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Article

Epithelial AhR Suppresses Allergen-Induced Oxidative Stress and Senescence via c-Myc Regulation

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Abstract

Environmental allergens trigger epithelial reactive oxygen species (ROS) production and cellular senescence, contributing to airway inflammation. The aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor responsive to environmental stimuli, may modulate this process. Single-cell transcriptomics from allergen-challenged bronchoalveolar brushings of allergic asthma and non-asthmatic allergic control subjects were analyzed for ROS, senescence, and AhR activity. Club cell-specific p16 knockout (*p16^{ΔScgb1a1}*) and AhR-deficient (*AhR^{ΔScgb1a1}*) mice were used to assess epithelial senescence and AhR function. Single-cell analysis revealed epithelial senescence as a hallmark of allergen-induced asthma. *p16^{ΔScgb1a1}* mice exhibited reduced ROS levels and airway inflammation. Single-cell analysis also demonstrated an increased AhR activity and ROS generation in airway epithelial cells of allergen-treated asthmatics, and ROS correlated positively with AhR activity and senescence. The regulation of AhR on senescence was documented that VAF347 attenuated, whereas AhR deficiency exacerbated, ROS generation and inflammation in *AhR^{ΔScgb1a1}* mice. RNA-seq identified senescence as a key AhR-regulated pathway, implicating c-Myc, TGF-β2, and SERPINE1 as major targets. AhR binding to the c-Myc promoter was confirmed by ChIP-PCR, and pharmacologic inhibition of c-Myc with EN4 reduced allergen-induced ROS, senescence, and inflammation. These findings demonstrate that epithelial AhR suppresses allergen-induced ROS generation and cellular senescence via direct regulation of c-Myc.

Keywords: allergen; asthma; ROS; aryl hydrocarbon receptor; c-Myc; cellular senescence; airway epithelial cells

1. Introduction

The prevalence of asthma has continued to rise despite increased awareness of its hazards and substantial efforts in prevention and treatment [1]. Notably, elderly patients (≥ 65 years) experience a significantly higher incidence of acute exacerbations compared with younger patients [2–4]. They also tend to exhibit more severe airway obstruction, poorer asthma control, and more frequent hospitalizations. Recently, increasing attention has been focused on the potential involvement of cellular senescence in asthma. Although data remain limited, emerging evidence suggests that cellular senescence is accelerated in asthma, particularly in elderly patients [5–9]. Cellular senescence is a complex state induced by factors such as cellular stress and DNA damage, leading to cell cycle

arrest and the release of senescence-associated secretory phenotype (SASP) factors [10–12]. The SASP includes chemokines, cytokines, growth factors, adhesion molecules, and lipids, which exert both local and systemic effects that contribute to disease pathogenesis [13–20]. Targeting senescence has therefore emerged as a promising therapeutic approach for preventing or treating disease [21]. However, the specific pathways driving cellular senescence and the potential of senescence-targeted therapies in asthma remain poorly understood.

Increasing evidence suggests that airway epithelial cells in asthma, particularly in elderly patients, may undergo accelerated cellular senescence [5–9]. Upon contact with aeroallergens such as cockroach, house dust mite, or pollen, epithelial cells generate excessive reactive oxygen species (ROS) through NADPH oxidase activation and mitochondrial dysfunction, leading to oxidative DNA damage and activation of p53-p21 and p16-RB senescence pathways [22–24]. Importantly, allergen-induced oxidative stress impairs autophagic flux, causing damaged mitochondria and proteins to accumulate, which amplifies ROS and SASP release [25], thereby driving epithelial barrier dysfunction, alarmin secretion (*e.g.*, TSLP, IL-33, and IL-25), and allergic airway inflammation [8,22].

Aryl hydrocarbon receptor (AhR), a cytoplasmic receptor and a ligand-activated transcription factor, has been suggested to regulated asthma phenotypes in elderly populations as part of the aging process [4,26]. Our research has established the AhR as a critical protective regulator in airway epithelial cells during allergic airway inflammation. Specifically, AhR activation suppressed mucus hyperproduction by inhibiting ROS-triggered NLRP3 inflammasome activation [27]. Furthermore, we demonstrated that AhR in type II alveolar epithelial (AT2) cells preserves autophagic flux, preventing the accumulation of damaged organelles and limiting inflammatory responses [28]. These findings align with emerging data demonstrating that environmental pollutants such as PM_{2.5} and vehicular exhaust particles exacerbate airway hyperresponsiveness and inflammation via AhR-dependent pathways, including ROS production and AhR-Notch signaling [29,30]. These findings suggest that AhR modulates key stress-response pathways in airway epithelial cells, oxidative stress, inflammasome activation, and autophagy, that may also be central drivers of allergen-induced cellular senescence.

In this study, we investigated the role of the AhR in regulating allergen-induced ROS generation and cellular senescence in bronchial epithelial cells and its contribution to allergic airway inflammation. Using both *p16^{ΔScgb1a1}* and *AhR^{ΔScgb1a1}* mice along with human bronchial epithelial cells, we demonstrated that AhR is a key regulator of epithelial ROS generation and senescence during allergen exposure and allergic airway inflammation. RNA-seq analysis identified cellular senescence as a major AhR-regulated pathway, with c-Myc emerging as a central target gene. These findings reveal that AhR modulates allergen-induced senescence via a c-Myc-dependent mechanism.

2. Methods

2.1. Single-Cell RNA-seq Data Acquisition and Pre-Processing

Raw count matrices for human asthma models were retrieved from the Gene Expression Omnibus (accession GSE1938161). The study provides pre-annotated single-cell transcriptomes from broncho-alveolar brushings collected at baseline, after diluent (solution) challenge and after allergen challenge in patients with allergic asthma (AA) and non-asthmatic allergic controls (AC)¹. Only the allergen-challenged cells (AA_Ag and AC_Ag) were retained for downstream analysis to focus on the immediate airway response to allergen exposure. Analyses were performed by Seurat (5.3.0), and gene counts were log-normalized and the top 2000 highly variable genes were identified with the vst method. Data was scaled to unit variance and zero meaning before dimensionality reduction. The harmony algorithm were used to remove the batch effect caused by sequencing channels².

2.2. Mice

Both *p16^{ΔScgb1a1}* and *AhR^{ΔScgb1a1}* mice on a C57BL/6 background were generated by crossing CC10-*Cre^{ERTM}* mice with *Cdkn2a^{fl}* or *AhR^{fl}* mice, respectively. All parental strains were obtained from The

Jackson Laboratory (Bar Harbor, ME, USA). Mice were maintained under specific pathogen-free conditions in the animal facilities of the Johns Hopkins University School of Medicine. All procedures were performed in accordance with institutional and National Institutes of Health guidelines and were approved by the Johns Hopkins University Animal Care and Use Committee. Experimental mice were used at ≥ 8 weeks of age, with age- and sex-matched littermates serving as controls.

2.3. Cockroach Allergen-Induced Asthma Mouse Model

Before induction of allergic asthma, $p16^{\Delta Scgb1a1}$ and $AhR^{\Delta Scgb1a1}$ mice received tamoxifen (75 mg/kg; Sigma) via intraperitoneal injection for 5 consecutive days, followed by a 1-week rest period, in accordance with the Jackson Laboratory protocol. Allergic airway inflammation was then induced using cockroach extract (CRE; Greer Laboratories) as shown in Figure 1A [31]. Briefly, under light isoflurane anesthesia, mice were intratracheally instilled with 20 μ g CRE (B46, Greer Laboratories) in 50 μ l phosphate-buffered saline (PBS) for sensitization and subsequently challenged with the same dose of CRE. Control mice received PBS alone. Mice were euthanized one day after the final challenge for collection of lung tissue, bronchoalveolar lavage fluid (BALF), and blood. BALF was obtained by flushing the lungs twice with 1.0 ml ice-cold PBS. Blood samples were centrifuged to obtain serum for further analysis. In selected experiments, mice were treated with the AhR agonist VAF347 (30 mg/kg; MCE) or the c-Myc inhibitor EN4 (50 mg/kg; MCE), administered by gavage or intraperitoneal injection 1 hour prior to each allergen challenge [32,33]; control mice received saline vehicle.

