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RESEARCH ARTICLE

Aryl hydrocarbon receptor deficiency causes the development of chronic obstructive pulmonary disease through the integration of multiple pathogenic mechanisms

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Abbreviations: AHR, Aryl hydrocarbon receptor; AHRR, Aryl hydrocarbon receptor repressor; ANOVA, Analysis of variance; ARNT, AhR nuclear translocator; ATII, alveolar type II epithelial; BD, bronchodilator; CanCOLD, Canadian cohort chronic obstructive lung disease; COPD, Chronic obstructive pulmonary disease; COX-2, cyclooxygenase-2; CPD, Cumulative particulate density; CS, Cigarette smoke; CYP, Cytochrome P450; DAB, 3,3'-diaminobenzidine; ddPCR, Droplet digital polymerase chain reaction; DRE, Dioxin response element; ECL, Enhanced chemiluminescence; eQTL, Expression quantitative trait loci; FDR, False Discovery Rate; FEV₁, Forced expiratory volume in 1 second; FRC, Functional residual capacity; FVC, Forced vital capacity; GWAS, Genome-wide association study; H&E, Hematoxylin-Eosin; HO-1, Heme oxygenase 1; HRP, Horseradish peroxidase; HuR, human antigen R; IBD, Inflammatory Bowel Disease; IC/TLC, Inspiratory capacity/Total lung capacity; Lm, Mean linear intercept; mIHC, Multiplex immunohistochemistry; MMP, Matrix metalloproteinase; NF-κB, nuclear factor-κB; NRF2, nuclear factor erythroid 2-related factor 2; PAH, polycyclic aromatic hydrocarbon; PCDD, polychlorinated dibenzodioxin; PV, Pressure-volume; PVDF, Polyvinylidene difluoride; RIPA, Radio-immunoprecipitation assay; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; SNP, Single nucleotide polymorphism; SOD-2, Superoxide dismutase-2; TIMP-1, Tissue inhibitor of metalloproteinase-1.

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Abstract

Emphysema, a component of chronic obstructive pulmonary disease (COPD), is characterized by irreversible alveolar destruction that results in a progressive decline in lung function. This alveolar destruction is caused by cigarette smoke, the most important risk factor for COPD. Only 15%-20% of smokers develop COPD, suggesting that unknown factors contribute to disease pathogenesis. We postulate that the aryl hydrocarbon receptor (AHR), a receptor/transcription factor highly expressed in the lungs, may be a new susceptibility factor whose expression protects against COPD. Here, we report that *Ahr*-deficient mice chronically exposed to cigarette smoke develop airspace enlargement concomitant with a decline in lung function. Chronic cigarette smoke exposure also increased cleaved caspase-3, lowered SOD2 expression, and altered MMP9 and TIMP-1 levels in *Ahr*-deficient mice. We also show that people with COPD have reduced expression of pulmonary and systemic AHR, with systemic *AHR* mRNA levels positively correlating with lung function. Systemic *AHR* was also lower in never-smokers with COPD. Thus, AHR expression protects against the development of COPD by controlling interrelated mechanisms involved in the pathogenesis of this disease. This study identifies the AHR as a new, central player in the homeostatic maintenance of lung health, providing a foundation for the AHR as a novel therapeutic target and/or predictive biomarker in chronic lung disease.

1 | INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is characterized by progressive and irreversible airflow obstruction. Worldwide, COPD affects an estimated 380 million people and is the third leading cause of death.¹ Despite the significant disease burden of COPD, few-to-no effective therapeutic options exist to stop or slow its progression, in part, because the mechanistic basis of this disease remains unknown. COPD is an umbrella term that encompasses chronic bronchitis and emphysema (alveolar airspace enlargement). The emphysema component of COPD is proposed to be mediated by several inter-dependent mechanisms including chronic inflammation, oxidative stress, a protease:anti-protease imbalance, and accelerated cell death in the lungs.² How these multiple pathogenic processes are regulated at the molecular level in COPD is unknown but is important to consider, especially as only 15-20% of all individuals who smoke develop clinically relevant COPD. This suggests that unknown molecular mediators contribute to COPD pathogenesis.^{3,4}

We postulate that the aryl hydrocarbon receptor (AHR) may be a new susceptibility factor whose expression protects against the development of COPD. The AHR is a

transcription factor that mediates the deleterious effects of the man-made toxicant dioxin. After binding dioxin, the AHR translocates to the nucleus and forms a heterodimer with the AHR nuclear transporter (ARNT). This AHR•ARNT complex binds to DNA sequences termed the dioxin response element (DRE), initiating the transcription of target genes such as cytochrome P450 (CYP) *CYP1A1* and the AHR repressor (*AHRR*), a negative regulator of the AHR pathway that competes with AHR for ARNT binding.⁵ Following gene induction, the AHR dissociates from ARNT, is shuttled back to the cytoplasm and degraded by the 26S ubiquitin-proteasome system.⁶ Although the AHR has largely been associated with xenobiotic metabolism leading to toxicity, a broad range of biochemical and genetic studies have now revealed that the AHR has important endogenous functions, including control over cell proliferation, differentiation, migration, and survival.⁷⁻¹⁰ We have published that the AHR controls acute cigarette smoke-induced pulmonary inflammation *in vivo* and controls oxidative stress and cell death pathways *in vitro*.^{8,11,12} There is also low AHR expression in many human disease, including inflammatory bowel disease (IBD)¹³ and Behcet's disease.¹⁴ Thus, lower than normal AHR levels raise the

possibility that reduced AHR expression may predispose some smokers to developing COPD, suggesting that AHR may offer protection in the lungs against the development of COPD.

Our data herein illustrate that the AHR controls the development of COPD through the integration of multiple pathogenic mechanisms, including control over anti-oxidant proteins and protease levels. We also show that there is a significant reduction in pulmonary and systemic AHR in COPD, and that there is a positive correlation between both AHR and ARNT expression with lung function in people with COPD. Collectively, these data suggest a protective role for the AHR in the pathogenesis of smoke-induced emphysema/COPD. Given that no effective therapeutic options currently exist to stop or slow disease progression, these findings could provide the basis for the development of new therapeutic agents or biomarkers for COPD.

2 | MATERIALS AND METHODS

2.1 | Animals

AhR-knockout (*Ahr*^{-/-}) mice (strain B6.129-*Ahr*^{tm1Bra}) were purchased from the Jackson Laboratory, bred and maintained as previously described.¹⁵ This strain carries a targeted deletion of exon 2 of the *Ahr* gene and was backcrossed for 12 generations onto C57BL/6. A breeding scheme of heterozygous *Ahr*^{+/-} to *Ahr*^{-/-} mice are used, rendering mice of the *Ahr*^{+/-} genotype as littermate controls. *Ahr*^{+/-} mice are phenotypically indistinguishable from wild-type (*Ahr*^{+/+}) mice and there are no differences between C57BL/6 and *Ahr*^{+/-} mice.¹¹ Finally, *Ahr*^{+/+} or *Ahr*^{+/-} mice do not exhibit any difference in AHR protein expression in lungs/lung cells (Figure 1)¹⁶ or differ in the ability to be activated by AhR ligands or cigarette smoke.^{11,15} Therefore, *Ahr*^{+/-} mice were used as controls in this study. All animal procedures were approved by the McGill University Animal Care Committee (Protocol Number: 5933).

2.2 | Cigarette smoke exposure

Mice (age 8-12 weeks) were exposed to cigarette smoke using a SCIREQ[®] InExpose Exposure System (SCIREQ) for 8 weeks or 4 months as previously described.¹⁵ Briefly, research cigarettes (3R4F; University of Kentucky, Lexington, KY) were smoked in groups of 4 at 1 puff/cigarette/15 seconds for a total of 1 hour (twice daily for 5 days per week). Mice received whole-body exposure of mainstream smoke diluted with air. The amount of smoke particulates was

monitored using a MicroDust Pro (Casella CEL) and maintained at a cumulative particulate density (CPD) of 300 g/L. Both male and female mice were used unless otherwise indicated. Twenty-four hours after the final exposure, mice were euthanized by exsanguination. Lung tissue was collected for protein/western blot analysis and/or measurement of airspace enlargement.

