Mechanistic role of RND3-regulated IL33/ST2 signaling on cardiomyocyte senescence

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Research Article

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Abstract

Objective: Hyperinflammatory responses are pivotal in the pathophysiology of cardiomyocyte senescence, with IL33 serving as a crucial pro-inflammatory mediator. Our previous findings highlighted RND3's suppressive effect on IL33 expression. This study delves into the influence of RND3 on IL33/ST2 signaling activation and cardiomyocyte senescence.

METHODS: AC16 cardiomyocytes were subjected to treatments involving recombinant IL33, NF-κB inhibitor PDTC, or ST2 antibody Astegolimab. SA-β-gal and γH2AX staining were utilized to monitor alterations in cell senescence and DNA damage, respectively. Western blot analysis was conducted to ascertain the expression of Senescence-Associated Secretory Phenotype (SASP) and NF-κB activation. Utilizing CRISPR/Cas9 technology, the *RND3* gene was knocked out in H9C2 cells, followed by senescence analysis and sST2 level detection in the culture medium supernatant via ELISA. Post-AAV9 injection overexpressing *RND3*in SD rats, IL33/ST2 and SASP expression in heart tissues, and serum IL33 and sST2 changes were evaluated using ELISA.

RESULTS: Exogenous IL-33 significantly induced IL-1α, IL6, and MCP1 expression, increased the p-p65/p65 ratio, and the proportion of SA-β-gal and γH2AX positive cells in AC16 cells. PDTC and Astegolimab application mitigated these effects. *RND3* knockout in H9C2 cells led to increased intracellular IL33, ST2L, IL1 α, IL6, and MCP1 expression, decreased sST2 in the supernatant, and increased SA-β-gal and γH2AX positive cells. *RND3* overexpression suppressed IL33, ST2L, IL-1α, IL6, and MCP1 expression in heart tissues, decreased serum IL33, and increased sST2 levels.

CONCLUSION: RND3 expression in cardiomyocytes modulates cell senescence by negatively regulating the IL33/ST2/NF-κB signaling pathway, underscoring its potential as a therapeutic target in cardiovascular senescence.

1.Introduction

Aging cardiomyocytes, characterized by structural damage, increased pacing frequency, systolic and metabolic dysfunction[1], secrete senescence-associated secretory phenotype (SASP) factors that induce senescence in neighboring cells, ultimately leading to heart failure[2]. Inflammation, a key determinant of aging, plays a central role in myocardial aging. Franceschi et al. coined the term "inflammaging" to describe the chronic, low-grade, systemic inflammation that develops with age in the absence of overt infection. This systemic inflammation is closely tied to cardiomyocyte failure[3], yet the molecular mechanisms mediating this relationship remain elusive.

Interleukin 33 (IL33), a member of the IL-1 family, is ubiquitously expressed in various cell types and is significantly elevated during various inflammatory diseases[4,5]. While exogenous IL33 has been confirmed as a key inducer of inflammation in several systems[6-9], its impact on the heart is underreported, and the mechanisms regulating IL33 expression remain largely unexplored.

The growth stimulating expression gene 2 (ST2) protein, a member of the IL-1 receptor family, comprises two key isoforms: the transmembrane receptor ST2L and the soluble receptor sST2. As a decoy receptor of IL33, sST2 binds to circulating IL33, blocking its binding to ST2L and exerting an inhibitory effect [10]. Despite numerous studies highlighting the diagnostic and prognostic significance of increased circulating sST2 in various cardiovascular conditions, the understanding of IL33/ST2 in the cardiovascular field remains contentious[10-15].

RND3, or RhoE, a member of the Rho GTP protein superfamily, has been implicated in various cardiovascular pathophysiological processes[16-19], suggesting the potential therapeutic value of restoring RND3 expression. Our group previously identified *IL33* as a potential downstream target gene of RND3 in rat H9C2 cardiomyocytes using CRISPR/Cas9 technology[20].

In this study, we demonstrate that exogenous IL33 induces cardiomyocyte senescence, promoting SASP expression in cardiomyocytes via NF-κB pathway activation. Blocking the ST2 receptor mitigates the effects of IL33 on cardiomyocyte senescence. Importantly, we identify RND3 as a key regulator of IL33/ST2 signaling. These findings suggest that RND3 modulates cardiomyocyte senescence by mediating the IL33/ST2/NF-κB pathway.

2.Materials and methods

2.1. Animal models

Six-week-old male SD rats, procured from SLAC Laboratory Animal Co. Ltd (Changsha, China), were used in accordance with Hainan Medical University's animal experimental ethics (Approval number KYLL-2020-048). Cardiac-specific *RND3* overexpressed rats were established by a one-time tail vein injection of exogenous *RND3* overexpressing AAV9 (GeneChem, Shanghai, China) at 10^{12} vg/rat, with control AAV9 particle-infused rats serving as negative controls. After 12 weeks of standard feeding, rats were anesthetized with isoflurane (#R510-22-10, RWD, Shenzhen, China), and blood was collected from the abdominal aorta. Following centrifugation at 4℃ at 1300 rcf for 10 min, the upper serum was used for ELISA to detect sST2 and IL33 levels. The hearts were then extracted, and left ventricular tissue was processed for total protein extraction and western blotting.

2.2 Cell culture

Human AC16 cardiomyocytes (ATCC, Manassas, USA) and rat H9C2 cardiomyocytes (Cell Bank of Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco's modified eagle medium (DMEM) (#PM150210A, Procell, Wuhan, China) supplemented with 10% fetal bovine serum (FBS) (#04-001-1ACS, BI, Iselin) and 1% Penicillin/Streptomycin(#BL505A, Biosharp, Hefei, China) at 37℃ with 5% CO_2. Medium was refreshed every two days. Upon reaching 80%-90% confluence, cells were detached with 0.25% trypsin and passaged at a 1:3 ratio. Cells were seeded in a 6-well plate or confocal dish at

densities of 2 × 10^5/ml. For ELISA assay, cells were switched to FBS-free culture medium 12 h in advance..