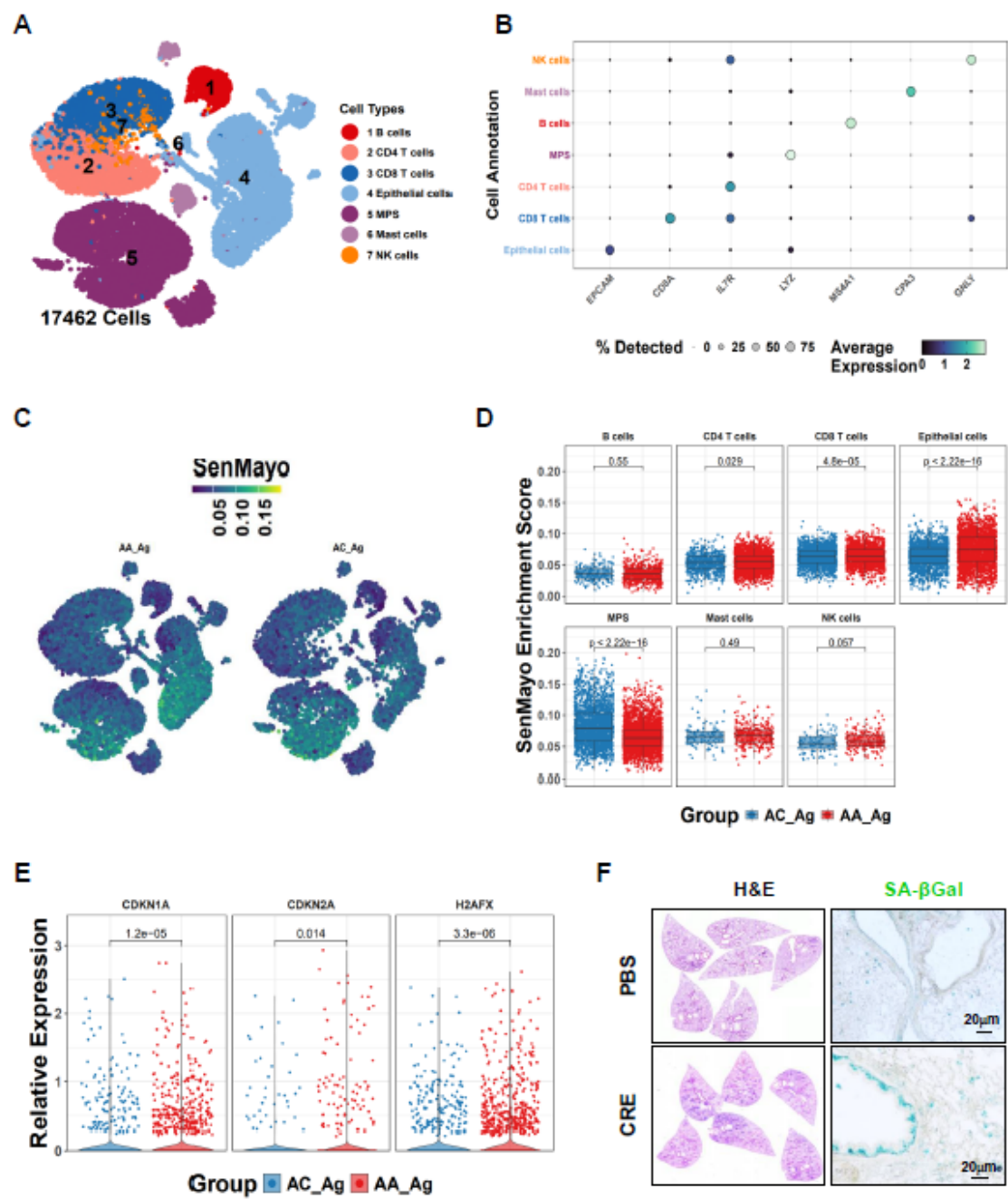


Figure 1. Single-cell transcriptomics reveal epithelial senescence as a key feature of allergen-induced asthma. (A) UMAP embedding of 17,462 single cells from allergic asthmatics (AA, n = 4) and allergic controls (AC, n = 4), color-coded by lineage. (B) Dot plot of canonical marker genes validating lineage identity. (C) UMAP embedding showing distribution of SenMayo senescence scores across cell subsets after allergen challenge. (D) SenMayo senescence scores in allergen-treated asthmatics (AA_Ag) versus allergen-treated allergic controls (AC_Ag). (E) Expression of senescence-associated genes (CDKN1A, CDKN2A, H2AFX) in airway epithelial cells. (F) H&E and SA-β-Gal staining of lung tissues of PBS and cockroach allergen (CRE)-challenged asthma mice. Data represent mean ± SEM.

2.4. Analysis of Lung Inflammation

The mouse lungs were perfused by injecting 10 mL of ice-cold PBS into the right ventricle. Subsequently, the left lung was excised, fixed with 4% formalin, and then embedded in paraffin or optimal cutting temperature (OCT) compound. Sections (4 μm) were prepared from these paraffin-

embedded lungs and stained with hematoxylin and eosin (H&E) as well as periodic acid-Schiff (PAS) to assess pulmonary pathology and mucus secretion [34]. The right lung was frozen at - 80 °C for further detection.

2.5. Flow Cytometry Analysis

The BALFs obtained in the previous step were centrifuged, and the supernatant was collected for later use. The cell pellet was resuspended with pre-cooled PBS to prepare a cell suspension. The total number of cells in the BALFs was counted using Countless II (ThermoFisher). The differential cell percentages in the BALFs were measured on an Accuri C6 Plus flow cytometer (BD Biosystems), and the data were analyzed using FlowJo software (Tree Star Inc.) [35]. The antibodies were provided in the online repository (Supplementary Table S1).

2.6. Immunofluorescence Staining

Immunofluorescence staining was performed as previously reported [36]. Briefly, first, lung tissue sections were blocked with 5% w/v bovine serum albumin (BSA) at room temperature for 1 hour. For cell samples, they were first fixed with 4% paraformaldehyde for half an hour and then blocked. Then, the samples were incubated with the primary antibodies listed in the online repository (Supplementary Table S2) at 4°C overnight. Subsequently, the samples were incubated with fluorescent-conjugated secondary antibodies at room temperature for 1 hour. Isotype-matched negative control antibodies were used under the same conditions. The sections were mounted with a fluorescent mounting medium (Sigma) containing DAPI (4',6-diamidino-2-phenylindole) (ThermoFisher) and then observed using a NIKON ECLIPSE Ti-U microscope (Nikon, USA) equipped with a DS-Fi2 camera.

2.7. ROS Measurement

For ROS in lung tissues, frozen sections were incubated with 5 µM of dihydroethidium (DHE, Thermo Fisher Scientific) 37°C for 30 min, and then fluorescent signal was observed and analyzed under microscope (Nikon, Chiyoda, Japan).

2.8. Senescence-Associated β -Galactosidase Staining

According to the manufacturer's instructions, the cultured cells and frozen lung tissue sections (10 µm) were fixed and stained using the SA- β -Gal staining kit (9860S, Cell Signaling Technology, USA).

2.9. Enzyme-Linked Immunosorbent Assay

Interleukin-4 (IL-4), IL-5, IL-13, IL-17, and IFN- γ in the cell-free BALF were detected using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions [37].

2.10. Cell Culture and Treatment

Human bronchial epithelial cells (HBECs) were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% v/v fetal bovine serum and 1% penicillin-streptoc-Mycin in a humidified atmosphere at 37°C and 5% CO₂. HBECs were treated with cockroach extract (CRE, Greer Laboratory) alone, or were pre-treated with the AhR agonist VAF347 (HY-135750, 20 µM, MCE), the AhR antagonist CH-223191 (C8124, 10 µM, Sigma), or the c-Myc inhibitor EN4 (HY-134761, 50 µM, MCE) 1 hour before CRE treatment [28,32,38].

2.11. RNA-seq Analysis

According to the manufacturer's instructions, the total RNA of each sample was processed using the Vazyme VAHTS Universal V10 RNA-seq Library Prep Kit, which selectively captures mRNA

transcripts through poly(A) enrichment. Subsequently, the enriched RNA was reverse-transcribed and constructed into a sequencing library following the manufacturer's protocol. The library was sequenced using paired-end 2×150 bp sequencing on the Element AVITI system, and approximately 20 million paired-end reads were obtained for each sample. Gene expression levels were defined as Salmon counts. Differential gene expression analysis was performed using the R package DESeq2 (version 1.46.0). Genes with an absolute log2 fold change [$\text{abs}(\log_2\text{FC})$] > 0.5 and $p < 0.05$ were defined as differentially expressed genes (DEGs). Gene set enrichment analysis was carried out using clusterProfiler version 4.14.4[39]. Upregulated or downregulated DEGs were visualized using ComplexHeatmap (version 2.22.0) and ggplot2 (version 3.5.1) [40]. All downstream analyses and visualizations based on RNA-seq were conducted in R version 4.4.2.

2.12. RNA Isolation and Quantitative Real-Time PCR Analysis

Total RNA was extracted from lung tissues or HBECs using the Monarch Total RNA Miniprep Kit (New England Biolabs), and cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher). Quantitative real-time PCR analysis was performed on an ABI Prism 7300 Detection System using Power SYBR Green PCR Master Mix (Thermo Fisher). Data were analyzed using the $2^{-\Delta\Delta\text{CT}}$ method relative to the housekeeping genes β -actin or GAPDH. The primer sequences are provided in the online repository (Supplementary Table S3).

2.13. In Silico Prediction of AhR–c-Myc Binding Using AlphaFold 3

To predict the regulatory relationship between AhR and c-Myc, we obtained the protein sequence data from UniProtKB (<https://www.uniprot.org/>), with the UniProtKB IDs of P35869 for AhR and P01106 for c-Myc. Subsequently, we conducted molecular docking using the AlphaFold 3 Server platform. Finally, we visualized the results using PyMOL (Version 3.0.3).

2.14. Chromatin Immunoprecipitation

To investigate the impact of AhR on the promoter region of c-Myc, a ChIP kit (Abcam) was employed, and the experiments were conducted following the manufacturer's instructions. Briefly, after intervention, HBECs were cross-linked with 1% formaldehyde to stabilize protein-DNA interactions. Subsequently, the nuclei were isolated by centrifugation and lysis, and the DNA was fragmented by sonication. Then, the DNA-protein complexes were immunoprecipitated by incubating overnight at 4 °C with antibodies against AhR or rabbit IgG. After that, the DNA was eluted and purified. Finally, the obtained DNA was analyzed by qPCR. The primer sequences for c-Myc are provided in the online repository (Supplementary Table S4).