2.3 | Mean linear intercept

Following formalin fixation, the left lung was embedded in paraffin, sectioned, and stained with hematoxylin-eosin (H&E). Airspace enlargement was assessed by calculating the mean linear intercept (Lm) as described.¹⁷

2.4 | Lung function assessment

Each mouse was administered xylazine IP (10 mg/kg) 5 min before being anesthetized with 50 mg/kg of sodium pentobarbital. Mechanical ventilation was set at a frequency of 150 breaths/min, a tidal volume of 10 mL/kg, a 2:3 inspiratory:expiratory ratio and a 3 cm H₂O positive end expiratory ratio. Rocuronium (0.2 mg/kg) was then administered, followed by a deep lung inflation maneuver to 30 cm H₂O and a 2-3 min equilibration period. Next, deep lung inflation and a stepwise pressure-volume (PV) curve, both set to a final pressure of 35 cm H₂O, were run and the mechanical ventilation was switched from room air to 100% oxygen for 5 min to degas the lungs by oxygen absorption prior to the construction of a full-range PV curve.¹⁸

2.5 | Western blot

Lung tissue was homogenized in RIPA buffer supplemented with protease inhibitor cocktail (Roche). Ten-twenty micrograms of total protein was separated on SDS-PAGE gels, electroblotted onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories), and blocked with 5% nonfat dry milk in 0.1% Tween 20 (in PBS). Antibodies against AHR (1:1000, Enzo Life Sciences), cleaved-casepase-3 (1:1000; Cell Signaling), SOD2 (1:1000; R&D Systems), HO-1, MMP9 (1:1000; Abcam), TIMP-1 (1:1000; Abcam), tubulin (1:10,000; Sigma-Aldrich), and total actin (1:50,000, EMD Millipore) were used to assess changes in relative expression. Proteins were visualized using HRP-conjugated secondary antibodies (1:10,000) followed by enhanced chemiluminescence (ECL) and imaged using a ChemiDocTM XRS + System (Bio-Rad Laboratories).

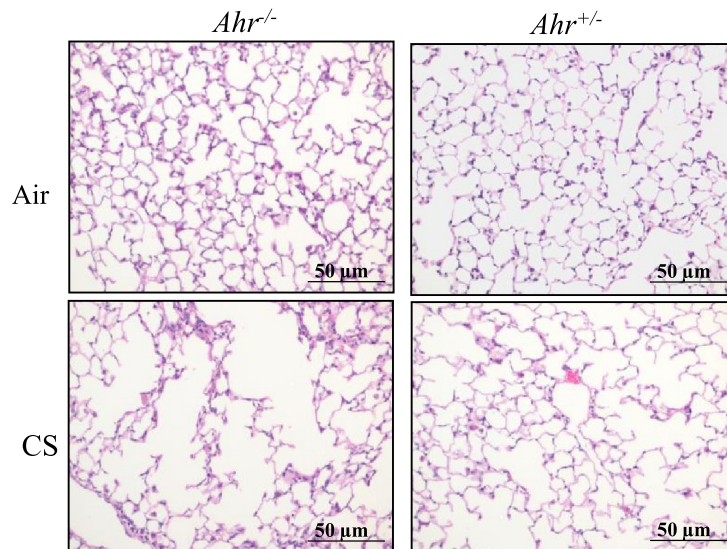
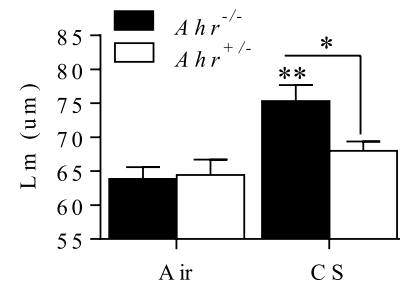
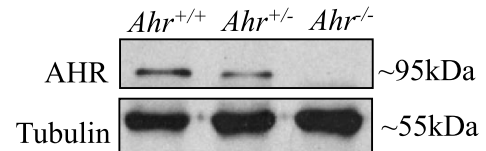
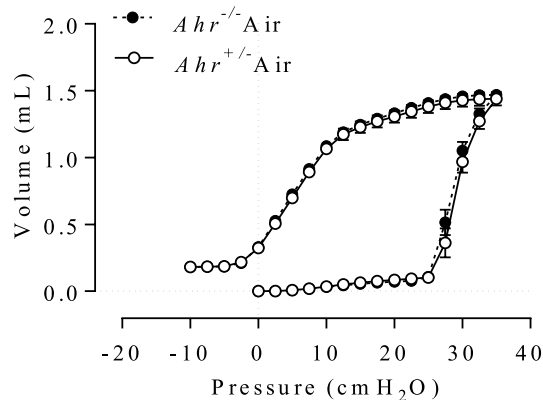
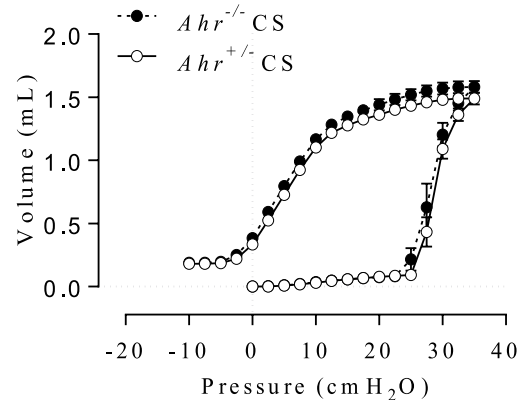
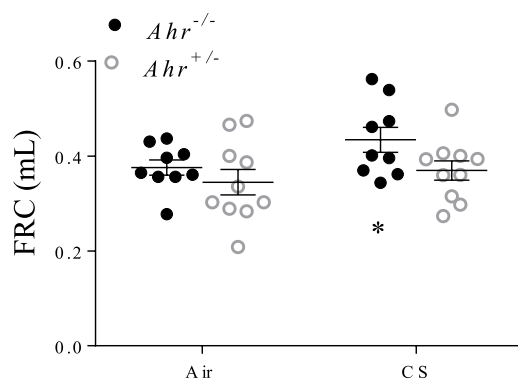
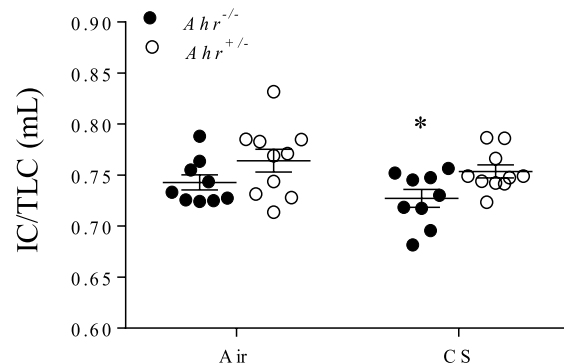
(A) Representative histology images**(B) Mean linear intercept****(C) Pulmonary AHR Protein****(D) PV curves- air exposure****(E) PV Curves- CS****(F) FRC****(G) IC/TLC**

FIGURE 1 AHR deficiency drives the development of cigarette smoke (CS)-induced emphysema in vivo. A, Representative histology images: Representative images from the lung parenchyma of *Ahr*^{-/-} and *Ahr*^{+/-} mice exposed to a 4-month CS regime are shown (magnification = 20x). B, Mean linear intercept: Lm was used to quantify airspace enlargement in the lung parenchyma. There was a significant increase in Lm in CS-exposed *Ahr*^{-/-} mice compared to air-exposed mice (***) as well as CS-exposed *Ahr*^{+/-} mice (**P* < 0.05). Results are expressed as mean ± SEM, n = 6-8 mice per group. C, Pulmonary AHR protein: Representative western blot showing that AHR protein levels are not different between *Ahr*^{+/+} and *Ahr*^{+/-} mice. Lung volume curves are shown comparing air-exposed (D) and CS-exposed (E) *Ahr*^{+/-} and *Ahr*^{-/-} mice. There were significant changes in functional residual capacity (FRC) (F), and the ratio of inspiratory capacity to the total lung capacity (IC/TLC) (G) in CS-exposed *Ahr*^{-/-} mice (**P* < .05). Results are expressed as mean ± the SEM, n = 9-10 mice per group.

2.6 | Lung tissue expression quantitative loci study

Gene expression profiling was performed using an Affymetrix custom array (GPL10379), which contained 51,627 non-control probesets and data were normalized using RMA.¹⁹ Genotyping was performed using the Illumina Human1M-Duo BeadChip array. Genotype imputation was undertaken using the 1000G reference panel. Following standard microarray and genotyping quality controls, data from 1111 patients were available, including 409 from Laval, 363 from Groningen, and 339 from UBC. Table 1 contains patient characteristics for the Laval cohort; as lung specimens for this cohort were obtained from patients that underwent surgery for lung cancer, post-bronchodilator (BD) spirometry was not performed. Association testing for each variant with mRNA expression in either cis (within 1 Mb of transcript start site) or in trans (all other combinations) was undertaken separately for each study sample, after which the results were meta-analyzed using inverse variance weighting. A genome-wide 10% false discovery rate (FDR) was applied to this analysis. A subset of these subjects was used to test for the association of *ARNT* gene with lung function. The lung tissue mRNA association testing with lung function parameters was restricted to 727 individuals who did not have any significant respiratory diseases, other than smoking-related conditions, and for whom lung function measurements were available. Gene expression data are available on GEO under accession number GSE23546.

