

RESEARCH ARTICLE

Aryl hydrocarbon receptor deficiency enhanced airway inflammation and remodeling in a murine chronic asthma model

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Abstract

The aryl hydrocarbon receptor (AhR) is a ligand-dependent-activated transcriptional factor that regulates the metabolism of xenobiotic and endogenous compounds. Recent studies have shown that AhR is a novel master regulator of the mucosal immune system, including lungs and intestine. To elucidate the role of AhR in chronic severe asthma, AhR wild-type and knockout mice (AhR^{-/-}) were sensitized and challenged with ovalbumin for 4 weeks. To uncover the underlying mechanisms, inflammatory cells profile and cytokines production were analyzed in bronchial lavage fluid (BALF) and lung tissue. Compared to wild-type mice, AhR^{-/-} mice had exacerbated asthma symptoms, including airway inflammation, mucus production, airway hyperresponsiveness, and airway remodeling. BALF monocytes, neutrophils, eosinophils, and lymphocytes were all enhanced in OVA-immunized AhR^{-/-} mice. In OVA-immunized AhR^{-/-} mice, T helper (Th) 17 cell-specific cytokine IL-17A, as well as airway remodeling factors, including epithelial-mesenchymal transition (EMT) markers and vascular endothelial growth factor (VEGF), were all enhanced in lung tissue. Moreover, human cohort studies showed that AhR gene expression in bronchial epithelial cells decreases in severe asthma patients. Loss of AhR leads to worsening of allergic asthma symptoms, indicating its importance in maintaining normal lung function and mediating disease severity.

KEYWORDS

airway remodeling, allergy, aryl hydrocarbon receptor, epithelial-mesenchymal transition

Abbreviations: AhR, aryl hydrocarbon receptor; ARNT, AhR-ligand complex, together with its nuclear partner; BAL, bronchoalveolar lavage fluid; DRE, dioxin response element; EMT, epithelial mesenchymal transition; H/E, hematoxylin/eosin; HBSS, Hank's balance salt solution; IHC, immunohistochemistry; IL, interleukin; iNOS, inducible NOS; i.t., intratracheal instillation; MCh, methacholine; MT, masson's trichrome; MH, multiple homology; OVA, ovalbumin; PAS, periodic acid-Schiff; Penh, parameter enhanced pause; PVDF, polyvinylidene fluoride membranes; TLR, toll-like receptor; Th, T helper type; VEGF, vascular endothelial growth factor.

[Correction added on September 28, 2020, after first Online publication: Affiliations 3 and 4 typo errors has been updated.]

1 | INTRODUCTION

Asthma is a chronic airway inflammatory disease characterized by three important clinical features, namely mucus production and infiltration of inflammatory cells around the airway, as well as airway hyperresponsiveness. According to the latest global estimates (2014), asthma is a chronic inflammatory disease affecting approximately 334 million people worldwide and the prevalence of asthma has increased in the past two decades. Approximately 10%-25% of patients with difficult-to-control asthma require high doses of corticosteroids to achieve efficient control of the disease, representing an unmet therapeutic need. Moreover, approximately 30% of severe asthmatics have steroid-refractory asthma, characterized by a frequent recurrence of symptoms even with high doses of inhaled and systemic steroid treatment,¹⁻⁵ suggesting that these patients could be resistant to corticosteroids (steroid resistance asthma).²⁻⁴ The mechanism of severe, steroid-resistant asthma is still unclear. However, it is commonly associated with mixed typed CD4⁺ T helper (Th) cells activation, as well as innate immune activation (such as toll-like receptor [TLR] 2 and 4, nucleotide-binding oligomerization domain [NOD]-like receptor [NLR] family, and interleukin [IL]-1 β) upon stimulation with allergens, pathogens, and pollutants exposure. Therefore, the underlying mechanism of this disease needs to be investigated in order to develop novel therapeutics and better treatments.

The Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor belonging to the family of basic helix-loop-helix transcription factors. The canonical biological and toxicological role of AhR is in the metabolism of xenobiotic toxicants, including polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and others, through binding of AhR. The AhR complex (two heat shock proteins 90,⁶ the AhR-interacting protein, p23, and the c-SRC protein kinase) is subsequently translocated into the nucleus where the AhR-ligand complex, together with its nuclear partner (ARNT), binds to the dioxin response element (DRE), thereby activating downstream genes, including phase I xenobiotic-metabolizing enzymes Cyp1a1 and Cyp1b1, as well as phase II enzymes, including NAD(P)H quinone oxidoreductase 1.⁷ Through activation of phase I and II enzymes, AhR induces toxicity or detoxification effects. In a noncanonical role, AhR can act as an E3 ubiquitin ligase⁸ or associate with pRb⁹ or KLF6¹⁰ to control the cell cycle, thereby playing a role in cancer formation and metastasis. In addition, the release of the c-SRC kinase results in the phosphorylation of multiple targets.^{11,12} During the past decade, AhR was shown to have a crucial role in regulating the differentiation of regulatory T cells (Treg) and T helper type 17 (Th17) cells. Different AhR-ligands affect Treg and Th17 differentiation. TCDD, ITE, Kyn, or Laquinimod metabolites induce AhR activation, leading to an increase of FoxP3⁺ Treg cells through different mechanisms,¹³⁻¹⁶