2.15. Statistical Analysis

All statistical analyses were conducted using GraphPad Prism version 10.1.2 (GraphPad Software, La Jolla, CA, USA). All data are presented as the mean $\pm$ SEM. For comparisons among more than two groups, an ordinary one-way analysis of variance (ANOVA) was performed, followed by Tukey's post-hoc test. A P value < 0.05 was considered to indicate a statistically significant difference.

3. Results

3.1. Single-Cell Transcriptomics Reveal Epithelial Senescence as a Key Feature of Allergen-Induced Asthma

To investigate the relevance of cellular senescence in asthma, we analyzed scRNA-seq data from the GSE193816 dataset, which included 17,462 lung cells obtained via endobronchial brushing from allergic asthmatics (AA, $n = 4$) and allergic controls (AC, $n = 4$) [41]. Unsupervised clustering identified seven major cell lineages using the Louvain algorithm (Figure 1A) [42], and lineage identity was validated through marker gene expression analysis, as illustrated by a dot plot showing both the

proportion of cells expressing canonical markers and their relative expression levels (Figure 1B). UMAP embedding of SenMayo senescence scores demonstrated broad enrichment of senescence signatures across cell subsets following allergen challenge (Figure 1C). Quantitative comparison revealed significantly higher senescence scores in allergen-treated asthmatics (AA_Ag) compared with allergen-treated allergic controls (AC_Ag), with airway epithelial cells exhibiting the strongest enrichment (Figure 1D). Consistent with these findings, senescence-associated genes, including CDKN1A, CDKN2A, and H2AFX, were significantly upregulated in airway epithelial cells from AA_Ag compared with AC_Ag (Figure 1E). Furthermore, SA- β -Gal staining of lung tissues confirmed enhanced senescence, particularly within airway epithelial cells, in an allergen-challenged asthma mouse model (Figure 1F). Together, these results demonstrate that allergen exposure induces increased cellular senescence, with airway epithelial cells being the primary compartment affected.

3.2. Deletion of Senescent Club Cells Attenuates Allergen-Induced ROS and Airway Inflammation

To examine the functional significance of epithelial senescence in allergen-induced airway inflammation, we generated $p16^{\Delta Scgb1a1}$ mice by crossbreeding $p16^{fl/fl}$ with $Scgb1a1-CreER^{TM}$ mice (Figure E1A). Selective deletion of $p16^+$ cells in Club cells was confirmed by genotyping (Figure E1B) and immunostaining (Figure E1C). These mice were then subjected to the CRE-induced asthma protocol (Figure 1A). In wild-type (WT) mice, CRE exposure markedly increased senescence markers, as demonstrated by SA- β -Gal staining and immunofluorescence for Cdkn2a (p16) and γ H2AX. In contrast, $p16^{\Delta Scgb1a1}$ mice exhibited substantially reduced expression of these markers (Figure 1B–C). The same pattern was also observed for ROS generation. RT-PCR further validated decreased transcript levels of p16, IL-6, and IL-1 β in lung tissues of $p16^{\Delta Scgb1a1}$ mice compared with WT controls (Figure 1D). Histological examination revealed attenuated peribronchial and perivascular inflammatory infiltrates (Figure 2E, upper) and reduced mucus hypersecretion in $p16^{\Delta Scgb1a1}$ mice (Figure 2E, lower). Consistently, bronchoalveolar lavage fluid (BALF) analysis showed significantly lower numbers of total inflammatory cells, with a marked reduction in eosinophils (Figure 2F, Figure E1D). Moreover, levels of cytokines IL-4, IL-5, IL-13, and IL-17 were significantly reduced in BALF from $p16^{\Delta Scgb1a1}$ mice compared with WT controls (Figure 2G). In contrast, CRE exposure suppressed the Th1 cytokine IFN- γ in WT mice, its levels were restored in $p16^{\Delta Scgb1a1}$ mice relative to WT controls. These findings demonstrate that selective deletion of senescent Club cells mitigate allergen-induced epithelial senescence, reduces mucus production, and alleviates allergic airway inflammation.

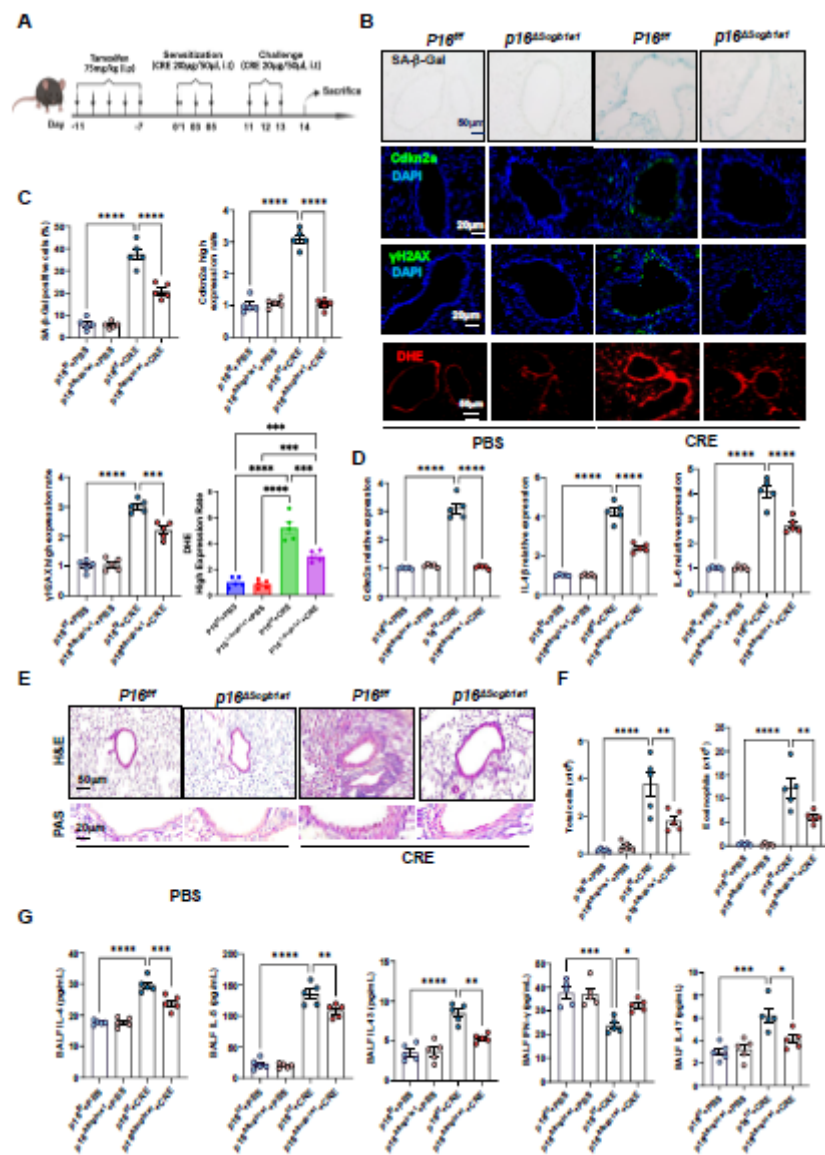


Figure 2. Deletion of senescent Club cells attenuates allergen-induced airway inflammation. **(A)** Experimental scheme of mouse asthma model. **(B)** Representative images of SA-β-Gal staining and Cdkn2a (p16), yH2Ax, and DHE immunostaining in lung tissue sections. **(C)** Quantitative analysis of relative fluorescence intensity for SA-β-Gal, Cdkn2a (p16), yH2Ax, and ROS. **(D)** RT-PCR analysis of Cdkn2a (p16), IL-6, and IL-1β in lung tissues. **(E)** Representative images of hematoxylin and eosin staining (H&E, upper) and periodic acid-Schiff staining (PAS, lower) of lung sections. **(F)** Total and eosinophil cell count in bronchoalveolar lavage fluid (BALF). **(G)** BALF cytokine levels measured by ELISA. n=5. Data represent mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

3.3. Allergen-Induced Upregulation of AhR Correlates with ROS Generation and Epithelial Senescence

To determine whether allergen challenge alters AhR expression, we analyzed single-cell transcriptomic profiles from the GSE193816 dataset. UMAP embedding revealed broad AhR expression across multiple cell lineages, with marked enrichment in epithelial subsets following allergen challenge (Figure 3A). Quantitative analysis showed significantly higher AhR activity scores in allergen-treated asthmatics (AA_Ag) compared with allergen-treated allergic controls (AC_Ag) (Figure 3B). To validate these findings, AhR expression was examined in HBECS. Treatment with CRE or the AhR agonist VAF347 increased AhR protein expression, with the strongest induction observed upon combined treatment (Figure 3C). Similarly, AhR expression was upregulated in lung tissues of WT mice exposed to CRE or VAF347, with the highest levels in mice receiving both

treatments (Figure 3D), indicating that allergen exposure enhances AhR expression predominantly in airway epithelial cells. Single-cell analysis further demonstrated increased ROS generation in allergen-treated asthmatics (Figure 3E), particularly within epithelial cells (Figure 3F). Notably, ROS levels correlated positively with AhR activity ($r = 0.531$, $P < 0.0001$; Figure 3G) and cellular senescence ($r = 0.453$, $P < 0.0001$; Figure 3H). Thus, allergen challenge promotes AhR expression and activation in airway epithelial cells, which correlates with elevated ROS and senescence signatures.