2.3 RND3 gene knockout in H9C2 cells

H9C2 cells were infected with lentiviruses (Genechem, Shanghai, China) carrying effective sgRNA targeting rat *RND3* gene and recombinant Cas9 protein, as per our previous method[20]. Puromycin-resistant cells were identified as H9C2 cells with *RND3* gene knockout (denoted as RND3_KO). RND3 protein expression was verified by western blotting.

2.4 IL33, Astegolimab and PDTC treatment

AC16 cells were treated with recombinant exogenous IL33 (0.5 μg/mL) (#C091, Nazyme, Nanjing, China), with PBS as a control, and cells were harvested 45 min later for related assays. To elucidate the role of NF-κB signaling in IL33-induced downstream gene expression, NF-κB inhibitor PDTC (10 μmol/L) (#S1809, Beyotime Biotechnology, Nanjing, China) and ST2 monoclonal antibody Astegolimab (10 μmol/L) (#2173054-79-8; MedChemExpress China, Shanghai) were applied to 0.5 μg/mL IL33-stimulated AC16 cells. Cells were harvested 48h later for subsequent experiments.

2.5 Senescence associated β-galactosidase staining

Senescence-associated (SA)-β-gal staining was performed using a kit (#C0602, Beyotime). Cells were seeded in 6-well plates and treated when confluence exceeded 60%. After 48 hours, cells were washed with PBS, fixed with β-galactosidase fixative for 15 min at room temperature, washed thrice with PBS, and incubated overnight at 37℃ with SA-β-galactosidase staining solution. Cells were then observed and photographed under an inverted microscope.

2.6 γH2AX immunofluorescence staining

DNA damage was assessed using a γ-H2AX Immunofluorescence Assay Kit(#C2035S, Beyotime). Post-fixation, cells were blocked, incubated with γH2AX antibody overnight at 4℃, washed with PBS, incubated with Alexa Fluor®488 for 1 hour at room temperature, and counterstained with DAPI. Images were captured under a confocal microscope.

2.7 Western blotting

Total protein from heart tissue and cardiomyocytes was extracted using RIPA (#P0013B, Beyotime) and quantified using BCA (#P0011, Beyotime). Proteins (40 μg) were subjected to PAGE, transferred to a PVDF membrane (#IPVH00010, Merck China, Shanghai), blocked with BSA, incubated with primary antibodies overnight at 4℃, washed, incubated with HRP-IgGs for 1 hour at room temperature, and developed using ECL (#BL520A, Biosharp). Primary antibodies used include RND3(1:1000; #66535-1-Ig, Proteintech, Wuhan, China); IL33 (1:1000; #DF8319, Affinity, Wuhan, China), p-p50(1:2000, #AF3219, Affinity), IL1α(1:2000; #DF6893, Affinity); ST2L (1:1000; (#PTM-6483; PTM, Hangzhou, China), p50(1:1000; #PTM-6483, PTM), β-actin (1:1000; #PTM-5706; #PTM-5028, PTM); MCP1 (1:1000; #bs-1955R, Bioss, Beijing, China) and IL6 (1:1000; #bs-4539R, Bioss).

2.8 ELISA

Rat ST2 (#JM-10483R1; FankeW, Shanghai, China) and IL-33 (#DL-IL33-Ra, FankeW) ELISA kits were used to analyze cell culture supernatant and serum samples, following the manufacturer's instructions.

2.9 Statistical processing

Data were expressed as mean ± SD. Comparisons between two groups were performed using unpaired t-tests, while multiple group comparisons were conducted using one-way ANOVA. GraphPad Prism (version 9.4.1) was used for data analysis and plotting. A P-value of less than 0.05 was considered statistically significant.

3. Results

3.1 Exogenous IL33 Induced Cardiomyocyte Senescence

We explored the impact of exogenous IL33 on cardiomyocytes by stimulating AC16 cardiomyocytes with recombinant human IL33 protein. Compared to the control group, exogenous IL33 effectively induced the expression of MCP1, IL1α, and IL6 in cardiomyocytes (Fig. 1A, B), while cycle-related SASP such as p16 and p53 remained unchanged. SA-β-gal staining revealed that IL33 stimulation significantly induced cardiomyocyte senescence (Fig. 1C), and γH2AX immunofluorescence staining demonstrated that IL33 significantly induced DNA damage in cells (Fig. 1D).

3.2 IL33 Induces SASP in Cardiomyocytes by Activating NF-κB Signaling

Previous reports suggest that IL33 can induce inflammation in vascular endothelial cells by activating the NF-κB pathway[21]. To ascertain whether IL33 operates through NF-κB signaling in cardiomyocytes, we treated IL33-stimulated AC16 cells with NF-κB inhibitor PDTC. Results showed an increase in p65 phosphorylation level in AC16 cardiomyocytes post-exogenous IL33 stimulation for 45 min, with no significant increase in p50 phosphorylation level. PDTC treatment downregulated p65 phosphorylation level and significantly inhibited MCP1, IL1α, and IL6 protein expression, while p16 and p53 protein levels remained unaffected (Fig. 2).

