated with 1% glacial acetic acid for 3 min before going through alcohol and xylene for dehydration. This technique facilitated the observation of the normal tissue in red, collagen in blue, and nucleus in black.

2.10. ***Statistical Analysis***

To analyze the data obtained, One-way ANOVA was used or t-test was used for a simple comparison between the mean value of SHAM and PAB groups. Statistical significance was determined by evaluating two-tailed p-values less than 0.05, and the results were presented as mean ± SEM.

Chapter 3 – Results

3.1. Effects of PAB on Right Heart Function and Structure

Echography provided important structural, functional, and hemodynamic data assessing the essential changes affecting the right atria, ventricles, and pulmonary artery vessels, during systolic and diastolic phases in SHAM compared to PAB rats, 21 days post-surgery. These non-invasive methods allowed to verify the efficiency of PAB surgery and evaluate possible atrial and ventricular morphological remodelling.

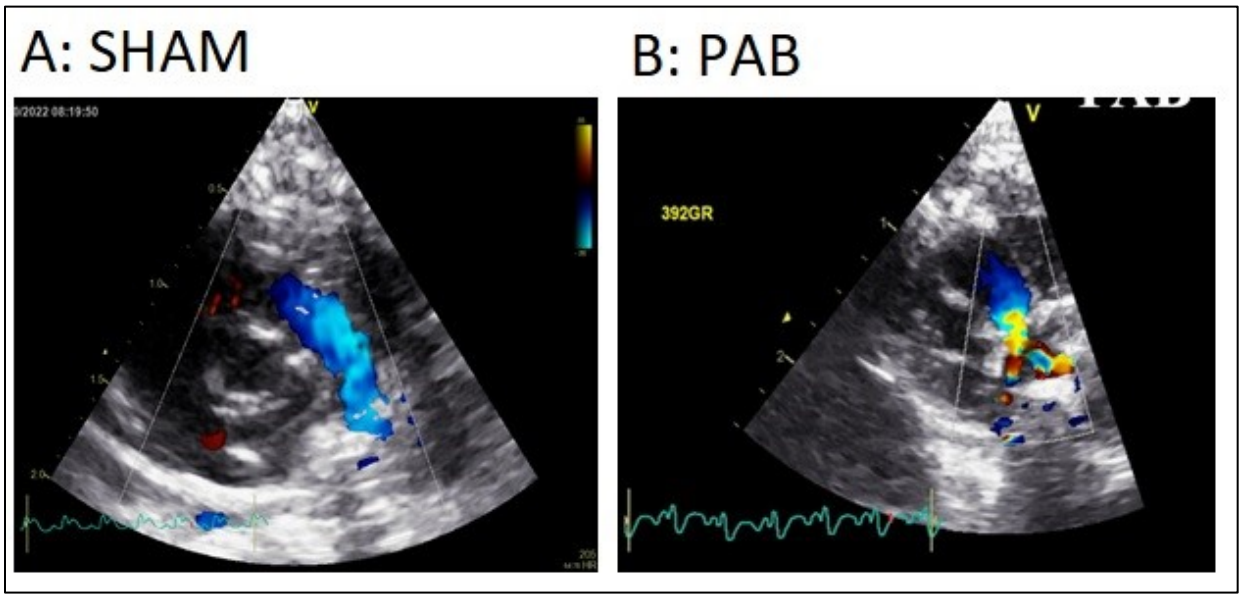

Figure 6: Pulmonary Artery Blood Outflow in SHAM vs PAB.

We measured the velocity of the blood passing from the right ventricle to the lungs through the pulmonary artery (PA) (*Figure 6*), and we found that the mean acceleration time **(AT)** of PA blood flow for 6 SHAM rats was 110.5 ± 47.66 cm/s, while for PAB rats the mean was significantly increased, more than two folds 351.3 ± 47.66 cm/s *(Figure 7A)*. This alteration is a consequent of the suture performed on the PA trunk. The limited blood flow in PAB at the banding site can clearly be observed in *Figure 6* (right panel), compared to the normal blood flow in SHAM (left panel).

Furthermore, RA size was evaluated in SHAM and PAB rats on day 21 post-surgery. We found that the mean right atrial dimension at end-systole **(RADs)** was 4.014 ± 0.3046 mm in SHAM. RADs were significantly increased in PAB rats, with an average dimension of 5.253 ± 0.3046 mm *(Figure 7B)*. The myocardial thickness was also evaluated. We observed that the right ventricular anterior wall thickness **(RVAWd)** increased significantly in PAB 0.8050 ± 0.0545 mm compared to SHAM 0.4943 ± 0.05453 mm *(Figure 7C).*

Finally, we evaluated the changes affecting the atrial and ventricular force of contraction, by looking at the atrial wall systolic contractility **(Sr)**. We observed that Sr was significantly decreased in PAB 5.054 ± 0.5013 cm/s compared to SHAM 7.901 ± 0.5013 cm/s *(Figure 7D).*

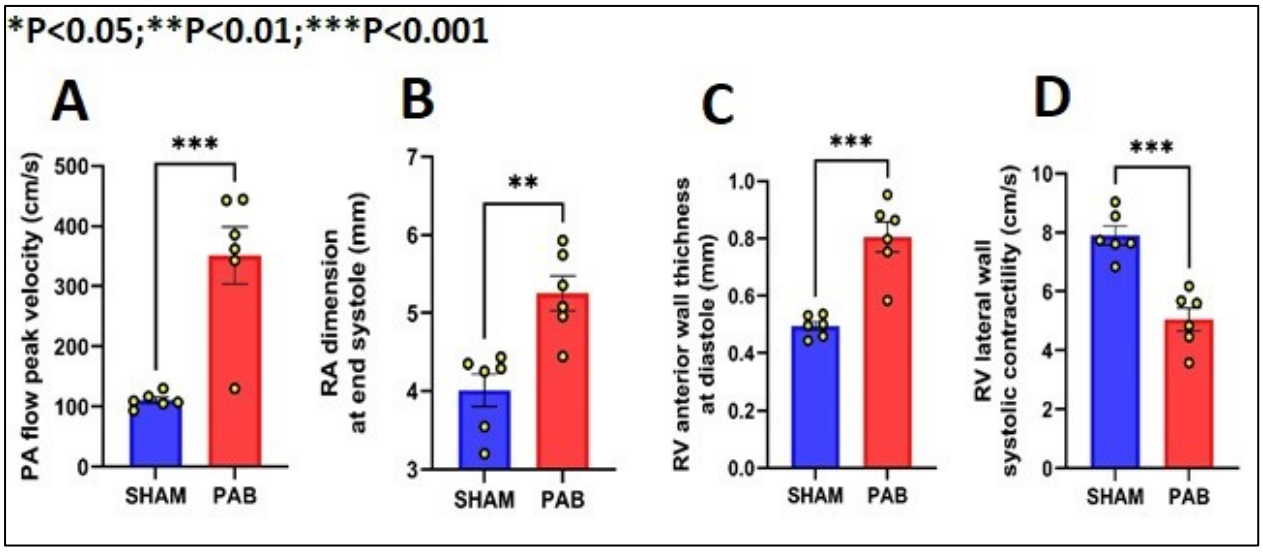

Figure 7: Severe RHD is Developed in Rats Three Weeks Post-PAB*. (A) Pulmonary artery (PA) blood flow peak velocity (cm/s) for 6 SHAM (in blue), and 6 PAB (in red). (B) Right Atrial dimension at end systole (mm). (C) Right Ventricular wall thickness at end systole (mm). (D) Right Ventricular lateral wall systolic contractility (cm/s). A-D results are MEAN ± SEM for 6 SHAM (blue bars) and 6 PAB (red bars). Statistical analysis: one-way ANOVA test.*

3.2. Effect of RHD on AF Vulnerability

Transoesophageal electrophysiological study performed on SHAM and PAB rats revealed that SHAM rats were not vulnerable to AF, whereas PAB rats developed significantly more episodes of AF *(Figure 8A and 8B)*. Among the 6 PAB rats, 3 were inducible to AF, while 2 others developed AF and atrial flutter (*Figure 8C*). All Sham rats conserved normal SR after burst pacing.

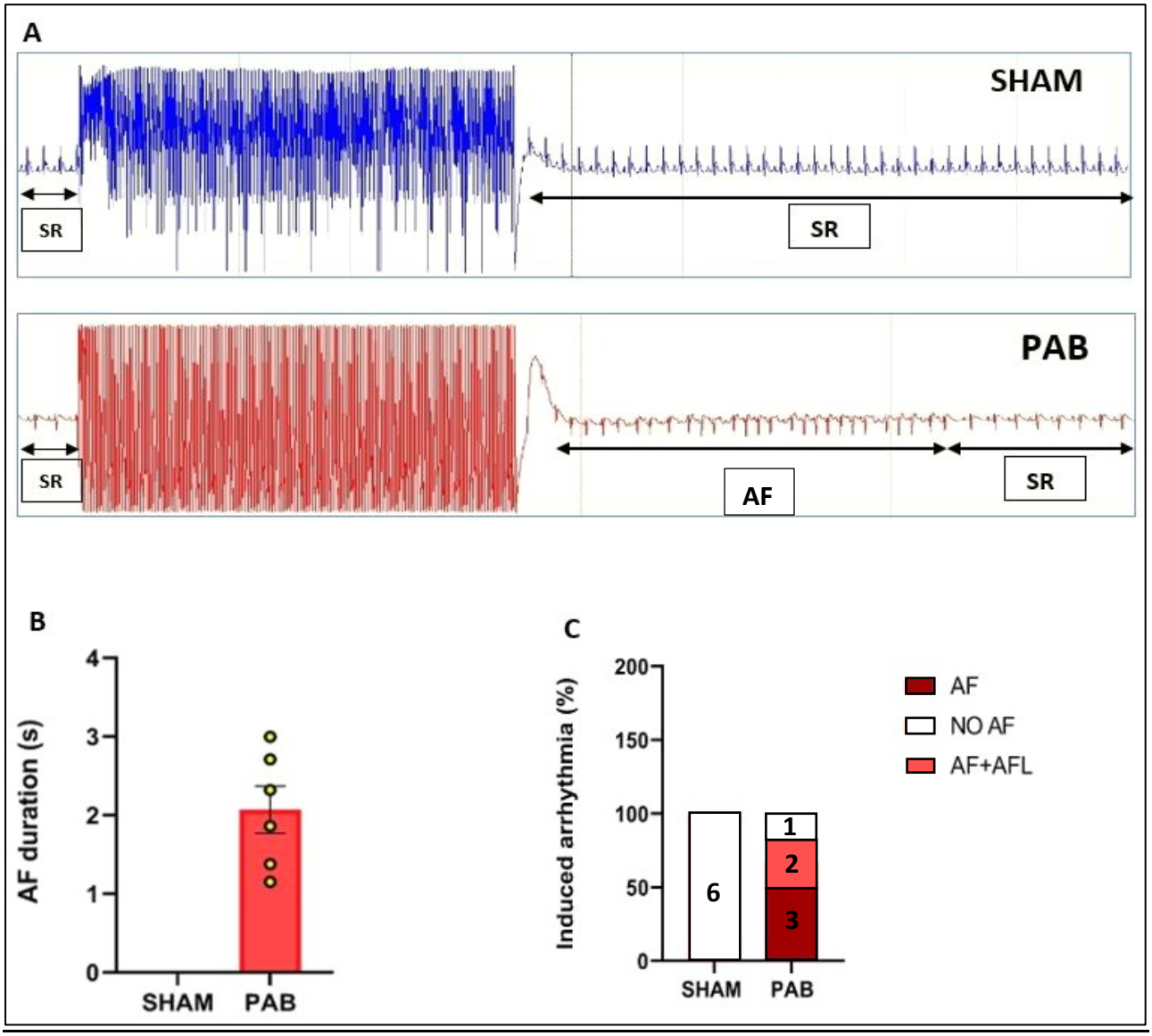

Figure 8: Electrophysiological Differences Between SHAM and PAB. *(A) ECGs presenting sinus rhythm (SR), 3s burst stimulation in SHAM (in blue), in addition to them, atrial fibrillation episode (AF) is showed in PAB (in red). (B) Mean ± SEM of AF duration in 6 SHAM and 6 PAB during transesophageal EPS. (C) Percentage number of rats that showed (AF), atrial flutter (AFL), both or no AF.*

3.3. Effect of RHD on the Expression of Fibrosis and Inflammation-Related Genes

The expression level of genes participating in the atrial inflammatory status during PAB was assessed to understand how they are associated with the incidence of AF. The results obtained were compared in SHAM and PAB using the ANOVA test to catch possible differences and calculate the level of significance.

3.3.1. Fibrosis -Related Genes

After treating the cultured FBs with the inflammatory cytokine (IL-6), the mean expression level of proliferation marker gene (ki67) of 9 SHAM rats was 0.9533 ± 0.4759 A.U, while for 7 PAB rats this value had increased to 1.815 ± 0.4298 A.U (*Figure 9A*). As well as the differentiation marker gene (Acta2) that provides instructions for making a-SMA filaments, the mean expression level of this gene for 10 SHAM was 1.253 ± 0.5763 A.U, comparing to 7 PAB this value increased to 2.147 A.U ± 0.5763 *(Figure 9B)*. On the opposite side, by adding the anti-inflammatory cytokine IL-37, the mean expression level of COL3A1 that helps to build extracellular matrix during the remodelling phase of the disease of 7 PAB decreased to 0.8568 A.U ± 0.2904 , while it was 1.163 A.U ± 0.2904 for 10 SHAM rats, on the same side, the inhibition of inflammation by IL-6 antibody slightly decreased the expression level of COL3A1 into 0.9622 ± 0.2881 A.U in PAB compared to SHAM that was 1.015 ± 0.2881 A.U**,** *(Figure 9C)*.