2.7 | Multiplex Immunohistochemistry

Human subjects were approved by the Research Ethics Board of St Joseph's Healthcare Hamilton (00-1839). Subject characteristics are in Table 2; post-BD spirometry lung function

TABLE 1 Clinical characteristics of the lung eQTL study (Laval cohort)

Variable	Control (n = 168)	COPD (n = 27)	P-value
Age (y)	60.3 ± 10.6	65.6 ± 8.8	2.50E-07
Male (n)	86	131	0.09
FEV1% predicted	90.8 ± 16.3	72.6 ± 17.4	<2.2E-16
FVC % predicted	91.9 ± 15.8	88.2 ± 16.2	0.03
FEV ₁ /FVC	75.5 ± 4.2	61.3 ± 7.9	<2.2E-16
Smoking (%)			
Smoker	28 (16.7)	59 (27.2)	0.0001
Former	117 (69.6)	151 (69.6)	
Never	23 (13.7)	7 (3.2)	

Note: Data are presented as the mean ± SD.

values are indicated. Lung tissue was fixed in 10% formalin and embedded in paraffin. Five- μ m thick sections were first incubated in primary AHR antibody (Abcam #2770; 1:100) for 16 min at 37°C, followed by incubation in secondary antibody OMap anti-Ms HRP (Roche #760-4310) for 16 min at 37°C. Sections were next incubated in primary vimentin antibody (Cell Signaling #3932; 1:50) for 16 min at 37°C, followed by incubation in secondary antibody UltraMap Anti-Rb Alk Phos (Roche #760-431) for 16 min at 37°C. Lastly, sections were incubated in pre-diluted primary cytokeratin-19 antibody (Roche #760-4281) for 16 min at 37°C, followed by incubation in secondary antibody OMap anti-Ms HRP (Roche #760-4310) for 16 min at 37°C. Sections were counter-stained using hematoxylin. Detection was accomplished using a 3,3'-diaminobenzidine (DAB) detection kit.

2.8 | Droplet digital PCR

Analysis of systemic *AHR* expression was analyzed by Droplet Digital PCR (ddPCR) using biological samples from the CanCOLD cohort; CanCOLD has been described in detail.²⁰ Briefly, CanCOLD is a population-based longitudinal study that aims to understand the heterogeneity of COPD presentation and disease progression. Subjects were sampled from the general population and data collected includes pulmonary function and cardiorespiratory exercise tests, CT scans, and blood sampling.²⁰ Patient characteristics of the samples analyzed in this study are in Table 3, which includes lung function, smoking status, emphysema and bronchiolitis score assessed by CT, as well as co-morbidities. Peripheral blood was collected using PAXgene blood RNA tubes (PreAnalytiX GmbH, Hombrechtikon, Germany) at initial visit and frozen at -80°C until analysis.²¹ Total RNA was isolated using a PAXgene blood RNA kit (PreAnalytiX GmbH) according to the supplier protocol. RNA was reverse transcribed to cDNA, diluted, and primers added in QX200 ddPCR Eva Green[®] Super-mix (Bio-Rad) as previously described.²² Samples were then partitioned into 20,000 droplets and PCR amplification was performed using the QX100 Droplet Digital[™] PCR System (Bio-Rad). Primer sequences for human *AHR* are: AGAGGCTCAGGTTATCAGTTT (f) and AGTCCATCGGTTGTTTTTTT (r). Primer sequences for β -actin are CTACCATGAGCTGCGTGTG (f) and TGGGGTGTGAAGGTCTC (r). Gene expression data were normalized to β -actin.

2.9 | Bronchial airway epithelial gene expression study

In the study by Steiling *et al*,²³ bronchial brushings were obtained from sixth to eighth generation bronchi from heavy

TABLE 2 Clinical characteristics of the St Joseph's Healthcare Hamilton subjects

Variable	Non-Smoker 11	At-Risk 11	COPD 13	p-value
Age (y)	68.1 ± 10.3	57.8 ± 8.7	65 ± 10.7	Non-smoker vs smoker $P = .056$ Non-smoker vs COPD $P = .733$ Smoker vs COPD $P = .20$
Male (n)	5	6	7	N/A
Female (n)	6	5	6	N/A
FEV1/FVC (%)	80.5 ± 8.2	75.5 ± 6.6	57.7 ± 10.4	Non-smoker vs smoker $P = .28$ Non-smoker vs COPD $P < .0001$ Smoker vs COPD $P = .0001$ Non-smoker vs smoker $P = .28$ Non-smoker vs COPD $P < .0001$ Smoker vs COPD $P = .0001$
Pack years*	0 ± 0	39.5 ± 16.2	48.1 ± 21.6	Non-smoker vs smoker $P < .0001$ Non-smoker vs COPD $P < .001$ Smoker vs COPD $P = .39$

Note: Data are presented as the mean ± SD.

current and ex-smokers who participated in an early lung cancer detection study at the British Columbia Cancer Agency between June 2000 and May 2009.^{24,25} In this dataset, gene expression profiling of the bronchial specimens was performed in 238 current and former smokers with (n = 87) and without COPD (n = 151). RNA was processed and hybridized to Affymetrix Human Gene 1.0 ST Arrays (Affymetrix, Santa Clara, CA). Gene expression estimates were derived using the RMA algorithm.¹⁹ Microarray data have been deposited in GEO under accession number GSE37147.

2.10 | GWAS of lung function in the UK biobank

To test if expression quantitative loci (eQTLs) for the *ARNT* gene were associated with lung function in large-scale human genetic studies, we used summary statistics from the UK Biobank GWAS of lung function measures in the general population (n = 48 943 individuals).²⁶ GWAS summary data were available through approved access to the UK Biobank.

2.11 | Single cell RNA-sequencing analysis

Raw single cell RNA-seq expression matrices for cells from 18 COPD and 28 lungs from²⁷ were filtered using SCANPY software.²⁸ Cells with less than 200 expressing genes or more than 40% of mitochondrial reads were filtered. Genes that were only expressed in less than three cells were also removed. Genes were further filtered if they exhibited low dispersion (<0.15) or very low-level expression (<0.0125). Gene expression was then converted to log₂ space (log₂expression+1).

2.12 | Statistical analyses

Statistical differences between group mean values were determined by two-way ANOVA followed by the Tukey's multiple comparisons test unless otherwise indicated. Systemic *AHR* mRNA expression in CanCOLD subjects were analyzed using a Kruskal-Wallis non-parametric one-way ANOVA (2+ comparison groups) or a Kolmogorov-Smirnov non-parametric *t* test (2 comparison groups). Statistical analysis was conducted for CanCOLD subjects (Table 3) using SAS v9.4 (SAS Institute Inc). These subject data were analyzed either using a one-way ANOVA (for a normal distribution) or a Kruskal-Wallis test, and a chi-squared test for categorical variables. Statistical analysis was conducted for St Joseph's Healthcare Hamilton subjects (Table 2) using a Kruskal-Wallis non-parametric one-way ANOVA. Genetic associations in the eQTL study (Laval cohort-Table 1) were evaluated using chi-square tests in PLINK. A $P < .05$ was considered significant.

3 | RESULTS

3.1 | AHR deficiency promotes the development of cigarette smoke-induced emphysema and reduces lung function in mice

As chronic exposure to cigarette smoke is the main risk factor for the development of COPD, we first utilized our preclinical mouse model to causally address the contribution of the AHR to the development of airspace enlargement (Lm), a surrogate marker of emphysema. *Ahr*^{+/-} and *Ahr*^{-/-} mice were exposed to cigarette smoke for 4 months and Lm was calculated. Only *Ahr*^{-/-} mice had a significant