3.3 Astegolimab Blocks the Effect of Exogenous IL33 on Cardiomyocyte Senescence

To further confirm the role of membrane ST2L receptor in exogenous IL33-induced cardiomyocyte senescence, we treated AC16 cardiomyocytes with Astegolimab, an ST2 IgG2 monoclonal antibody, to block exogenous IL33-ST2L binding. Results showed that ST2 blockade downregulated p65 phosphorylation level and significantly inhibited inflammatory SASP expression such as MCP1, IL1α, and IL6. Interestingly, Astegolimab-stimulated cardiomyocytes exhibited significantly lower p53 expression than baseline (Fig. 3A, B), suggesting Astegolimab may exert additional pharmacological effects beyond IL33 blockade. SA-β-gal and γH2AX staining results suggest that Astegolimab effectively mitigates IL33-induced cardiomyocyte senescence and DNA damage (Fig. 3C, D).

3.4 *RND3* Knockout Induced Cardiomyocytes Senescence and DNA Injury

We designed a mature lentivirus carrying sgRNA and recombinant Cas9 protein targeting rat *RND3* gene to infect H9C2 cells, successfully obtaining RND3_KO H9C2 cells with *RND3* gene knockout. Western blot analysis revealed that RND3_KO significantly increased the expression of SASP genes such as MCP1, IL1, p53, p16, and IL6 compared to the control group (Fig. 4A, B). Concurrently, SA-β-galactosidase staining suggested that RND3_KO promoted cardiomyocyte senescence (Fig. 4C), accompanied by a significant increase in DNA damage (Fig. 4D).

3.5 RND3 Knockout Activates the IL33/ST2 Signal

We further investigated the regulatory role of RND3 on the IL-33/ST2 axis. Western blot results demonstrated that *RND3* gene knockout led to increased expression levels of IL33 and ST2L proteins in H9C2 cells, accompanied by elevated phosphorylation levels of NF-κB p65 (Fig. 5A, B). Concurrently, the level of the decoy receptor sST2 of IL33 in the cell culture supernatant significantly decreased following *RND3* knockout (Fig. 5C).

3.6 *RND3* Overexpression Inhibits Cardiomyocyte SASP

In this study, AAV9 particles carrying the overexpression of exogenous *RND3* was injected into the tail vein to achieve the overexpression of *RND3* in the heart tissue. Western blot results showed that the level of RND3 protein in RND3_OE group was significantly increased, along with the inhibition of NF-κB pathway and the expression levels of SASP factor including MCP1, IL1α and IL6 were significantly decreased. Meanwhile, overexpression of *RND3* inhibited the expression of IL33 and ST2L proteins (Figure 6A, B). ELISA results showed that compared with the control group, plasma IL33 level of rats in RND3_OE group decreased, sST2 level increased (Figure. 6C), and plasma IL33/ sST2 ratio decreased (Figure. 6D).

4. Discussion

RND3, a member of the Rho GTPase superfamily, plays diverse biological roles. While it has been extensively studied in the context of cancer [15], recent research has focused on its role in cardiovascular diseases [16-19, 23-25]. Our previous gene chip screening revealed that RND3 gene knockout in cardiomyocytes was accompanied by IL33 signal activation [20]. Given the ongoing debate about the role of IL33/ST2 signaling in cardiovascular diseases, this study aimed to explore the relationship between IL33/ST2 signaling and cardiomyocyte senescence. Our findings suggest that down-regulation of the RND3 gene in cardiomyocytes promotes the activation of IL33/ST2 signaling, which further mediates cardiomyocyte senescence through NF-κB signaling regulation of SASP gene expression. This study is the first to propose that activation of the IL33/ST2 signaling axis promotes cardiomyocyte senescence, expanding research on the relationship between RND3 and cardiovascular disease, and providing a new foundation for elucidating the pathophysiological role of the IL33/ST2 signaling system in cardiomyocytes.

IL33 can be expressed in various tissue cells, including cardiomyocytes. Under normal conditions, IL33 is expressed in the cell nucleus and is secreted extracellularly in cases of infection, inflammation, tissue injury, and cell necrosis [5, 26]. IL33 binds to its transmembrane receptor, ST2L, and the activated IL33/ST2 signal participates in critical pathophysiological processes including inflammation, fibrosis, and heart failure [27-30], suggesting the IL-33/ST2 pathway has broad clinical translational potential. The mechanism of increased intracellular IL33 expression remains unclear, but some studies suggest that Yap/Taz can directly regulate the *IL33* promoter and promote its expression in cardiac fibroblasts [31]. In this study, we found that IL33 and ST2L expression in cells significantly increased after *RND3* gene knockout, suggesting that RND3 is an upstream regulator of IL33, but the mechanism by which RND3 regulates IL33 expression requires further clarification.

The biological role of IL33/ST2 signaling in cardiomyocytes is controversial. It has been reported that IL33 released after acute kidney injury can induce cardiomyocyte hypertrophy, demonstrating a pathogenic effect [29], while in diabetic cardiomyopathy mice, IL33 administration can block autophagy and enhance cardiac diastolic function, thus having a protective effect [30]. In this study, we found that cardiomyocytes exhibited a significant SASP after administration of exogenous IL33, which was manifested as increased expression of SASP such as MCP1, IL1α, and IL6, as well as an increased number of SA-β-galactosidase active cells and a trend of cellular DNA damage, suggesting that increased IL33 has a damaging effect on cardiomyocytes. Concurrently, we found that administration of Astegolimab can effectively treat cardiomyocyte senescence and DNA damage caused by IL33, further confirming the role of IL33/ST2 signaling activation in cardiomyocyte senescence. In terms of mechanism, this study suggests that elevated IL33 increases the phosphorylation of p65 through myocardial cell membrane ST2L and activates NF-κB signaling to mediate the expression of aging-related inflammatory proteins, which is highly consistent with recent studies on obstructive sleep apnea patients [32].