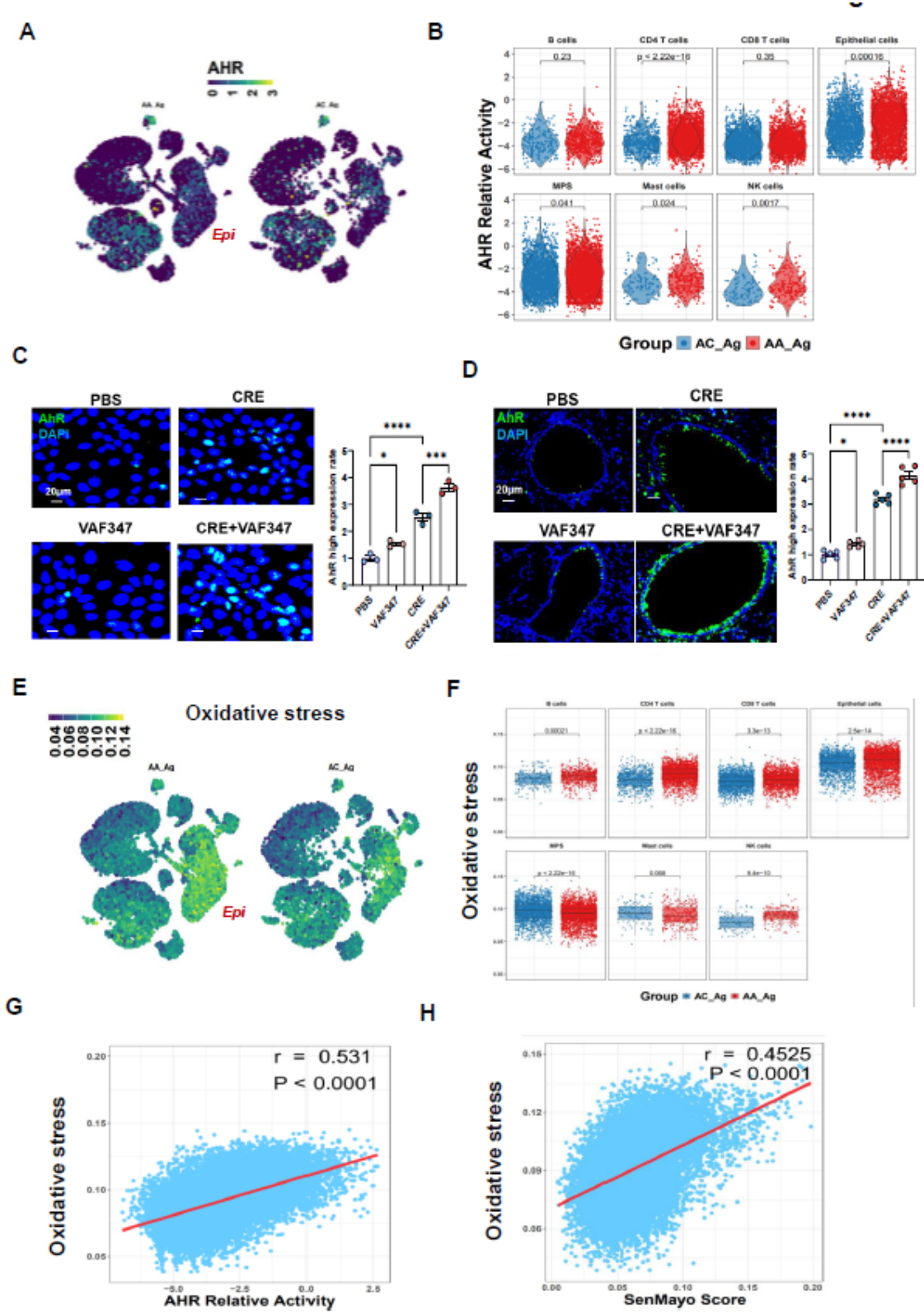


Figure 3. Enhanced AhR signaling in airway epithelial cells following allergen exposure. (A) UMAP embedding of single-cell data showing AhR expression across cell lineages following allergen challenge. (B) AhR activity scores in allergen-treated asthmatics (AA_Ag) versus allergen-treated allergic controls (AC_Ag). (C-D) Immunostaining and quantification of AhR expression in HBECs (C) and lung tissues of mice (D) treated with cockroach extract CRE), AhR agonist VAF347, or CRE+VAF347. (E-F) UMAP embedding single-cell data showing ROS expression across cell lineages following allergen challenge (E) and ROS scores in allergen-treated asthmatics (AA_Ag) versus allergen-treated allergic controls (AC_Ag) (F). (G-H) Correlations between ROS levels and AhR activity (G) and cellular senescence (H). Data represent mean $\pm$ SEM; * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

3.4. Enhanced AhR Signaling Protects Against Allergen-Induced Senescence in HBECs

Given the enhanced AhR signaling observed in airway epithelial cells, we hypothesized that AhR signaling may serve as a central driver of allergen-induced cellular senescence. To test this, HBECs were exposed to CRE (50 μ g/mL) for 24 h in the presence or absence of the AhR agonist VAF347 (20 μ M) or antagonist CH223191 (10 μ M). Expression of AhR and senescence-related markers was assessed by immunostaining (Figure E2A, B) and RT-PCR (Figure E2C). As expected, CRE increased SA- β -Gal activity in HBECs, which was further enhanced by CH223191 but suppressed by VAF347. Similar patterns were observed for immunofluorescent staining of Cdkn2a, Cdkn1a, and γ -H2AX. In addition, IL-1 β and IL-6, representative SASP factors, were elevated after CRE exposure, augmented by CH223191, and reduced by VAF347 (Figure E2D). These *in vitro* findings indicate that AhR activation protects against allergen-induced cellular senescence.

3.5. Club cell-Specific Deletion of AhR Exacerbates Cockroach Allergen-Induced Epithelial Senescence and Airway Inflammation

Next, we examined whether loss of AhR in Club cells would have the opposite effect. AhR^{AScgb1a1} mice were generated by crossbreeding AhR^{fl/fl} mice with Scgb1a1-CreERTM mice (Figure E3A), and efficient deletion of AhR in Club cells was confirmed by genotyping (Figure E3B) and immunostaining (Figure E3C). Following CRE exposure as illustrated in Figure 2A, AhR^{AScgb1a1} mice exhibited markedly increased SA- β -Gal activity and stronger immunofluorescent staining for senescence-associated markers compared with AhR^{fl/fl} controls (Figure 4A). Quantitative fluorescence intensity analysis demonstrated significant upregulation of all three markers in AhR-deficient Club cells (Figure 4B). The same pattern was also observed for ROS generation. Consistent with these histologic observations, qRT-PCR analysis revealed elevated expression of Cdkn2a, and SASP-related cytokines (IL-1 β , IL-6) in lung tissues from AhR^{AScgb1a1} mice compared with controls (Figure E3D). Histopathologic evaluation showed more severe peribronchial and perivascular inflammatory infiltrates and increased mucus production in AhR^{AScgb1a1} mice (Figure 4C). BALF analysis confirmed significantly higher total inflammatory cell counts, particularly eosinophils (Figure 4D, Figure E3E), along with increased levels of cytokines IL-4, IL-5, IL-13, and IL-17 in BALF from p16^{AScgb1a1} mice compared with WT controls (Figure 4E). In contrast, CRE exposure suppressed the Th1 cytokine IFN- γ in WT mice, its levels were further reduced in p16^{AScgb1a1} mice relative to WT controls. Collectively, these results suggest that pharmacologic activation of AhR protects against allergen-induced ROS, cellular senescence, and alleviates allergic airway inflammation.

