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# Resolvin E1 attenuates doxorubicin-induced cardiac fibroblast senescence: A key role for IL-1β

Jenaro A. Espitia-Corredor [a,b,c,d,1], Licia Shamoon [b,c,e,1], Francisco Olivares-Silva [d], Constanza Rimassa-Taré [a], Claudia Muñoz-Rodríguez [d], Claudio Espinoza-Pérez [a], Carlos F. Sánchez-Ferrer [b,e,*], Concepción Peiró [b,e,*], Guillermo Díaz-Araya [a,d,**]

[a] Laboratorio de Farmacología Molecular, Department of Pharmaceutical and Toxicological Chemistry, Faculty of Chemical Sciences and Pharmacy, Universidad de Chile, Santiago, Chile
[b] Department of Pharmacology, Faculty of Medicine, Universidad Autónoma de Madrid, Madrid, Spain
[c] Ph.D. Programme in Pharmacology and Physiology, Doctoral School, Universidad Autónoma de Madrid, Madrid, Spain
[d] Advanced Center for Chronic diseases (ACCDiS), Faculty of Chemical Sciences and Pharmacy, Universidad de Chile, Santiago, Chile
[e] Instituto de Investigaciones Sanitarias (IdiPAZ), Madrid, Spain

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ABSTRACT

Cardiac fibroblasts (CFs) undergo senescence in reaction to different stressors, leading to a poor prognosis of cardiac disease. Doxorubicin (Doxo) is an antineoplastic drug with strong cardiotoxic effects, which induces IL-1β secretion and thus, triggers a potent pro-inflammatory response. Doxo induces CFs senescence; however, the mechanisms are not fully understood. Different pharmacological strategies have been used to eliminate senescent cells by inducing their apoptosis or modifying their secretome. However, Resolvin E1 (RvE1), a lipid derivative resolutive mediator with potent anti-inflammatory effects has not been used before to prevent CFs senescence.

CFs were isolated from adult male C57BL/6J mice and subsequently stimulated with Doxo, in the presence or absence of RvE1. Senescence-associated β-galactosidase activity (SA-β-gal), γ-H2A.X, p53, p21, and senescence-associated secretory phenotype (SASP) were evaluated. The involvement of the NLRP3 inflammasome/interleukin-1 receptor (IL-1R) signaling pathway on CFs senescence was studied using an NLRP3 inhibitor (MCC950) and an endogenous IL-1R antagonist (IR1A).

Doxo is able to trigger CFs senescence, as evidenced by an increase of γ-H2A.X, p53, p21, and SA-β-gal, and changes in the SASP profile. These Doxo effects were prevented by RvE1. Doxo triggers IL-1β secretion, which was dependent on NLRP3 activation. Doxo-induced CFs senescence was partially blocked by MCC950 and IR1A. In addition, IL-1β also triggered CFs senescence, as evidenced by the increase of γ-H2A.X, p53, p21, SA-β-gal activity, and SASP. All these effects were also prevented by RvE1 treatment.

*Conclusion:* These data show the anti-senescent role of RvE1 in Doxo-induced CFs senescence, which could be mediated by reducing IL-1β secretion.

## 1. Introduction

The primary function of cardiac fibroblasts (CFs) in healthy and diseased hearts is extracellular matrix (ECM) homeostasis, and in cardiac tissue, excess ECM deposition leads to cardiac fibrosis [1]. CFs act as sentinel cells of cardiac tissue by responding to chemical, cellular and non-cellular signals, such as peptides, cytokines, and growth factors, and they also respond to drugs used for the treatment of different diseases [2].

Cellular senescence is the permanent arrest of cell cycle arrest that limits the growth and proliferation of mammalian cells and is paralleled by alterations in function, morphology, and gene expression. Senescent

* Corresponding authors at: Department of Pharmacology, Faculty of Medicine, Universidad Autónoma de Madrid, Calle Arzobispo Morcillo 4, C.P. 28029 Madrid, Spain.
** Correspondence to: G.D. -Araya, Department of Pharmaceutical and Toxicological Chemistry, Faculty of Chemical Sciences and Pharmacy, Universidad de Chile, Sergio Livingstone 1007, Santiago 8380492, Chile.
*E-mail addresses:* carlosf.sanchezferrer@uam.es (C.F. Sánchez-Ferrer), concha.peiro@uam.es (C. Peiró), gadiaz@ciq.uchile.cl (G. Díaz-Araya).
[1] These authors contributed equally to this study.

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cells exhibit an increase in senescence-associated β-galactosidase activity (SA-β-gal), up-regulation of several protein molecular markers (such as p53 and p21) [3], and increased senescence-associated secretory phenotype (SASP), which is mainly composed of cytokines, proteases, chemokines, and growth factors. SASP promotes a pro-inflammatory environment that, through a bystander effect, can propagate cell senescence to neighboring cells, thus amplifying the pro-senescence phenotype [4]. In cultured cells, senescence can also be induced by different types of stimuli, including stresses caused by culture shock, high serum content [5,6], oxidative stress with $H_2O_2$ [7], and anticancer drugs [8]. In the heart, CFs senescence is one of the main mechanisms that contributes to cardiac fibrosis. Furthermore, the accumulation of senescent CFs could create a cardiac environment that is suitable for adverse remodeling and gradual progression to heart failure [9–13].

Several anticancer drugs, including doxorubicin (Doxo), have shown adverse cardiac effects [14,15]. The complexities of how Doxo damages the heart and triggers a disease process that can last for decades in a person's life are not fully known. A largely unexplored area is the effect of Doxo on CFs, a key cell type in the cardiac injury response that regulates the homeostasis of the ECM and wound healing. Doxo induces cellular senescence and ultimately drives cells into a death pathway [8,16]. However, the cellular consequences of Doxo-induced stress in CFs, such as CFs senescence and the mechanism involved, are not fully understood. As previously reported, Doxo activates NLRP3 and induces IL-1β secretion in CFs [17], cardiomyocytes, and H9c2 cells [18]. Therefore, Doxo-induced cardiac effects could probably be IL-1β-dependent.

IL-1β is a potent cytokine, which signals through IL-1R to produce a strong pro-inflammatory effect and recruit myeloid cells, such as neutrophils, to the inflammation sites [19]. Previously, we have shown that CFs secrete IL-1β after LPS plus ATP treatment [20]. As a SASP component, IL-1β interacts with IL-1R, and activates the nuclear factor κB (NF-κB) signaling pathway, inducing transcriptional activation of other SASP components, such as IL-6, IL-8, and IL-1β thus magnifying the pro-inflammatory microenvironment [21,22].

Therefore, the role of IL-1β in identifying pharmacological targets against senescence and the possible deleterious effects of Doxo must be considered. In this context, full attention has been focused on mediators of inflammation resolution [23,24]. These include resolvin E1 (RvE1), which reduces pro-inflammatory responses and activates restorative pathways in various chronic inflammatory processes [23,24]. In particular, we have shown that pretreatment with RvE1 prevents the pro-inflammatory effects of LPS in CFs [25].

In this study, we sought to establish a foundation for further research focused on resolvins or other SPM in the prevention of cellular senescence, and in the role that they might play in healthy aging.

## 2. Materials and methods

### 2.1. Reagents

Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 culture medium (DMEM-F12, Ref: 17101-015, GIBCO™), type II collagenase (Ref: 17101-015, GIBCO™), and MTT (Ref: M6494, Invitrogen) were from Thermo-Fisher, USA. Fetal bovine serum (FBS) (Ref: 04-127-1A) and Penicillin, streptomycin, and amphotericin B solution (Ref: 03-033-1B) were from Biological Industries, Israel. RGB Plus Prestained Protein Ladder (Ref: 02102-250) was purchased from Maestrogen, Taiwan. Bovine serum albumin (BSA, Ref: BM-0150) was obtained from Winkler Ltda., Chile. The sterile plastic material was obtained from Falcon Inc. (New York, NY). Doxorubicin (Ref: D1515), human recombinant IL-1 receptor antagonist (Ref: SRP3327), and Bradford reagent (Ref: B6916) were obtained from Sigma-Aldrich, USA. IL-1β (Ref: 5204SC) was obtained from Cell Signaling Technology, USA. Resolvin E1 was purchased from Cayman Chemical, USA (Ref: 10007848). MCC950 was purchased from Merck, USA (5.38120, Calbiochem®).