TABLE 3 Clinical characteristics of CanCOLD subjects

Variables	Total	Normal	At-Risk	COPD	P-values
	N = 63	N = 16	N = 15	N = 32	
Age, in years [mean(sd)]	67.00 ± 9.17	67.75 ± 5.84	65.53 ± 8.46	67.31 ± 10.86	0.974
Sex, male gender, n (%)	27 (42.86)	4 (25.00)	5 (33.33)	18 (56.25)	0.087
Tobacco smoking status, n (%)					
Never	26 (41.27)	16 (100.00) ^a	—	10 (31.25) ^a	<.001*
Ex-smokers	27 (42.86)	—	13 (86.67) ^a	14 (43.75) ^a	<.001*
Current smokers	10 (15.87)	—	2 (13.33)	8 (25.00)	0.066
Cigarette smoker pack-years	15.16 ± 22.36	—	13.40 ± 10.61 ^a	23.57 ± 27.49 ^a	<.001*
Post-bronchodilator spirometry					
FEV ₁ , L	2.32 ± 0.68	2.38 ± 0.48	2.60 ± 0.60	2.16 ± 0.76	.18
FEV ₁ , % predicted	91.73 ± 22.10	101.28 ± 11.33 ^a	100.18 ± 11.11 ^b	82.72 ± 26.52 ^{ab}	.002*
FEV ₁ /FVC, %	68.99 ± 12.70	76.39 ± 4.22 ^a	78.61 ± 4.36 ^b	60.52 ± 12.68 ^{ab}	<.001*
Emphysema score	0.89 ± 1.89	0.00	0.15 ± 0.55 ^a	1.63 ± 2.37 ^a	.001*
Emphysema (score ≥ 1)	15 (24.59)	0 (0.0) ^a	1 (7.69) ^{ab}	14 (43.75) ^{ab}	<.001*
Bronchiolitis (score ≥ 2)	7 (11.48)	0 (0.0)	0 (0.0)	7 (21.88)	.194
Self-reported comorbidities, n (%)					
Bronchiectasis	2 (3.2)	0 (0.0)	0 (0.0)	2 (6.3)	.738
Pneumonia	8 (12.7)	0 (0.0)	1 (6.7)	7 (21.9)	.078
Osteoporosis	12 (19.0)	1 (6.3)	3 (20.0)	8 (25.0)	.344
Any musculoskeletal	23 (36.5)	2 (12.5) ^a	9 (60.0) ^a	12 (37.5)	.023*
Angina	5 (7.9)	0 (0.0)	1 (6.7)	4 (12.5)	.419
Hypertension	20 (31.7)	5 (31.3)	7 (46.7)	8 (25.0)	.33
CVD (excluding Hypertension)	13 (20.6)	2 (12.5)	2 (13.3)	9 (28.1)	.426
Depression	3 (4.8)	1 (6.3)	1 (6.7)	1 (3.1)	.8
Cataract	12 (19.0)	5 (31.3)	2 (13.3)	5 (15.6)	.48
Glaucoma	7 (11.1)	2 (12.5)	2 (13.3)	3 (9.4)	.883
Diabetes	5 (7.9)	2 (12.5)	1 (6.7)	2 (6.3)	.831
Asthma	18 (28.6)	2 (12.5) ^a	2 (13.3)	14 (43.8) ^a	.029*

Note: Data are presented as the mean ± SD. ^{a,b}Means with same letters are significantly different from each other after Tukey's adjustment for multiple comparisons ($P < .05$).

* $P < .05$.

increase in Lm from chronic cigarette smoke exposure (Figure 1A,B); this increase was also significant compared to smoke-exposed *Ahr*^{+/-} mice. Verification of pulmonary AHR protein expression is shown in Figure 1C; note that there is no difference in AHR levels between *Ahr*^{+/-} and *Ahr*^{+/+} mice. We also assessed if chronic cigarette smoke exposure led to corresponding alterations in lung function using flexiVent. The parameters evaluated were derived from PV curves obtained using a full volume lung maneuver. PV curves are virtually super-imposable between the air-exposed *Ahr*^{+/-} and *Ahr*^{-/-} mice (Figure 1D). In *Ahr*^{-/-} mice exposed to cigarette smoke for 4 months, there is an upward/leftward shift of the PV curve compared to the smoke-exposed *Ahr*^{+/-} mice (Figure 1E). This shift in the

PV curve of the smoke-exposed *Ahr*^{-/-} mice is consistent with the PV curve shift in patients with emphysema due to the loss of elastic tissue and parenchymal destruction.²⁹ From these curves, we calculated the functional residual capacity (FRC). FRC is a parameter of lung function that increases in emphysema as a result of elastic destruction.²⁹ The FRC was significantly increased in the smoke-exposed *Ahr*^{-/-} mice (Figure 1F). The ratio of the inspiratory capacity to the total lung capacity (IC/TLC) is also reduced in emphysema³⁰ and was significantly reduced in the smoke-exposed *Ahr*^{-/-} mice (Figure 1G). Taken together, these data support that the AHR protects against the development of a cigarette smoke-induced emphysema-like phenotype, as evidenced by alterations in lung structure and function.

3.2 | AHR deficiency exacerbates pathogenic mechanisms implicated in the development and progression of COPD

The pathogenesis of emphysema is mediated by a variety of inter-related mechanisms, including chronic inflammation, heightened oxidative stress, a protease-anti-protease imbalance, and accelerated death of lung cells.² We have previously published that the AHR suppresses pulmonary inflammation in response to cigarette smoke.^{11,15} This inflammatory response is believed to underlie COPD pathogenesis, as it is postulated that oxidants released by recruited immune cells, along with oxidants in cigarette smoke, cause accelerated cell death (i.e., apoptosis) that leads to emphysema.^{31,32} We have also previously shown in vitro that the AHR suppresses smoke-induced oxidative stress, in part, due to the upregulation of key anti-oxidant proteins such as superoxide dismutase-2 (SOD2), a mitochondrial enzyme that converts superoxide anions to hydrogen peroxide and water and thus protects cells against oxidative damage leading to apoptosis.⁸ Whether dysregulation of anti-oxidant proteins/apoptosis also occurs in the lungs of *Ahr*^{-/-} mice exposed chronically to smoke has not been examined. Therefore, to assess whether the AHR is a global regulator of these pathogenic mechanisms, we also now evaluated select anti-oxidants, activated caspase-3, and as well as MMP levels in mice exposed to cigarette smoke for 8 weeks. Analyses of samples from this exposure time allowed us to compare whether dysregulation of these mechanisms occurs prior to airspace enlargement in *Ahr*^{-/-} mice. Our novel data show that there is significant induction of cleaved caspase-3 in response to an 8-week smoke exposure regime in *Ahr*^{-/-} mice compared to smoke-exposed *Ahr*^{+/-} mice (Figure 2A). Although cigarette smoke significantly increased the expression of SOD2 in both *Ahr*^{+/-} and *Ahr*^{-/-} mice, the level of induction was significantly less in the lungs of *Ahr*^{-/-} mice (Figure 2B). There was no difference in the induction of heme oxygenase 1 (HO-1), a cyto-protective enzyme that catalyzes the rate-limiting step in the degradation of heme, between smoke-exposed *Ahr*^{+/-} and *Ahr*^{-/-} mice (Figure 2C).

Lastly, a protease/antiprotease imbalance within the lungs is also a mechanism thought to underlie the pathogenesis of emphysema, with an increased matrix metalloproteinase 9 (MMP9) to tissue inhibitor of metalloproteinase 1 (TIMP-1) ratio commonly observed in patients with emphysema.² Therefore, MMP9 and TIMP-1 protein expression were assessed by western blot in whole lung homogenate from *Ahr*^{-/-} and *Ahr*^{+/-} mice. MMP9 expression was significantly elevated in the lungs of both the air- and smoke-exposed *Ahr*^{-/-} mice relative to the *Ahr*^{+/-} mice (Figure 2D,E), while TIMP-1 expression was only elevated in the smoke-exposed *Ahr*^{-/-} mice (Figure 2D,F). This resulted in a significantly

higher MMP9:TIMP1 ratio in the lungs of the air-exposed *Ahr*^{-/-} mice relative to the *Ahr*^{+/-} mice (Figure 2G). Collectively, these preclinical data support that the AHR controls multiple pathogenic mechanisms associated with the development of a COPD-like phenotype, pointing toward its role in the homeostatic regulation of lung health in response to cigarette smoke.

3.3 | Correlation of lung function with expression of AHR and ARNT in COPD subjects

There is a reduction of AHR expression in several human diseases that have an underlying inflammatory etiology.^{13,14} This, combined with our experimental data showing that absence of the AHR results in heightened cigarette smoke-induced inflammation and development of a COPD-like phenotype, led us to wonder if AHR expression was altered in people with COPD. To determine this, we analyzed AHR expression using biological samples obtained from COPD and control subjects from four separate cohorts (Tables 1-3) and as in.²⁷ In our first cohort (Table 1), analysis revealed that there was significantly less *AHR* mRNA in the lungs of COPD subjects compared to subjects without COPD (i.e., smokers) (Figure 3A). In the second cohort (Table 2), we found that there appeared to be less intense staining with an anti-AHR antibody in COPD-derived lung specimens as determined by multiplex IHC compared to the relatively uniform staining seen in lung samples from non-smokers as well as smokers without COPD (Figure 3B,C). The reduction in staining intensity was particularly noticeable in the bronchial epithelium of COPD subjects compared to At-Risk smokers (Figure 3C). This suggests that AHR protein expression may be reduced in the lungs of those with COPD relative to both non-smokers and smokers without COPD ("At-Risk").