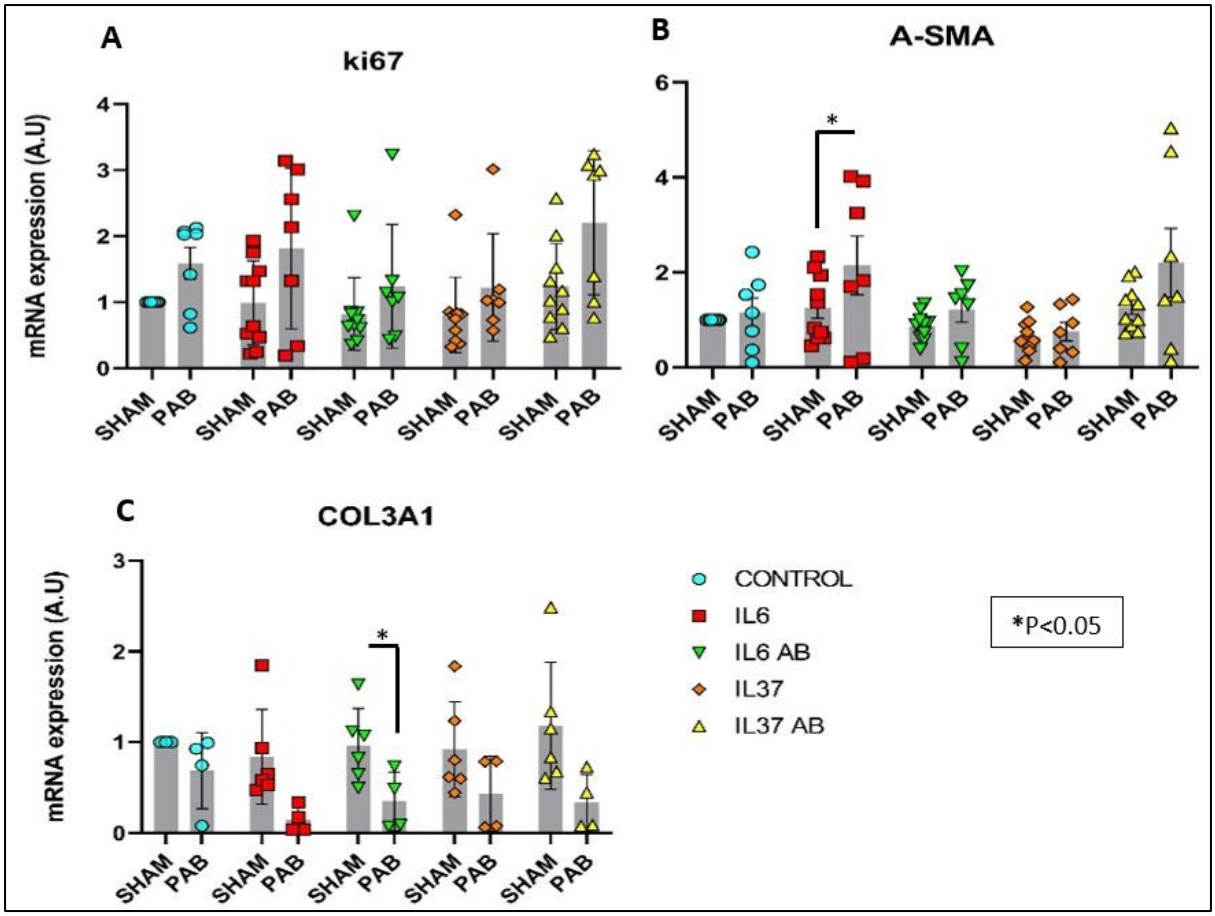

Figure 9: Expression Levels of Fibrosis-Related Genes. *MEAN ± SEM (A.U) of messenger RNA expression of (A) KI67 (SHAM: n=10, PAB: 7), (B) α-SMA (smooth muscle actin) (SHAM: n=10, PAB: n=7), and (C) COL3A1 (Collagen) (SHAM: n=6, PAB: n= 4) obtained by qPCR. FBs cultured without treatment (blue), with IL-6 (red), IL-6 AB (green), IL-37 (orange), and IL-37 AB (yellow). Results analysis: One-way ANOVA test.*

3.3.2. Inflammasome-Related Genes

Several genes implicated in the inflammasome pathway were examined to understand the regulatory mechanism orchestrating this process and to discover the key effectors capable of inducing changes in the normal inflammatory pathway.

The expression level of the CASP1 gene that activates many inflammatory cytokines was increased in PAB rats into 4.9 ± 1.178 A.U after treating the FBs with IL6, while it was 1.643 A.U ± 1.178 in SHAM (*Figure 10 A*). As well as for NLRP3 gene, it was increased more than 2-folds in PAB compared to SHAM (PAB: 2.708 ± 0.7434 A.U vs SHAM: 1.232 ± 0.7434 A.U) *(Figure 10 B)*.

Similarly, a slight increase was observed in these genes after the inhibition of the anti-inflammatory pathway using IL-37 antibodies in PAB rats compared to SHAM, in particular, the expression of CASP1 was 1.689 ± 0.8379 A.U in PAB rats vs 0.8843 ± 0.8379 A.U in SHAM.Whereas, ASC, which is an adaptor protein in many inflammasome complexes, was 0.8177 ± 0.1992 A.U, slightly lower than SHAM 0.8965 ± 0.1992 A.U *(Figure 10 C).*

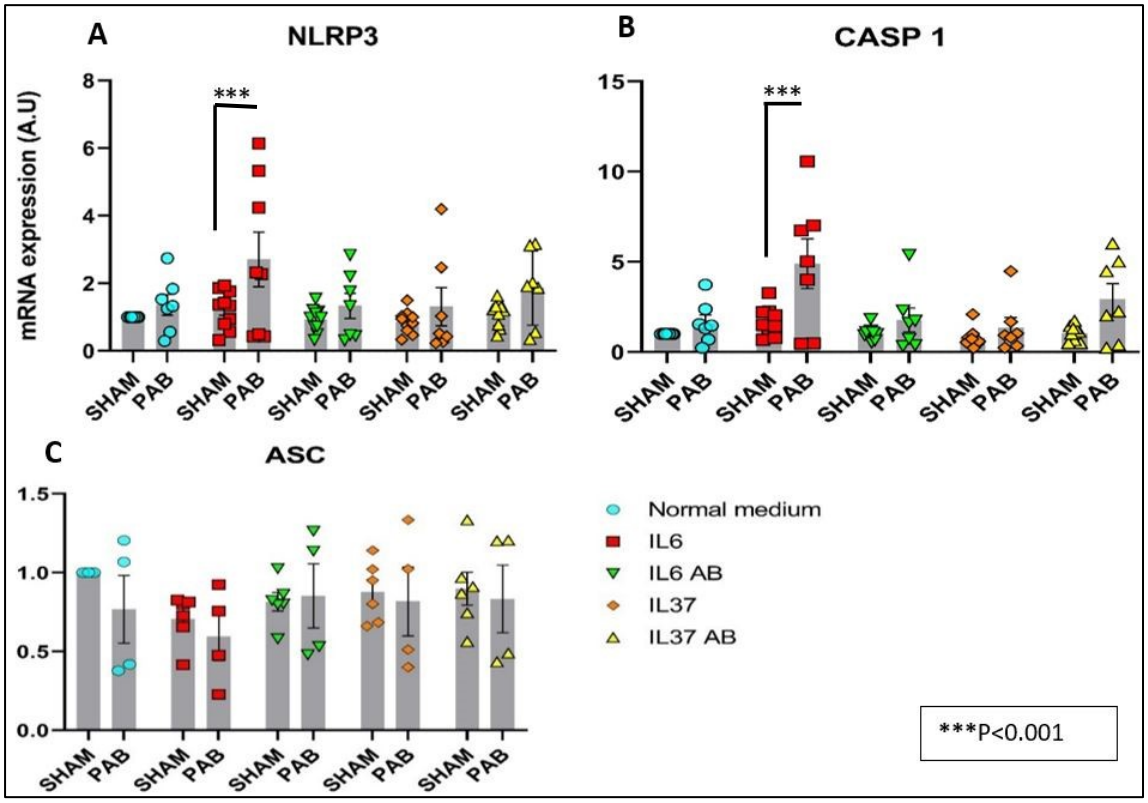

Figure 10: Expression Levels of Inflammasome-Related Genes. *MEAN ± SEM (A.U) of messenger RNA expression of (A) NLRP3 (SHAM: n=10, PAB: 7), (B) CASP1 (SHAM: n=7, PAB: n=7), and (C) ASC (SHAM: n=6, PAB: n= 4) obtained by qPCR. FBs cultured without treatment (blue), with IL-6 (red), IL-6 AB (green), IL-37 (orange), and IL-37 AB (yellow). Results analysis: One-way Anova test.*

3.3.3. Pro-Inflammatory Interleukins

After The addition of anti-inflammatory treatment (IL-37), the mean expression level of IL-6 for 6 SHAM was 0.7987 ± 0.3297 A.U, while a significant reduction was observed for 4 PAB 0.2055 ± 0.3297 A.U *(Figure 11A*). In like manner, with anti-IL6 the mean expression level of IL-6 among 6 SHAM was 0.7314 ± 0.3461 A.U , whereas it was 0.3002 ± 0.3461 A.U for 4 PAB, which also demonstrated a decrease among PAB.

On the other side, the mean expression level of Il-1ß which is considered a key mediator of the inflammation for 7 PAB was increased to 3.528 ± 0.9657 A.U, compared to 9 SHAM 1.342 ± 0.9657 A.U. Likewise, with anti-Il37, the expression level of IL-1b for 4 PAB increased to 5.391 ± 1.153 A.U, although the mean for 8 SHAM was lower 1.921 ± 1.153 A.U. *(Figure 11B).*

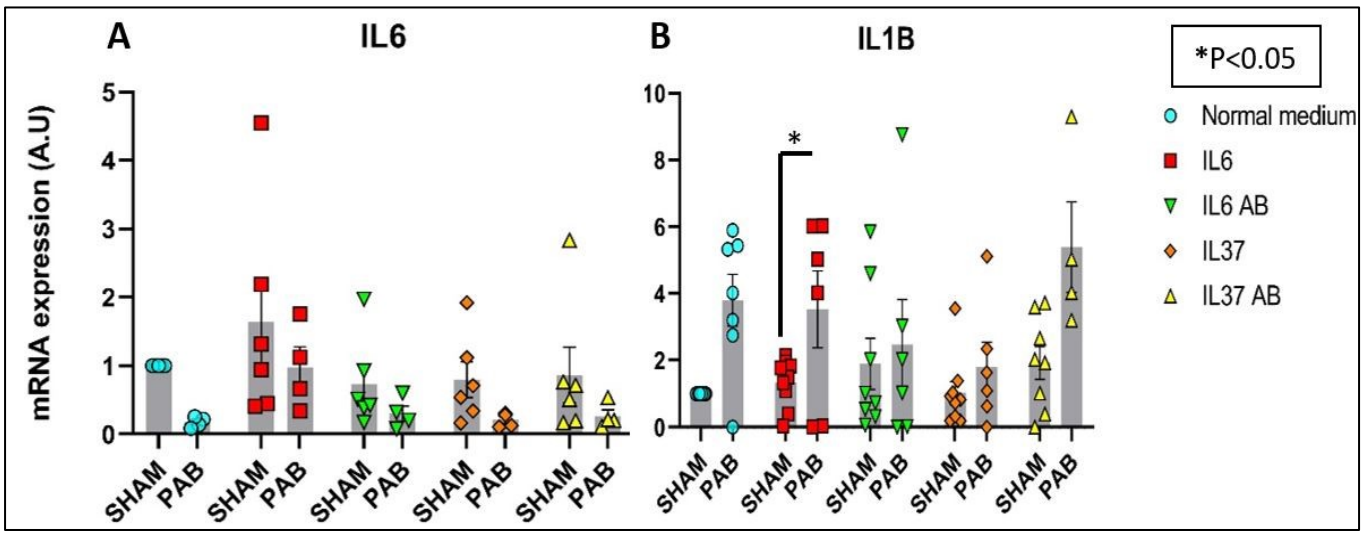

Figure 11: Expression Levels of Pro-Inflammatory Interleukins. *MEAN ± SEM (A.U) of messenger RNA expression of (A) IL6 (SHAM: n=6, PAB: 4), (B) IL1ß (SHAM: n=8, PAB: n=6) obtained by qPCR. FBs cultured without treatment (blue), with IL-6 (red), IL-6 AB (green), IL-37 (orange), and IL-37 AB (yellow). Results analysis: One-way ANOVA test.*

3.3.4 Anti-Inflammatory Biomarkers

At the end stage of the disease, the inflammatory pathway is suppressed by the activation of a gene responsible for producing anti-inflammatory signals (IL-10), so that the immune system can switch to the resolution phase, the mean expression level of this cytokine for 6 SHAM was 2.821 ± 2.126 A.U after the addition of IL-37, while it was increased more than 3-Folds in PAB rats 7.183± *2.126 A.U (Figure 12).*

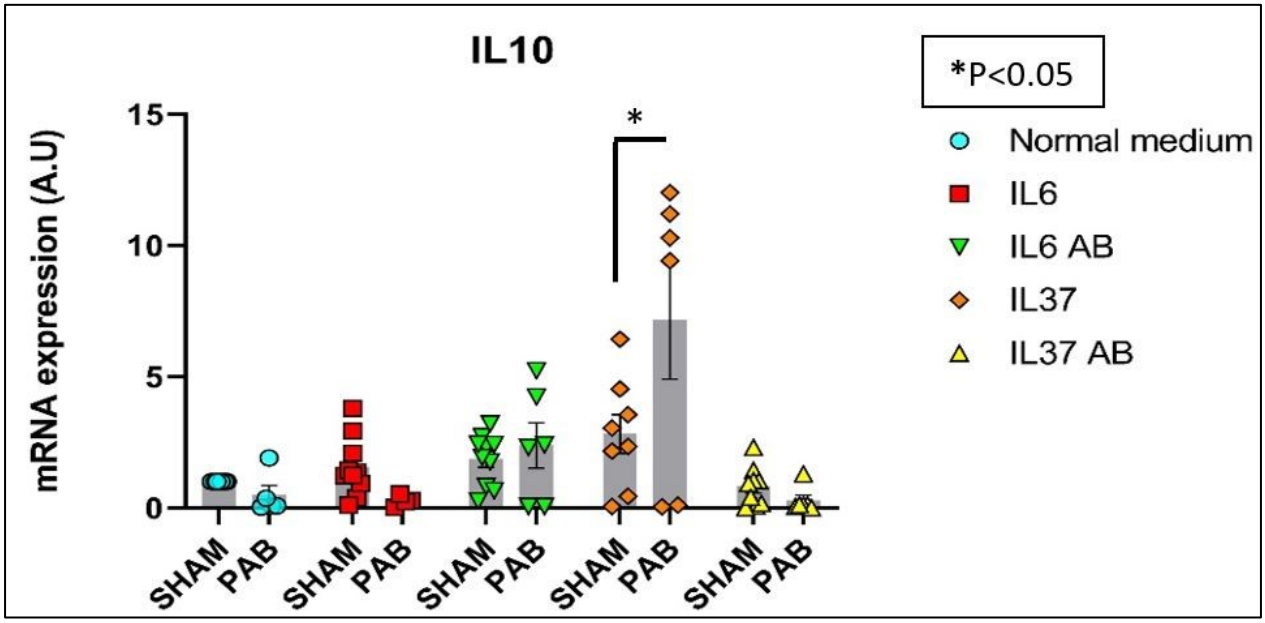

Figure 12: Expression Level of Il-10. *MEAN ± SEM (A.U) of messenger RNA expression of IL-10 (SHAM: n=8, PAB: 6) obtained by qPCR. FBs cultured without treatment (blue), with IL-6 (red), IL-6 AB (green), IL-37 (orange), and IL-37 AB (yellow). Results analysis: One-way ANOVA test.*

3.4. Effect of RHD on Fibroblasts Differentiation and Proliferation

In response to cardiac death and extracellular degradation during the inflammatory phase, FBs play an essential role in the compensation of devoid area to prevent cardiac rupture. Anti-inflammatory and pro-fibrotic factors regulate the proliferation of FBs [27]. To detect the cytokines involved in FBs proliferation and suppression, we looked at these cells in culture after the incubation 48h with 4 different conditions.

For PAB-induced RHD rats, when we cultured the right atrial FBs with IL6, we noticed an increase in the proliferation of these cells to 90 ٪ compared to the cells cultured in normal medium 75 %; otherwise this proliferation was suppressed with anti-IL6 in PAB to 77.33 %. Conversely, in the presence of IL-37, the mean percentage of confluency for the PAB rats was 58.33 %, this decrease was prevented with anti-Il37 to 75%.