### 2.2. Animals

Male adult C57BL/6J mice were obtained from the animal breeding facility located at the Faculty of Dentistry Sciences, University of Chile. The animals were kept in cages (12 h light/dark cycles) with ad libitum access to rodent chow and water. All studies were developed in compliance with the NIH Guide for the Care and Use of Laboratory Animals, updated in 2011 [26]. Experimental protocols were approved by the CICUA Ethics Committee (Protocol code: 18161-ODO-UCH, approved on May 29, 2018).

### 2.3. Isolation of cardiac fibroblasts

CFs were isolated from C57BL/6J mice (6–8 weeks old) using enzymatic digestion, as previously described [27]. Briefly, mice were terminally anesthetized with saline containing Ketamine at 160 mg/kg and Xylazine at 16 mg/kg by I.P. injection. Hearts were extracted in an aseptic environment, and the tissue was digested before isolating the fibroblasts. In brief, the atria were removed and the ventricles were cut into small pieces (1–2 mm), and digested with type II collagenase (585 units/mL) in DMEM-F12 with BSA (0.1 %) at 37 °C for 90 min, with mechanical movement every 10 min. Then the cells were centrifuged twice at 600*g*/4 °C/6 min and the resulting pellet was resuspended in 10 mL of DMEM-F12 supplemented with 20 % FBS and antibiotics (100 μg/mL streptomycin, 100 IU/mL penicillin, and 0.25 μg/mL amphotericin B), and cultured in a 5 % $CO_2$ atmosphere at 37 °C for at least 4–5 days. For the experiments, CFs having 1–3 passages were incubated with different treatments in DMEM-F12 medium during specific time points.

### 2.4. Cell treatment

CF were treated either with Doxo (10 nM) or IL-1β (2.5 ng/mL) for 24 h. In some experiments, RvE1 (100 nM), MCC950 (10 μM), or IR1A (10 ng/mL) were added 30 min before the stimulation with either Doxo (10 nM) or IL-1β (2.5 ng/mL). Stock solutions for Doxo, IL-1β, MCC950, and IR1A were prepared in Milli-Q® water under dark light conditions, while the stock solution of RvE1 was prepared in absolute ethanol accordingly to manufacturer instructions. Doxo and RvE1 intermediate working solutions were prepared in DMEM-F12 medium, and the MCC950 working solution was done with Milli-Q® water before its addition to the plates. IL-1β was added directly from the stock solution to the plates.

### 2.5. MTT assay

CFs viability was determined using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Cells were seeded into 24-well plates at a density of 10,000 cells/well with different concentrations of Doxorubicin (10–1000 nM). After 24 h, the cells were incubated with 0.5 mg/mL MTT for 4 h at 37 °C in a humidified 5 % $CO_2$ atmosphere. Following medium aspiration, the formazan crystals were dissolved in 300 μL DMSO by pipetting thoroughly. From each well, 100 μL were transferred to 96-well microplates in duplicate and the absorbance was measured at 540 nm using a microplate reader (EPOCH Microplate Spectrophotometer, BioTek Instruments Inc., USA).

### 2.6. Senescence associated-β-galactosidase activity

Cells were seeded into 6-well plates at a density of 100,000 cells/well and were treated with different stimuli. After 24 h, the senescence-associated-β-galactosidase (SA-β-gal) activity was measured using the Senescence Cells Histochemical Staining Kit (CS0030, Sigma-Aldrich, St. Louis, MO, USA), as previously described [28]. The ratio of SA-β-gal positive cells/Total cells was calculated by treatment-blinded manual counting of at least 1000 total cells in 12 random fields under phase contrast illumination, using an inverted microscope Nikon Eclipse TS2R

(Nikon, Tokyo, Japan). Representative images were obtained with a Lanoptik camera WiFi 5G (MC4KW-G1, Lanoptik, Guangzhou, China).

### 2.7. Western-blot assays

Cells were seeded into 60 mm plates at a density of 200,000 cells/plate for different treatments. After 24 h, the samples were prepared through mechanical scraping with ice-cold lysis buffer. Protein concentration was measured by colorimetric quantification using the Bradford assay. 20 μg protein from each sample was resolved by 15 % SDS-PAGE and then transferred to polyvinyl fluoride membranes (Bio-Trace™ PVDF Transfer Membrane 7569G, PALL Life Sciences, México). After blocking the membrane, it was incubated overnight with primary antibodies against phosphorylated (Ser139) histone H2A.X (γ-H2A.X) (1:1000; 2577S, Cell Signaling Technology, USA), p21 (1:200; sc-6246, Santa Cruz), p53 (1:1000; 2524S, Cell Signaling Technology, USA), and GAPDH (1:1000; 2118, Cell Signaling Technology, USA). Subsequently, the membrane was incubated with the corresponding horseradish peroxidase-conjugated anti-mouse (1:5000; 7076, Cell Signaling Technology, USA) or anti-rabbit (1:5000; 7074, Cell Signaling Technology, USA) secondary antibodies. GAPDH was used as a loading control. Bands were visualized using the enhanced chemiluminescence detection kit (Cat #1705062, Bio-Rad; USA) and quantified by densitometry analysis using the NIH ImageJ 1.52a program (National Institutes of Health; USA).

### 2.8. Cytokine determinations by multiplex assay

CFs supernatants obtained from cultured cell plates were centrifuged at 21,380*g* and 4 °C for 15 min, and then stored at −80 °C. Before the cytokine analysis, samples were thawed and analyzed for cytokine levels using the Milliplex™ MAP-Mouse Cytokine/Chemokine Magnetic Bead Panel Immunology Multiplex Assay, according to the manufacturer's instructions. The cytokines that were measured included IL-1β, IL-6, IL-10, KC (keratinocytes-derived chemokine in mice), MCP-1 (monocyte chemoattractant protein-1), and TNF-α, using a Luminex 200 System,