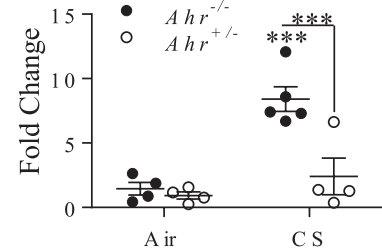
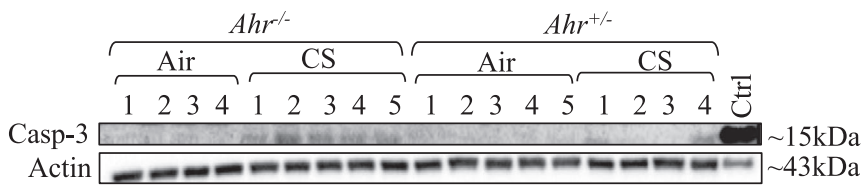
We also analyzed scRNA-seq data from control and COPD subjects using data available from.²⁷ After preprocessing, we obtained 165,755 cells in total, of which 58,604 were COPD cells (35.45%); the remainder were Control cells. Based on the processed and normalized expression matrix, we generated a dot plot (Figure 4A), a matrix plot (Figure 4B), and a bar plot (Figure 4C) to compare the difference between *AHR* in COPD and Control cells. We found that *AHR* expression level is significantly lower (one-sided Mann-Whitney U test; P -value = $1.38e-12$) in COPD cells compared with Control cells. We also annotated the cell type for all cells we obtained using the same strategy described as in the original study,²⁷ and examined the percentage difference of AHR positive cells between COPD and Control cells for each cell type. We found that the largest positive difference is observed in alveolar type

II epithelial (AII) cells (more AHR positive cells in COPD vs Control), while the most negative difference (fewer AHR positive cells in COPD vs Control) is in mast cells and to a lesser extent myofibroblasts (Figure 4D).

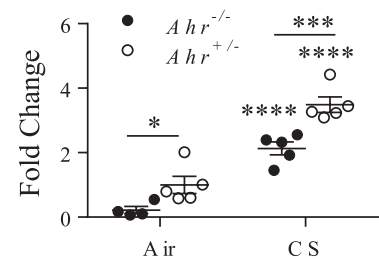
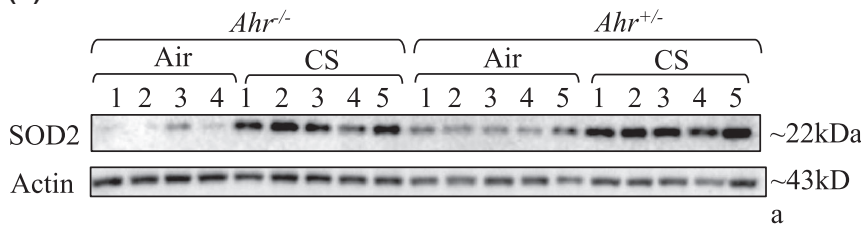
Finally, in subjects participating in CanCOLD²⁰ (Table 3), we found that there was significantly less systemic *AHR*

mRNA in subjects with COPD relative to At-Risk subjects (smokers; Figure 5A) as well as in COPD-Never Smokers (no history of smoking) (Figure 5B). Subjects with an emphysema score of 1 or more as assessed by computed tomography (CT) scan also have significantly less *AHR* mRNA relative to the At-Risk subjects (Figure 5C). Lastly, systemic

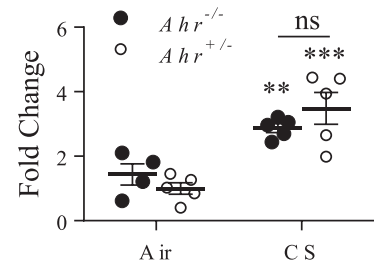
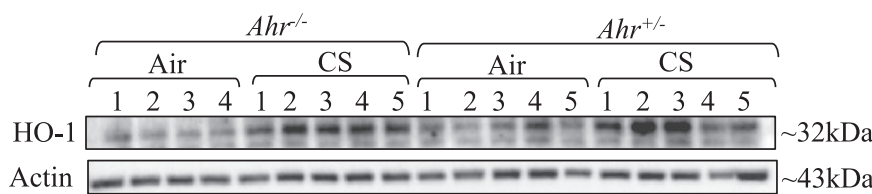
(A) Cleaved caspase-3



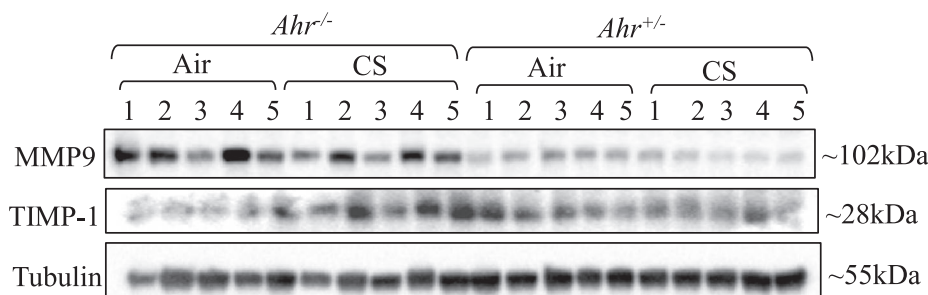
(B) SOD2



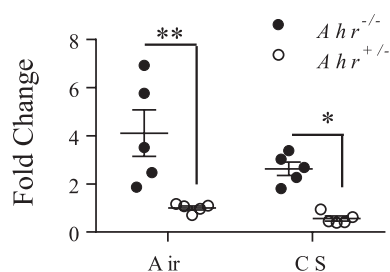
(C) HO-1



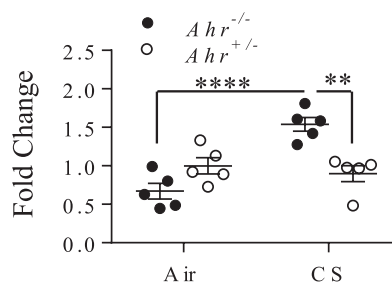
(D) MMP9/TIMP-1



(E) MMP9 quantification



(F) TIMP-1 quantification



(G) MMP9/TIMP-1 ratio

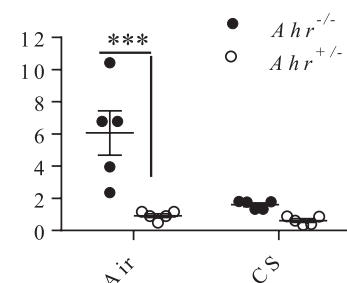


FIGURE 2 AHR deficiency results in a dysregulated anti-oxidant and apoptotic response as well as a protease:anti-protease imbalance. *Ahr*^{+/-} and *Ahr*^{-/-} mice were exposed to CS for 8 weeks and protein expression analyzed by western blot. A, Cleaved caspase-3—there was a significant increase in cleaved caspase-3 in *Ahr*^{-/-} mice exposed to CS (****P* < .001); this increase in cleaved caspase-3 was significantly higher compared to smoke-exposed *Ahr*^{+/-} mice. B, SOD2—there was a significant increase in the protein expression of SOD2 in *Ahr*^{+/-} and *Ahr*^{-/-} mice exposed to CS (*****P* < .0001); the increase in smoke-exposed *Ahr*^{-/-} mice was higher than *Ahr*^{+/-} mice (****P* < .001). C, HO-1—there was an increase in HO-1 expression in the lungs of smoke-exposed *Ahr*^{+/-} and *Ahr*^{-/-} mice (***P* < .01; ****P* < .001); there was no difference between the genotypes (ns). D, MMP9/TIMP-1—Western blot of MMP9 and TIMP-1 in lung homogenates; number of mice is indicated. E, MMP9 quantification—there was significantly more MMP9 at baseline in the lungs of *Ahr*^{-/-} mice that did not change significantly in response to CS (**P* < .05; ****P* < .01); levels remained higher compared to *Ahr*^{+/-} mice. F, TIMP-1 quantification-- TIMP-1 expression significantly increased only in CS-exposed *Ahr*^{-/-} mice (***P* < .01; *****P* < .001). G, MMP9: TIMP-1 ratio—the ratio of MMP9:TIMP-1 is significantly higher in the air-exposed *Ahr*^{-/-} mice relative to the air-exposed *Ahr*^{+/-} mice. Results are expressed as mean ± SEM.

AHR mRNA levels were positively correlated with the lung function parameter FEV₁/FVC (%) (Figure 5D).