including direct transactivation, the induction of epigenetic modifications that control Foxp3 transcription, and modulation of dendritic cells (DCs). AhR is highly expressed in Th17 cells and its activation by FICZ enhances Th17 cell differentiation and promotes IL-22 production.¹⁷ In addition, AhR also controls DCs activation. Thatcher et al found that in the OVA-immunized acute allergic asthma model, AhR-deficient mice exhibited a higher inflammatory response, as well as higher Th2 cell differentiation and MHC-II and CD86 expression in DCs.^{18,19} Recent studies have shown that AhR shows as a novel key regulator of the mucosal immune system, including in the lungs.^{13,14} In case of allergic asthma, AhR knockout mice showed aggravated asthma symptoms^{19,20} and activation of AhR through ligand treatment led to epigenetic regulation of epithelium-derived Th2 cytokines.²¹ However, the role of AhR in severe asthma is still unclear. However, the role of AhR in a chronic severe asthma mouse model still unclear. In particular, the mechanism of mixed type Th cells activation and airway remodeling which contributes the steroid-resistant effects remain unknown. In this study, we created a chronic severe asthma mouse model using AhR knockout (AhR^{-/-}) mice and showed that AhR^{-/-} accelerated asthma symptoms through activation of Th17 cells and enhancement of EMT in the airways.

2 | MATERIALS AND METHODS

2.1 | Animal experiments

Six-week-old female C57BL/6 mice were purchased from the National Laboratory Animal Center, National Research Laboratories, Taiwan. C57BL/6-Ahr^{tm1.2Arte} (AhR knockout) mice were purchased from Taconic Biosciences, Inc (NY, USA) and did not breed. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the China Medical University (Protocol No. 103-127-N). On day 0, 6-week-old female C57BL/6 mice were immunized with 50 μ g of OVA and 2 mg of alum adjuvant dissolved in PBS to a total volume of 200 μ L via intraperitoneal injection (i.p.). Boosters composed of 25 μ g of OVA combined with 2 mg of alum were administered on days 4 and 7.²² Subsequently, sensitized mice were exposed to/challenged with 5 mL of aerosolized 4% of OVA in PBS using a nebulizer (AG-AL1000, Aeroneb^R Lab Nebulizer Unit, Standard VMD) once a day, 5 days per week, for 4 weeks (Figure 1A), as previously described.²³

2.2 | Bronchoalveolar lavage (BAL) and lung histology

Bronchoalveolar lavage (BAL) was performed by injecting 1 mL of HBSS and gently aspirating 1 mL of BAL. The