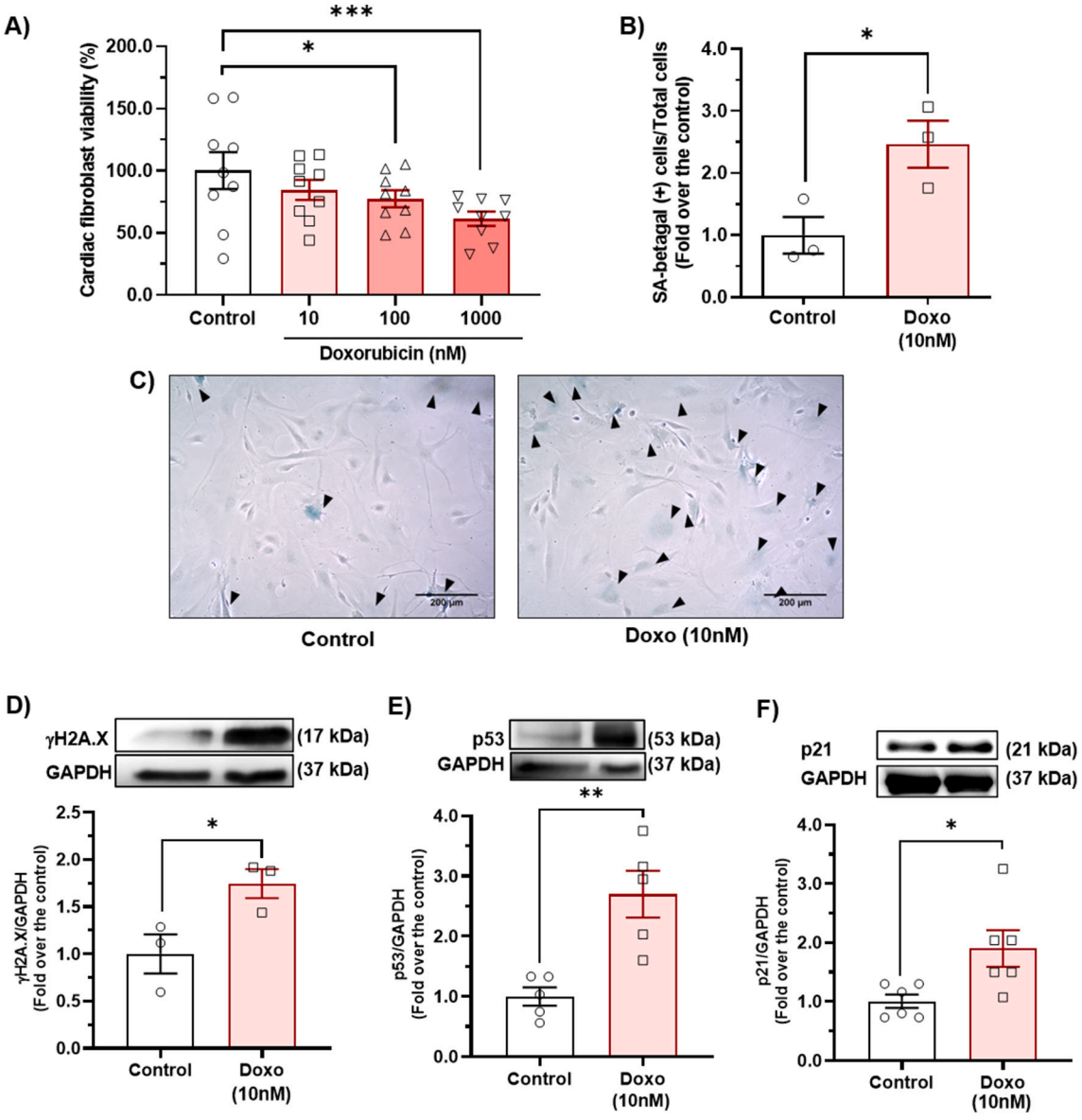


**Fig. 1.** Doxorubicin induces adult mouse CFs senescence. (A) Cell viability of CFs treated with Doxo (10, 100, and 1000 nM) for 24 h. (B) SA-β-gal positive cells/total cells were quantified by manual scoring in CFs stimulated with Doxo (10 nM) for 24 h. (C) Representative phase-contrast images of SA-β-gal positive cells (blue staining) indicated with arrowheads (20×, bar = 200 μm). (D) γ-H2A.X, (E) p53, and (F) p21 protein levels in CFs treated with Doxo (10 nM) for 24 h. Data are presented as the mean values ± SEM from 3 to 9 independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. untreated control cells. Doxo: doxorubicin.

Multiplex Bio-Assay. Standard curves were performed for each cytokine (0–10,000 pg/mL). Values were standardized as pg of cytokine/μg of total cell protein.

### 2.9. Statistic analysis

All the data are presented as mean ± SEM of 3–9 independent experiments. First, normality and homoscedasticity were checked for each variable using the Shapiro-Wilk and Brown-Forsythe tests, respectively. The two-tailed unpaired Student's *t*-test (parametric variables) or the Mann-Whitney test (non-parametric variables) were used to determine differences between two groups. One-way analysis of variance (ANOVA), followed by Dunnett T3 post hoc test (parametric variables) or Kruskal-Wallis, followed by Dunn's multiple comparison post hoc test (non-parametric variables) were used to determine differences among three groups or more. Statistical significance was defined as $p \leq 0.05$. The analysis was done using GraphPad Prism 9.0.2 software (California, USA).

## 3. Results

### 3.1. Doxorubicin induces senescence in adult mouse cardiac fibroblasts

Our first aim was to determine the cell viability of the primary cultures of adult mouse CFs by exposure to increasing Doxo concentrations (10–1000 nM). As shown in Fig. 1A, Doxo (10 nM) was the only concentration that did not significantly compromise CFs viability; while concentrations of 100 and 1000 nM reduced cell viability by 30 and 40 %, respectively (Fig. 1A). Therefore, we decided to choose the 10 nM concentration in the following experiments, since this concentration did not compromise CFs viability. Subsequently, we stimulated CFs with Doxo (10 nM for 24 h) and observed a 2.4-fold increase in SA-β-gal positive cells (Fig. 1B, C). Similarly, γ-H2A.X, p53, and p21 protein levels significantly increased by 1.7-, 2.5- and 1.7-fold over control, respectively (Fig. 1D–F).

### 3.2. Doxorubicin induces pro-inflammatory cytokine secretion in adult mouse cardiac fibroblasts

Since SASP is characteristic of senescent cells, we then evaluated the secretion of a panel of cytokines in CFs stimulated with Doxo. To test this, we evaluated Doxo (10 nM) effects at 24 h. Doxo increased the secretion of IL-1β, KC, and MCP-1 significantly (Fig. 2A, D, E); however, it was not able to promote changes in the secretion of IL-6, IL-10, and TNF-α (Fig. 2B, C, F). Moreover, we evaluated the role of NLRP3 inflammasome in Doxo-induced IL-1β secretion. The increase of IL-1β secretion induced by Doxo was prevented in presence of MCC950 (10 μM, an NLRP3 oligomerization inhibitor) (Supplementary Fig. 1).

### 3.3. In senescent cardiac fibroblasts, β-galactosidase activity induced by doxorubicin is mediated by the NLRP3 inflammasome and IL-1 receptor

Considering that IL-1β is a strong inductor of cellular senescence and that Doxo 10 nM was able to increase IL-1β secretion in an NLRP3 inflammasome-dependent manner, we next studied whether the NLRP3 inflammasome and, consequently, IL-1β, played a role in senescence induced by Doxo. To test this, we studied whether pretreatment of CFs with Doxo either in the presence of MCC950 (10 μM), or IR1A (10 ng/mL, an endogenous antagonist of the IL-1 receptor) could modulate the increase in SA-β-gal activity induced by Doxo. The results showed that both inhibitors significantly attenuated the SA-β-gal staining induced by 10 nM Doxo at baseline (Fig. 3A, B), indicating that NLRP3 and the IL-1 receptor are involved in the pro-senescent effects produced by Doxo.

### 3.4. Interleukin-1β induces senescence in adult mouse cardiac fibroblasts

Next, we studied whether IL-1β by itself could induce CFs senescence. According to our results, exogenous IL-1β (2.5 ng/mL for 24 h)

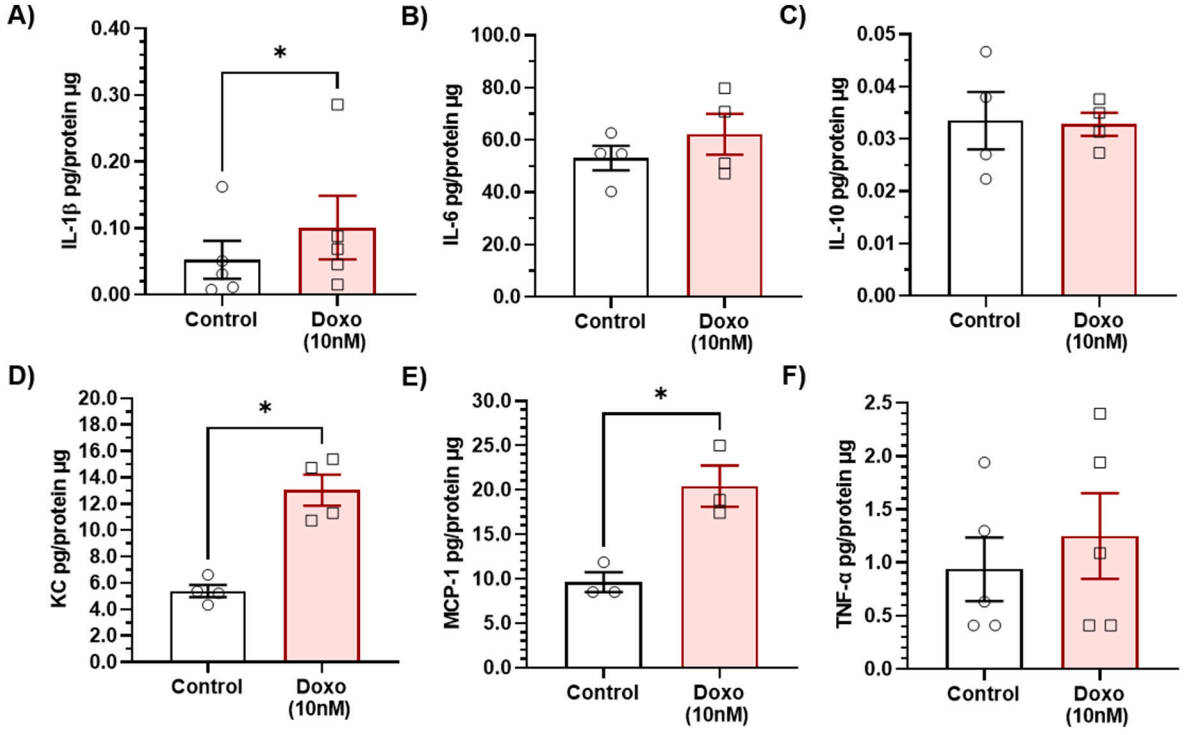


**Fig. 2.** Doxorubicin induces the secretion of proinflammatory cytokines in adult mouse CFs. Secreted cytokine levels were measured at 24 h, after Doxo (10 nM) treatment: (A) IL-1β, (B) IL-6, (C) IL-10, (D) KC, (E) MCP-1, and (F) TNF-α protein levels in adult mouse CFs supernatants. Data are presented as the mean values ± SEM from 3 to 5 independent experiments. *p < 0.05 vs untreated control cells. Doxo: doxorubicin, IL-1β: interleukin-1β, IL-6: interleukin-6, IL-10: interleukin-10, KC: keratinocytes-derived chemokine in mice, MCP-1: monocyte chemoattractant protein-1, TNF-α: tumor necrosis factor-α.