In addition, *ARNT* expression was positively associated with FEV₁ after adjusting for age, gender, and smoking status (beta = 0.0056, *P* = .0005). In human airway epithelium, *ARNT* expression was also positively associated with FEV₁ (beta = 0.002, *P* = .00001). In lung tissue, *ARNT* is under genetic control with the top eQTL SNP rs72237606 (eQTL *P* = 7.38E-59). This same SNP was also positively associated with FEV₁ in the UK Biobank GWAS (*P* = 1.24E-05), suggesting a correlation between the expression of *ARNT* in lung tissue and lung function. Overall, these data show that there is dysregulation of AHR and *ARNT* expression in COPD and that there is a positive correlation with their expression and lung function.

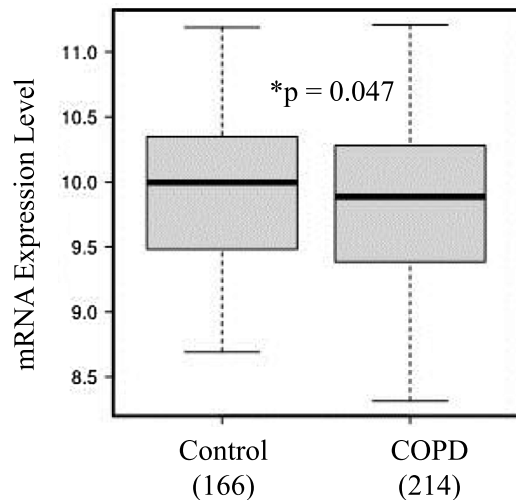
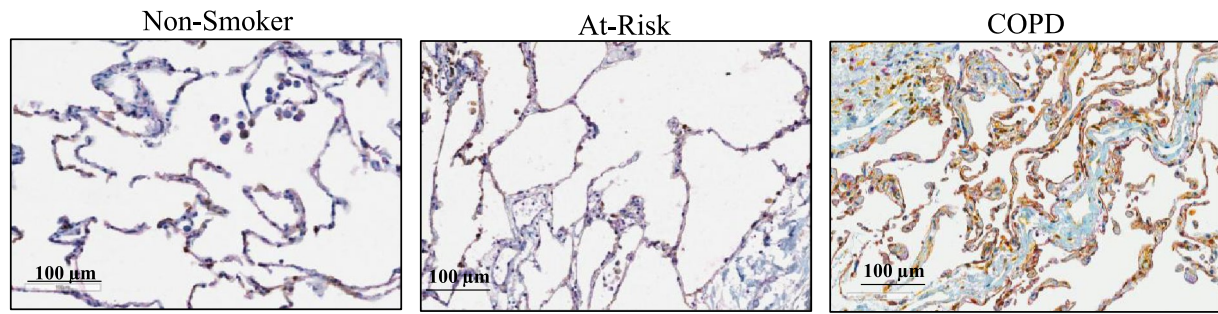
4 | DISCUSSION

COPD is characterized by abnormal airway inflammation and irreversible airflow obstruction. While the main treatment option for targeting inflammation is glucocorticoids, these medications have little effect on the inflammatory response caused by cigarette smoke.^{33,34} Other approaches to reduce inflammation have included targeting specific mediators involved in the chemotaxis and accumulation of neutrophils (e.g., CXCL8 and TNF-α) or downstream signal transduction pathways (e.g., p38) known to be activated by cigarette smoke, but these have either had little clinical benefit or have been associated with significant side effects.^{35,36} Thus, the development of safe and effective therapies with disease-modifying effects for smokers with and without obstructive lung disease remains a priority. Cigarette smoke is a complex mixture that activates numerous biological pathways in the lung and other organs to ultimately contribute to the pathophysiology of COPD. However, we speculated that the AHR is an essential protein in the homeostatic control in the lung, capable of mitigating the damaging effects of tobacco smoke. Herein, we show that the AHR protects against emphysematous lung destruction and preserves lung function of mice in response to chronic smoke exposure. We also show

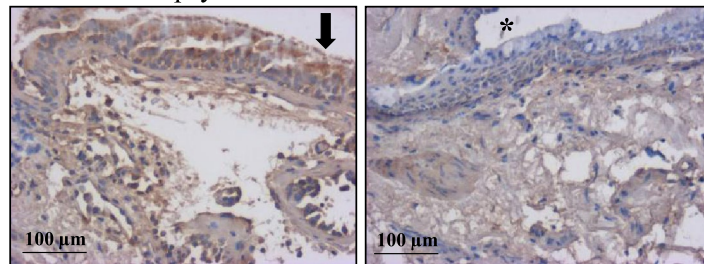
that the AHR controls other key pathogenic mechanisms involved in COPD development, including anti-oxidant levels and MMP9 expression. Thus, defining the AHR as an important protein that integrates multiple protective mechanisms in the lung may enable the development of more effective approaches to treat complex diseases such as COPD.

Our data support that the AHR protects against the development of the emphysema component of COPD. The fact that *Ahr*^{-/-} mice have extensive lung parenchymal destruction after only a 4-month smoke-exposure is striking, as the *Ahr*^{-/-} mice are on the C57BL/6 background, a strain that requires ~6 months of cigarette smoke exposure to develop the features of emphysema.³⁷ Airspace enlargement was not evident in air-only *Ahr*^{-/-} mice, suggesting that the emphysematous changes in response to cigarette smoke are not attributable to impaired lung development or a congenital defect due to absence of the AHR. However, our observation of a significantly elevated MMP9:TIMP1 ratio in the air-only *Ahr*^{-/-} mice suggests that this protease:antiprotease imbalance may predispose these mice to the development of emphysema upon exposure to subsequent pulmonary insults, such as cigarette smoke. In addition to the lung structural damage, smoke-exposed *Ahr*^{-/-} mice also exhibited emphysema-like changes in lung function, including a significant increase in the FRC and a significant decrease in the IC/TLC ratio. This is in accordance with lung function in humans with emphysema, where lung parenchymal destruction results in an outward recoil of the lung, leading to hyperinflation, air trapping, and an increased FRC.²⁹ Moreover, a reduced IC/TLC ratio is associated with worse clinical outcomes in COPD patients as well as with more severe disease, increased exacerbations and increased mortality. Overall, our data suggest that the AHR globally protects the lungs from the deleterious effects of smoking.

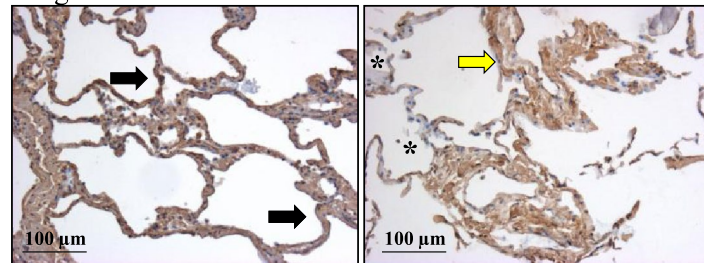
It could be that AHR dysregulation (altered function and/or reduced expression) may predispose a susceptible individual to the development of emphysema and consequent deterioration in lung function. In line with this notion is our findings that lung and systemic AHR expression is reduced in COPD subjects—even those that are never-smokers—and that low AHR levels correlate with worse lung function. Why

(A) GWAS analysis of lung *AHR* mRNA**(B) mIHC of relative *AHR* expression (purple)****(C) IHC of *AHR* levels (brown)**

Bronchial Biopsy



Lung



At-Risk

COPD

FIGURE 3 *AHR* expression is reduced in lung tissue from human COPD subjects. A, There was significantly less *AHR* mRNA in the lungs of COPD subjects ($n = 214$) compared to subjects without COPD (Control) ($n = 166$). B, mIHC was used to detect *AHR* (purple), vimentin (fibroblasts; yellow), and cytokeratin-19 (epithelial cells; brown) in human lungs. Note the relative decrease in *AHR* (purple) in COPD subjects relative to Non-smokers and those At-Risk (smokers without COPD); representative images are shown. C, Bronchial biopsy (top panels) and lung tissue (bottom panels)—there is less intense *AHR* expression (brown) in lung tissue from COPD subjects compared to those considered At-Risk (smokers); regions of noticeably less *AHR* are denoted by an asterisk (*). Representative images are shown.