It has been reported that on one hand, the expression of RND3 is reduced in the heart tissue of chronic heart failure [26], on the other hand, the decreased expression of RND3 promotes the inflammatory response in the local myocardium of myocardial infarction by activating NF-κB signaling [18]. Considering that some inflammatory factors such as IL1, IL6, TNF-α, and MCP1 are consistent with secreted phenotypic factors related to cell aging in the inflammatory response after myocardial infarction, it is necessary to further elucidate the relationship between RND3 and IL33/ST2 signaling. In vitro experiments revealed that *RND3* gene knockout significantly increased the expression of IL33 and ST2L in cells, while reducing the level of sST2 in the cell supernatant. Concurrently, the senescence-related factors MCP1, IL1α, p16, p53, and IL6 also increased, suggesting that the decreased expression of RND3 activated IL33/ST2 signaling and promoted the age-related inflammatory response in cardiomyocytes. In vivo experiments found that overexpression of *RND3* in myocardial tissue inhibited the expression of IL33/ST2 signal and SASP such as MCP1, IL1α, and IL6 in myocardial tissue, which confirmed the negative regulatory effect of RND3 on IL33/ST2 signal from another level.

It should be noted that in animal experiments, the serum sST2 level of rats in the overexpressed *RND3* group was significantly increased compared with the control group, while the level of IL33 and the ratio of IL33/sST2 also decreased. In this study, there was no statistical difference between the two groups in ejection fraction, short contraction fraction, and E/A peak 2 months after the administration of AAV9, but after induction into diabetic state, the over-expression of *RND3* group had better cardiac function, and a significant negative correlation between plasma sST2 level and ejection fraction was also found (unpublished data). At present, whether sST2 is a marker of heart failure has aroused certain attention and controversy [13, 15, 34], and our results support the conclusion that sST2 level is a marker

of heart failure. As for how cardiac overexpression of RND3 leads to a decrease in IL33 and an increase in sST2 levels in animal serum, the mechanism is still unclear.

In conclusion, this study is the first to show that IL33 can induce cardiomyocyte senescence. RND3 negatively regulates the IL33/ST2/NF-κB signaling axis in cardiomyocytes and is involved in the regulation of cardiomyocyte senescence-related phenotypes. Our study provides an experimental basis for future interventions targeting the RND3/IL33/ST2/NF-κB signaling axis to address heart.

Declarations

Author Contributions

Linxu Wu, Xinglin Zhu, Cai Luo, Yangyang Zhao, Shanshan Pan and Kaijia Shi performed the experiments and collected data. Zhihua Shen searched literature and performed statistical analyses. Zhihua Shen, Junli Guo and Wei Jie conceived of and coordinated the study and drafted the manuscript. All of the authors read and approved the final manuscript.

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Disclosure

The authors have no conflicts of interest related to this work.