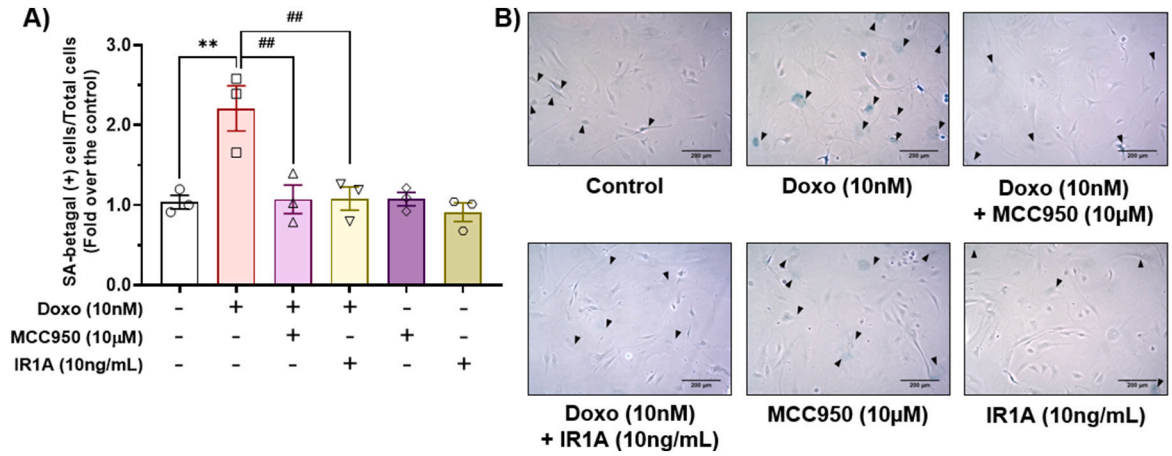


**Fig. 3.** Doxorubicin-induced SA-β-gal is mediated by NLRP3 inflammasome activation and the IL-1 receptor. (A) SA-β-gal positive cells/total cells were quantified by manual scoring in CFs treated for 24 h with Doxo (10 nM), either alone, or in the presence of an NLRP3 inhibitor, MCC950 (10 μM), or an endogenous antagonist of the IL-1 receptor, IR1A (10 ng/mL). (B) Representative phase-contrast images of SA-β-gal positive cells (blue staining) indicated with arrowheads (20×, bar = 200 μm). Data are presented as the mean values ± SEM from 3 independent experiments. $^{**}p < 0.01$ vs. untreated control cells; ##p < 0.01 vs. Doxo alone. Doxo: doxorubicin, MCC950: NLRP3 oligomerization inhibitor, IR1A: endogenous antagonist of the interleukin – 1 receptor.

induced CFs senescence similarly to Doxo, increasing SA-β-gal positive cells by 1.7-fold, compared to the control (Fig. 4A, B). In addition, γ-H2A.X, p53, and p21 protein levels increased by 2.5-, 1.5- and 1.7-fold over control, respectively (Fig. 4C–E). These results confirm that treatment of cultured CFs with IL-1β reproduces the pro-senescent effects observed with Doxo (10 nM) at 24 h.

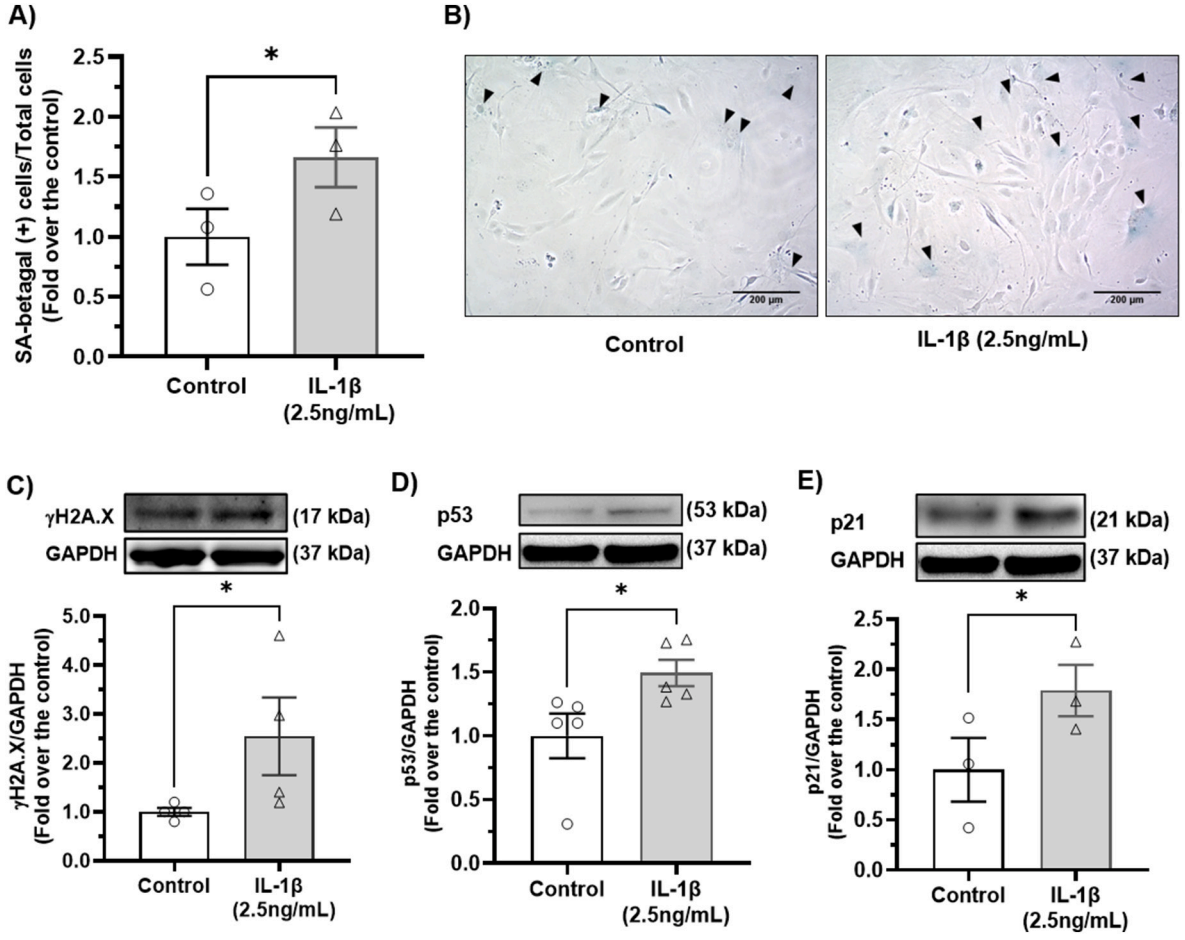


**Fig. 4.** Interleukin-1β induces adult mouse CFs senescence. (A) SA-β-gal positive cells/Total cells were quantified by manual scoring in CFs stimulated for 24 h with IL-1β (2.5 ng/mL). (B) Representative phase-contrast images of SA-β-gal positive cells (blue staining) indicated with arrowheads (20×, bar = 200 μm). (C) γ-H2A.X, (D) p53, and (E) p21 protein levels in CFs treated for 24 h with IL-1β (2.5 ng/mL). Data are presented as the mean values ± SEM from 3 to 5 independent experiments. $^{*}p < 0.05$ vs. untreated control cells. IL-1β: interleukin-1β.

### 3.5. Interleukin-1β induces secretion of pro-inflammatory cytokines in adult mouse cardiac fibroblasts

Since IL-1β secreted after stimulation with Doxo (10 nM) could be associated with CFs senescence, we then evaluated whether IL-1β increased SASP associated with senescent CFs. Our results showed that IL-1β (2.5 ng/mL for 24 h) was able to induce a strong secretion of IL-1β, IL-10, KC, MCP-1, and TNF-α in CFs (Fig. 5A–F); additionally, the levels were higher than those observed after stimulation with Doxo.