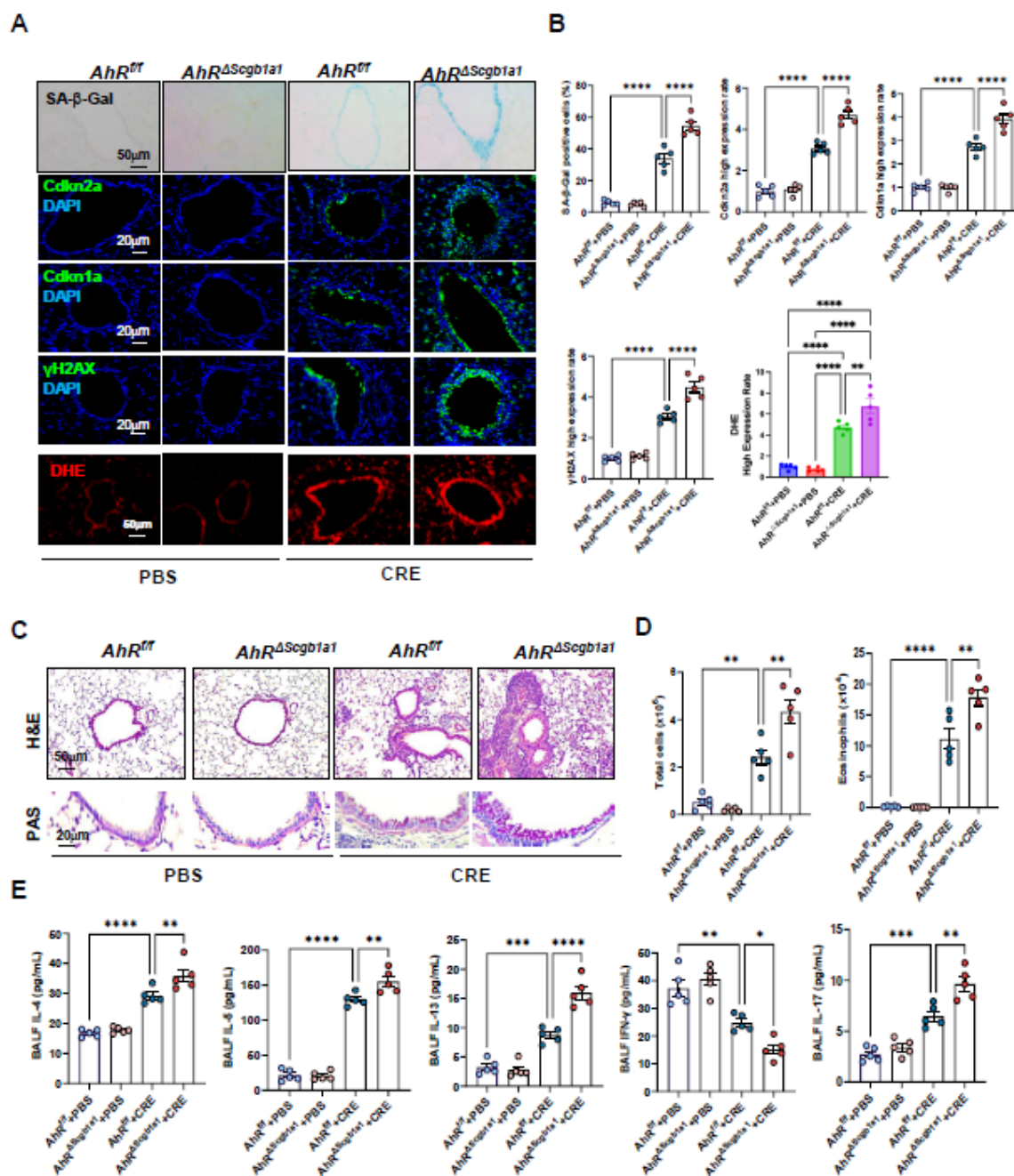


Figure 4. Club cell-specific deletion of AhR exacerbates cockroach allergen-induced epithelial senescence and airway inflammation. **(A)** Representative immunofluorescence staining for SA- β -Gal activity, senescence-associated markers, and ROS in lung sections. Nuclei were counterstained with DAPI. **(B)** Quantitative analysis of relative fluorescence intensity for SA- β -Gal, Cdkn2a (p16), γ -H2AX, and ROS from images in **(A)**. **(C)** Representative images of hematoxylin and eosin staining (H&E, upper) and periodic acid-Schiff staining (PAS, lower) of lung sections. **(D)** Total and eosinophil cell count in bronchoalveolar lavage fluid (BALF). **(E)** BALF cytokine levels measured by ELISA. n=5. Data represent mean $\pm$ SEM; * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

3.6. AhR Activation Suppresses Cockroach Allergen–Induced Senescence and Airway Inflammation

To determine whether AhR activation modulates allergen-induced cellular senescence, we employed the CRE-induced asthma mouse model in the presence or absence of the AhR agonist VAF347 (Figure E4A). CRE exposure markedly increased SA- β -Gal activity and expression of senescence-associated markers [Cdkn2a (p16), Cdkn1a (p21), γ -H2AX] in the lung tissues, whereas VAF347 treatment significantly suppressed these responses (Figure E4B, C). qRT-PCR analysis

confirmed that VAF347 reduced CRE-induced expression of Cdkn2a, IL-6, and IL-1 β in lung tissues (Figure E4D). Histological analyses revealed that VAF347 attenuated peribronchial and perivascular inflammation and decreased mucus production (Figure E4E). Consistent with these histologic findings, VAF347 treatment significantly reduced total and differential inflammatory cell counts in BALF, particularly eosinophils (Figure E4F), and lowered BALF IL-4, IL-5, IL-13, and IL-17 but increased IFN- γ levels (Figure E4G). These results indicate that pharmacologic activation of AhR protects against allergen-induced cellular senescence and alleviates allergic airway inflammation.

3.7. Transcriptomic Profiling Identifies AhR-Regulated Gene Expression in HBECs

To define the transcriptional programs regulated by AhR during allergen exposure, we performed RNA-seq on CRE-treated HBECs with or without the AhR agonist VAF347. Volcano plot analysis revealed a distinct transcriptional separation between the CRE and CRE+VAF347 groups (Figure 5A). A total of 585 mRNAs were differentially expressed in HBECs, including 566 down-regulated genes and 19 up-regulated genes. Heatmap visualization of the top differentially expressed genes confirmed robust activation of AhR signaling by VAF347, as evidenced by increased expression of canonical targets AHRR, CYP1B1, and CYP1A1 (Figure 5B). KEGG pathway analysis identified cellular senescence as one of the most prominently altered pathways following AhR activation, along with focal adhesion, tight junctions, extracellular matrix-receptor interaction, and the Rap1 signaling pathway (Figure 5C). Within this pathway, key genes including MYC, TGF β 2, IGFBP3, and SERPINE1 were markedly modulated by VAF347, as illustrated in the senescence-focused heatmap (Figure 5D). RT-PCR validation confirmed significant reduction of MYC, TGF β 2, IGFBP3, and SERPINE1 in CRE-treated HBECs exposed to VAF347 (Figure 5E). Collectively, these results demonstrate that AhR activation reprograms the allergen-induced transcriptome in HBECs, selectively suppressing senescence-driving pathways and their associated effector genes.

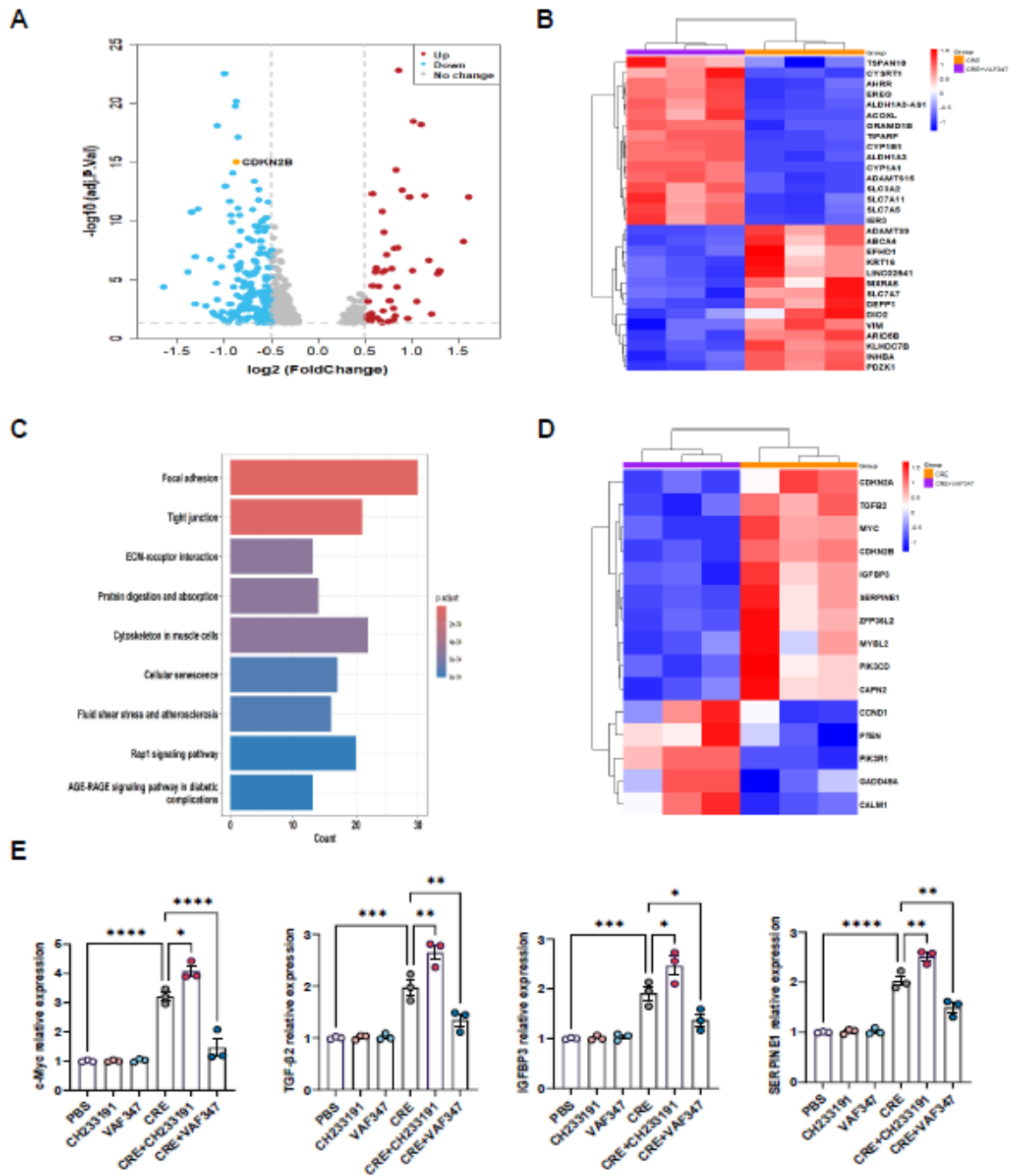


Figure 5. Transcriptomic profiling identifies AhR-regulated gene expression in HBECs. (A) Volcano plot of differentially expressed mRNAs in CRE-treated HBECs with or without the AhR agonist VAF347. (B) Heatmap of the top-ranking differentially expressed mRNAs in CRE-treated HBECs with or without VAF347. (C) KEGG pathway enrichment map of differentially expressed genes comparing the CRE and CRE+VAF347 groups. (D) Heatmap of differentially expressed genes enriched in the cellular senescence pathway. (E) Validation of selected senescence-related genes (MYC, TGF β 2, IGFBP3, and SERPINE1) by RT-PCR. n=3. Data represent mean $\pm$ SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.8. AhR Regulates c-Myc Expression Via Direct Promoter Binding