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Figures

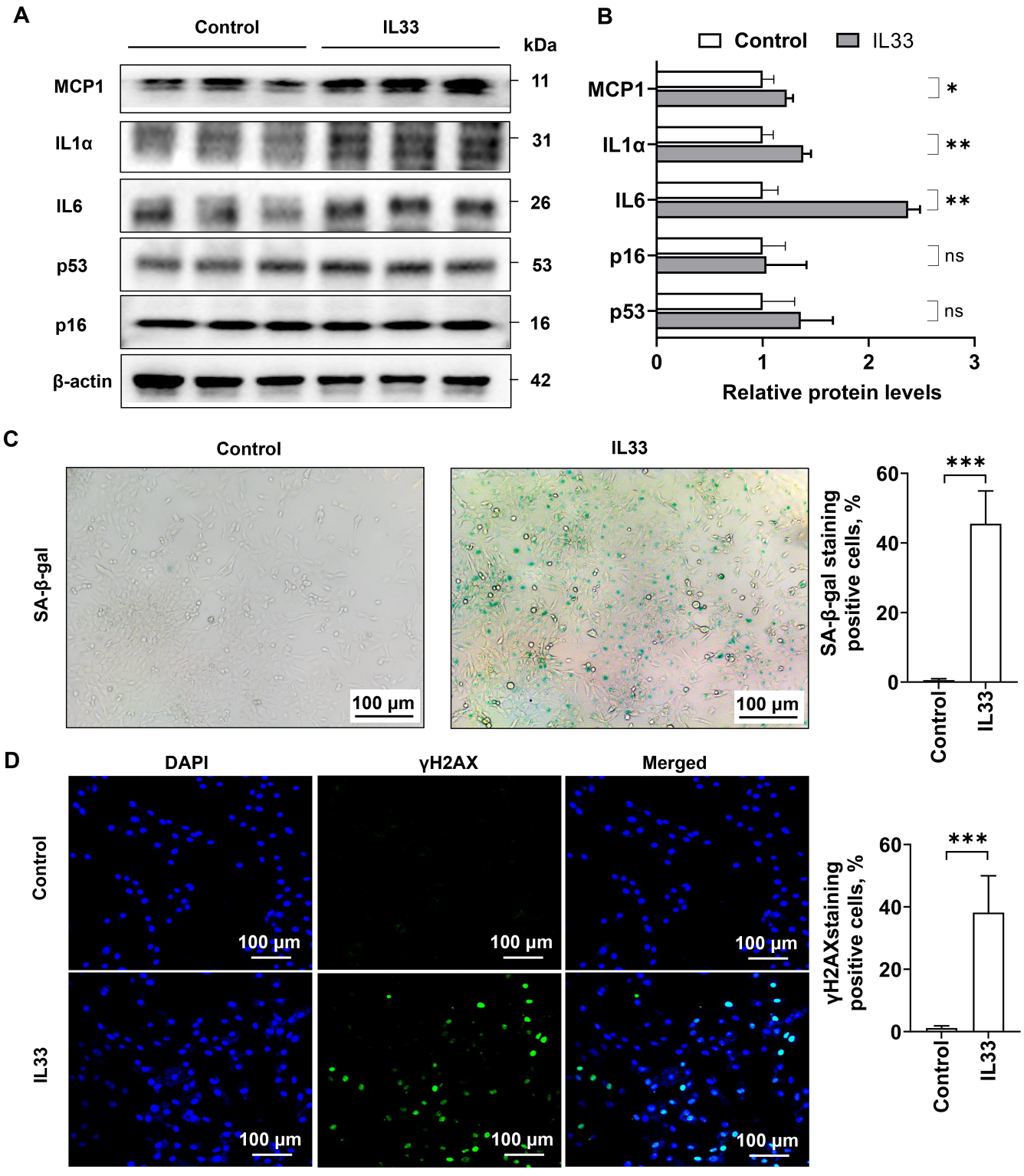

Figure 1

Exogenous IL33 induces senescence related phenotype and DNA damage in cardiomyocytes. (A-B) Representative western blots for the expression of SASPs (MCP1, IL1α, IL6, p16 and p53) in AC16 cells. mean ± SD, *n*=3, **P*<0.05, ***P*<0.01, ns, no significance. (C) Representative image for SA-β-gal staining, Scale Bar = 100 μm, mean ± SD, *n*=5, ****P*<0.001. (D) Representative images for DNA injury assessed by γH2AX staining in AC16 cells. Bar = 100 μm, mean ± SD, *n*=5, ****P*<0.001.

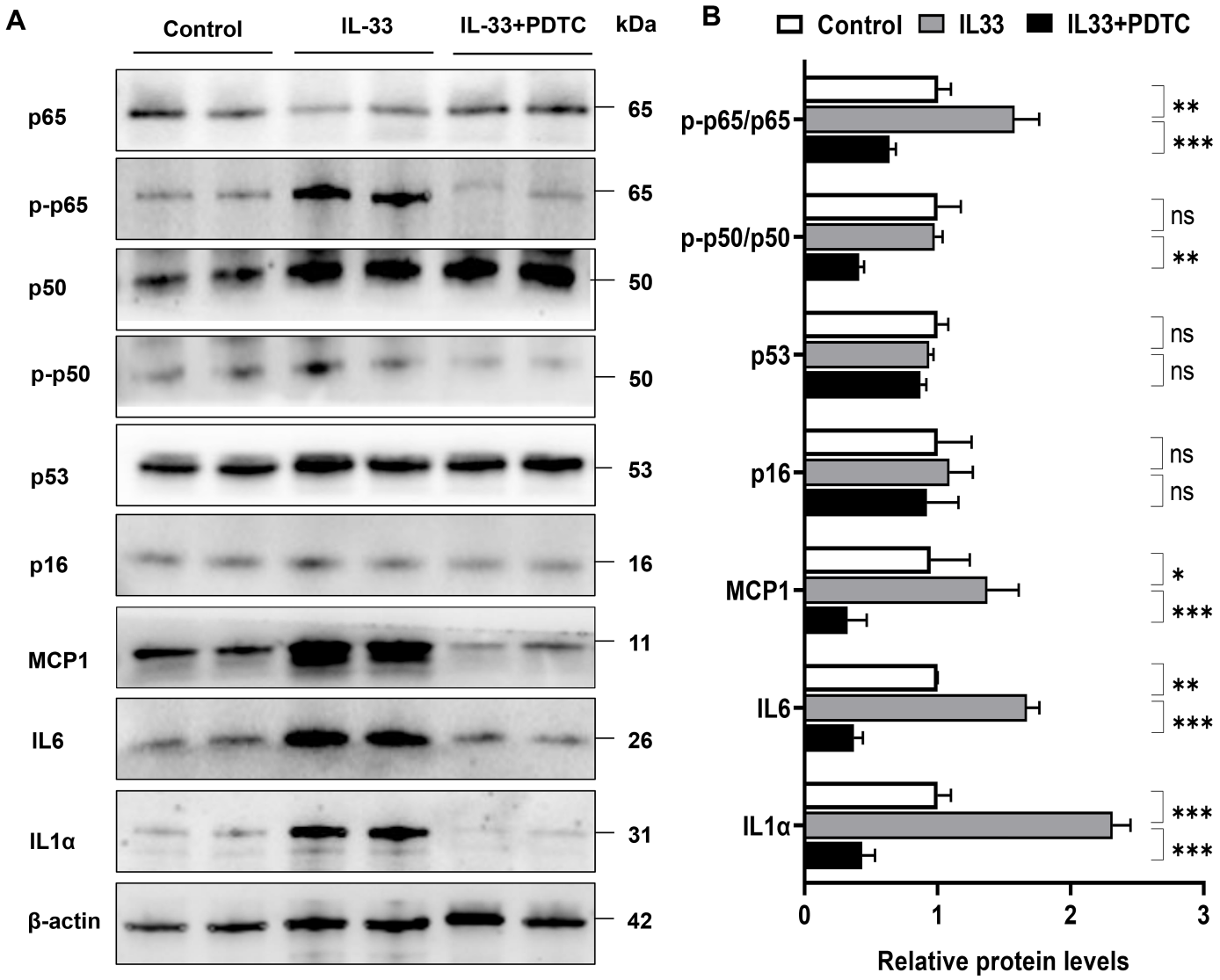

Figure 2

Exogenous IL33-activated NF-κB signaling induces senescence related phenotype in cardiomyocytes. (A-B) Representative western blots for IL33-induced NF-κB activation (p-p65/p65, p-p50/p50) and SASPs (p53, p16, MCP1, IL1α, IL6) in AC16 cells, mean ± SD, n=3, *P<0.05, **P<0.01, ***P<0.001, ns, no significance.