3.5. Histological Analysis of PAB-Induced RHD Compared to SHAM

3.5.1. Effect of RHD on the Development of Atrial Fibrosis Content

Fibrosis is the accumulation of extracellular matrix (ECM) material composed of connective tissue, mainly collagen. Fibrosis was detected with Masson's Trichrome coloration that allowed differentiation of normal cardiac tissue into fibrous areas [28]. In right atrial samples from SHAM compared to PAB rats *(Figure 14 A and 14B).*

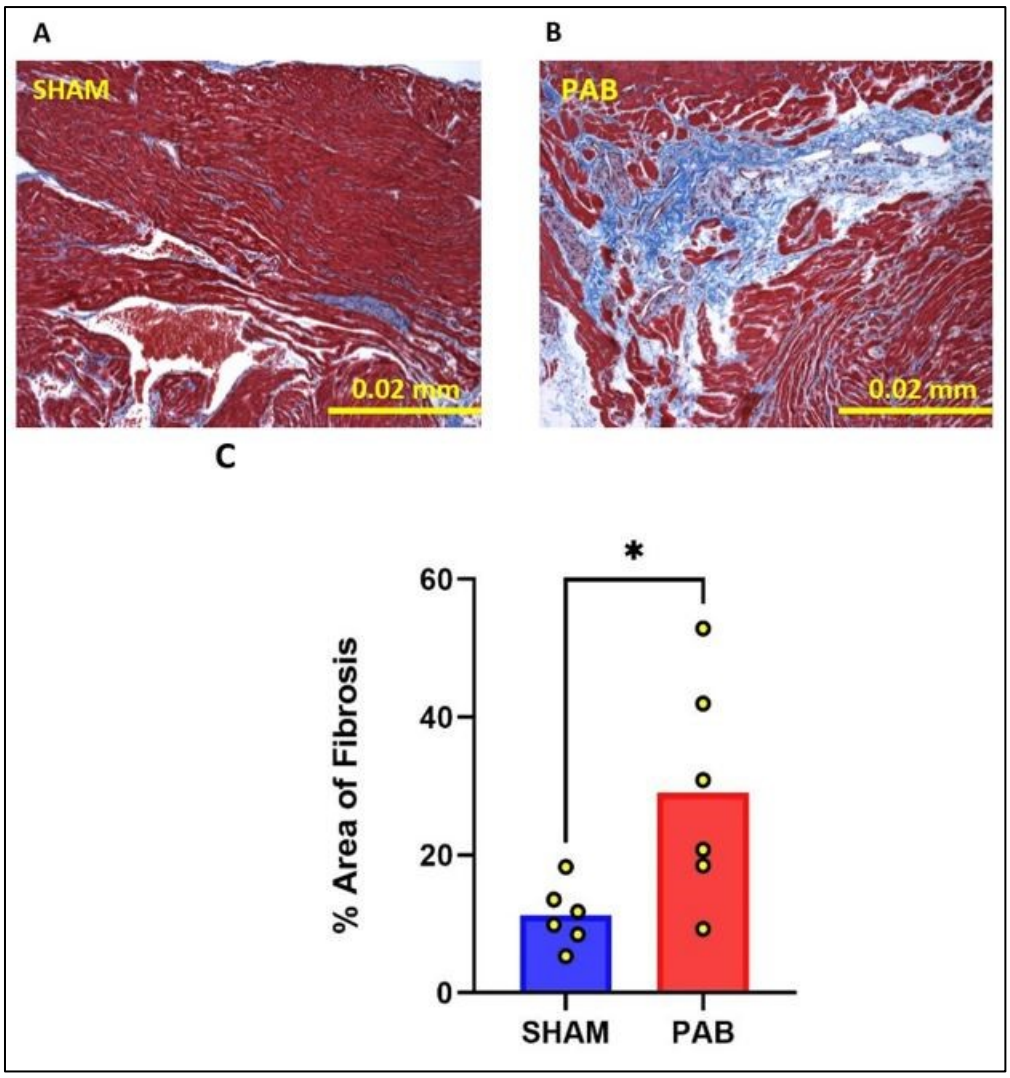

Figure 13: Masson Trichrome´s Staining of RA Tissue*. Representative histological image showing RA fibrotic area in (A) SHAM, and (B) PAB. (C) MEAN ± SEM percentage RA fibrotic area in SHAM (blue bar; n=6) and PAB (red bar; n=6).*

Our data showed that the mean percentage of fibrous area in RA from SHAM rats was 11.22 ± 6.85 %. Fibrosis was significantly increased in RA tissue from PAB rats (29.04 ± 6.857 %), *(Figure 14 C)*

3.5.2. Effect of RHD on the Differentiation of *Fibroblasts into Myofibroblasts*

Alpha smooth muscle actin (α-SMA) is present in certain types of cells, such as smooth muscle cells, blood vessels, and myo-FBs, giving these cells the contractile ability. It is well accepted that α-SMA expression can be used to indicate the differentiation of fibroblasts into myo-FBs transition, in addition to the inflammation, scar formation, and fibrosis [29].

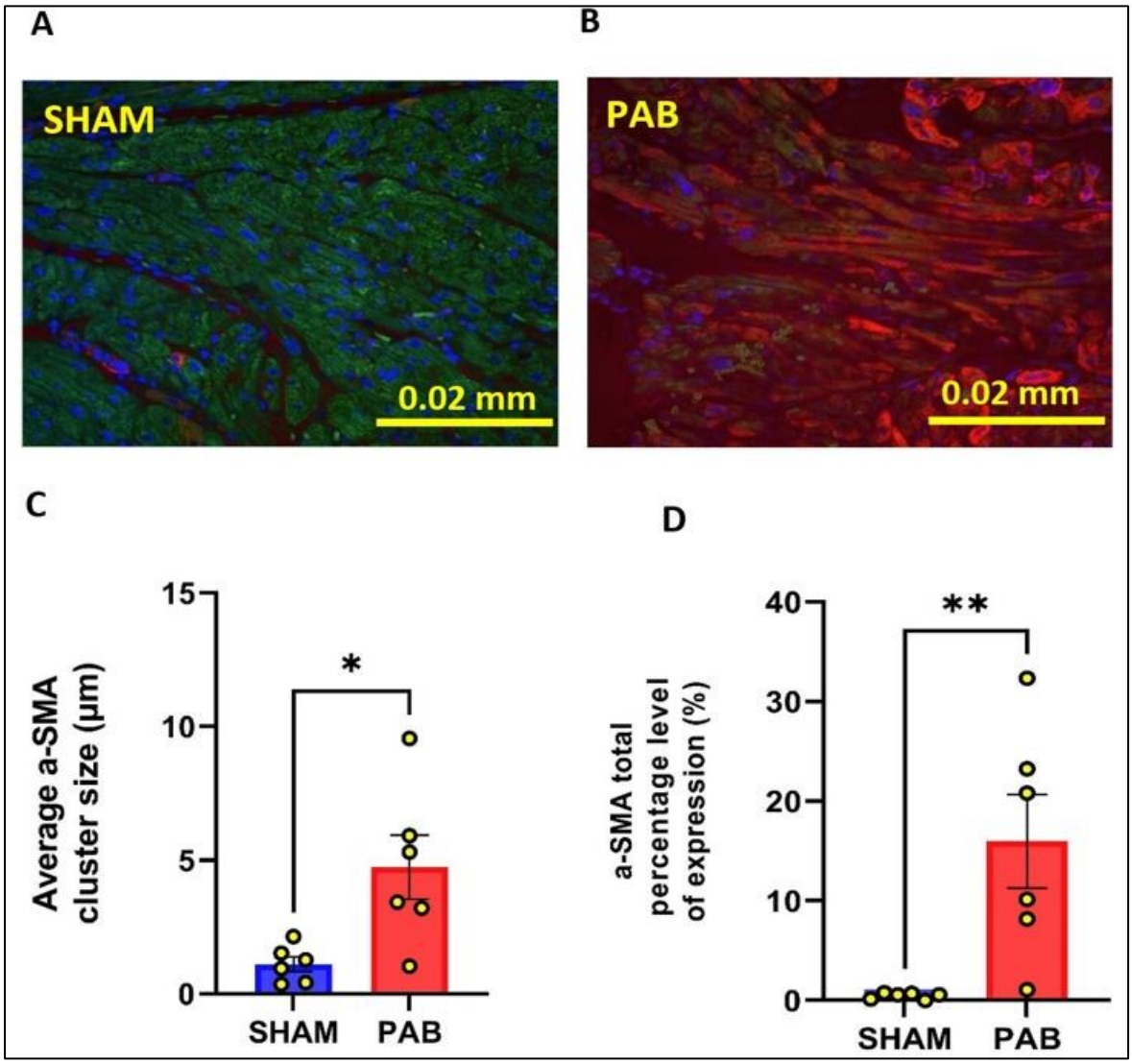

Figure 14: Immunofluorescence of α-SMA. *Representative histological images showing RA alpha smooth muscle actin (α-SMA) in (A) SHAM, and (B) PAB rats. (C) average α-SMA cluster size (µm), (D) α-SMA percentage level of expression (٪) in SHAM (blue bars; n=6), PAB (red bars; n=6).*

Our data showed that α-SMA wasn't expressed in RA from SHAM tissue, while it was highly expressed in RA from PAB *(Figure 16 A and 16B).* Also, the mean of average cluster size of α-SMA for 6 SHAM rats was 1.108 ± 1.226 µm, however for the PAB rats, the mean was increased more than 4-folds reaching 4,743 µm ± 1.226 µm (*Figure 15 C*).

As well as for the mean of total percentage level of α-SMA, it was between 0.4730% in SHAM rats, while in PAB it was significantly higher at 15.97% (*Figure 15 D*).

3.5.3. Impact of RHD on Macrophage Recruitment and Polarization

Immunohistochemistry was used to detect the presence of M1-macrophages in the right atrial tissue of both SHAM and PAB rats 21 days post-surgery. The protein CD11c was selected as the target marker for the identification and quantification of this cell subset [30].

A total of 6 SHAM and 6 PAB rats were used. For each rat, five distinct fields of view were captured using a light microscope at 20x, and within each field of view, quantification of the stained proteins was performed. The average count of CD11c protein detected was calculated and illustrated (*Figure 16 E).* As we can see, the mean number of CD11c in SHAM rats was 0.5097 cells/mm², while it was slightly higher in PAB at 0.7939 cells/mm².

In like manner, we used the INOS as a target protein to detect M1-macrophages, it was only present in PAB rats 1.667 cells/mm² (*Figure 16 F*).

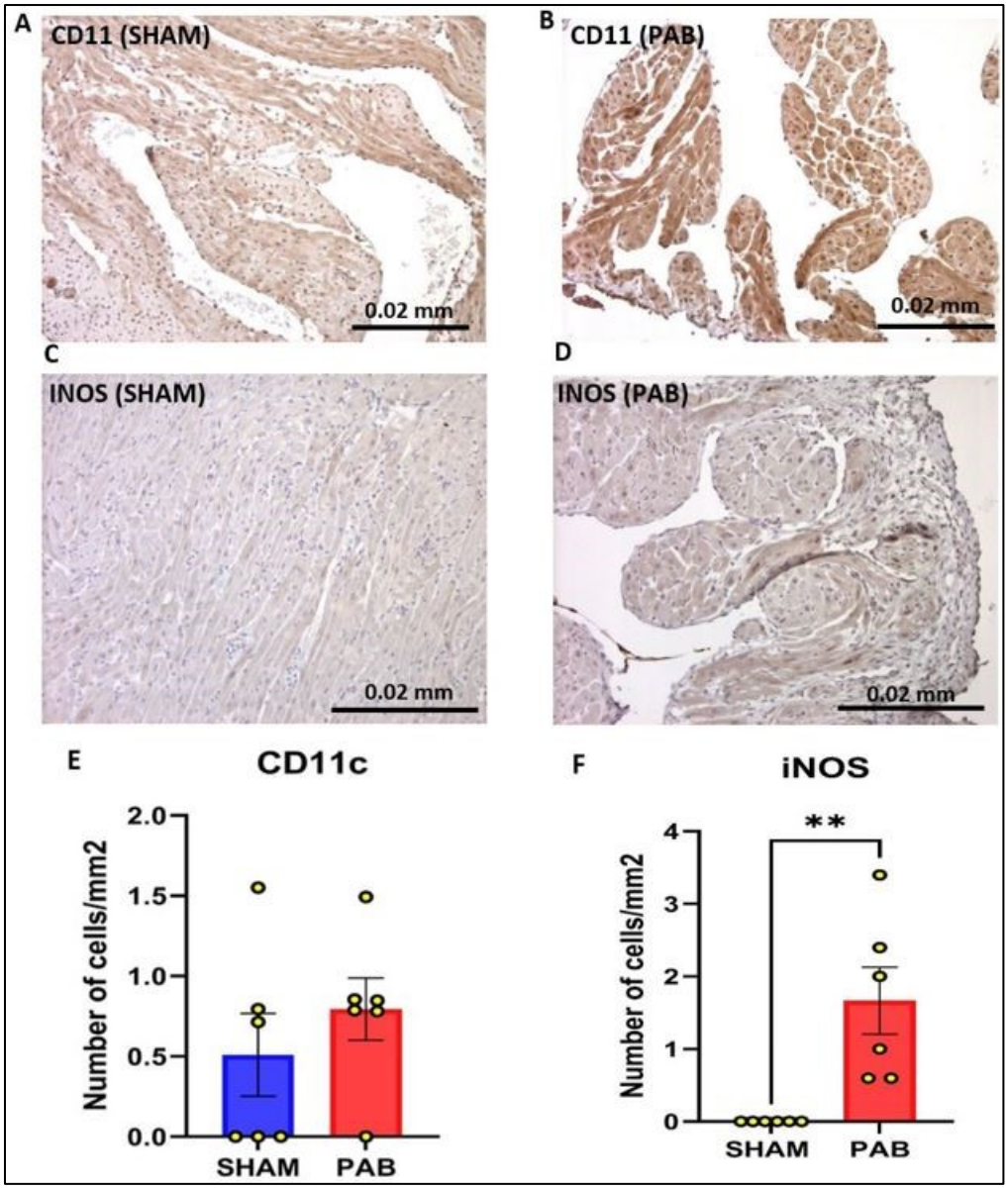

Figure 15: CD11c and iNOS Detected in The RA of SHAM and PAB rats. *Histological images show the presence of protein CD11 in RA tissue of (A) PAB, and (B) SHAM rat. iNOS enzyme in (C) SHAM, and (D) PAB. (E) MEAN ± SEM number of cells with CD11 protein in SHAM (blue bar; n=6), and (F) in SHAM (red bar; n=6 rats). (F) number of cells with iNOS (SHAM: n=6, and PAB: red bar; n=6).*

CD206 is a protein expressed on the surface of M2 macrophage lineage, and therefore it's used as a specific marker to detect these cell [30]. The results showed that the number of M2-macrophages was 53.49 cells/mm² significantly higher than in SHAM 13.74 cells/mm² (*Figure 17 C*).