Within the senescence network, the proto-oncogene c-Myc emerged as a central candidate [43–45]. To examine whether AhR regulates c-Myc expression, HBECs were exposed to CRE (50 $\mu\text{g/mL}$, 24 h) in the presence or absence of the AhR agonist VAF347 (20 μM) or antagonist CH223191 (10 μM). c-Myc expression was evaluated by immunostaining (Figure 6A) and quantified (Figure 6B). CRE

exposure increased c-Myc expression, which was further elevated by CH223191 and inhibited by VAF347. The VAF347-induced downregulation of c-Myc was confirmed in vivo: lung tissues from CRE-exposed mice treated with VAF347 showed decreased c-Myc expression compared with controls (Figure 6C, D). Conversely, lung tissues from CRE-treated *AhR*^{ΔScgb1a1} mice exhibited significantly higher c-Myc expression than those from *AhR*^{fl/fl} controls (Figure 6E, F). To explore a potential regulatory interaction, molecular docking analysis predicted binding interfaces between AhR and c-Myc (Figure 6G). This interaction was experimentally validated by co-immunoprecipitation (Co-IP), where c-Myc was detected in AhR immunoprecipitates (Figure 6H). We further hypothesized that AhR modulates c-Myc transcription via direct promoter binding. Analysis of the JASPAR database identified a conserved AhR-binding motif in the c-Myc promoter. ChIP-PCR using primers flanking this site (Figure 6I) demonstrated significantly enriched AhR binding after CRE exposure (Figure 6J), indicating that AhR directly associates with the c-Myc promoter and regulates its transcriptional activity.

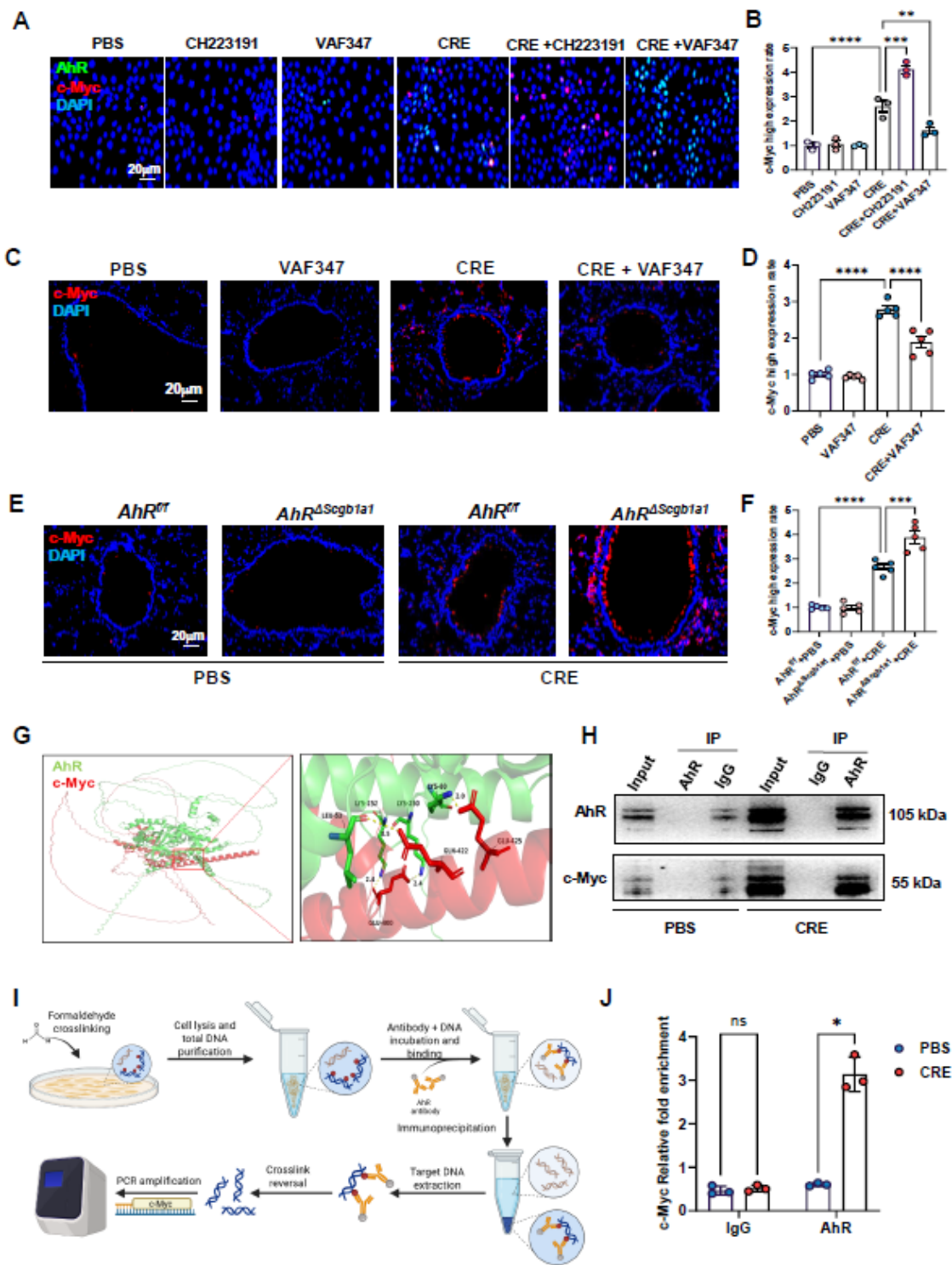


Figure 6. AhR regulates c-Myc expression via direct promoter binding. (A) Immunofluorescence staining of c-Myc in HBECs exposed to CRE (20 μ g/mL, 24 h) with or without the AhR agonist VAF347 (20 μ M) or antagonist CH223191 (10 μ M). (B) Quantification of c-Myc fluorescence intensity from (A). (C, D) Immunostaining and quantification of MYC expression in lung tissues from CRE-exposed mice treated with or without VAF347. (E, F) Immunostaining and quantification of MYC expression in lung tissues from CRE-treated *AhR^{Scgb1a1}* and *AhR^{fl}* control mice. (G) Predicted molecular docking structure showing interaction sites between AhR and c-Myc using the AlphaFold 3 Server platform and visualized using PyMOL (Version 3.0.3). (H) Co-immunoprecipitation (Co-IP) of MYC with an anti-AhR antibody. (I) Schematic showing the protocol for ChIP-PCR. (J) The bindings of AhR to the c-Myc promoter following CRE exposure. n=3. Data represent mean $\pm$ SEM; * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

3.9. Inhibition of c-Myc Suppresses Allergen-Induced Senescence and SASP Expression in HBECs

Given that c-Myc emerged as a downstream target of AhR, we hypothesized that it contributes to allergen-induced cellular senescence. HBECs were exposed to CRE (50 $\mu\text{g/mL}$, 24 h) with or without the c-Myc inhibitor EN4 (50 μM). Immunostaining revealed that EN4 effectively suppressed CRE-induced c-Myc expression (Figure 7A, B). Notably, EN4 treatment reduced CRE-induced senescence, as evidenced by decreased SA- β -Gal activity and reduced expression of senescence markers Cdkn2a (p16), Cdkn1a (p21), and γ -H2AX. These inhibitory effects were further confirmed by RT-PCR (Figure 7C). Consistently, EN4 also reduced CRE-induced expression of SASP-related proinflammatory cytokines IL-1 β and IL-6 (Figure 7D). These in vitro findings indicate that c-Myc is a functional mediator of allergen-induced cellular senescence and SASP activation.