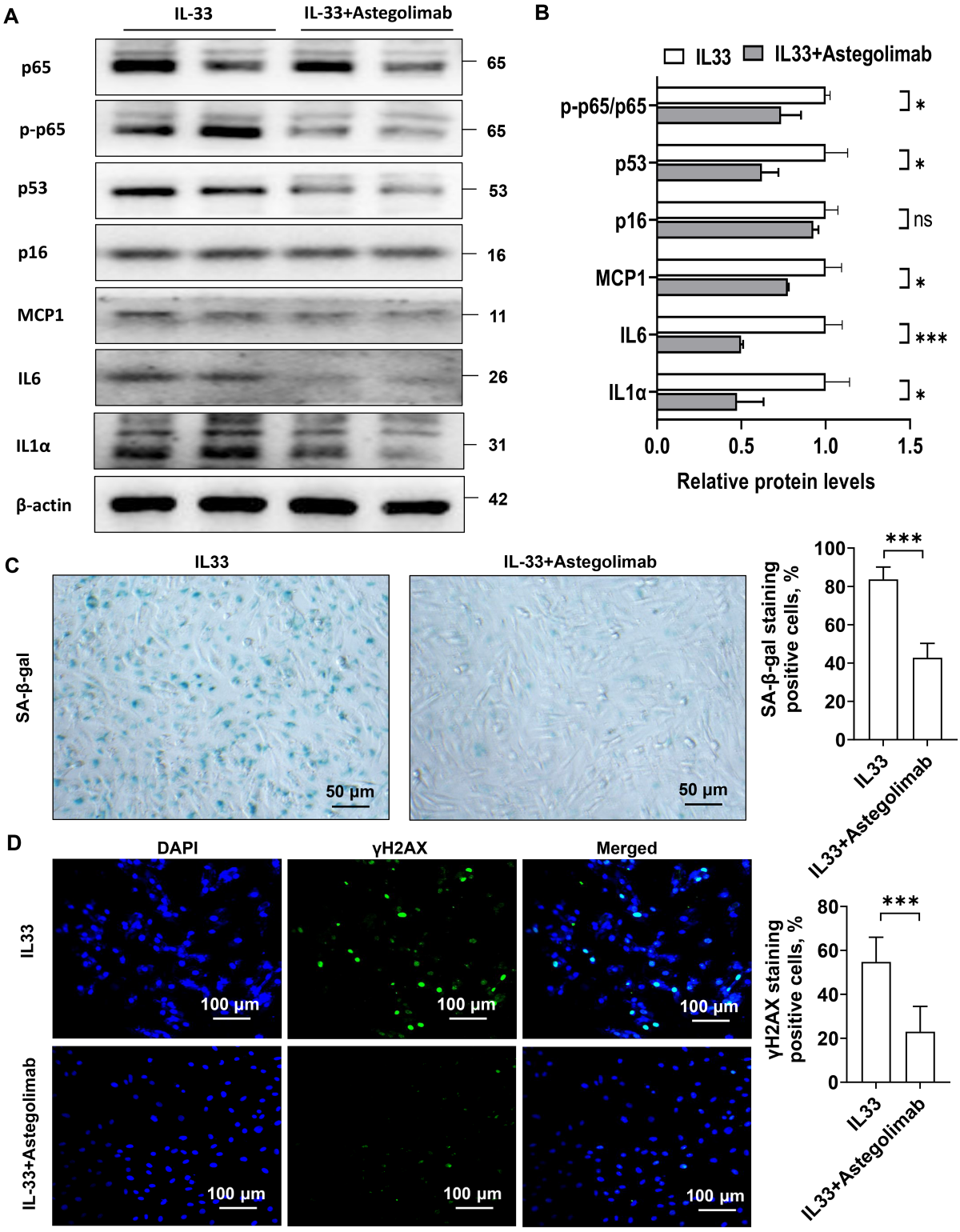

Figure 3

Astegolimab blocks IL33-induced senescence. (A-B) Representative western blots for the activation of NF-κB and expression of SASPs in AC16 cells. mean±SD, *n*=3, **P*<0.05, ****P*<0.001, ns, no significance. (C) Representative SA-β-gal staining images, Scale Bar = 50 μm, mean ± SD, *n*=5, ****P*<0.001. (D) Representative images for DNA injury assessed by γH2AX staining in AC16 cells. Bar = 100 μm, mean ± SD, *n*=5, ****P*<0.001.