### 3.6. Resolvin E1 attenuates doxorubicin-induced senescence of adult mouse cardiac fibroblasts

Once establishing that the pro-inflammatory mechanisms triggered by Doxo, such as the assembly of the NLRP3 inflammasome, IL-1β secretion, and activation of the IL-1R, were involved in CFs senescence induced by Doxo, we studied whether specialized pro-resolutive mediators (SPM) could attenuate CFs senescence. For this aim, we used RvE1, a recognized SPM that reduces inflammation in CFs. Our results showed that RvE1 (100 nM) significantly inhibited the SA-β-gal stain induced by Doxo in CFs (Fig. 6A, B). Additionally, RvE1 also decreased protein expression of γ-H2A.X, p53, and p21 in Doxo-stimulated CFs (Fig. 6C–E). Finally, we evaluated the ability of RvE1 to modulate the Doxo-induced SASP in CFs. The lipid mediator was only able to attenuate the increase in IL-1β secretion induced by Doxo (10 nM at 24 h) (Fig. 7A); however, it did not modify the increase in KC or MCP-1 induced by Doxo (Fig. 7D and E). Furthermore, RvE1 by itself was also unable to modulate the levels of IL-10, IL-6, and TNF-α (Fig. 7B, C, F).

### 3.7. Resolvin E1 attenuates IL-1β-induced senescence of adult mouse cardiac fibroblasts

We finally studied whether RvE1 could attenuate CFs senescence induced by IL-1β. Our results showed that RvE1 significantly inhibited the SA-β-gal stain induced by IL-1β in CFs (Fig. 8A, B). Additionally, RvE1 also decreased the protein expression of γ-H2A.X, p53, and p21 in IL-1β-stimulated CFs (Fig. 8C–E). Finally, RvE1 was able to completely attenuate the increase in IL-1β, IL-10, MCP-1, and TNF-α secretion induced by IL-1β (Fig. 9A, C, E, F); and partially attenuate the increase of KC (Fig. 9D), but was not able to modify the upregulated IL-6 levels induced by IL-1β (Fig. 9B).

## 4. Discussion

We demonstrated that RvE1 protects adult mouse CFs against Doxo-induced senescence in this work. We also discovered that the harmful CFs senescence induced by Doxo could be IL-1β dependent, at least in part. Furthermore, we demonstrated that RvE1 could protect CFs by attenuating the effects of released IL-1β.

Senescence induction of CFs can be detrimental to the establishment of myocardial fibrosis during aging [9,29]. Extracellular stimuli like as cytokines, vasoactive peptides, and chemotherapeutic drugs could induce premature CFs senescence [8–10]. The success of Doxo as an anticancer drug was the motivation for using it as a senescence stressor in this study. Doxo's biggest drawback is its cardiotoxic side effects caused by DNA intercalation, which result in cellular senescence, apoptosis, cardiomyopathy, and, ultimately, premature death in a significant percentage of patients [30]. In this regard, CFs senescence has been proposed in recent years as a potential mechanism for the cardiovascular damage associated with anthracycline treatment [30,31].

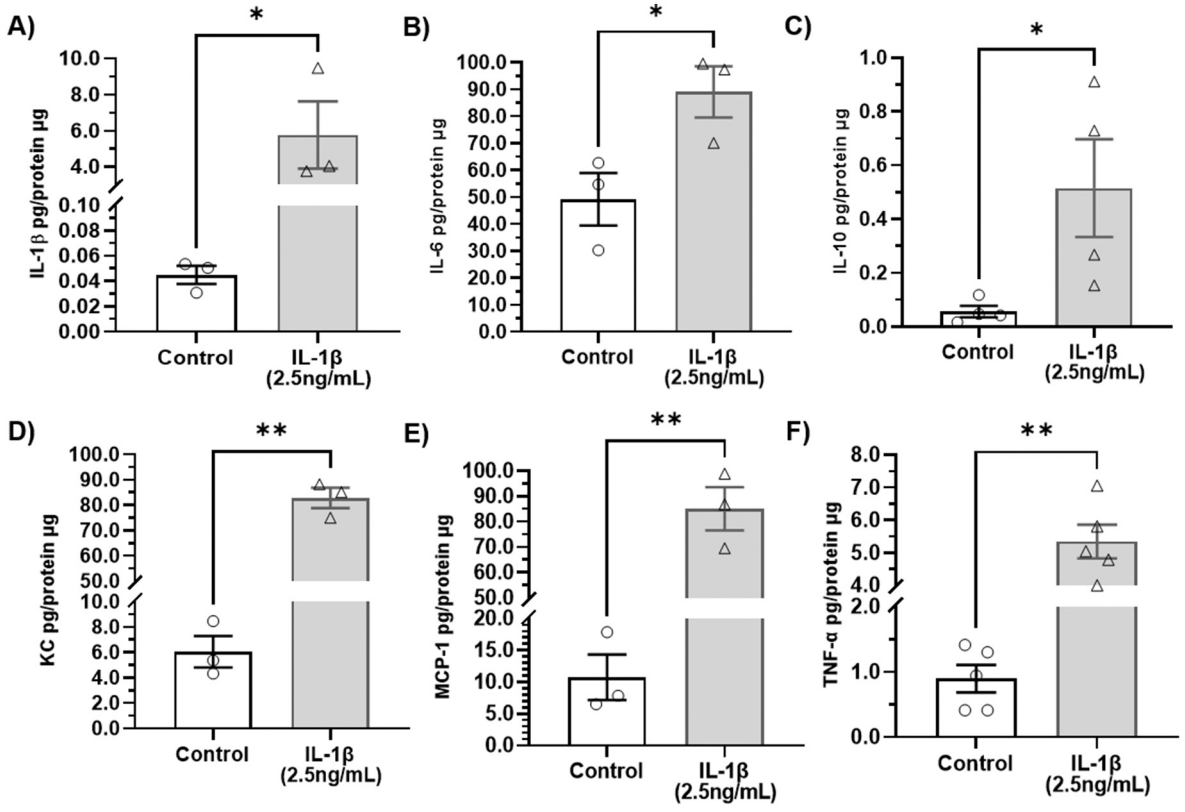


**Fig. 5.** Interleukin-1β induces the secretion of proinflammatory cytokines in adult mouse CFs. Secreted cytokine levels were measured at 24 h after IL-1β (2.5 ng/mL) treatment: (A) IL-1β, (B) IL-6, (C) IL-10, (D) KC, (E) MCP-1, and (F) TNF-α protein levels in adult mouse CFs supernatants. Data are presented as the mean values ± SEM from 3 to 5 independent experiments. *$p < 0.05$, **$p < 0.01$ vs. untreated control cells. IL-1β: interleukin-1β, IL-6: interleukin-6, IL-10: interleukin-10, KC: keratinocytes-derived chemokine in mice, MCP-1: monocyte chemoattractant protein-1, TNF-α: tumor necrosis factor-α.