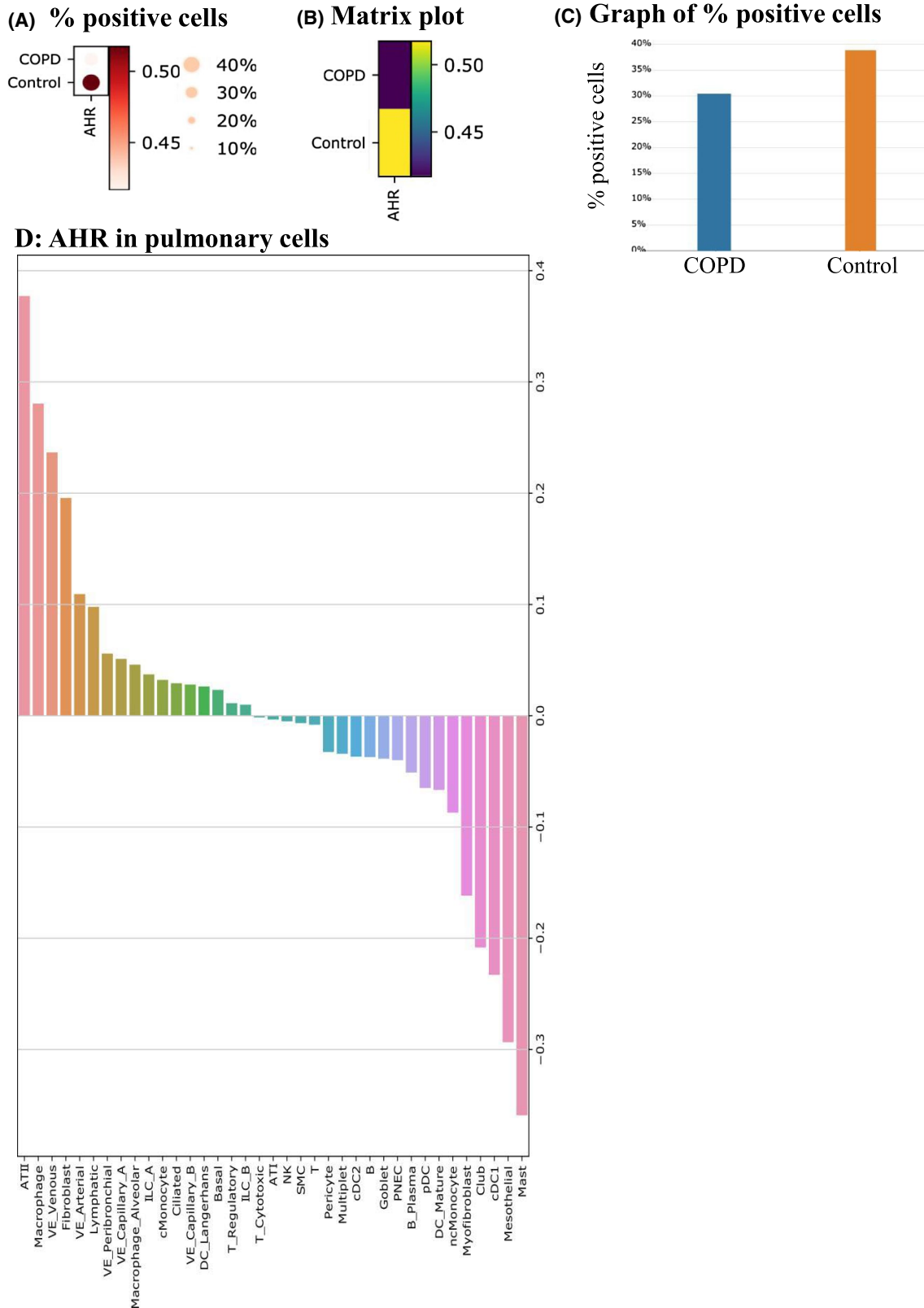


FIGURE 4 AHR expression is significantly lower in COPD cells compared to Control cells. A, % positive cells—AHR is lower in COPD, as indicated in a dot plot. The size of the dot represents the % of cells with positive AHR expression. The grade of the dot color represents the AHR expression level. B, Matrix Plot—A matrix plot for AHR in COPD vs Control. C, Graph of % positive cells—COPD has 30.5% AHR positive cells, while 38.9% of Control cells are AHR positive. D, Percent difference of AHR positive cells between COPD and Control for each pulmonary cell type. The most considerable positive difference is observed in ATII cell type (many more AHR positive cells in COPD vs Control), while the largest negative difference (much less AHR positive cells in COPD vs Control) is found in mast cells.

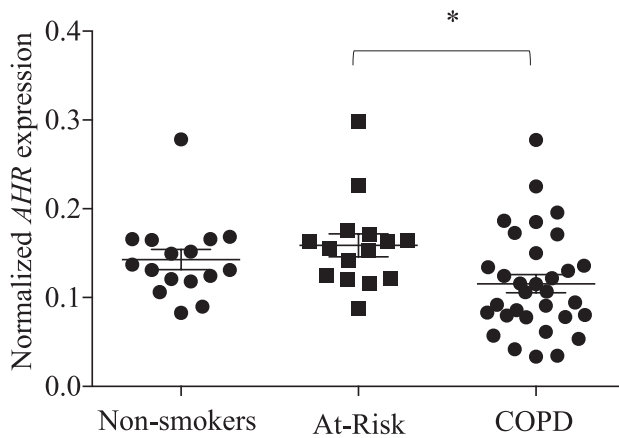
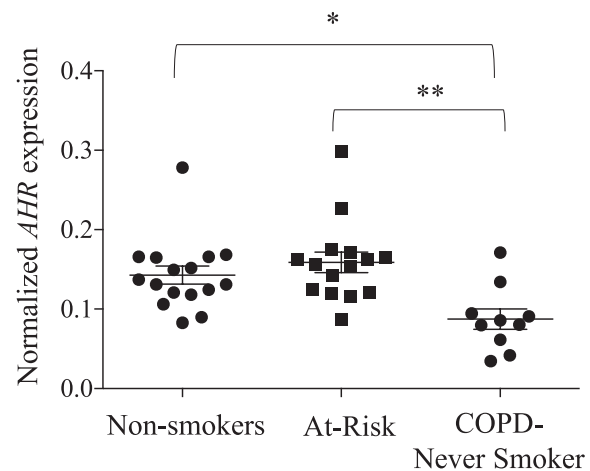
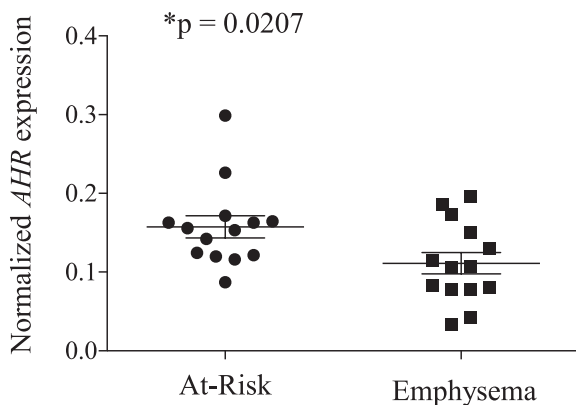
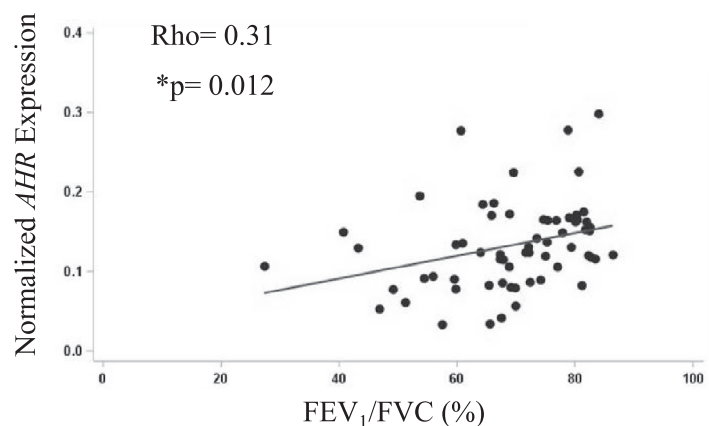
(A) Systemic *AHR* levels(B) *AHR* levels COPD never smokers(C) Systemic *AHR* in emphysema(D) *AHR* correlation with lung function

FIGURE 5 *AHR* expression is significantly reduced in the blood of human COPD subjects from CanCOLD. A, There was significantly less systemic *AHR* mRNA in COPD subjects relative to the “At-Risk” subjects ($*P < .05$). B, *AHR* mRNA expression was significantly less in COPD subjects with no history of smoking (COPD-Never smokers) relative to both Non-smokers and At-Risk control subjects ($*P < .05$, $***P < .01$). Results are expressed as mean \pm SEM. C, *AHR* mRNA expression was significantly less in COPD subjects that had also been diagnosed with emphysema via CT (Emphysema) relative to At-Risk control subjects. Results are expressed as mean \pm SEM. D, There was a significant positive correlation between systemic *AHR* mRNA expression and lung function (FEV_1/FVC) as assessed using a Pearson’s correlation coefficient.