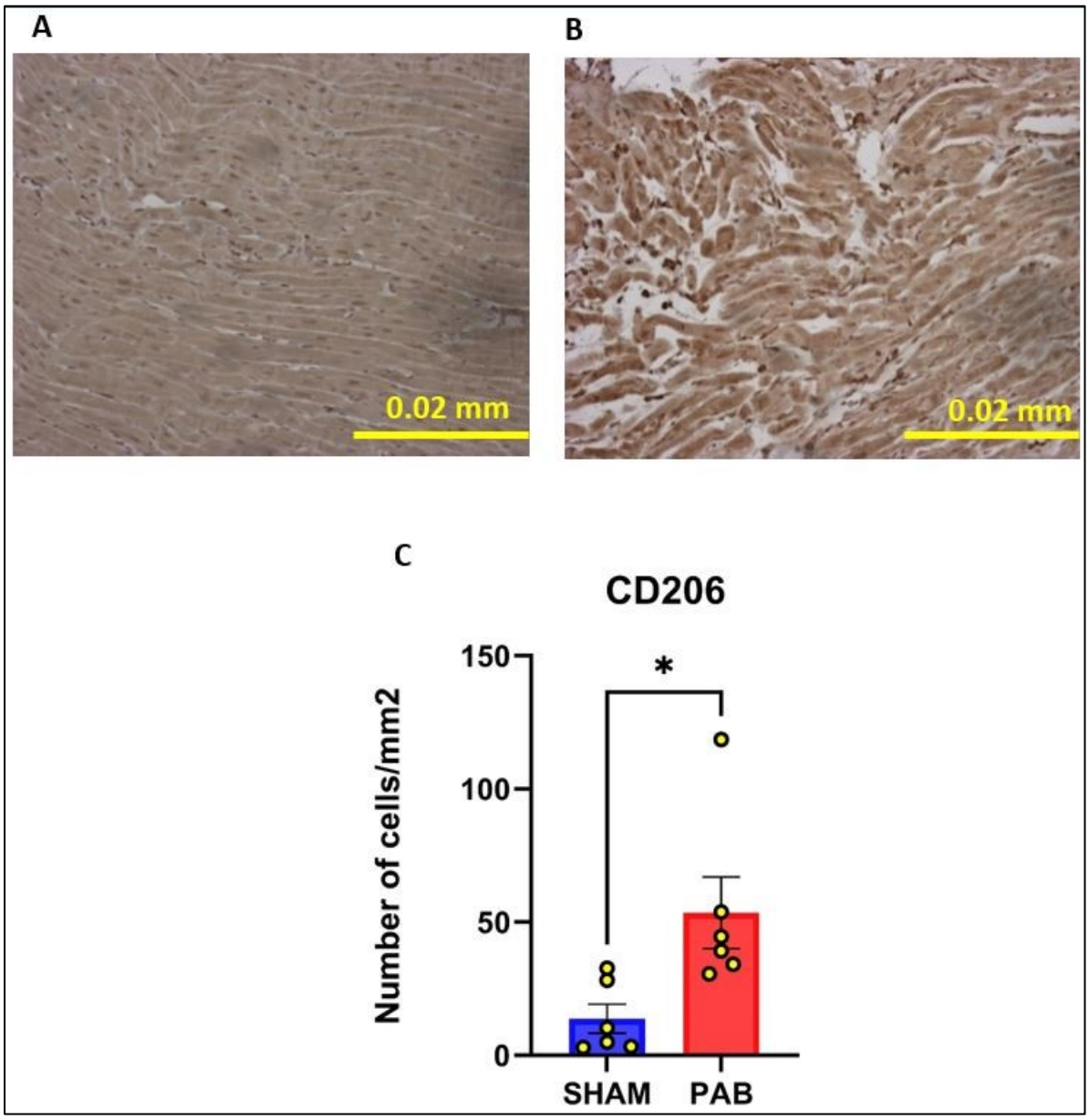

Figure 16: CD206 Detected in RA in SHAM and PAB Rats. *Histological images show the presence of protein CD206 in RA tissue of (A) SHAM, and (B) PAB rats. (D) MEAN ± SEM number of cells expressing CD206 in SHAM (blue bar; n=6), and PAB (red bar; n=6).*

Chapitre 4 – Discussion

4.1. Main Discoveries

After PAB surgery, the partial restriction of the pulmonary artery disrupted the normal blood circulation through this vessel since the acceleration time (AT) of blood flow is increased. We can clearly say that there is some resistance to the blood circulating from the right ventricle to the lungs, this condition is known as pulmonary arterial hypertension. The high pressure requires more effort from the right ventricle to maintain the normal blood output, and overcome the stress, so as a compensatory mechanism, the extra strain triggers an increase in the number of CMs. This phenomenon is known as hypertrophy, and it was observed through the increase in right ventricular anterior wall thickness in PAB rats.

Consequently, the right ventricle becomes stiff and unable to totally overcome the chronic resistance, leading to an increase in right ventricular afterload, systolic impairment, right ventricular failure, and death, converting to a pathological maladaptive response [31]. Eventually, deceased CMs initiate an inflammatory response orchestrated by the cytokine IL-6. This response involves the recruitment of M1 macrophages also the upregulation of specific gene expressions in FBs responsible for cellular differentiation, proliferation, and production of fibrotic substances to compensate for the loss of CMs. Thereby, the inflammation is suppressed by the upregulation of M2 macrophages, and the secretion of anti-inflammatory cytokine IL-10 stimulated by IL-37.

4.2. Role of FBs in the Inflammatory Response

Fibroblasts play a crucial role in the context of heart diseases, since they are considered a primer trigger of the inflammatory cascade in response to various pathological stimuli, such as tissue injury and CM death. Once these cells are activated, they can orchestrate a reparative process via extracellular matrix remodelling to compensate for the functional impairment, and to maintain cardiac tissue architecture. To display the role of FBs in detail during RHD, we published a review paper that explains the reparative process, and how the maladaptive response can lead to AF before looking for possible strategies to modulate their function. *A detailed discussion has been developed in my published article attached to this memoir and visible in the Appendix*.

4.2.1. Concept of 'Failed Inflammation-Resolution Mechanism' in Cardiac Remodeling.

FBs have a pivotal role in responding to cardiac injuries by closely interacting with activated inflammatory cells and participating in the tightly regulated healing process that aims to limit tissue damage [32]. These FBs are essential for activating reparative mechanisms that are important for maintaining the proper structure and function of the heart [32,33]. Prolonged and uncontrolled inflammation can lead to failure of the FBs in the resolution phase of the disease, provoking further cardiac remodeling and leading to complications such as congestive heart failure, cardiac dysfunction, arrhythmias, and sudden death [34,35].

4.2.2. Initiation of Inflammation and FBs Activation.

When a cardiac injury occurs, the extracellular matrix (ECM) undergoes degradation, producing damage-associated molecular patterns (DAMPS) [36]. This leads to disruption of cardiomyocytes' cellular membranes and subsequent release of inflammatory cytokines [36]. DAMPs bind on pattern recognition receptors (PRRs) present on various cells (leucocytes, macrophages, endothelial cells…), triggering an innate inflammatory response. This response starts from the activation of a cascade of intracellular proteins ending in the activation of transcription factors such as the nuclear factor-kappa B (NF-κB) [37]. NF-κB activates the transcription of genes coding for proinflammatory agents involved in facilitating the recruitment and differentiation of inflammatory cells. Such proinflammatory markers include cytokines (IL-6, IL-18), and chemokines (ICAM-1, VCAM-1). In addition, they stimulate the upregulation of FBs-associated expression of degradation enzymes such as proteases [38]. If sustained, the activation of proinflammatory markers contributes to cardiac fibrosis development, ultimately leading to impaired cardiac function and increased arrythmogenicity [39,40,38].

4.2.3. FBs Activation and Upregulation of Critical Fibrosis Biomarkers.

NLRP3-inflammasome has imerged as a significant player in the developement and progression of various cardiac diseases, including cardiac arrhythmia [41,42]. Extensive evidence supports the pivotal rôle of NLRP3-inflammasome in exacerbating cardiac inflammation [43,44]. For instance, in patients experiencing acute myocarditis, initiation of inflammation coincides with the activation of inflammasome in polymorphonuclear leukocytes (PMN), FBs and cardiomyocytes,

showing correlation with heart failure severity [45]. On the other side, in myocardial ischemia, the inflammasome exacerbates tissue damage and contributes to cardiac failure, while the inhibition of NLRP3 improved cardiac function [46]. Also, genetic deficiency of NLRP3 was associated with reduced expression of proinflammatory cytokines (TNF-α, IL-1β, IL-6), and increased secretion of anti-inflammatory LXA4 and LXB4 [47]. This suggests that the presence of NLRP3 promotes fibrosis during the progression of inflammation [47].

The secretion of IL-1β by leukocytes is known to play a significant role in activating both inflammatory and fibrogenic pathways during the healing process [48]. It contributes to the pathogenesis of cardiac remodeling by inducing the synthesis of inflammatory mediators by the activated leukocytes [49]. In a study conducted on mice lacking IL-1β receptors subjected to coronary occlusion/reperfusion, immunohistochemical staining revealed reduced infiltration of inflammatory cells in the infarct area [50]. These mice exhibited lower levels of secreted cytokines and chemokines compared to wild-type animals [50]. The administration of anti-IL-1β resulted in decreased collagen secretion and scar formation [50]. These findings suggest that IL-1β plays a crucial role in the development of cardiac fibrosis.

During myocardial injury, IL-6 plays an important in the regulation of cardiac FBs differentiation and secretion of inflammatory factors, which implificates the inflammation-response and remodeling [51]. In details, at the site of injury, when IL-6 binds to its receptor, it regulates the proliferation and differentiation of FBs. This phenomenon promotes the synthesis of hyaluronan synthase (HAS1, HAS2) to increase hyaluronan providing strength, lubrication and hydration to the extracellular-matrix [52,53]. Some studies performed in mice subjected to myocardial infarction, revealed that the administration of anti-IL-6 significantly inhibited HAS1 and HAS2 [54]. Moreover, they noticed a decrease in α-SMA secretion in the affected area [54].

Transforming growth factor-beta (TGF-β), expressed by macrophages and FBs [55], initiates non-canonical pathways upon binding to its receptors TBRI and TBRII, activating p38-kinase and subsequently the serum response factor (SRF) [56]. This leads to the transcription and upregulation of TRPC6, facilitating calcium entry and activating calcineurin, thereby enhancing FBs' conversion into myofibroblasts by NFAT [57].

Studies using recombinant adenovirus expressing TRPC6 in mice demonstrated increased α-smooth muscle actin (αSMA) levels, augmented FBs activation, and enhanced fibronectin domain ED-A expression [58]. Conversely, TRPC6 knockout in a murine myocardial infarction (MI) model reduced FBs conversion into myofibroblasts but led to higher mortality due to smaller scar formation [58]. These findings suggest that TGF-β-induced FBs differentiation into myofibroblasts contributes to fibrosis-related morbidity and promotes arrhythmogenic fibrosis [58].

In our study, PAB-induced RHD was associated with increased expression of αSMA and pro-inflammatory macrophages. In right atrial FBs, we observed that IL6 could stimulate the overexpression of fibrosis- (*Col1a1, αSMA*) and inflammation-related genes (*Nlrp3, Asc, Casp1, Il6, Il1b*).

4.3. Potential Impact of IL37 in Inflammation Therapy

IL-37 is known as an anti-inflammatory cytokine due to its function in limiting inflammation. Many studies were done to understand the potential impact of IL-37 in inflammatory and autoimmune diseases [59], since the elevation of this cytokine was associated with attenuation of atherosclerotic plaque expansion, and the decreased level of IL-37 was accompanied by severe and chronic diseases, such as acute coronary syndrome and heart failure [60].

4.3.1. IL37 Suppresses Inflammation and Fibrosis.

IL-37 recruits IL18 antagonists to block the activation of the inflammasome pathway, so it interferes with the NF-kb signaling pathway and the subsequent production of pro-inflammatory cytokines [57]. This suppression was observed after we treated the cultured FBs with IL-37, the expression level of IL-6 decreased significantly, which reflects the ability of IL-37 to down-regulate inflammatory genes in the cardiac FBs. Moreover, IL-37 has affected the proliferation of fibroblast in rats with RHD, thus we observed a decrease in the percentage of the proliferation of the cells treated with IL-37, and downregulation of COL3A1 gene, although it upregulates the anti-inflammatory cytokine IL-10, so it contributes to a more controlled tissue repair process.

4.3.2. IL37 Modulates Immune Cell Proliferation.

IL-37 facilitate the transition from macrophage subtype M1 that is usually activated during the inflammatory phase of the disease toward the anti-inflammatory subtype M2 activated during the resolution phase [60]. Therefore, this can reflect that IL-37 is able to accelerate the resolution of the disease and prevent persistent inflammation that aggravates AF and leads to heart failure.

IL-37 is an interesting target that helps to construct an anti-inflammatory therapy, due to its ability to control the inflammation through multiple mechanisms. Starting from the beginning of the disease to the resolution phase, IL-37 helps in maintaining immune homeostasis and prevents excessive inflammation, in addition to the prevention of maladaptive responses that may occur due to excess fibrosis, so it can be considered as a protective agent from inflammation, fibrosis, and AF.

5. Limitations

While our experimental design offers valuable insights on the impact of pro- and anti-inflammatory treatments on fibroblasts and macrophages-subtypes' behaviours, some limitations could be addressed in the short, medium, and long term to improve our demonstration.

The scratch wound healing assay will be performed to study fibroblasts' migration and offer a detailed *in vitro* representation of cellular proliferation and migration following the above-described treatment groups.

More protein assays will be necessary to evaluate the level of expression of targeted markers following our treatment conditions. We plan to perform protein quantification using the Luminex xMap technology, with ProcartaPlex ImmunoAssays. Western blot analyses were performed, but the data showed a lot of variability and various technical challenges that we will overcome by using the *ProcartaPlex ImmunoAssays.*

Future experiments will also focus on extending our approach to *in vivo* experiments, consisting of injecting the rats with IL37 to evaluate its efficiency in preventing atrial fibrosis and AF inducibility in PAB rats compared to sham.

The *in vivo* model will provide a new avenue to study the activation of M1 and M2 macrophages at different stages of the disease (ex: day 7 post-surgery, day 14 post surgery…) to evaluate whether IL-37 can modulate macrophage infiltration, recruitment, and polarization in the atrium from PAB compared to sham rats.

Although we evaluated known inflammatory markers involved in AF inducibility. Further investigations would consist of studying other pro and anti-inflammatory cytokines (i.e.: IL4, IL-17A, IL10) to better elucidate the cellular mechanisms and inflammatory pathways that may lead to, or prevent, atrial fibrillation and fibrosis in RHD. This approach will contribute to discovering more efficient targets to ultimately develop new treatments and therapeutic strategies.

6. Perspectives

In the pursuit of advancing our understanding of the mechanisms of atrial remodeling, AF, and the development of new treatments, this study aims to unveal new experimental and therapeutic strategies. This includes conducting *in vivo* experiments involving direct injection of the IL-37 treatment to experimental models of AF, including rats or mice. Such *in vivo* investigation could provides valuable insights on the efficacy and safety of IL-37 in preventing inflammation-induced arrhythmogenic substrate. Furthermore, given the higher incidence of AF complications in elderly females, and the differential impact of Pulmonary Artery Banding on each gender, we recognize the importance of comparing gender-specific responses to inflammatory and anti-inflammatory treatments. Additionally, to go deeper in elucidating the mechanistic role of inflammation in AF incidence, we can evaluate the impact of IL6 and IL37 on the atrial contractility levels by studying the effect of IL-6 and IL-37 on isolated cardiomyocytes.

Conclusion

In summary, PAB initiates a cascade of hemodynamic alterations, resulting in increased afterload on the right ventricle, and subsequent modifications in cardiac morphology and function. Chronic pressure overload requires adaptive changes, including concentric ventricular and atrial hypertrophy and dilation. Over time, loss of cardiomyocytes initiates the immune responses, activates FBs proliferation and secretory activity, and promotes excess fibrotic tissue deposition. This deleterious cascade contributes to disrupting cardiac cells connectivity, and predisposes individuals to cardiac arrhythmias, including atrial fibrillation.

Current therapeutic approaches for AF predominantly target symptoms, primarily focusing on heart rate regulation. Nevertheless, a potential avenue for more comprehensive and effective interventions lies in addressing the underlying mechanisms of AF.

Our study supports the idea that modulating the activation of inflammation and the production of atrial fibrosis, using IL-37 treatment, may contribute to attenuating AF vulnerability in RHD.

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Appendix

Review article

Right Heart Disease-induced Failed Resolution Mechanisms promote Chronic Inflammation, Fibrosis and Arrhythmias.