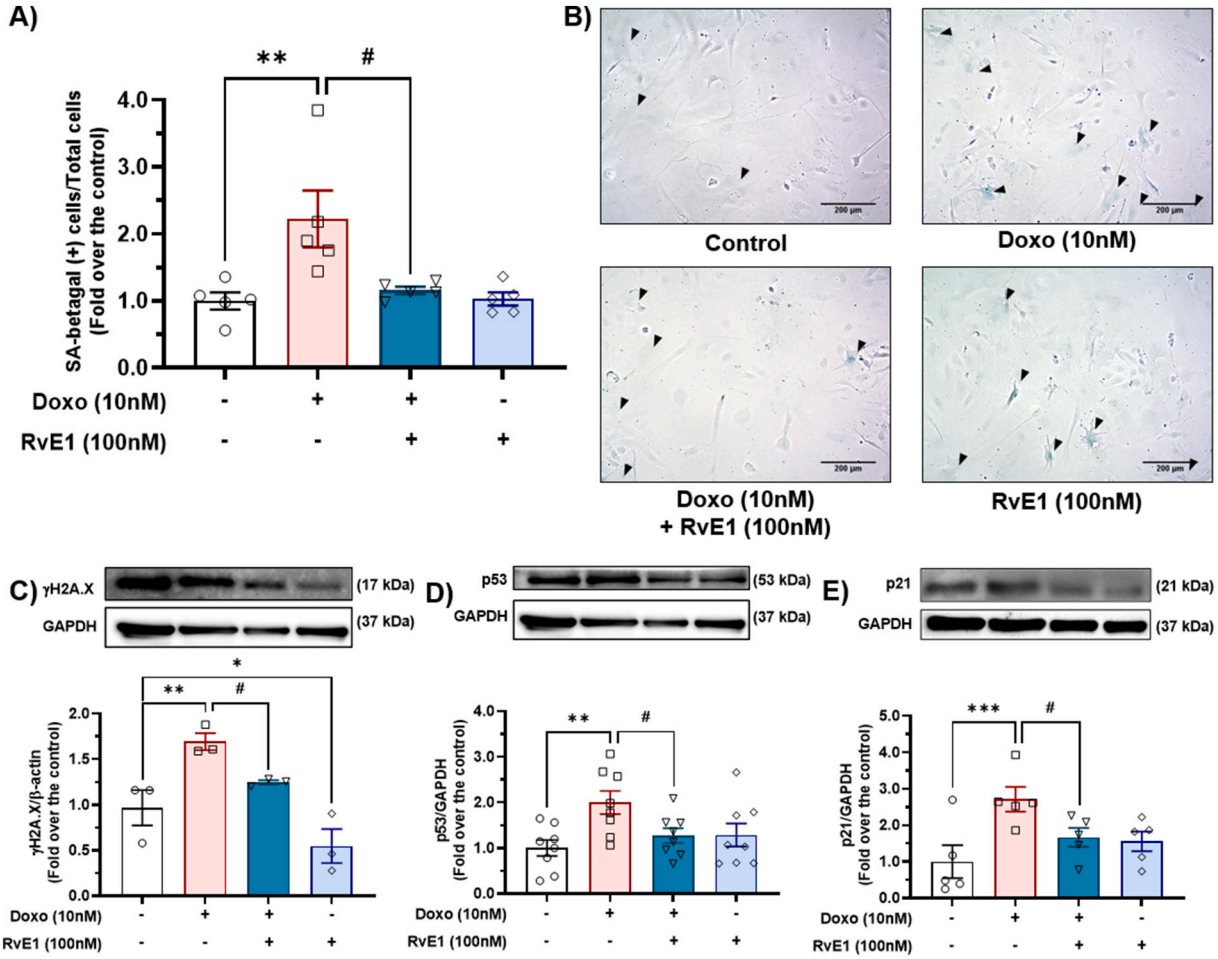


**Fig. 6.** Resolvin-E1 attenuates adult mouse CFs senescence induced by doxorubicin. (A) SA-β-gal positive cells/total cells were quantified by manual scoring in CFs treated for 24 h with Doxo (10 nM), alone, or in combination with RvE1 (100 nM). (B) Representative phase-contrast images of SA-β-gal positive cells (blue staining) indicated with arrowheads (20×, bar = 200 μm). (C) γ-H2A.X, (D) p53, (E) p21 protein levels in CFs treated for 24 h with Doxo (10 nM), alone or in combination with RvE1 (100 nM). Data are presented as the mean values ± SEM from 3 to 8 independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. untreated control cells; #$p < 0.05$ vs. Doxo alone. Doxo: doxorubicin, RvE1: resolvin E1.

To avoid senescence and apoptosis-associated cardiomyopathy and aberrant cardiac function, as well as fibrosis, finding a druggable new therapeutic inhibitor of senescence is a critical challenge.

In this work, we showed the effects of lower Doxo concentrations, which do not compromise CFs viability, on the induction of CFs senescence. We showed that Doxo increases the expression of p53, p21 and γ-H2A.X, and SA-β-gal, which are key in inducing senescence. Doxo has been demonstrated in recent studies to have a similar effect in CFs [8], in other fibroblasts from a different origin [32,33], and in other cell types from the cardiac origin [33,34]. Furthermore, our concentration range is similar to the plasma Doxo concentration in the slow elimination phase, which is the major exposure moment to the antineoplastic drug after its administration [35].

The mechanisms driving Doxo-induced senescence have previously been explored [33,36]. In this context, oxidative stress has a potential involvement in p53 activation through its phosphorylation at serine 15 as a hallmark of cellular senescence [37], and ROS generation plays a significant role in the onset of premature senescence of cancer cells [18]. A growing body of evidence suggests that anthracycline's clinically relevant toxicity is linked to its nuclear effects, which come from direct interactions with single- and double-stranded DNA [38,39]. Double-stranded DNA breaks are, in fact, a major cause of premature senescence. They trigger histone H2A.X activation by phosphorylation and converting it to γ-H2A.X, the most sensitive marker of double-stranded DNA breaks. Following that, the γ-H2A.X stimulates downstream ATM/ATR-regulated DNA damage stress responses (such as phosphorylation of the kinases Chk-1 and Chk-2), which propagate and activate the p53 and p21, resulting in cell cycle arrest [40].

Some anthracycline-induced deleterious cardiac effects have been linked to systemic and local inflammation [41,42]. In this regard, the SASP (a hallmark of senescence) is mostly constituted of proinflammatory cytokines, growth factors, and proteases [43]. In line with this, Wan et al. (2021) recently showed that Doxo triggers IL-1β release in a rat model of heart injury [44]; similarly, Huang et al. (2021) reported that Doxo induces senescence and increases cytokine secretion in H9c2 cells [34]. In the case of CFs senescence and SASP, Solokova et al. (2017) [45] showed that palmitate induces CFs senescence and increases IL-1β. One possible hypothesis is that Doxo-induced activation of the NLRP3 inflammasome is caused either by the formation of reactive oxygen species (ROS) or by the reduction of intracellular potassium levels. In this regard, LPS-primed murine bone marrow-derived macrophages treated with Doxo, either in the presence or absence of *N*-acetylcysteine, or high extracellular potassium, showed significantly reduced activation of the NLRP3 inflammasome and secretion of IL-1β [46]. Collectively, with these findings, we hypothesized that Doxo-induced local release of IL-1β could be the basis of premature CFs senescence and contribute to cardiac damage. To support this hypothesis, our results showed that Doxo induces IL-1β secretion, and, in addition, MCC950 (NLRP3 inhibitor) and ILR1A (endogenous antagonist of the IL-1 receptor) completely blocked Doxo-induced increased SA-β-gal.