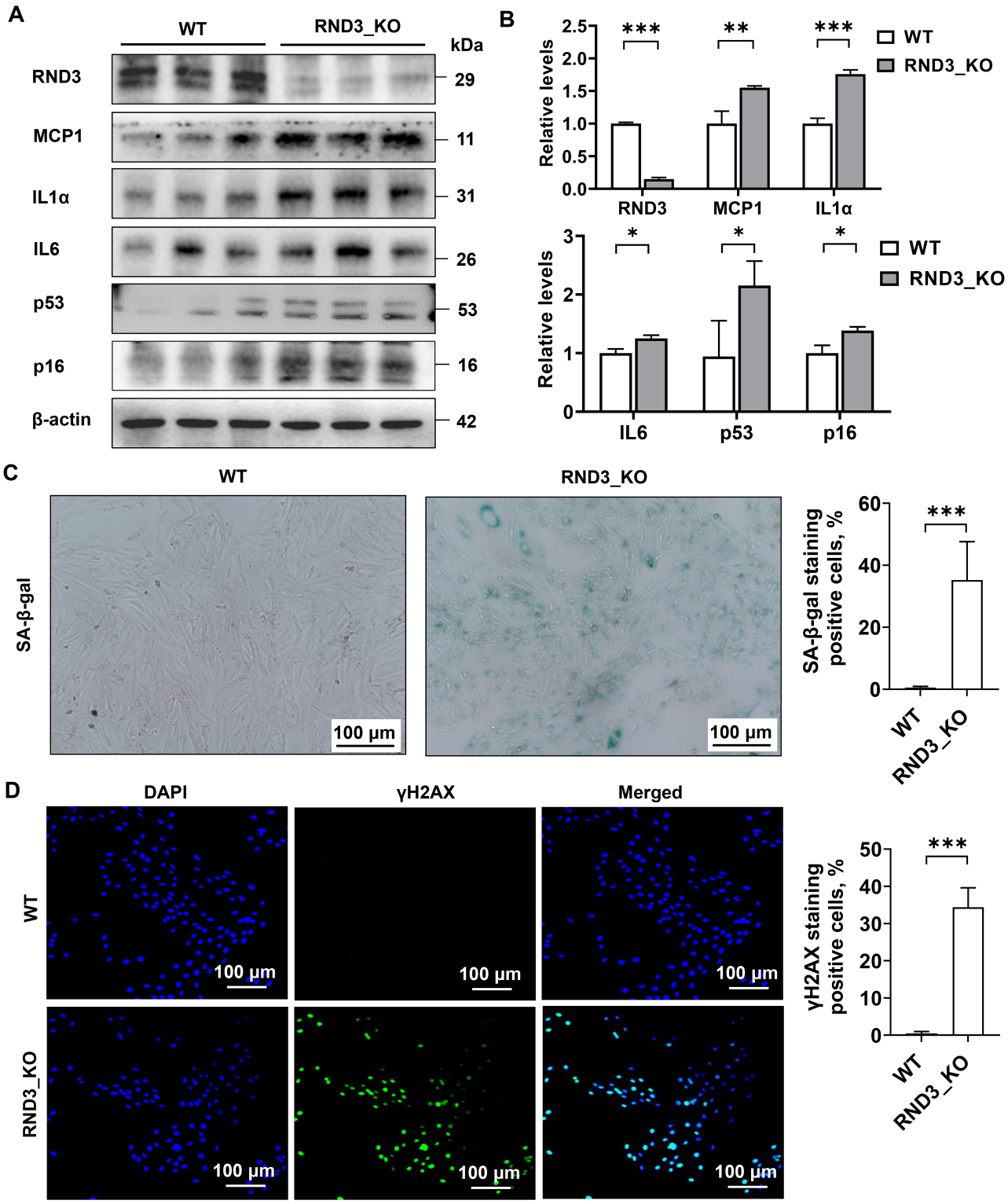

Figure 4

***RND3* gene knockout accelerates SASP in cardiomyocytes.** (A-B) Representative western blots for the expression of RND3 and SASPs in H9C2 cells with or without *RND3* knockout. mean ± SD, n=3, *P<0.05, **P<0.01, ***P<0.001. (C) Representative SA-β-gal staining images, Scale Bar = 100 μm, mean ± SD, n=5, ***P<0.001. (D) Representative images for DNA injury assessed by γH2AX staining in H9C2 cells. Bar = 100 μm, mean ± SD, n=5, ***P<0.001.

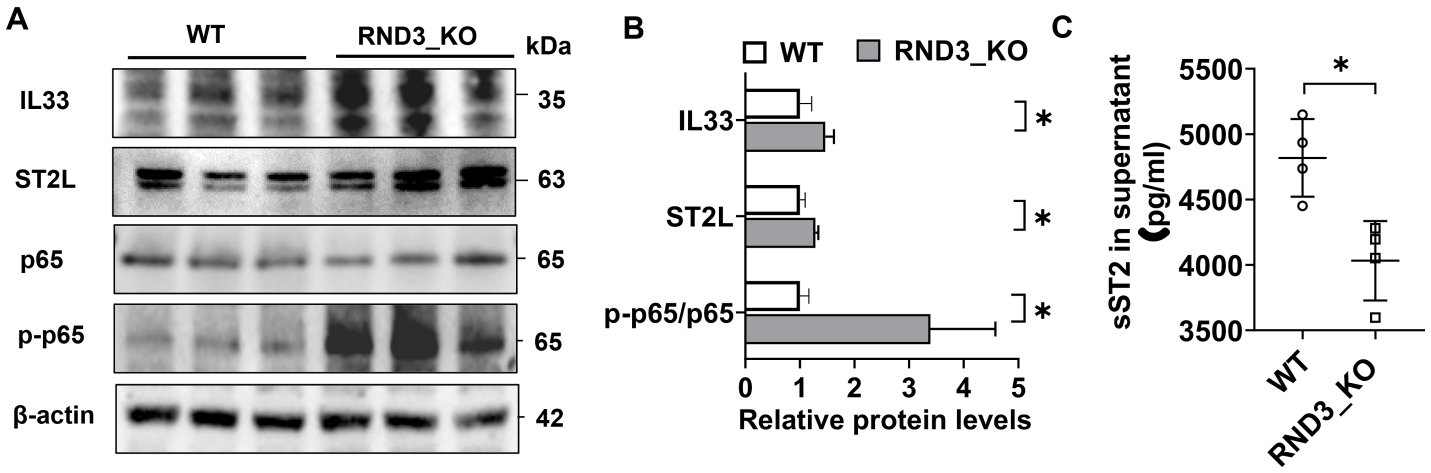

Figure 5

***RND3* gene knockout activates IL33/ST2L signaling in cardiomyocytes.** (A-B) Representative western blots for the expression of IL33, ST2L and activation of NF-κB(p-p65/p65) in H9C2 cells, mean ± SD, *n*=3, **P*<0.05. (C) ELISA was used to detect the expression of sST2 in the supernatant of cell culture medium, mean ± SD, *n*=4, **P*<0.05.