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Abstract: Inflammation is a complex program of active processes characterized by the well-orchestrated succession of an initiation and a resolution phase aiming to promote homeostasis. When the resolution of inflammation fails, the tissue undergoes an unresolved inflammatory status which, if remains uncontrolled, can lead to chronic inflammatory disorders due to aggravation of structural damages, development of fibrous area, and loss of function. Various human conditions show a typical unresolved inflammatory profile. Inflammatory diseases include cancer, neurodegenerative disease, asthma, chronic obstructive pulmonary disease, atherosclerosis, myocardial infarction, or atrial fibrillation. New evidence has started to emerge on the role and pro-resolution involvement of chemical mediators in the acute phase of inflammation. Although flourishing knowledge is available about the role of specialized pro-resolving mediators in neurodegenerative diseases, atherosclerosis, obesity, or hepatic fibrosis, little is known about their efficacy to combat inflammation-associated arrhythmogenic cardiac disorders. It has been shown that resolvins, including

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RvD1, RvE1, or Mar1, are bioactive mediators of resolution. Resolvins can stop neutrophil activation and infiltration, stimulate monocytes polarization into anti-inflammatory-M2-macrophages, activate macrophage phagocytosis of inflammation-debris and neutrophils to promote efferocytosis and clearance. This review aims to discuss the paradigm of failed-resolution mechanisms (FRM) potentially promoting arrhythmogenicity in right heart disease-induced inflammatory status.

Keywords: Inflammation; Fibrosis; Resolution; Right Heart Disease; Atrial Fibrillation

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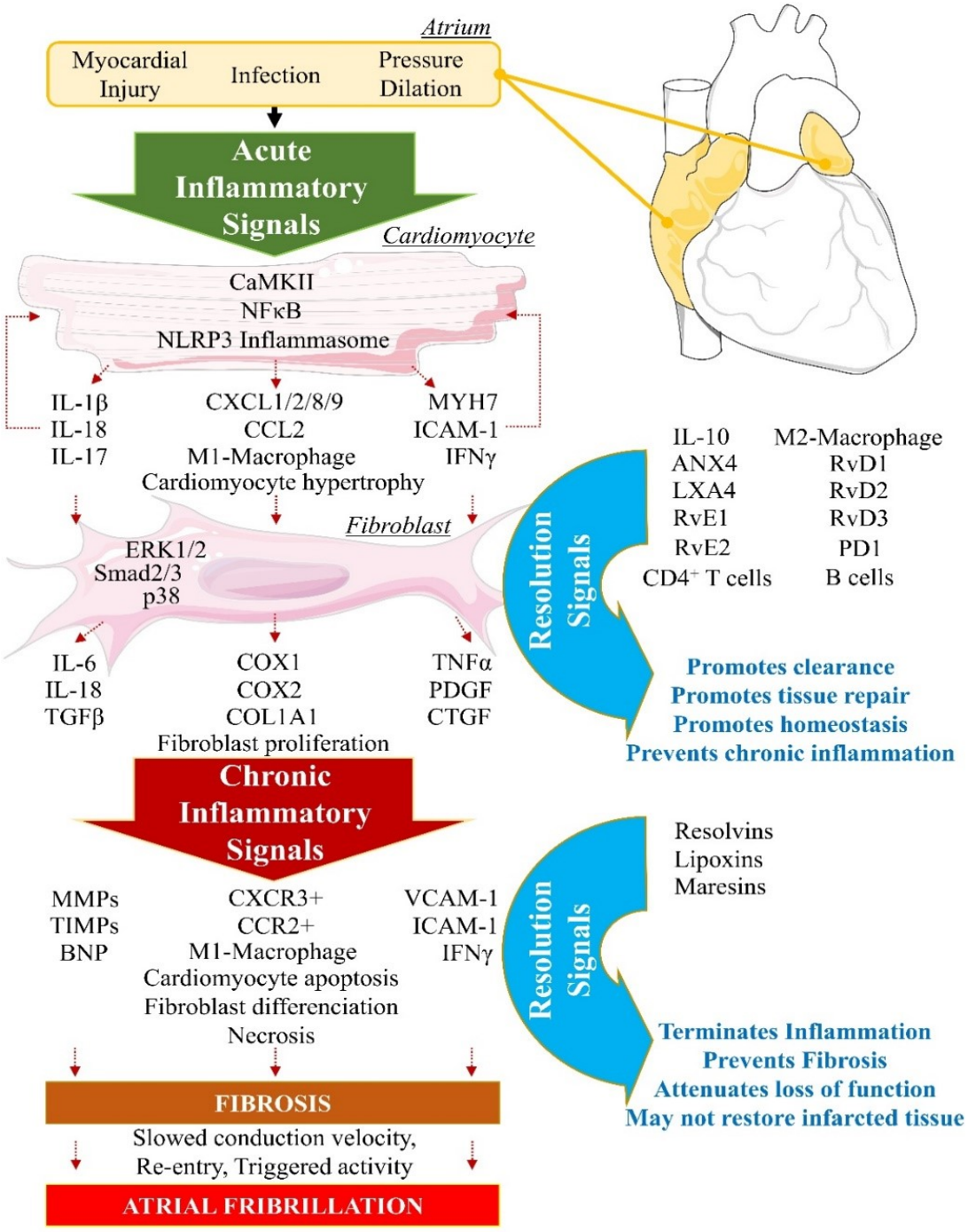
Highlights

- Initiation of inflammation is required to combat cardiac insults and injury.
- Arrhythmogenic events may include inhibition of bio-molecularly active processes of lipid-mediator class-switching and resolution.
- Future therapeutic strategies targeting cardiac inflammation must consider the complex equation of not inhibiting the required initiation-processes of inflammation while promoting resolution mechanisms.

Abbreviations

AF	**:**	Atrial Fibrillation
CM	**:**	Cardiomyocyte
ECM	**:**	Extracellular Matrix
FB	**:**	Cardiac Fibroblast
FRM	**:**	Failed Resolution Mechanisms
IL	**:**	Interleukin
MI	**:**	Myocardial Infarction
RA	**:**	Right Atrium
RHD	**:**	Right Heart Disease
SPMs	**:**	Specialized Pro-Resolving Mediators



Graphical Abstract. Biomolecular Orchestration of Cellular Events from Cardiac Insult to Resolution Opposed to Arrhythmogenic Chronic Inflammation. Longstanding exposure of the right and left atrium (RA and LA) to myocardial injuries, infections or chronic pressure and dilation provokes the normal initiation of acute inflammation. In cardiomyocytes (CM), intracellular inflammatory response involves CamKII, NF-kB or NLRP3 inflammasome pathways activation, which contribute to CM deregulation of structural genes (*Myh7*), and secretion of proinflammatory

cytokines including interleukins (IL-1β, IL-18) and chemokines (CXCL, CCL), leading to promotion of M1-macrophage infiltration. Proinflammatory signals contribute to the activation of cardiac fibroblasts (FB). FB activate additional pro-inflammatory signals (TGFβ, TNFα, PDGF) provoking FB differentiation into myo-FB, aiming to promote repair and wound healing, if the resolution signals are properly activated in response to inflammation initiation. Resolution mediators, including IL-10, LXA4, D- and E-series resolvins, contribute to terminate M1-macrophages infiltration, facilitate M2-macrophages polarization and phagocytosis, while activating CD4+ T cells and B cells efferocytosis, leading to homeostasis. When Resolution fails to occur, inflammation is perpetuated via FB and myoFB secretion of chronic-inflammation-promoting mediators (MMPs, IFNγ, CXCR3+, M1-macrophages) leading to CM necrosis, and loss of function. Resolution signals can be promoted to limit chronic inflammation-induced damages. If failed resolution mechanisms persist, the myocardium is exposed to the development of fibrosis, slowed conduction velocity, triggered activity, re-entry and increased susceptibility to arrhythmias, including atrial fibrillation.

1. Introduction

Cardiac diseases, including atrial fibrillation (AF), the most common form of arrhythmia, are characterized by an unresolved inflammatory status [1,2,3]. In response to cardiac injury, apoptotic cardiomyocytes (CM) contribute to activate the inflammatory status regulated by pro-inflammatory signals released by cardiac cells and recruited inflammatory cells [4]. These events characterize the acute phase of inflammation aiming to promote wound cleaning and to start the healing process [5]. Resolution-promoting signals are then secreted to stop acute inflammation to allow the initiation of the resolution phase and the maintenance of homeostasis [6]. Cardiac fibroblasts (FBs) are sensitive to circulating and CM-originated inflammatory signals [7]. When resolution is successfully activated, pro-resolution processes promote FBs-secreted collagenous material to consolidate the extracellular matrix, compensate loss of apoptotic CM, and preserve the mechanical stability of the myocardium to protect the heart from rupture and failure [5,8]. In contrast, myocardial remodeling could become dangerous when the acute inflammatory period is prolonged and when the resolution response fails to occur [5,9]. This can lead to the switch into a chronic inflammatory status instead of resolving the inflammation [5,10]. Chronic inflammatory signals promote fibrotic tissue deposition, constituting a “stiff” layer on the myocardium [11]. Such fibrous zones are non-contractile and electrical insulator areas that disrupt the normal propagation of action potential causing conduction slowing, refractoriness and AF [12,13]. Pro-resolution therapeutic strategies are poorly described in the field of anti-arrhythmic drug-development and arrhythmia-management.

Among cardiac disorders with an important inflammatory impact, right heart disease (RHD) is a pathological condition in which the right ventricle (RV) suffers from a structural and electrical remodeling that strongly affects cardiac physiological functions [14]. Right heart structure, heart chambers, and circulatory system are vulnerable to morphological modifications that may result from hypertension-promoting cardiac conditions including pulmonary artery hypertension (PAH), chronic obstructive pulmonary disease (COPD) or pulmonary embolism [15]. Volume- and pressure-overload conditions associated with structural remodeling negatively impacts the cardiac function, particularly because of the induced inflammatory status and potentially resulting myocardial fibrosis in response to a chronic rise in blood pressure, myocardial tissue stretching, or myocardial injury [16]. In the RV and the right atrium (RA), electrical remodeling is at the origin of potential tachycardia and arrhythmias, including ventricular fibrillation or/and atrial fibrillation (VF and AF) [17,18]. In response to structural remodeling, pro-inflammatory cytokines, and chemokines such as IL-1β, IL6, IL18, TGF-β, or CXCL1/2 stimulate fibroblasts (FBs) differentiation into myofibroblasts (myo-FBs) associated with a gradual loss of function in the myocardium [19,20]. Events and conditions promoting the development of cardiac fibrosis in the atrial tissue are associated with arrhythmogenic structural and functional modifications promoting AF [3,21,22].

The current article aims to review: (I) the general biochemical paradigm orchestrating the active mechanisms of resolution and the relevance of the concept of failed resolution mechanisms (FRM) in cardiac disorders; (II) the clinical and experimental investigations who tried to understand the role of cardiac FBs in the different phases following cardiac injury including initiation of inflammation, resolution, chronic inflammatory phase, and arrhythmogenic cardiac remodeling including RHD; (III) the importance of considering FRM in the understanding and therapeutic management of hypertrophic cardiomyopathy and RHD associated to arrhythmogenic atrial remodeling. We finally discuss the relevance of novel molecular targets that could potentially help to switch chronic inflammation into resolution and homeostasis, to prevent cardiac arrhythmias and AF.

2. Biomolecular Paradigm of Active Resolution Mechanisms in the heart.

2.1. Initiation Phase of Inflammation: Central Regulatory Role of Arachidonic Acid.

During the initiation of acute inflammation, phospholipase A2 (PLA2) levels are increased at the site of injury [23]. PLA2 produces arachidonic acid (AA: 5, 8, 11, 14-eicosatetraenoic acid) by hydrolyzation of the sn-2 ester bond of cellular phospholipids [23]. Patients with coronary artery disease show increased levels of lipoprotein-associated PLA2 (Lp-PLA2) [24]. Elevated level of Lp-PLA2 has been suggested as an important risk factor of cardiovascular diseases [25]. Paradoxically, when Lp-PLA2 hydrolyzes platelet-activating factor (PAF), its enzymatic activity is associated with anti-inflammatory properties [26]. The underlying mechanisms governing this paradox will be discussed below.

2.1.1. Arachidonic Acid Metabolism by Cytochrome P450.

AA is an essential polyunsaturated fatty acid (omega-6 PUFA) that can interact with cytochrome P450 (CYP450) enzymes to undergo monooxygenation or epoxidation and produce hydroxyeicosatetraenoic acids (19- and 20-HETEs) and dihydroxyeicosatrienoic acid (diHETrEs) [27]. These molecules act as hormone-like autocrine and paracrine agents to promote vasoconstriction, vascular permeability, polymorphonuclear leukocytes (PMN), and proinflammatory (M1)-macrophages chemotaxis, and proinflammatory signaling [28](Figure 1A).

2.1.2. Arachidonic Acid Metabolism by COX1 and COX2.

AA can directly interact with COX1 and COX2 to produce prostaglandin H2 (PGH2), an intermediate metabolite that is converted into bioactive proinflammatory lipid mediators such as thromboxane A_2 (TXA_2), prostaglandin A_2 (PGA_2), PGB_2, PGE_2, PGI_2 (**Figure 1A**). These AA metabolites have been shown elevated in various cardiovascular conditions including hypertension, atherosclerosis, vasculopathy, and myocardial infarction [29]. AA-derived lipids mediate vasoconstriction, increase vascular permeability, and stimulate expression of proinflammatory chemokines (complement component [C]: C3b, C5a; chemokine C-X-C motif ligand 1 [CXCL1], CXCL2, CXCL8) and interleukins (IL1β, IL6, IL8, IL18, tumor necrosis factor alpha [TNFα]) to promote PMN and M1-macrophages chemotaxis and adhesion by increasing

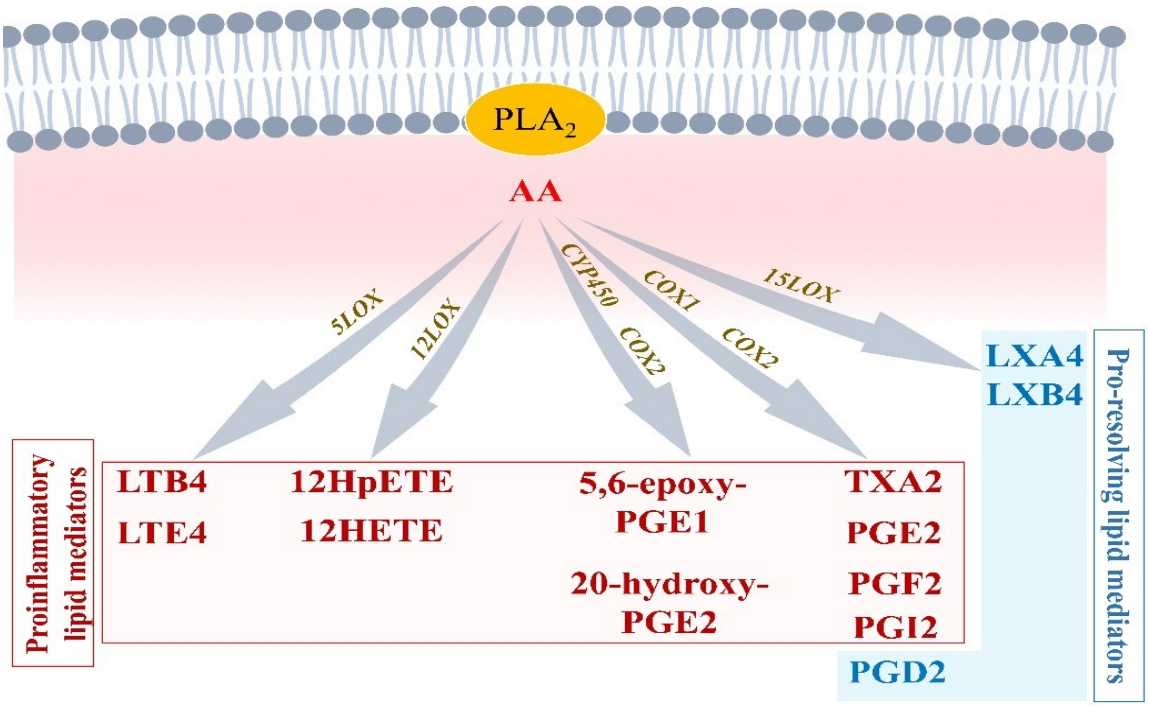
expression of intercellular adhesion molecule 1 (ICAM1), vascular cell adhesion molecule 1 (VCAM1) and e-selectin (SELE), which act on endothelial cells to promote the adherence of neutrophils to the blood vessels wall [29,30]. These inflammatory biomarkers have been described to promote the development and progression of cardiovascular diseases including cardiac arrhythmias and atrial fibrillation [11,12,22].