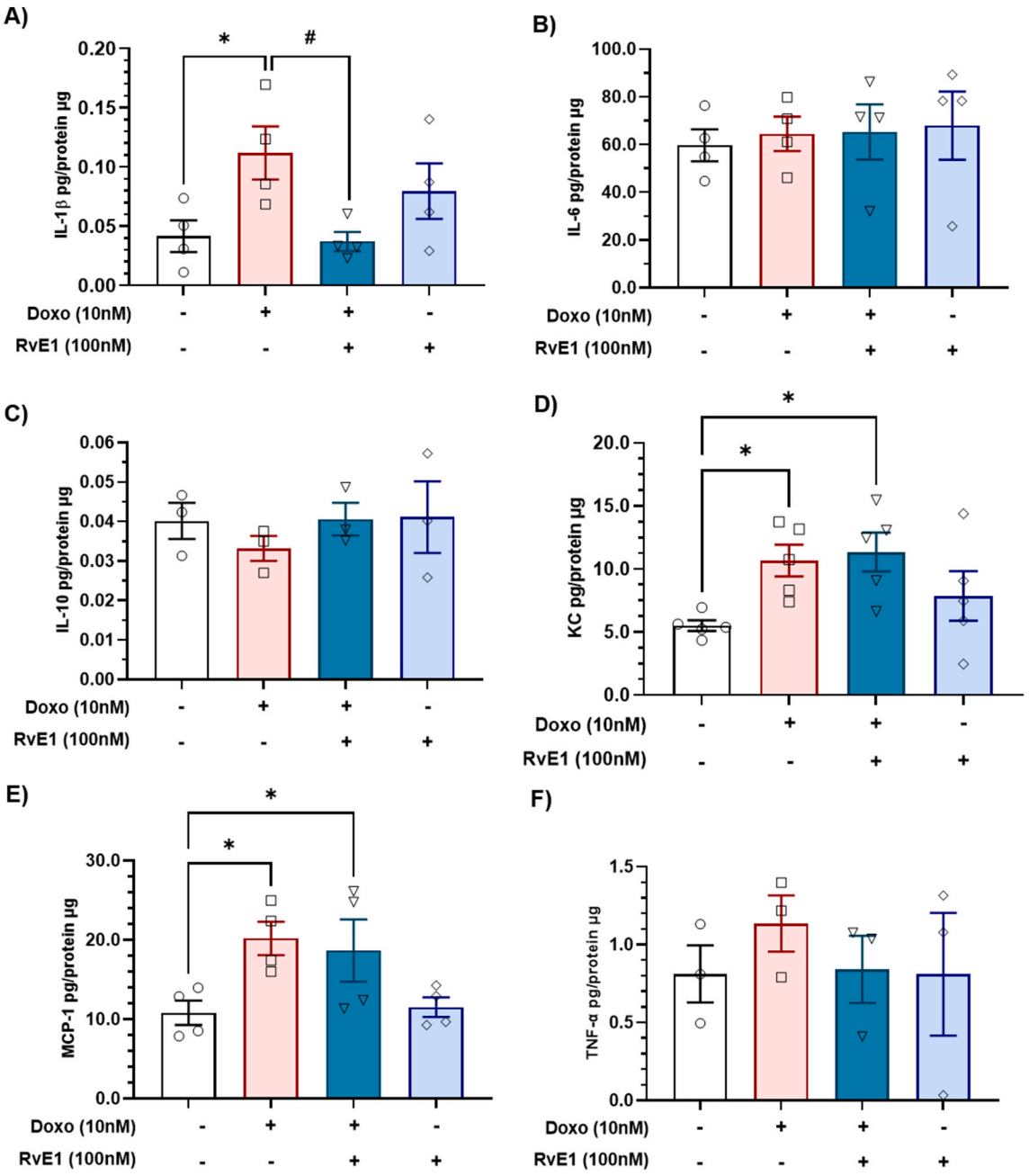


**Fig. 7.** Resolvin-E1 effect on the doxorubicin-induced secretion of proinflammatory cytokines in adult mouse CFs. Secreted cytokine levels were measured at 24 h after treatment with Doxo (10 nM), alone, or in combination with RvE1 (100 nM): (A) IL-1β, (B) IL-6, (C) IL-10, (D) KC, (E) MCP-1 and (F) TNF-α protein levels in adult mouse CFs supernatants. Data are presented as the mean values ± SEM from 3 to 5 independent experiments. *$p < 0.05$ vs. untreated control cells; #$p < 0.05$ vs. Doxo alone. Doxo: doxorubicin, RvE1: resolvin E1, IL-1β: interleukin-1β, IL-6: interleukin-6, IL-10: interleukin-10, KC: keratinocytes-derived chemokine in mice, MCP-1: monocyte chemoattractant protein-1, TNF-α: tumor necrosis factor-α.

However, we cannot exclude other IL-1β-independent mechanisms, which could be associated with Doxo-induced cardiotoxicity. For example, Kobayashi et al. (2016) reported that, regardless of IL-1β production, NLRP3 could promote Doxo-induced cardiotoxicity by regulating the production of IL-10 in macrophages [47].

No previous data has considered IL-1β as the link between Doxo and CFs senescence; thus, this is the first report about this mechanism triggered by Doxo to induce CFs senescence. Moreover, we have previously described that CFs produce and release IL-1β through the activation of the NLRP3 inflammasome by LPS and ATP [20]. Other authors have shown that palmitate activates NLRP3 and releases IL-1β [45]. In the current study, we showed that the inhibition of the NLRP3 inflammasome/IL-1 receptor axis by MCC950 and ILR1A was able to prevent CFs senescence induced by Doxo, suggesting that IL-1β secretion could be related to CFs senescence induced by Doxo. In line with these findings, recently, we have demonstrated that priming and activation of NLRP3 inflammasome and IL-1β secretion mediate at least in part the Doxo-induced senescence in endothelial cells [48].

Moreover, chronic inflammation induced by stimulation of the IL-1/IL-1R/NF-B axis promotes telomere damage and accelerates cellular senescence in mice [22]. Furthermore, the production of pro-inflammatory cytokines has been linked to the acquisition of a cellular SASP, which has ramifications locally. SASP has also been linked to the spread of the senescent phenotype to surrounding cells through a

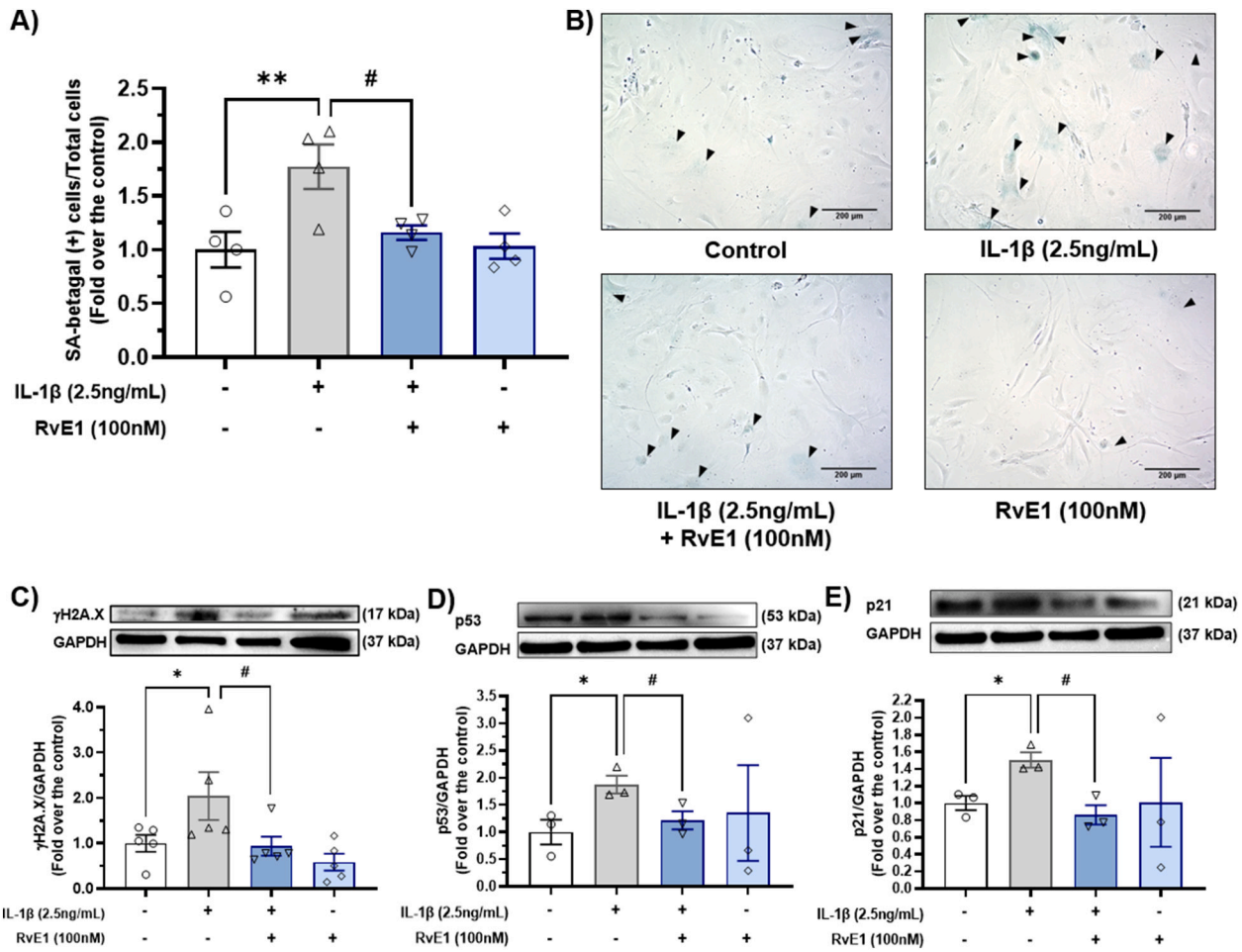


**Fig. 8.** Resolvin-E1 attenuates adult mouse CFs senescence induced by interleukin-1β. (A) SA-β-gal positive cells/total cells were quantified by manual scoring in CFs treated for 24 h with IL-1β (2.5 ng/mL), alone, or in combination with RvE1 (100 nM). (B) Representative phase-contrast images of SA-β-gal positive cells (blue staining) indicated with arrowheads (20×, bar = 200 μm). (C) γ-H2A.X, (D) p53, (E) p21 protein levels in CFs treated for 24 h with IL-1β (2.5 ng/mL), either alone, or in combination with RvE1 (100 nM). Data are presented as the mean values ± SEM from 3 to 4 independent experiments. *$p < 0.05$, **$p < 0.01$ vs. untreated control cells; #$p < 0.05$ vs. IL-1β alone. RvE1: resolvin E1, IL-1β: interleukin-1β.

bystander effect in this regard [37].