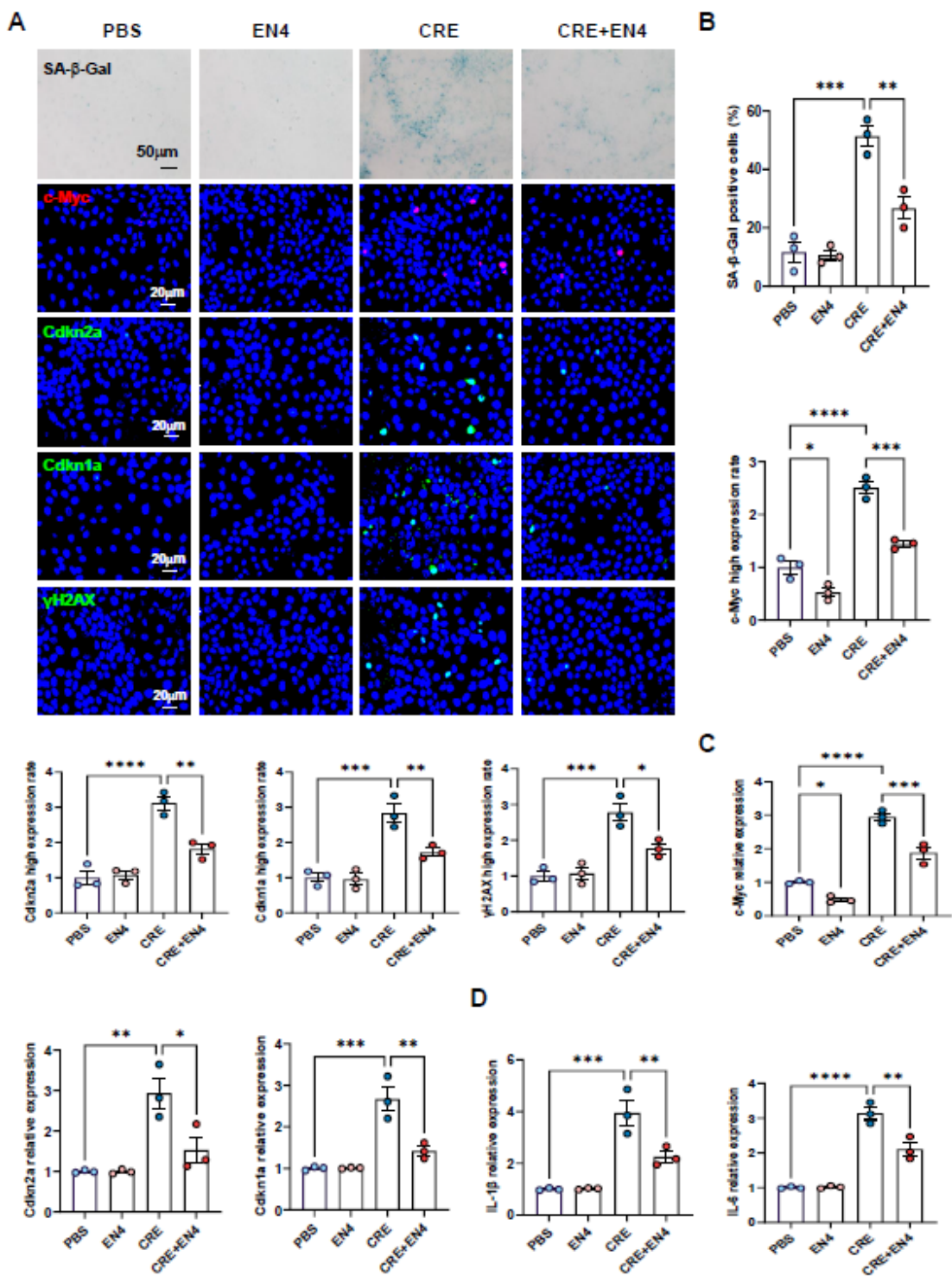


Figure 7. Inhibition of c-Myc suppresses allergen-induced senescence and SASP expression in HBECs. (A) Immunofluorescence staining of c-Myc and senescence markers (SA-β-Gal, p16, p21, and γ-H2AX) in HBECs exposed to CRE (20 μg/mL, 24 h) with or without the c-Myc inhibitor EN4 (50 μM). (B) Quantification of c-Myc and senescence marker fluorescence intensity from (A). (C-D) RT-PCR analysis of senescence-associated markers (C) and SASP-related cytokines (D) in HBECs. n=3. Data represent mean ± SEM; **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

3.10. Inhibiting c-Myc Attenuates Allergen-Induced ROS, Cellular Senescence, and Allergic Airway Inflammation

Next, we investigated whether c-Myc inhibition mitigates allergen-induced cellular senescence and airway inflammation. To do this, we established a CRE-induced asthma mouse model with or without the c-Myc inhibitor EN4 (Figure E5A). CRE exposure markedly increased SA- β -Gal activity and expression of senescence markers [Cdkn2a (p16), Cdkn1a (p21), and γ -H2AX] in lung tissues, whereas EN4 treatment significantly suppressed these responses (Figure 8A, B). The same pattern was also observed for ROS generation. qRT-PCR analysis confirmed reduced expression of Cdkn2a and Cdkn1a in EN4-treated lungs (Figure E5B). Histological examination revealed that EN4 attenuated peribronchial and perivascular inflammation and decreased mucus hypersecretion (Figure 8C). Consistent with these findings, EN4 lowered total and differential BALF inflammatory cell counts (Figure 8D, Figure E5C), particularly eosinophils, and reduced BALF IL-4, IL-5, IL-13, and IL-17 but increased IFN- γ levels (Figure 8E). Collectively, these results identify c-Myc as a functional mediator of allergen-induced epithelial senescence and airway inflammation

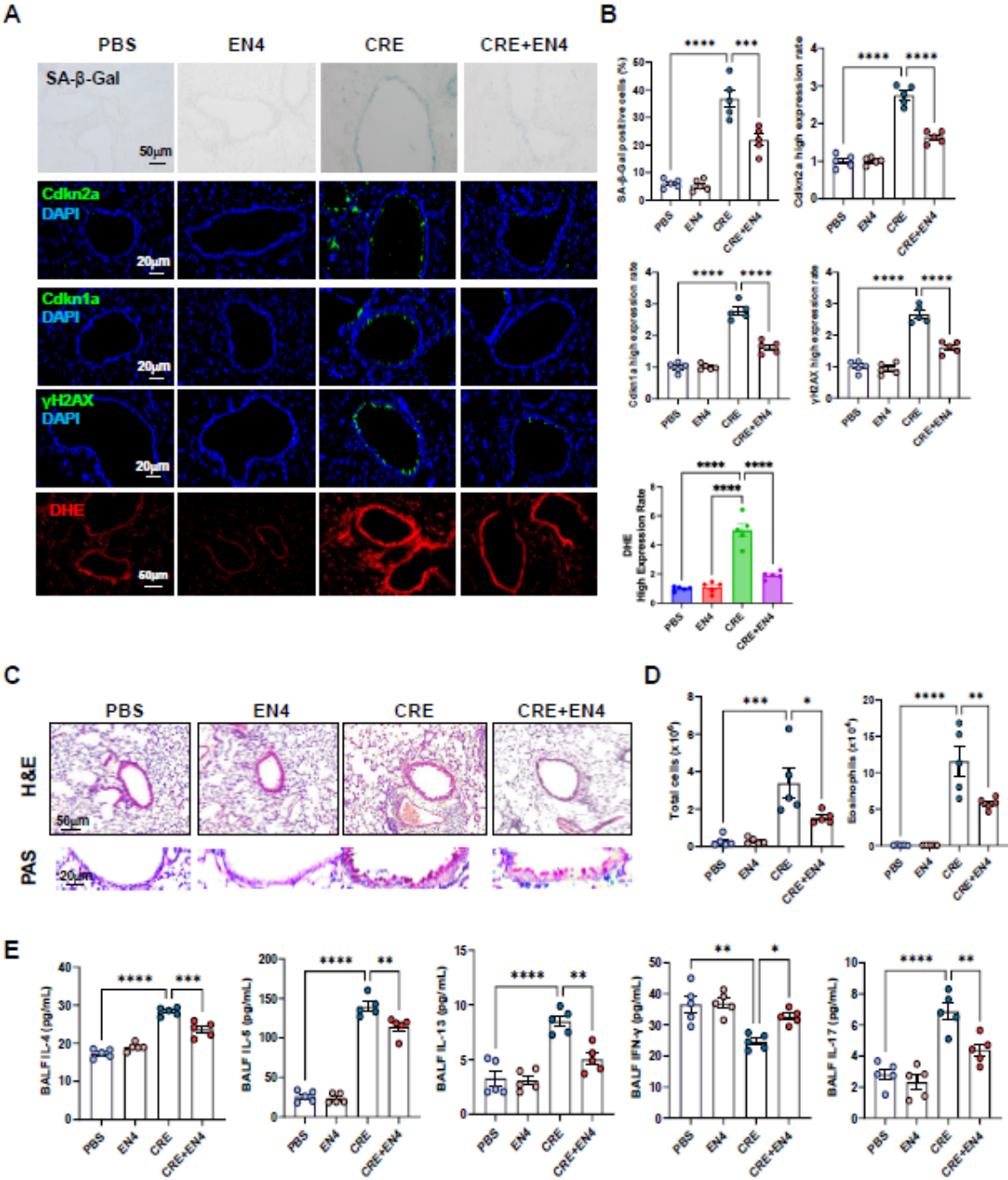


Figure 8. Inhibition of c-Myc attenuates allergen-induced cellular senescence and allergic airway inflammation in vivo. (A) Representative immunofluorescence staining for SA-β-Gal activity, senescence-associated markers, and ROS in lung sections. Nuclei were counterstained with DAPI. (B) Quantitative analysis of relative fluorescence intensity for SA-β-Gal, Cdkn2a (p16), γ-H2AX, and ROS from images in (A). (C) Representative images of hematoxylin and eosin staining (H&E, upper) and periodic acid–Schiff staining (PAS, lower) of lung sections. (D) Total and eosinophil cell count in bronchoalveolar lavage fluid (BALF). (E) BALF cytokine levels measured by ELISA. n=5. Data represent mean ± SEM; **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

4. Discussion

Compared with younger patients, elderly asthmatics are at a disproportionately higher risk of morbidity and mortality, reflecting age-related physiological and immunologic changes that complicate disease presentation and management[46–48]. This is supported by asthma mouse models showing that allergen-challenged aged mice exhibit greater eosinophilia in BALF [49], as well as

enhanced mucus secretion and parabronchial collagen deposition [50], compared with young mice. However, the mechanisms driving airway inflammation in the aged population remain poorly understood and may differ from those in younger individuals. Our study provides new mechanistic insight by showing that cockroach allergen exposure induces excessive ROS production and cellular senescence in bronchial epithelial cells, processes that synergistically amplify airway inflammation. Importantly, we demonstrate that epithelial AhR plays a critical role in restraining allergen-induced ROS generation, senescence, and inflammation. Furthermore, we identify c-Myc as a novel downstream mediator of AhR signaling, revealing that the AhR–c-Myc axis constitutes a previously unrecognized regulatory pathway controlling epithelial ROS homeostasis, senescence, and airway inflammation, likely in elderly asthmatics.