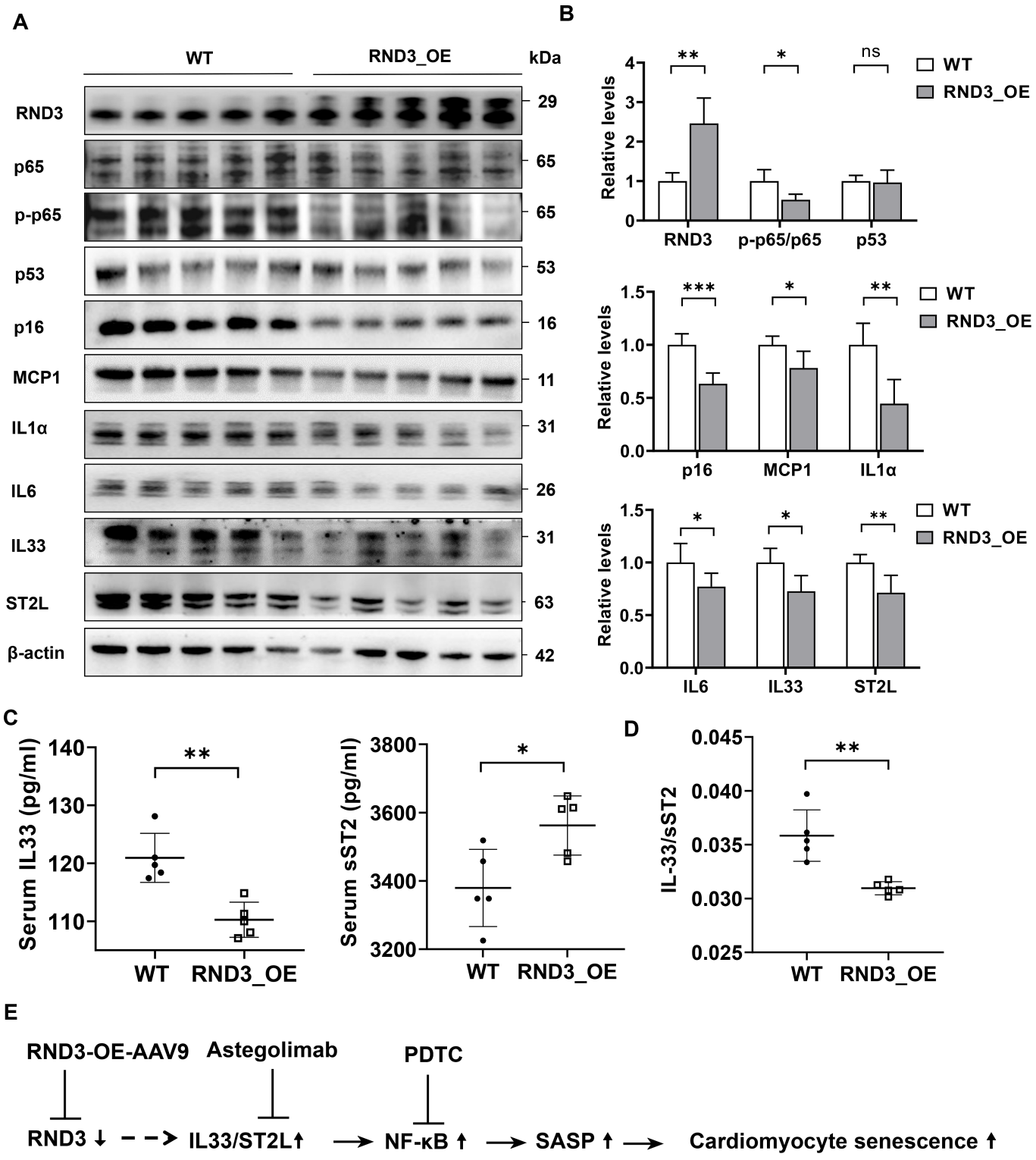

Figure 6

Overexpression of *RND3* in heart tissues inhibits IL33/ST2L signaling and SASPs. (A-B) Representative western blots for the expression of RND3, NF-κB, p53, p16, MCP1, IL1α, IL6, IL33 and ST2 in AAV9 infected heart tissues. mean ± SD, *n*=5, **P*<0.05, ***P*<0.01, ****P*<0.001, ns, no significance. (C) ELISA was used to detect the expression of IL33 and sST2 in the rat serum, mean ± SD, n=5, **P*<0.05, ***P*<0.01. (D) Ration of IL33/sST2 in the rat serum, mean ± SD, n=5, ***P*<0.01. (E) Schematic diagram of the

mechanism of cardiomyocyte senescence induced by down-regulation of RND3 expression. After down-regulation of RND3 expression, it activates IL33/ST2 signal through an unknown mechanism, and then activates NF-κB signal to induce SASP phenotype and promote cardiomyocyte senescence. Overexpression of RND3 by cardiomyocytes, or administration of ST2 antibody Astegolimab, or NF-κB inhibitor PDTC can inhibit cell senescence.