2.1.3. Arachidonic acid metabolism by 5-LOX.

AA can also interact with 5-LOX to produce 5-Hydroperoxyeicosatetraenoic acid (5-HpETE) which promotes vasoconstriction. 5-HpETE can be metabolized either by leukotriene (LT) C-synthase to produce LTC4, LTD4, and LTE4 or by LTA-hydrolase to produce LTB4 via LTA4, which are all leukotrienes playing proinflammatory properties by amplifying PMN and M1 macrophages influx in the injured tissue [31] (**Figure 1A**). HETEs have been shown to activate nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signaling to promote abnormal cardiomyocyte hypertrophy [32].

Evidence show that AA-derived metabolites' receptors are expressed on most cardiac cells including cardiomyocytes and fibroblasts [33]. In cardiomyocytes, inflammation signaling promotes NFκB activity and the assembling of the NACHT, LRR, and PYD domains-containing protein 3 (NLRP3) inflammasome leading to secretion of IL-1β and increased inflammatory status [34]. Patients with atrial fibrillation have shown increased expression of IL-1β and NLRP3 inflammasome components [35]. Normal initiation of inflammation must be followed by bio-molecularly orchestrated cellular processes aiming to terminate inflammatory state and promote resolution [36]. In this purpose, lipid-mediator class switching is a key event that could be defined as a transition phase between the end of acute inflammation and the beginning of resolution [37].

A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5LOX
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LXA4
LXB4
Pro-resolving lipid mediators
Proinflammatory lipid mediators
LTB4
LTE4
12HpETE
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20-hydroxy-PGE2
TXA2
PGE2
PGF2
PGI2
PGD2

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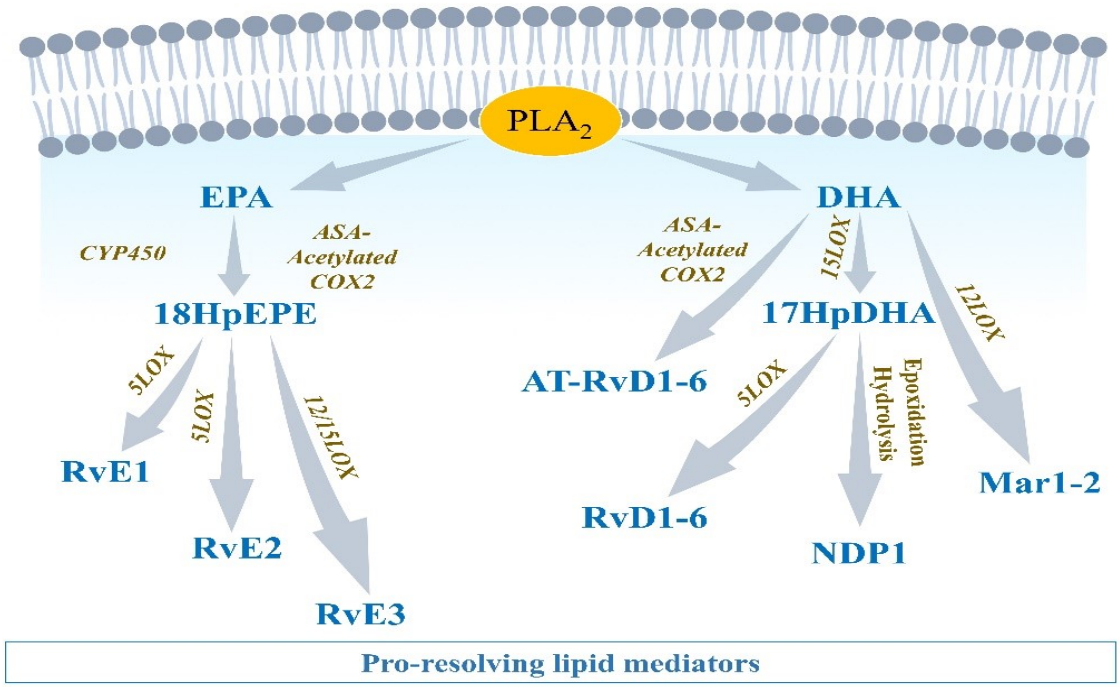
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ASA-Acetylated COX2
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17HpDHA
5LOX
15LOX
12/15LOX
AT-RvD1-6
5LOX
Epoxidation Hydrolysis
RvE1
RvE2
RvE3
RvD1-6
NDP1
Mar1-2
Pro-resolving lipid mediators

Figure 1. Arachidonic acid, Eicosapentaenoic acid and Docosahexaenoic acid-derived Lipid Mediators. [**A**] Arachidonic acid (AA) interaction with COX1, COX2, 5LOX, 12LOX, or CYP450 enzymes mainly leads to production of proinflammatory lipid mediators including leukotrienes, thromboxanes, and prostaglandins. AA interaction COX1/2 or 15LOX can generate pro-resolution mediators including PDG2, LXA4, and LXB4. [**B**] Eicosapentaenoic acid and docosahexaenoic acid compete with AA in interacting with COX1/2, 5LOX, 12LOX, and 15LOX. Lipids produced from EPA and DHA metabolism include E-series resolvins (RvE1-3) and D-series resolvins (RvD1-6) respectively, involved in pro-resolution mechanisms.

2.2. Lipid-Mediator Class Switching: Transition from Pro-Inflammatory to Pro-Resolution Signals.

During the initiation phase, neutrophils play intense apoptotic and phagocytic activity [38]. This activates intracellular accumulation and extracellular secretion of 12/15-LOX in the damaged tissue. This accumulation of 12/15-LOX activates lipid-mediator-class switching from proinflammatory to pro-resolution mediators [39]. AA is then enzymatically metabolized by 12/15-LOX into lipoxins, including lipoxin (LX) A4 and LXB4 (**Figure 1A**). LXA4 activates its transmembrane specific-receptor lipoxin A4 receptor or formyl peptide receptor 2 (ALX/FPR2) expressed on PMNs and macrophages to limit further leukocyte trafficking, stimulate monocyte recruitment, promote anti-inflammatory (M2)-macrophage polarization, and activate phagocytosis and elimination of debris [39]. LXA4 has been shown to be significantly decreased in patients with chronic heart failure [40]. Recent studies have shown that LXA4 attenuates myocarditis by inhibiting NFκB and PI3K/Akt signaling pathways, and 15-epi-LXA4 promotes initiation of resolution after myocardial infarction [41,42]. This activity of LXA4 suggests that, in arrhythmogenic conditions, anti-resolution signals promote the diminution of LXA4 production or/and LXA4-associated activity and signaling [42]. Promoting LXA4 could be an interesting candidate in the prevention of inflammation-induced substrate of arrhythmias including AF.

2.3. Resolution of Inflammation: SPMs-Mediated Efferocytosis and Homeostasis.

Along with AA, other essential n3PUFAs are delivered with edema fluids in the site of injury. Among them, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) compete with AA to be enzymatically metabolized by either CYP450, 5LOX, 12LOX, 15LOX, or aspirin acetylated COX2 [43]. Knowing that AA-derived metabolites are crucial in the initiation of normal inflammation,

and that EPA and DHA products are important in resolution, it is understandable that optimal healthy conditions must promote a fair balance between AA, EPA and DHA concentrations. In opposition to what has long been though, it is not recommended to completely annihilate AA-derived metabolites (i.e. by using COX inhibitors) to guaranty homeostasis [44] (**Figure 1** and **2**).

2.3.1. EPA-derived Specialized pro-resolving mediators.

EPA is metabolized by CYP450 or aspirin-acetylated COX-2 into 18-HpEPE (18R-hydroperoxy-5Z, 8Z, 11Z, 14Z, 16E-eicosapentaenoic acid) which itself can interact with either 5LOX to produce E-series resolvins (Rvs), RvE1 and RvE2 or 15LOX to produce RvE3. E-series Rvs activate specific receptors such as chemokine-like receptor 1 (CMKLR1), also known as chemerin receptor 23 (ChemR23) (receptor of RvE1) or antagonize proinflammatory leukotriene receptors, such as leukotriene B4 receptor 1 (BLT1), expressed on PMN cell membrane, to stop the expression of chemoattractants, limit neutrophils adhesion/infiltration, and promote phagocytosis of apoptotic neutrophils and efferocytosis [45] (**Figure 1B**).

2.3.2. DHA-Derived Specialized Pro-Resolving Mediators.

DHA can be metabolized by 12LOX to produce maresins (MaR1-2), or 15LOX to produce D-series resolvins (RvD1-6) and 67ôle67ving67ction D1 (NPD1) [46]. DHA interaction with aspirin-acetylated COX2 results in aspirin-triggered resolvins (AT-RvD1-6) which have been described to have similar properties as their classic homologs of the D-series Rvs [47] (**Figure 1B**). D-series Rvs activate specific receptors such as ALX/FPR2 (receptor of RvD1), G-protein coupled receptor 32 (GPR32: receptor of RvD1 and RvD3), and GPR18 (receptor of RvD2) that are expressed on vascular endothelial cells [37]. The activation of these signals promotes eNOS and P-ERK1/2 signaling, vascular permeability to non-phlogistic monocytes, cessation of PMN infiltration, macrophage polarization into M2-macrophages, M2-macrophages phagocytosis of cellular debris, and maintenance of homeostasis [45,37].

2.3.3. Arachidonic Acid-Derived Specialized Pro-Resolving Mediators.

The metabolism of AA by COX does not only generate proinflammatory components. PGD2 has been shown to play an important role in resolution of inflammation [48]. PGD2 interacts with prostaglandin-D2-receptor 1 and 2 (DP1/2) expressed on T helper type (Th2) cells and dendritic

cells that are involved in efferocytosis, phagocytosis, and clearance, to promote elimination of debris and pathogens, and induce complete resolution [48]. The interaction of AA with CYP450 can lead to production of epoxyeicosatrienoic acids (EETs) that are converted by soluble epoxide hydrolase (I) into dihydroxyeicosatrienoic acids (DiHETrEs). Although DiHETrEs are toxic, it has been shown that EETs mostly play beneficial role by promoting vasorelaxation, and cardioprotective effects [49] (**Figure 1A**).

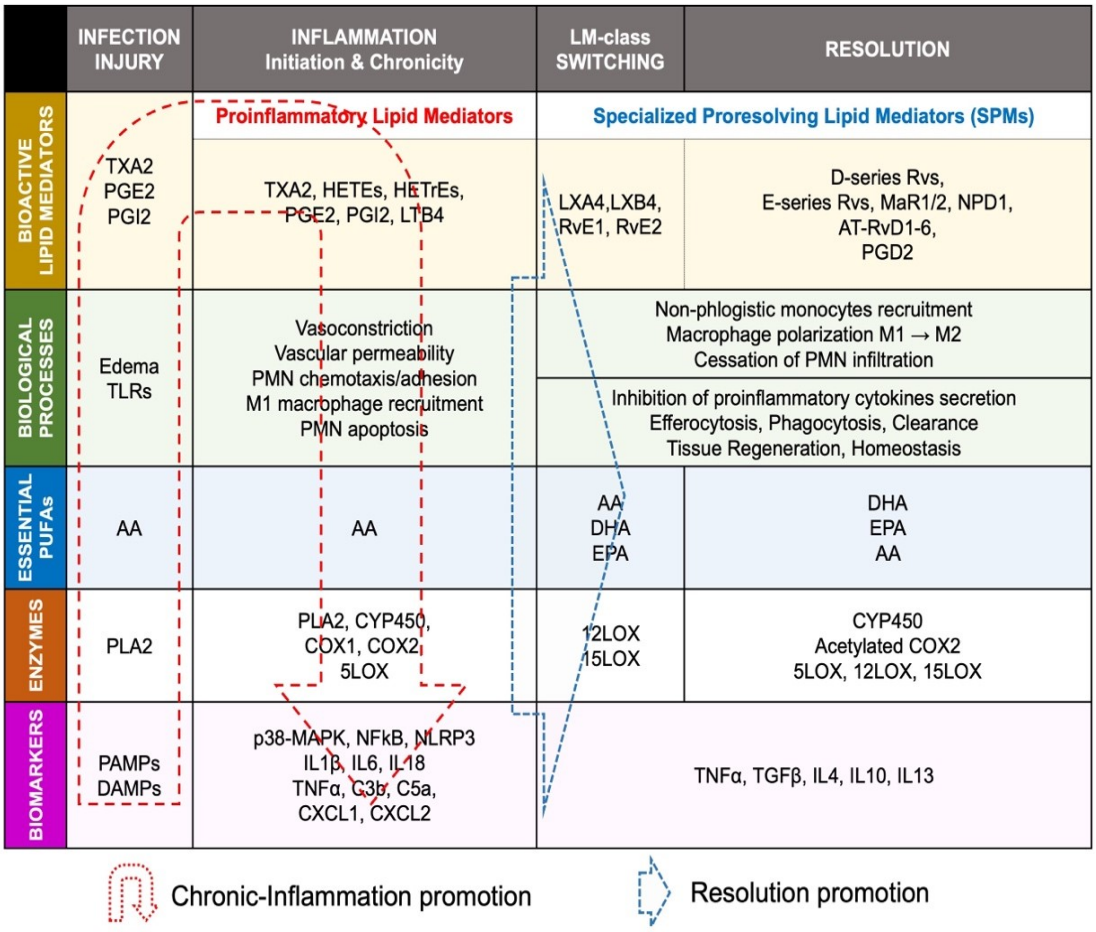


Figure 2. Categorization of Inflammation and Resolution-Promoting Agents. In response to pathogens or injury stimuli (PAMPs, DAMPs), phospholipase A2 (PLA2)-induced biosynthesis of arachidonic acid (AA) leads to production of proinflammatory lipid mediators including thromboxanes, leukotrienes, prostaglandins. Such events mark the initiation phase of acute inflammation characterized by PMN chemotaxis, pro-inflammatory-M1-macrophages recruitment, and enhanced proinflammatory signals (NLRP3 inflammasome, NF-κB, IL-1β, CXCL1/2). Phagocytic M1-macrophages release 12/15 LOX which promotes the activation of lipid-mediators (LM) class switching, where AA, docosahexaenoic acid (DHA) amd eicosapentaenoic acid (EPA) interact with 12LOX and 15LOX enzymes to be metabolized into specialized pro-resolving mediators (SPMs) including (PGD2 from AA, D-series resolvins from DHA, or E-series resolvins from EPA). SPMs promote anti-inflammatory (M2)-macrophage recruitment, inhibition of proinflammatory cytokines' secretion, termination of inflammation and regeneration of optimal functions. When the

resolution mechanisms fail to occur, inflammation is perpetuated. Inflammatory mediators are overexpressed, leading to persistence of cellular damages, necrosis, fibrosis, loss of function and cardiac vulnerability to arrhythmias and heart failure. Strategies promoting augmentation of pro-resolution mechanisms can potentially limit or eventually reverse some chronic-inflammation-induced cardiac disorders.

2.4. 'Failed Resolution Mechanisms' in the Development of Chronic Inflammation and Heart Diseases.