there is a correlation between systemic *AHR* levels and lung function is not clear, but could reflect an overall reduction in *AHR* due to genetic factors. Given the now-recognized homeostatic role for the *AHR* in inflammation, cell proliferation, differentiation, and survival, including our own data, this reduction in *AHR* may, therefore, alter lung function/disease through perturbation of multiple mechanisms. In these analyses, it was important to distinguish if the reduced *AHR* expression in COPD was a consequence of smoking, given that reduced pulmonary *AHR* protein expression occurs from smoke exposure both in vitro and in vivo^{15,38} due to *AHR* degradation by the proteasome. In assessment of systemic and pulmonary *AHR* expression, our data show that *AHR* expression was reduced in COPD subjects relative

to subjects without COPD who were current smokers. We also observed that there was significantly less systemic *AHR* in COPD never-smokers. Numerous studies now show that never-smokers comprise a substantial proportion of people with COPD,³⁹⁻⁴¹ with Tan *et al* showing a COPD prevalence in never smokers of 6.4% in the general population.⁴⁰ COPD in never-smokers could be due to age, exposure to other environmental toxicants or existing conditions (e.g., asthma). Our data, that there is reduced *AHR* in individuals with clinically defined COPD, further supports that the *AHR* is important in preventing a COPD phenotype that is independent of smoking. Finally, *AHR* levels were also decreased at the mRNA level. This is an important distinction because ligand-induced proteasomal degradation of the *AHR* protein has little effect

on AHR gene expression. The sum of these data support that the reduced AHR protein expression observed in COPD is not a consequence of smoking, but rather a reflection of the disease state. Although the focus of this manuscript was on the AHR and COPD, airflow obstruction in non-smokers can occur from other conditions including asthma, bronchiectasis, and obliterative bronchiolitis. It is, therefore, interesting to speculate that the AHR may play a more global role in preserving lung function outside COPD.

One of the strengths of this study is the consistency in which we observed a decrease in AHR expression among the different cohorts of subjects. Although the cohorts differed substantially in the patient population, biological samples collected (lung vs blood), number of participants, and clinical information available, our collective data show that in people diagnosed with COPD according to current GOLD guidelines, AHR expression is significantly reduced. This suggests an important role for the AHR in the pathogenesis of COPD, which we were able to demonstrate using *Ahr*-deficient mice. However, this study is not without its limitations, including the cross-sectional nature in which these data were obtained. It is also possible that additional variables in these cohorts contribute to alterations in AHR levels. For example, the presence of co-morbidities may also impact AHR, as reduced expression has been observed in other chronic diseases.¹³ Our results may also be influenced by the presence of other lung conditions such as asthma, bronchiectasis and/or bronchiolitis. It is possible, therefore, that reduced AHR expression is more broadly applicable to lung diseases associated with inflammation and obstruction; further studies would be needed to address its expression in this context. Finally, it is possible that medication used by the subjects in these cohorts is impacting AHR expression. Notable to this is downregulation of the AHR protein in response to dexamethasone in vitro in non-pulmonary cells.^{42,43} The extent to which glucocorticoids (or other medications) is impacting AHR levels in COPD remains to be determined. Nonetheless, in spite of these limitations, the totality of our data from different cohorts of subjects strongly support that there is reduced AHR in COPD.

Cigarette smoke is also a mixture of gases and particulate matter composed of metals (iron and nickel), gases, biological agents (endotoxins), and organic chemicals such as polychlorinated dibenzodioxins (PCDDs) and polycyclic aromatic hydrocarbons (PAHs).⁴⁴⁻⁴⁷ Many of these organic chemicals are ligands of the AHR.^{48,49} Perhaps unsurprisingly, there is considerable overlap in cellular signaling pathways that are modulated by both cigarette smoke and AHR; these include nuclear factor- κ B (NF- κ B), human antigen R (HuR), and nuclear factor erythroid 2-related factor 2 (NRF2). The NF- κ B family is comprised of five members: RelA (p65), RelB, c-Rel, NF- κ B1 (p50), and NF- κ B2 (p52).⁹ Cigarette smoke potently activates the canonical NF- κ B pathway,⁵⁰ whereby RelA interacts with

p50, which are sequestered in the cytoplasm via the inhibitory protein I κ B α . Phosphorylation of I κ B α leads to its degradation via the proteasome, allowing RelA and p50 heterodimers to translocate to the nucleus for subsequent activation of target genes involved in inflammation and cell survival, among others. However, the AHR can physically interact with RelB,⁵¹ a component of the alternative NF- κ B pathway that exerts anti-inflammatory and anti-apoptotic control. Although AHR-RelB dimers can initiate transcription of target genes via binding to a unique and DRE-independent response element termed the RelBAHRE,⁵¹ we have shown that AHR-mediated suppression of smoke-induced neutrophilia in the murine lung is associated with the nuclear retention of RelB.¹⁵ These findings raise the possibility that the AHR-mediated nuclear retention of RelB may represent one way through which the AHR can suppress acute CS-induced inflammation. Another example of an AHR-dependent protein is the regulation of HuR, an RNA-binding protein that functions to stabilize target mRNA when localized to the cytoplasm. The AHR attenuates smoke-induced cyclooxygenase-2 (COX-2) expression via the nuclear sequestration of HuR, resulting in the destabilization and degradation of *Cox-2* mRNA in vitro.⁵² Moreover, another established target gene of HuR is *Mmp9*.⁵³ Thus, it is not unreasonable to postulate that similar to COX-2, perhaps the elevated protease expression (i.e., increased MMP9:TIMP1 ratio) we observed in the lungs of the *Ahr*^{-/-} mice is a consequence of the cytoplasmic shuttling of HuR, resulting in enhanced *Mmp9* mRNA stability and expression. It should also be considered whether the AHR may be interacting with NRF2, a master regulator of antioxidants. This consideration is relevant based on our finding that the upregulation of SOD2 is impaired in smoke-exposed *Ahr*^{-/-} mice.¹² Although the AHR and NRF2 regulate several common antioxidant genes such as *Nqo1*, we speculate that the AHR regulation of antioxidant defenses in the murine lung is likely independent of NRF2. This assertion is supported by previous reports demonstrating no difference in either the expression or nuclear localization of NRF2 between AHR-deficient and expressing lung structural cells.⁸ However, it may be that dampened upregulation of the mitochondrial antioxidant SOD2 in the lungs of the smoke-exposed *Ahr*^{-/-} mice is an indirect result of mitochondrial dysfunction that ensues as a consequence of AHR ablation. In addition to interaction with numerous cellular proteins, the AHR may control the pathogenesis of COPD via its regulation of noncoding (nc) RNA. One type of ncRNA is miRNA, which target mRNA for degradation. Changes in miRNA levels in response to cigarette smoke exposure is well-noted in vitro as well as in vivo and in individuals with COPD.⁵⁴⁻⁵⁷ We have shown that the AHR controls the expression of a number of these miRNAs, including miR-196a, miR-96, miR-137, and miR-133b.⁴⁸ In line with this, the attenuation of smoke-induced apoptosis is mediated by the AHR-dependent regulation of miR-196a⁷ and control over inflammation is related to expression of miR-96.⁴⁸ While

it remains to be established how the AHR controls diverse pathogenic mechanisms such as inflammation, oxidative stress, and cell death, it seems plausible that the AhR exerts its protective effects via noncanonical interactions with other cellular proteins and/or control over epigenetic pathways implicated in disease development.

Since its discovery as the receptor responsible for the induction of CYP1A1 expression in response to dioxin,⁵⁸ the endogenous role of the AHR has remained enigmatic. The relatively high expression of the AHR in the human lung, coupled with the identification of endogenous AHR ligands, raises the possibility that the AhR plays important physiological roles independent of its response to dioxin. Within the lung—an organ continuously exposed to the external environment—we establish herein that the AHR is a critical component of the homeostatic response to cigarette smoke. Not only do we report that lung and systemic AHR expression is reduced in COPD subjects, and that low AHR levels correlate with worse lung function, but we also establish that low AHR expression is causally implicated in COPD pathogenesis by demonstrating that absence of the AHR drives the development of smoke-induced emphysema. Collectively, our data position the AHR as a central player in the homeostatic maintenance of lung health.⁵⁹ These findings lay the foundation for the eventual use of the AHR as a novel therapeutic target or biomarker in identifying those individuals susceptible to the development of COPD.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Data curation and/or analysis: NG, HT, ARS, JD THT, YB, JD, LS, CJB, MZ, AR, AB, PZL, MO, DDS, CAB, and DCN; Funding acquisition: CJB and J.B; Investigation: NG, HT, and CJB; Methodology: NG, HT, YB, AR, LS., THT, and S.P; Project administration: NG, HT, and CJB; Resources: YB, PZL, JB, WCT, RPP, PJS, AR, and P.N; Supervision: NG and CJB; Intellectual contributions: NG, HT, CJB, BMS, QH, DHE, AB, AR, and Y.B; Manuscript writing, review, and editing: NG, HT, CJB, YB, THT, AR, RPP, PJS, P.N, and BMS.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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