In the present study, we showed that IL-1β itself can also promote CFs senescence, as evidenced by increases in SA-β-gal activity, γ-H2A.X, p53, and p21. Notoriously, IL-1β-triggered SASP was more complex in composition, and the magnitude of the cytokine levels increase. We observed a strong increase in IL-1β, TNF-α, IL-6, IL-10, KC, and MCP-1. Indeed, these results suggest that IL-1β can induce a strong chronic inflammation, which could lead to major cardiac remodeling. CFs senescence induced by IL-1β has not been published before, although Koudssi et al. (1998) [49] showed that IL-1β increases p21 and p27 protein levels. This leads to cell cycle arrest in CFs, which corresponds to another hallmark of cellular senescence; however, these authors did not study or describe senescence as a mechanism involved in IL-1β effects. The CFs-released pro-inflammatory molecules may promote a low-grade chronic inflammation, causing tissue damage and triggering the recruitment of circulating immune cells, exacerbating cardiac inflammation [25,50]. These results suggest that senescent CFs could also promote immune cell recruitment, amplifying even more cardiac inflammation induced by Doxo.

We investigated if a druggable molecule that regulated the resolution of inflammatory responses may decrease the premature CFs senescence associated with the accelerated aging process, especially because we recognized pro-inflammatory IL-1β as a promoter of cell senescence caused by Doxo. The key findings of this work give strong evidence in favor of RvE1's protective effect against Doxo-induced CFs senescence in this context. RvE1 inhibited CFs' senescence as well as SASP secretion produced by Doxo and IL-1β. To our knowledge, this is the first study to link RvE1 to the prevention of CFs senescence produced by Doxo or IL-1β, indicating that resolving lipid mediators may have a protective impact against anthracycline toxicity mediated through CFs senescence induction. In respect of resolvins and senescence, a recent study showed that RvD1 suppresses skin fibroblast senescence induced by $H_2O_2$ [7]. Similarly, RvE1 also reduces Doxo-induced cardiotoxicity via altering AKT/mTOR signaling-mediated oxidative stress, autophagy, and apoptosis, according to a recent study [51]. In summarizing, these observations point out that RvE1 is a key disruptor of CFs senescence induced by the auto-inflammatory loop Doxo/IL-1β.

### 4.1. Limitations of the work

This work was conducted entirely in vitro and the effect of RvE1 on in vivo cardiac senescence models should be evaluated in the future. These results would help establish the key role of IL-1β as an inductor of cardiac senescence and could further support the key role of RvE1 as a pharmacotherapeutic strategy in cardiac senescence. Furthermore, because of the lacking commercially available ChemR23-specific antagonist, we were unable to experimentally demonstrate the involvement of ChemR23 in the effects of RvE1.

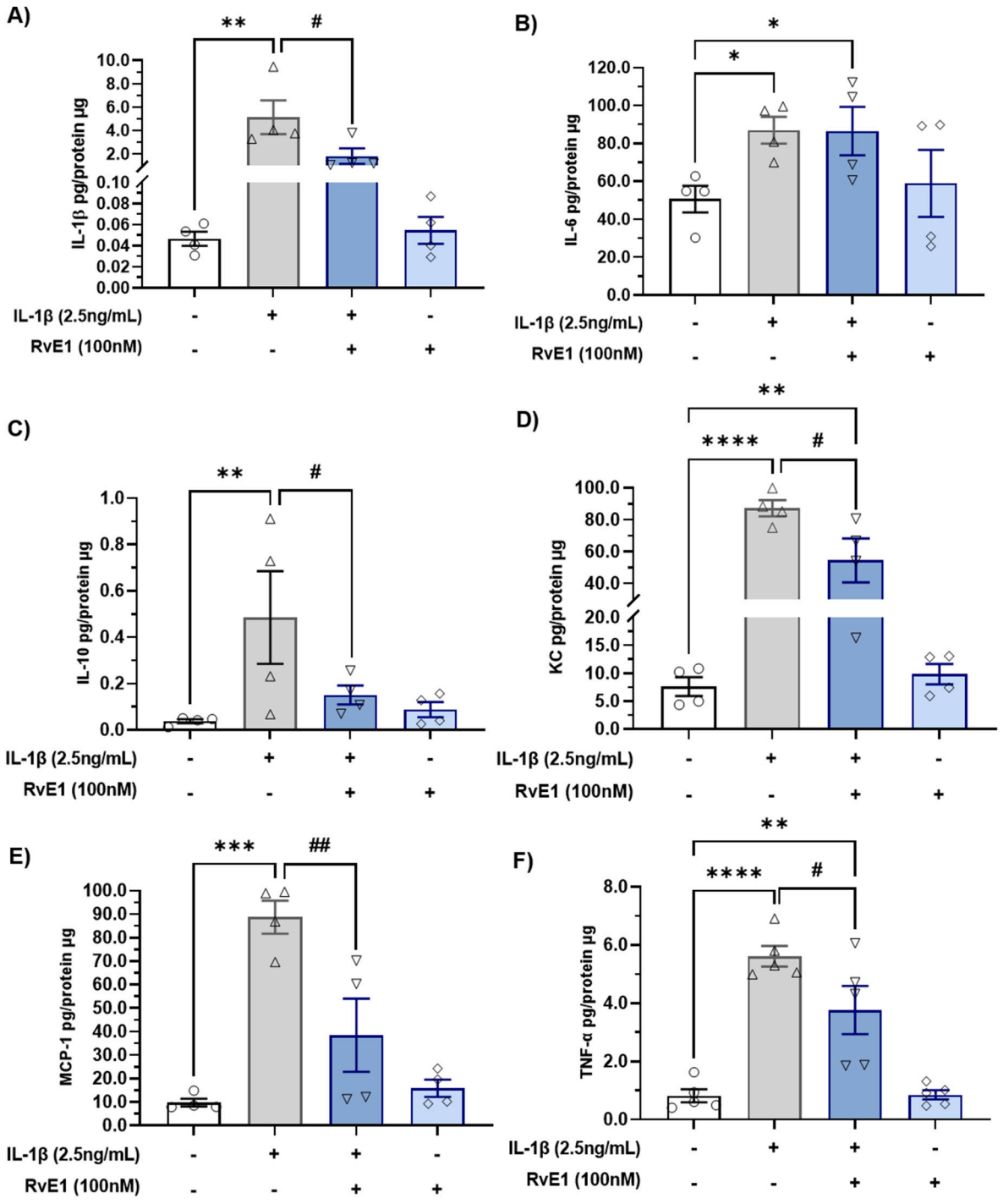


**Fig. 9.** Resolvin-E1 effect on the interleukin-1β-induced secretion of proinflammatory cytokines in adult mouse CFs. Secreted cytokine levels were measured at 24 h after treatment with IL-1β (2.5 ng/mL), alone, or in combination with RvE1 (100 nM): (A) IL-1β, (B) IL-6, (C) IL-10, (D) KC, (E) MCP-1 and (F) TNF-α protein levels in adult mouse CFs supernatants. Data are presented as the mean values ± SEM from 4 to 5 independent experiments. *p < 0.05, **p < 0.01, ***$p$ < 0.001, ****$p$ < 0.0001 vs. untreated control cells; #p < 0.05, ##p < 0.01 vs. IL-1β alone. RvE1: resolvin E1, IL-1β: interleukin-1β, IL-6: interleukin-6, IL-10: interleukin-10, KC: keratinocytes-derived chemokine in mice, MCP-1: monocyte chemoattractant protein-1, TNF-α: tumor necrosis factor-α.

## 5. Conclusion

Based on our results, Doxo (10 nM) and IL-1β (2.5 ng/mL) can induce CFs senescence by increasing the expression of γ-H2A.X, p53, p21, and SA-β-gal, and also increasing inflammatory markers (SASP). Moreover, the Doxo CF senescence response is dependent on the NLRP3 inflammasome/IL-1R axis activation. RvE1, at a dose of 100 nM, attenuates CFs senescence induced by Doxo and IL-1β, evidenced by a reduction in γ-H2A.X, p53, p21, SA-β-gal, and SASP. SASP induced by IL-1β was more pro-inflammatory than the effect induced by Doxo. Our results, along with the anti-inflammatory effects of RvE1 on senescent cells, invite us to further assess the safety of this therapeutic agent in preclinical settings using aging animal models.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2022.166525.

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## CRediT authorship contribution statement

**Jenaro A. Espitia-Corredor:** Formal analysis, Investigation, Writing – original draft. **Licia Shamoon:** Formal analysis, Investigation, Writing – original draft. **Francisco Olivares-Silva:** Investigation. **Constanza Rimassa-Taré:** Investigation. **Claudia Muñoz-Rodríguez:** Investigation. **Claudio Espinoza-Pérez:** Investigation. **Carlos F. Sánchez-Ferer:** Conceptualization, Writing – review & editing, Supervision. **Concepción Peiró:** Conceptualization, Writing – review & editing, Supervision. **Guillermo Díaz-Araya:** Conceptualization, Writing – review & editing, Supervision.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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