Lipid mediators (LM) production and signaling are fundamental in the regulation of the normal process of acute inflammation from its initiation to its resolution [46]. When the cardiac tissue undergoes chronic inflammatory status, the crucial phase of LM class switching, which promotes the end of PMN infiltration and the activation of efferocytosis, may have failed to promote the shift of the cellular and lipidic accumulation from proinflammatory to pro-resolution mediators in the injured tissue [39]. Pathologic failure in production of 12/15 LOX by immune cells including eosinophils, PMN, lymphocytes and macrophages, leads to lack of metabolization of AA into lipoxins (LXA4, LXB4) [38]. Lipoxins are essential to activate the cessation of neutrophil recruitment and the infiltration of non-phlogistic monocytes in the site of injury which is the first step of resolution [38,39]. Moreover, lack of 12/15LOX prevents the production of D-series Rvs from DHA and RvE3 from EPA [38,50]. This may contribute to annihilate resolution. Then, more proinflammatory LM (Prostaglandins, leukotrienes) are produced from AA enzymatic interaction with the other enzymes available (COX2, CYP450, 5LOX) [50]. Abnormal accumulation of proinflammatory signaling promotes the prolongation of the initiation phase, characterized by persistence of the external, cellular, and molecular signs of inflammation. This chronic inflammatory status leads to development of fibrosis and loss of function [40,50]. If the local production of 12/15LOX is restored or if bioavailability of resolvins and lipoxins is increased (from diet or endogenous biosynthesis) in the site of injury, the tissue may enter the resolution phase by the termination of proinflammation signaling, reduction of fibrosis, wound healing, and restoration of homeostasis [39,51,52] (**Figure 2**).

The detailed biomolecular characterization of inflammation-resolution remains partially understood in cardiac conditions. Moreover, each cardiac disease may display specific biomarkers involved in the incidence of the disorder. Although recent studies suggest a role of SPMs in ischemia-reperfusion [42,53] and pulmonary arterial hypertension-induced right atrial

arrhythmogenic substrate [54], further fundamental research and clinical studies are required to assess whether resolution promoting strategies and which cytokine therapies could be an efficient approach to prevent and treat cardiac diseases including hypertrophic cardiomyopathy, dilated cardiomyopathy, valvopathy, myocardial infarction, or arrhythmias.

3.Description of 'Failed Resolution Mechanisms' in Cardiac Arrhythmogenic Remodeling.

In cardiac arrhythmias, the available knowledge about the pathophysiology of ventricular or atrial fibrillation and their risk factors suggests that not only inflammation signals but also specific breakdown in the active resolution machinery that we define as "failed resolution mechanisms" (FRM), may play a key role in the occurrence and development of the arrhythmogenic substrate [20,22,55]. Although inflammatory cytokines seem involved in arrhythmogenic cardiomyopathy, pharmacological strategies targeting inflammatory and resolution systems are not standardized as antiarrhythmic medications [56]. Mounting evidence suggest that such therapeutic strategies may represent a promising avenue to explore in clinical management of cardiac arrhythmias [13,56].

3.1. FRM Associated with Cardiac Electrical Conduction Abnormalities.

Inflammatory cytokines have been reported to directly affect cardiac remodeling by promoting electrical changes early after initiation of inflammation [22,56]. If unresolved, inflammatory cytokines' release promote alteration of the CM transmembrane activity of inward depolarizing cation currents, including sodium current Ina, and calcium current IcaL [56,57]; and the perturbation of outward depolarizing potassium currents, including IK1, Ito, Ikr, Iks, IKACh, IKATP, Ikur [56,58]. Dysfunction of these ion channels is associated with abnormal action potential duration (APD) leading to myocardial refractoriness, promoting arrhythmogenicity [59]. In addition to electrical remodeling, inflammation signals promote gap-junctions' downregulation leading to lateralization and decreased expression of connexin (Cx) 40 and Cx43 [60]. These inflammation-induced channelopathies contribute to slowed conduction velocity promoting re-entry [53-60]. FRM-mediated chronic inflammation and CM remodeling promote abnormal intracellular Ca2+-handling due to pathological increase in Ca2+-loading, RyR2-opening, and/or dysfunctional sarco-endoplasmic reticulum Ca2+-ATPase (SERCA) activity contributing to ectopy

and triggered activity [61-63]. It is suspected that prevention of FRM and control of inflammation and fibrosis may play cardioprotective effects to preserve normal myocardial conduction properties and limit arrhythmia occurrence [54,63] (**Figure 3**).

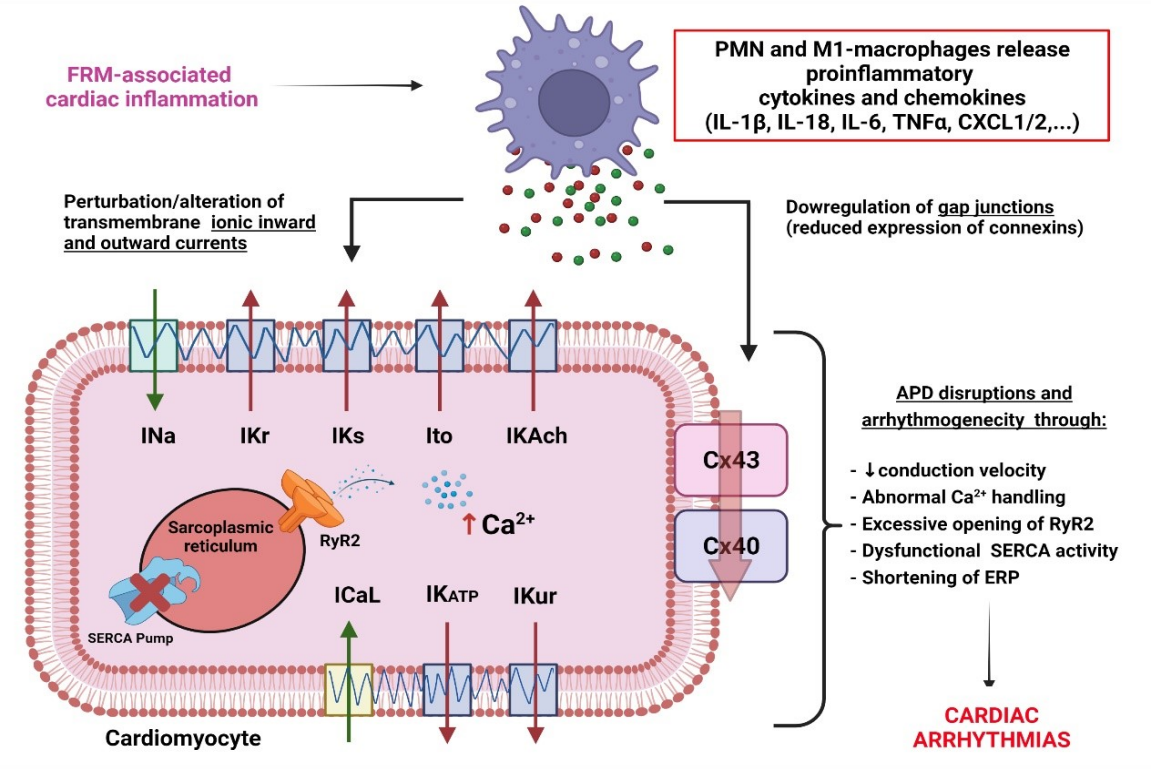


Figure 3. FRM-induced Arrhythmogenic Channelopathy in Cardiac Myocytes. In the myocardium, failed resolution mechanisms are associated with increased infiltration of polymophonuclear leukocytes (PMN), enhanced M1-macrophages phagocytic activity and augmentation of secretion of proinflammatory cytokines. Chronicity of proinflammatory signals provokes deregulation of genes coding for key ion channels and connexins involved in the establishment of cardiac action potential (AP) and conduction velocity. Inflammation-associated channelopathy induces: abnormal calcium-(Ca^{2+})-handling, effective refractory period shortening, reduced AP duration, slowed conduction, vulnerability to re-entry and triggered activity leading to increased risk of cardiac arrhythmias and fibrillation.

3.2. FRM Associated with Cardiac ECM Arrhythmogenic Structural Remodeling.

The extracellular matrix (ECM) is a complex network consisting of glycoproteins, proteoglycans, and glycoaminoglycans including fibers, collagen, fibronectin, laminin, and elastin, surrounding cardiac cells to provide structural support and strength [64]. FBs perform important secretory activity to maintain the integrity and regulation of the ECM [65]. After the initiation of inflammation, resident FBs are activated and recruited to the site of injury to initiate reparative processes [64,65]. Inhibitors of metalloproteinases are activated to limit acute inflammation and

protect newly synthetized ECM from degradation [66]. When resolution and termination of inflammation fail, FRM consist in persistence of inflammatory signaling promoting FBs differentiation into myo-FBs by acquiring various phenotypic changes, including higher cytoplasmic volume, increased microfilament bundles and upregulated αSMA filament expression [67]. Myo-FBs can secrete further ECM components contributing to build-up the myocardium structure and compensate disease-induced CM necrosis. In this context, myo-FBs are also able to provide contractile force, stiffening of the ECM and expansion of fibrotic area [68]. At later stage of cardiac remodeling, secreted collagen is subjected to cross linking to consolidate the scar [69]. When healing processes fail to promote neither regeneration of the tissue nor repairment, chronicity of inflammation and FRM lead to chronic wound formation characterized by upregulation in the expression of lysyl-oxidase and pro-fibrotic signaling responsible for the maturation of the scar associated with outrageous degradation of the ECM [70,71] (**Figure 4**).

Chronic inflammation-induced loss of myocardial thickness is associated with cardiac loss of function and aggravation of cardiac disease [72]. FRM-associated degradation of ECM and CM-death is a major challenge in cardiac disease management [73]. It is unclear whether specialized pro-resolving mediators could regenerate wounded myocardium. Further studies investigating the impact of resolvins after irreversible scar formation in ischemic cardiac disorders are required to assess or not their potential regenerative effects and efficiency (**Figure 4**).

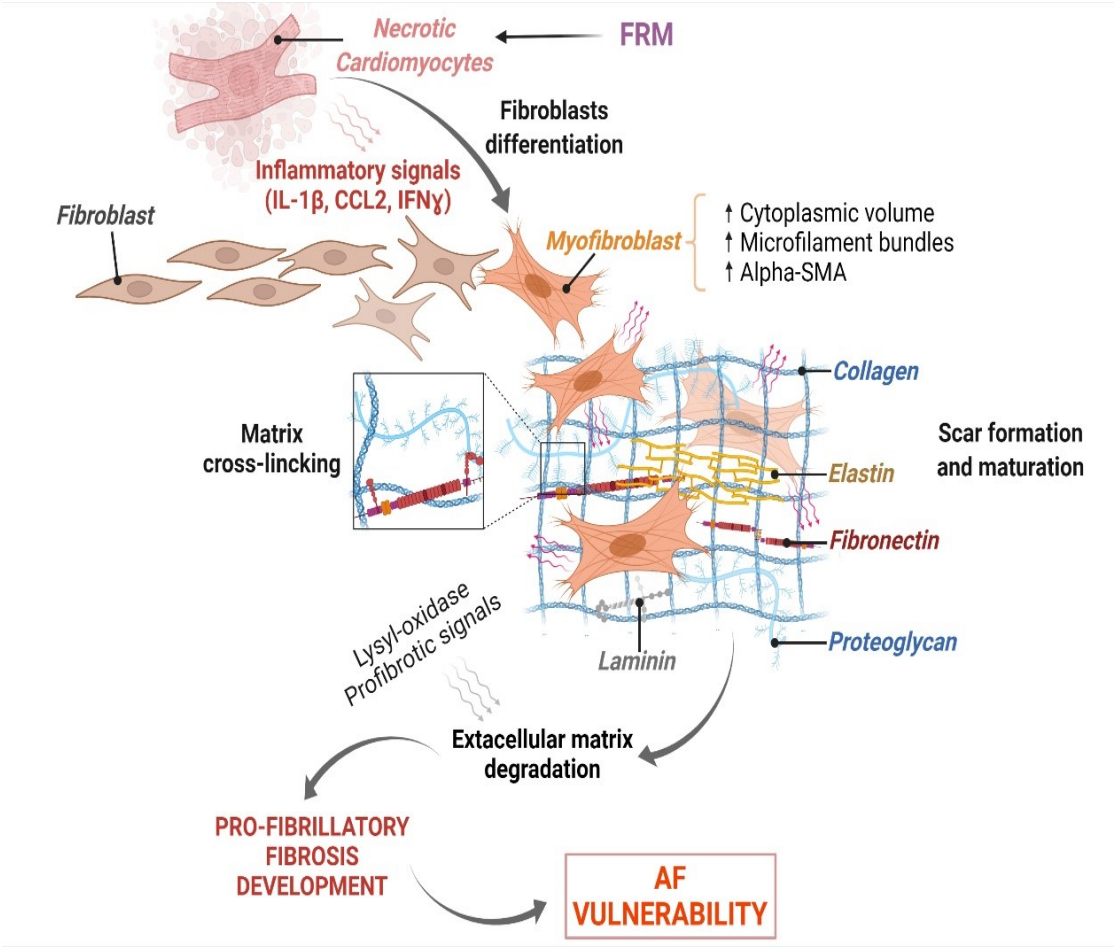


Figure 4. Cardiomyocytes and Fibroblasts' Orchestration of Arrhythmogenic Extracellular Matrix Degradation. During acute inflammation, failure in the occurrence of resolution mechanisms (FRM) promotes exacerbation of inflammatory signals generated from apoptotic cardiac cells and activated cardiac fibroblasts, leading to aggravation of myocardial damages. The extracellular matrix (ECM) is intensively remodeled and degraded promoting persistence of fibrosis, scar formation, abnormal cardiac function and increased risk of arrhythmias including atrial fibrillation.

3.3. FRM Associated with Abnormal Cardiac Fibroblasts' Remodeling and Atrial Fibrosis.

Cardiac FBs are sensitive to cardiac immune response to injury via close interactions with activated inflammatory cells and the tightly regulated healing process initiated to limit tissue damage [69]. FBs are involved in the activation of reparative processes essential to preserve the proper structure and function of the heart [66,69]. Longstanding and uncontrolled inflammation can lead to FRM, cardiac remodeling, congestive heart failure, cardiac dysfunction, arrhythmia, and sudden death [54,72].

3.3.1. Fibroblast Response to Inflammation Initiation.

Injury-induced degradation of ECM generates damage-associated molecular patterns (DAMPs) [74]. Heart injury disrupts cardiomyocytes' cellular mem.brane and leads to the release of inflammatory cytokines [74]. The binding of DAMPs to their specific pattern recognition receptors (PRR) present on leukocytes, macrophages, endothelial cells, and resident FBs initiates the innate immune response by the activation of a cascade of intracellular proteins which culminate in the activation of transcription factors such as NF-κB [75]. This molecule is then able to translocate to the nucleus to initiate the transcription of genes involved in the inflammation and immune response, such as cytokines (Il-6, Il-18), chemokines (Cxcl-1, Cxcl-2), and adhesion molecules (Icam-1, Vcam-1) to induce the recruitment, activation, differentiation of inflammatory cells, and upregulate the FBs expression of proteases to degrade dead cellular debris [76]. If perpetuated these changes contribute to the differentiation, proliferation, and migration of FBs leading to the development of fibrosis contributing to loss of cardiac function and arrhythmogenicity [13,22,76] (**Figure 5**).