Cellular senescence has emerged as a fundamental biological process contributing to tissue dysfunction and chronic inflammation in aging-related diseases such as COPD and IPF, largely through its promotion of inflammation and tissue remodeling [8]. Senescence is a complex state marked by cellular stress, DNA damage, irreversible cell cycle arrest, and the secretion of SASP factors [10–12]. Given the heterogeneity and context-dependent nature of senescence across cell types and disease stages [8,51,52], a focused investigation is needed to clarify how senescence contributes to allergic airway inflammation and identify potential regulatory pathways and therapeutic targets. To define the cell-type context of senescence in asthma, we analyzed scRNA-seq data from GSE193816 (17,462 endobronchial-brushing cells from allergic asthmatics and allergic controls) and found that senescence and ROS signatures were significantly higher in allergen-treated asthmatics, most prominent in airway epithelial cells. Consistently, our allergen-challenged asthma mouse model confirmed enhanced epithelial senescence—evidenced by SA- β -Gal staining, increased ROS accumulation, and upregulation of senescence-associated genes (*CDKN1A*, *CDKN2A*, *H2AFX*). Given the elevated epithelial ROS and senescence, we hypothesized that these processes amplify inflammatory cascades, particularly in the elderly where oxidative burden is higher. Indeed, selective deletion of p16⁺ Club cells markedly attenuated epithelial ROS and senescence, reduced peribronchial and perivascular inflammation, and decreased mucus hypersecretion, BALF total cells, eosinophils, and Th2/17 cytokines, indicating that Club cell–derived ROS and senescence are functional drivers of allergen-induced airway inflammation. We targeted p16^{INK4a}-expressing senescent cells because p16 is a well-validated senescence effector and a gold-standard surrogate marker of cellular senescence [53]. Clearance of p16^{high} cells has been shown to ameliorate pathology in multiple organs, including the lung [54]. While our approach targeted p16, it may have been limited in fully identifying and removing all senescent cells. Additionally, Club cells were prioritized because they serve as epithelial progenitors, pollutant and allergen sensors, and key sources of inflammatory mediators [55,56]. Their well-established genetic manipulability (e.g., *Scgb1a1-CreER^{T2}*) makes them ideal for dissecting how epithelial ROS and senescence contribute to allergic airway inflammation.

AhR is abundantly expressed in airway tissues and activated by diverse endogenous and exogenous ligands, exerting both pro- and anti-inflammatory effects [57–59]. Endogenous ligands such as tryptophan metabolites promote anti-inflammatory responses by inducing IL-10, enhancing CD4⁺FoxP3⁺ Treg generation, and promoting macrophage polarization toward regulatory phenotypes [57]. In allergic models, AhR activation attenuates Th2-driven inflammation and protects against chronic airway diseases such as COPD [60]. Our previous studies demonstrated that epithelial AhR preserves lung homeostasis by controlling autophagy in AT2 cells [28] and suppressing ROS-triggered NLRP3 inflammasome activation that drives mucus hypersecretion [27]. Consistent with these findings, the present study shows that AhR is broadly expressed across lung cell types but is most strongly induced in airway epithelial subsets following allergen challenge, with upregulation confirmed in HBECs and mouse lungs treated with CRE or the AhR agonist VAF347. VAF347, a high-affinity AhR agonist, binds to AhR and initiates canonical AhR-driven transcriptional signaling like the prototype agonist 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) [33,38]. These results underscore the airway epithelium as a central site where AhR integrates environmental cues to restrain ROS generation and modulate downstream immune and inflammatory responses.

Our study extends the protective paradigm of AhR by demonstrating its essential role in suppressing allergen-induced ROS generation and cellular senescence in bronchial epithelial cells and mouse lungs. While previous reports have linked AhR signaling to senescence in contexts such as stemness, atherosclerosis, and renal fibrosis, its role in regulating epithelial senescence and senescence-associated airway inflammation has not been explored. Using *AhR* ^{Δ Scgb1a1}, we found that loss of AhR resulted in markedly elevated ROS accumulation, enhanced epithelial senescence, exaggerated airway inflammation, and mucus hypersecretion following CRE exposure. Conversely, pharmacologic activation of AhR with the selective agonist VAF347 significantly attenuated ROS production, epithelial senescence, and airway inflammation. These findings provide direct evidence that epithelial AhR functions as a key regulator restraining allergen-induced oxidative stress and senescence, thereby maintaining epithelial integrity and limiting allergic airway inflammation in asthma.

Mechanistically, transcriptomic analysis revealed that AhR activation profoundly reprograms the allergen-induced epithelial response. Pharmacologic stimulation with VAF347 strongly induced canonical AhR targets (*AHRR*, *CYP1B1*, *CYP1A1*), confirming effective pathway engagement, while broadly suppressing allergen-driven transcriptional programs. Pathway enrichment analysis identified cellular senescence among the most significantly altered pathways, along with focal adhesion, tight junction, and extracellular matrix–receptor interactions. Within the senescence network, the proto-oncogene c-Myc emerged as a central regulatory node, consistent with previous evidence linking AhR to c-Myc regulation [43–45]. We confirmed this relationship through complementary in vitro and in vivo approaches. In HBECs, CRE-induced c-Myc expression and ROS generation were both reduced by VAF347 and enhanced by the AhR antagonist CH223191, indicating that AhR negatively regulates c-Myc and its associated oxidative stress program. Molecular docking and co-immunoprecipitation confirmed a physical association between AhR and c-Myc, and ChIP-PCR demonstrated AhR occupancy at a conserved motif within the c-Myc promoter after allergen exposure.

c-Myc, a well-characterized proto-oncogene and transcription factor, orchestrates diverse cellular processes including proliferation, metabolism, and apoptosis [61]. Beyond these canonical roles, c-Myc maintains stemness by promoting cancer stem cell self-renewal, thereby contributing to tumorigenesis and chronic inflammation [62–64]. Importantly, c-Myc also modulates cellular senescence—a tumor-suppressive program that limits uncontrolled proliferation. Environmental carcinogens such as benzo[a]pyrene (BaP) can stabilize c-Myc and suppress senescence, driving oncogenic programs associated with growth, metastasis, and therapy resistance [44]. Notably, c-Myc is overexpressed in peripheral group 2 innate lymphoid cells (ILC2s) from asthma patients, where it enhances type 2 inflammation, and its inhibition dampens allergen-induced type 2 responses [65]. Consistent with these findings, our study shows that c-Myc is upregulated in CRE-treated HBECs and mouse lungs, linking allergen exposure to epithelial ROS generation and senescence. Particularly, pharmacologic inhibition of c-Myc with EN4 effectively suppressed epithelial senescence, SASP secretion (e.g., *IL-1 β* , *IL-6*), ROS production, and airway inflammation. These results identify c-Myc as a functional mediator linking AhR activation to epithelial ROS regulation, senescence, and downstream inflammatory responses.

This study demonstrates that allergen exposure induces epithelial ROS generation and senescence, establishing senescence as an active driver of airway inflammation through SASP-mediated cytokine release. We identify the AhR-c-Myc signaling axis as a key regulator of this process, providing a direct mechanistic link between environmental sensing and pathological epithelial responses. Pharmacologic inhibition of c-Myc effectively reduced ROS accumulation, epithelial senescence, airway inflammation, and Th2/Th17 cytokine production, underscoring the translational potential of targeting this pathway. Nonetheless, certain limitations should be acknowledged, including possible off-target effects of EN4, the focus on acute rather than chronic allergen exposure, and the need for further mechanistic dissection of AhR-c-Myc cross-regulation in senescence and immune modulation.

Author Contributions: Z.C., W.G., and Y. Z. performed experiments. Z.C performed RNA-seq analysis. R.W. performed scRNA-seq analysis. X.X., R.U., and P.G. designed and supervised the study. Z. C., P.G. wrote manuscript.

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Institutional Review Board Statement: All procedures were performed in accordance with institutional and National Institutes of Health guidelines and were approved by the Johns Hopkins University Animal Care and Use Committee. Protocol Number: MO25M140.

Informed Consent Statement: Not applicable

Data Availability Statement: The data presented in this study are available on request from the corresponding author. We preserve our datasets on our laboratory.

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Conflicts of Interest: The authors have declared that no conflict of interest exists.

Abbreviations

CRE	Cockroach extract
BALF	Bronchoalveolar lavage fluid
AhR	Aryl hydrocarbon receptor
SASP	Senescence-associated secretory phenotype
ROS	Reactive oxygen species
RNA-seq	RNA sequencing
scRNA-seq	Single-cell RNA-seq
HBEC	Human bronchial epithelial cell
DEG	Differentially expressed genes
GSEA	Gene set enrichment analysis
KEGG	Kyoto encyclopedia of genes and genomes
Cdkn2a	Cyclin dependent kinase inhibitor 2A
Cdkn1a	Cyclin dependent kinase inhibitor 1A
IP	Immunoprecipitation
ChIP	Chromatin immunoprecipitation
qRT-PCR	Quantitative reverse transcription-polymerase chain reaction
SA-β-Gal	Senescence β-Galactosidase
OCT	Optimal cutting temperature
DAPI	4',6-diamidino-2-phenylindole
ANOVA	An ordinary one-way analysis of variance

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