3.3.2. FBs-induced Expression of key FRM-Promoting Biomarkers.

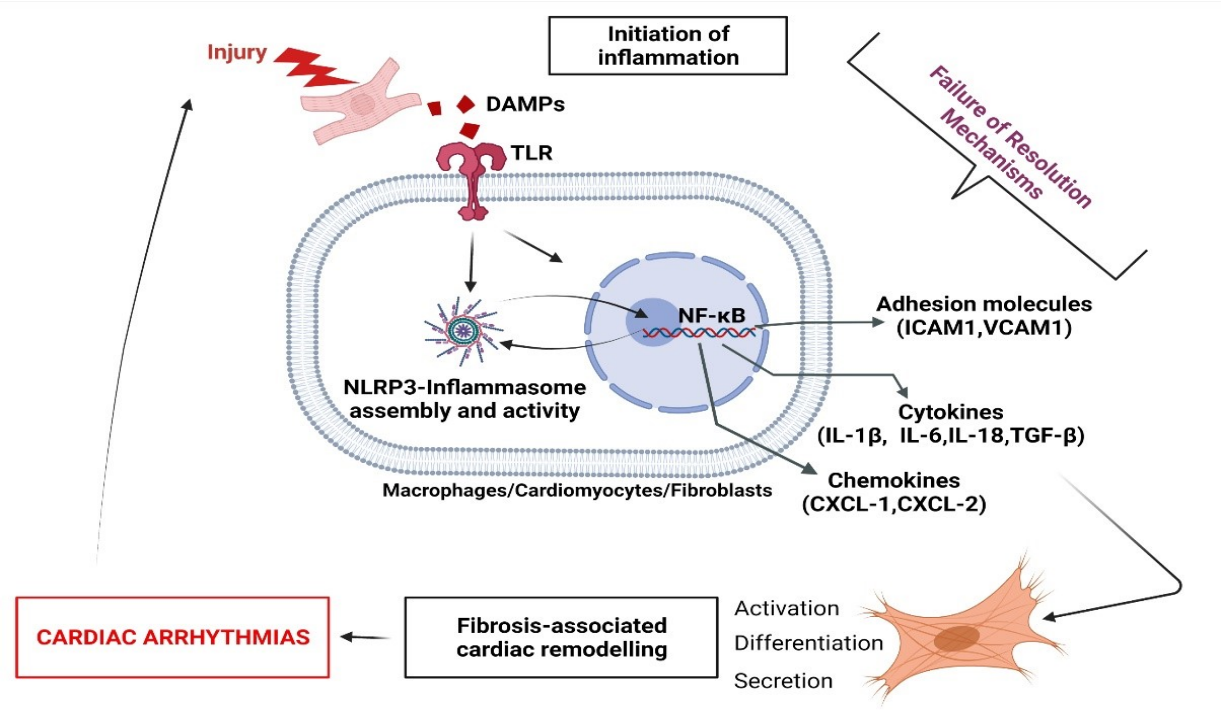
NLRP3 inflammasome. Chronic inflammation is suspected to be responsible for the development and progression of various cardiac diseases including coronary arterial disease (CAD), myocardial infarction (MI), valvulopathy, and cardiac arrhythmias [77,78]. Mounting evidence suggest that the NLRP3 inflammasome plays a central role in modulating chronic inflammation and aggravation of heart disease progression [35,79,80]. In patients with acute myocarditis, initiation of inflammation is associated with activation of the inflammasome in PMN, fibroblasts and cardiomyocytes, correlating with the severity of HF [81]. In myocardial ischemia, the inflammasome aggravates tissue injury and promotes cardiac failure while absence or inhibition of the NLRP3 inflammasome leads to improvement of cardiac function in preclinical studies [82]. In the CANTOS trial, inhibition of IL-1β maturation was efficacious in secondary prevention for cardiovascular events in patients with previous MI [83]. Genetic deficiency of NLRP3 was associated with reduced expression of proinflammatory cytokines (TNFα, IL1β, IL6), reduced secretion of proinflammatory PGE2 and LTB4, and increased expression of LXA4 and LXB4, which

suggests that presence of NLRP3 may negatively influence the LM-class switching and promote FRM during inflammation progression [84] (**Figure 2 and 5**).

Interleukin-1β. IL-1β secreted by leukocytes plays an important role in the activation of inflammatory and fibrogenic pathways during the healing process [85]. IL-1β is a contributor to the pathogenesis of cardiac remodeling through the induction of inflammatory mediators' synthesis by activated leukocytes [86]. In a study performed on IL-1R-/- knock-out mice (not expressing IL-1β receptor) subjected to coronary occlusion/reperfusion, the immunohistochemical staining of the infarct area with anti-alpha-smooth muscle actin (αSMA) antibodies, anti-macrophages and anti-neutrophils showed a reduced quantity of infiltrating immune cells and myofibroblasts [87]. Moreover, the authors observed that animals not expressing IL-1β receptor had lower levels of cytokines and chemokines secreted compared to wild type animals [87]. Furthermore, it has been shown that administration of an anti- IL-1β neutralizing antibody, in the acute phase of non-reperfused murine MI, resulted in reduced collagen accumulation in the scar and attenuated adverse remodeling [87]. Finally, IL-1R-/- mice had lower FBs-induced secretion of metalloproteinases (MMP-2, MMP-3) [87]. These data suggest that IL-1β is a crucial agent in FRM and fibrosis development – a deleterious event to combat in arrhythmia prevention.

Interleukine-6. In myocardial injury, IL-6 is considered as key regulator of cardiac FBs differentiation and FBs-induced release of inflammatory factors implicated in amplification of the inflammatory response and tissue remodeling [88]. In affected areas, when IL-6 binds to its specific receptor (IL-6R), it activates Hyaluronan (HA) synthase (I1, HAS2) responsible for the formation of HA-rich environment that provides strength, lubrication, and hydration within the ECM, while regulating FBs motility, proliferation, and differentiation [89,90]. In mice subjected to MI induced by LAD surgery (ligation of the left anterior descending coronary artery), antibodies against IL-6 inhibited the expression of HAS1 and HAS2 [91]. Moreover, it has been shown that the presence of αSMA positive cells in the border zone of the infarct was markedly reduced in mice pretreated with blocking IL-6 antibodies [91]. IL-6 binding to glycoprotein (GP130) on the surface of FBs result in a downstream phosphorylation of STAT3 (signal transducer and activator of transcription 3) that moves to the nucleus and activates the transcription of HA-synthase to

initiate the formation of HA. HA binding to its receptor CD44 on FBs' surface contributes to the release of proinflammatory molecules such as chemokine ligand CCL5 and monocyte



chemoattractant protein 1 (MCP1: also known as CCL2) and promotes myo-FBs phenotype – a FRM, profibrosis and proarrhythmogenic phenomenon [91].

Figure 5. Central Role of Unresolved Fibroblasts-associated Inflammatory Status in the Development of Arrhythmogenic Cardiac Fibrosis. During acute inflammation, the initiation of inflammation is normally followed by resolution, to promote homeostasis. Failure in the activation of pro-resolving signals contributes to exacerbate cardiac cells' release of proinflammatory stimuli. Fibroblasts are highly sensitive to inflammatory agents. When activated cardiac fibroblasts participate in increasing the production of inflammatory compounds leading to development of fibrosis, myocardial dysfunction and arrhythmogenicity.

Transforming Growth Factor-Bêta. TGF-β is expressed by macrophages and cardiac FBs [92]. TGF-β binds to its receptors TBRI and TBRII to initiate non-canonical pathways by the activation of p38 kinase in the cell which in turn can activate the serum response factor (SRF) in the nucleus, to start the transcription and upregulation of TRPC6 (the transient receptor potential cation channel, subfamily C, member 6) [93]. TRPC6, located on the surface of the cell facilitates calcium (Ca^{2+}) entry, leading to the activation of calcineurin (I) that enhances the myofibroblastic phenotype conversion by NFAT (Nuclear factor of activated T-cells) [94]. Studies have demonstrated that

mice infected with recombinant adenovirus expressing TRPC6, shown increased levels of αSMA, augmented activation of FBs, and enhanced expression of fibronectin (FN) domain ED-A [95]. In addition, TRPC6 knock-out in murine model of MI mice showed a lower count of FBs conversion into myofibroblasts associated, however, with a higher rate of mortality due to ventricular rupture because the scar formed was smaller compared to wild type mice [95]. These data suggest that events promoting TGF-β-induced FBs differentiation into myo-FBs contribute to FRM and promote development of arrhythmogenic fibrosis [95].

4. Arrhythmogenic FRM in the Context of Right Heart Disease.

4.1. Generalities on RHD-Induced Arrhythmogenicity.

Right ventricular (RV) hypertrophy is often accompanied by ventricular and/or supraventricular arrhythmias [14,17]. When not monitored, RHD associated with electrophysiological and hemodynamic pathologies could lead to heart failure, stroke, or sudden death [14-16]. PAH, COPD, obesity, and obstructive sleep apnea (OSA) are conditions inducing RV hypertrophy while also elevating the risk of AF [17,96-99]. Indeed, the chronic and deleterious rise of pressure and volume in the RV can induce tricuspid annulus plane deformation, and tricuspid regurgitation leading to direct structural and functional effects on the right and the left atrium (RA and LA) [15,16,99]. Longstanding exposure to elevated pressure and volume leads to RA and LA tissular stretch and dilation [96-99]. Prolonged pressure/volume overload activates inflammatory stimuli produced in response to atrial structural remodeling [100]. Atrial FBs play a crucial role in RHD-induced atrial remodeling, following the above-described mechanisms in section 3.3 [101]. Myo-FBs differentiation contribute the formation of atrial fibrotic tissue. Fibrosis-induced atrial remodeling includes perturbation of electrical circuits associated with AF [101,102](**Figure 3, 4 and 5**).

4.3. Resolution-Promoting Strategies in Monocrotaline and Sugen-Hypoxia Models of RHD and FRM Associated with Cardiac Arrhythmias.

Conditions affecting the right heart have the potential to trigger the chronic development of atrial fibrosis and AF [102]. Various experimental models have been using animals to better understand the underlying mechanisms of AF. Respectively, the choice of studying AF in rodent, dogs, pigs,

ewe, or horses have specific perks, advantages, and limitations [103]. RHD-induced cardiac arrhythmias have mostly been studied using models of provoked pulmonary embolism or pulmonary artery occlusion [101,104-106]. Technically, different approaches can be utilized to mimic or experimentally re-create pulmonary obstruction and sustained pressure overload-induced RV failure [107]. Among well described methods we found monocrotaline (MCT) [108], chronic hypoxia chambers [109], sugen-induced hypoxia (SuHx) [110], pulmonary artery banding [111,112], or chronic thromboembolic pulmonary hypertension [113].

Available knowledge about FRM-induced arrhythmogenicity is limited in RHD-induced models of myocardial remodeling. Here we report very novel studies that have specifically explored the effect of resolution-promoting molecules in animal models of arrhythmia only in the context of RHD. These investigations were performed in rodent's models of RHD induced by MCT and SuHx.

Monocrotaline. Monocrotaline (MCT) injection have been widely used to induce pulmonary arterial hypertension and right-sided cardiac hypertrophy and dilation in rats [107]. The ventricular arrhythmogenic aspects of MCT-induced RV hypertrophy has been described in rats [105]. Recently, it has been shown specialized pro-resolution treatment with RvD1 can attenuate atrial fibrous content and reduce AF inducibility in rats with MCT-induced RHD and atrial dilation [101]. In 2022, Tianyou Qin and collaborators have shown that administration of Dapaglifozin, a sodium-glucose cotransporter 2 (SGLT2) inhibitor, significantly decreased ventricular fibrosis and attenuated NFκB activity while preventing ventricular arrhythmias and VF vulnerability [114] (**Table 1**). These investigations consolidate the idea that atrial and ventricular arrhythmias associated with RHD could be prevented via prevention of FRM. More studies are required to evaluate the impact of pro-resolution biomarkers promoting anti-arrhythmogenic effects in RHD.

Sugen Hypoxia. Sugen (SU5416), an antagonist of vascular endothelial growth factor receptor 2 (VEGFR2), is used to induce pulmonary hypertension and RHD in rodents [115]. Sugen hypoxia (SuHx) has been shown to increase VF inducibility in rats [116]. Treatment with relaxin (RLX), a heterodimeric polypeptide hormone member of the insuline-like superfamily, prevented ventricular fibrosis and VF vulnerability in SuHx-induced RHD-rats [116]. RLX also prevented atrial fibrosis and AF in a rat model of spontaneous hypertension [117]. Anti-arrhythmogenic effects of RLX treatment was associated with decreased expression of TGF-β, matrix metalloproteases 2

and 9 (MMP2 and MMP9), collagenases 1 and 3 (COLI, COLIII) [116,117], suggesting that FRM occurring in cardiac hypertensive disorders could be targeted in the management of cardiac arrhythmias (**Table 1**).

Experimental models of RHD	Resolution strategies	Anti-Arrhythmogenic Effects	References
Monocrotaline (MCT)	Resolvin D1 (RvD1) (*i.p.: 2 µg/kg/d; 3w*)	• ↓ Atrial fibrosis • ↓ Expression of IL6, TGF-β, ICAM1, IL1β NLRP3-inflammasome • ↑ Expression of IL10, CHEMR23 • ↓ AF susceptibility	Hiram et al., 2021 [54]
	Dapagliflozin (DA) (*per os: 60 mg/L; 4w*)	• ↓ Ventricular fibrosis • Prevented channelopathy • ↓ TLR4 and NFκB activity • ↓ VF vulnerability	Qin et al., 2022 [114]
Sugen-Hypoxia (SuHx)	Relaxin (RLX) (*sc.: 30-400 µg/kg/d; 6w*)	• ↓ NRF2 and gluthathione transferase • ↓ Expression of TGF-β, MMP2, MMP9, COLI and COLIII • ↓ Ventricular fibrosis and VF • ↓ Atrial fibrosis and AF	Martin et al., 2021 [116] Parikh et al., 2013 [117]

Table 1. Recent Resolution strategies in experimental models of RHD. Review of recently tested resolution strategies in the treatment of arrhythmogenic structural and electrical remodeling resulting from severe pulmonary hypertension generated by MCT and SuHx in rats. RvD1 has been show to significantly reduce atrial fibrous content while decreasing AF vulnerability in MCT rats. Dapgliflozin prevented ventricular arrhythmias via reduces NFκB activity in MCT rats. Relaxin prevented atrial and ventricular fibrosis and fibrillation, and reduced the expression of notoriously proinflammatory cytokines and proteins. **Abbreviations**: **AF**: Atrial Fibrillation; **CHEMR23**: chemerin receptor 23; **COL**: Collagenase; **DA**: dapaglifozin; **ICAM1**: intracellular adhesion molecule 1; **IL**: interleukin; **MMP**: Matrix Metalloproteinase; **MCT**: monocrotaline; **NLRP3**: NACHT, LRR, and PYD domains-containing protein 3; **NFκB**: Nuclear Factor kappa B; **NRF2**: nuclear factor erythroid-derived 2; **RLX**: relaxin; **RvD1**: 79ôle79ving D1; **SuHx**: Sugen-Hypoxia; **TGF-β**: Transforming Growth Factor Beta; **TLR4**: toll-like receptor 4; **VF**: Ventricular Fibrillation.

5.Conclusions

Chronic inflammation is a consequence of inflammatory-response machinery's failure to switch from initiation phase of inflammation into the active resolution phase mediated by specialized pro-resolving lipid mediators (SPMs). Among other mechanisms, AA, DHA and EPA-derived SPMs

expressed by peripheral blood leukocytes directly target efferocytosis promotors to prevent FRM and chronic inflammatory status. Clinical evidence demonstrating decreased plasmatic levels of RvD1 in patients with chronic heart failure consolidate the concept of 'Failed Resolution Mechanisms' in progressive cardiac diseases including RHD and AF. SPMs are potential strong therapeutic candidates able to promote resolution in inflammation-associated arrhythmogenic cardiac disorders.

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