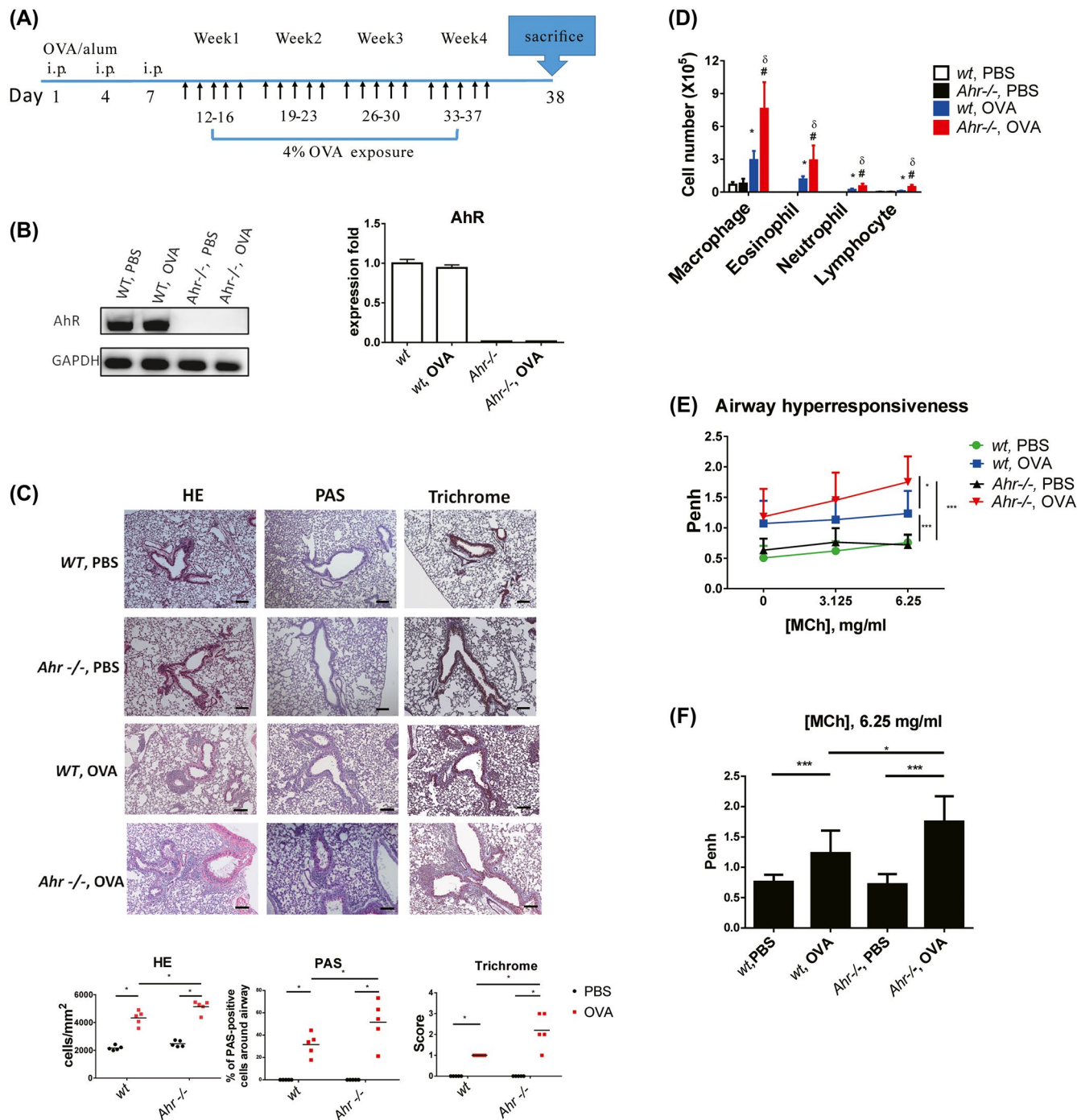


FIGURE 1 Effects of asthma symptoms in OVA-immunized mice. A, Animal protocol. Six-week-old female C57BL/6 mice were intraperitoneally (i.p.) immunized with 50 μ g of OVA and 2 mg of alum adjuvant at day 1, 4, and 7, and then, sensitized with aerosolized OVA in PBS using a nebulizer once a day, 5 days per week for 4 weeks. B, AhR protein expression in the lungs was detected by western blot and quantified by ImageJ software. C, Lung sections were stained through H&E, PAS, and MT staining to assess inflammatory cell infiltration, mucus production, and collagen deposition around the airway, respectively. D, BAL differential cell counts were assessed through May-Grünwald Giemsa staining. Data are expressed as mean $\pm$ SD. * P < .05, as compared with the wt, PBS group; # P < .05, as compared with the Ahr^{-/-}, PBS group; δP < 0.05, as compared with the wt, OVA group (E) Airway resistance (Penh) was determined via whole-body plethysmography and data of MCh 6.25 mg/mL was shown in (F). Data are expressed as mean $\pm$ SD (n = 5). * P < .05; *** P < .001

procedure was performed three times. All BAL samples were centrifuged 1500 rpm at 4°C for 5 min and the supernatant from the first BAL sample was collected and stored at -20°C

for subsequent cytokine analysis by enzyme-linked immunosorbent assay (ELISA). Total BAL cells were counted using a hemocytometer. Cytospin slides of the BAL cells were stained

with May-Grünwald Giemsa stain. The cell profile was determined for a total count of 300 cells in at least four fields of view under a light microscope.^{24,25} Lung tissue was fixed with 10% of formalin and sections were prepared for hematoxylin/eosin (H/E) staining, periodic acid-Schiff (PAS) staining, and Masson's trichrome (MT) staining to quantify the number of infiltrating cells, as well as mucus production and collagen deposition. Quantification of inflammatory cells, PAS-positive bronchial epithelial cells, and the area of collagen deposition were performed as previously published.²⁴

2.3 | Immunohistochemistry (IHC)

Lung tissue sections were deparaffinized and hydrated. Epitope retrieval was performed according to the manufacturer's protocol (Thermo Quanto Detection system, CA, USA). Briefly, the lung sections were stained with hydrogen peroxide and protein blockers, and then, stained with primary antibodies. The sections were then stained with secondary HRP-labeled polymer and DAB (Antibody amplifier + System-HRP, Thermo) and counterstained with hematoxylin. Immunostained sections were viewed under a Zeiss microscope (Stuttgart, Germany).

2.4 | Western blot analysis

Lung tissues were homogenized in the presence of a lysis buffer (25 mM Tris-HCl, 2 mM EDTA, 1 mM benzamidine, 1 mM PMSF; pH 7.4) containing protease and phosphatase inhibitors (Thermo Fisher Scientific, Waltham, MA USA). Appropriate volumes of the homogenates, such that each well contained 5 µg of proteins as quantified using a Bradford assay kit (cat: 500-0006, Bio-Rad, Hercules, CA), were separated by 10% of SDS-PAGE. The proteins were then transferred to polyvinylidene fluoride membranes (PVDF) and probed with primary antibodies. After incubation with horseradish peroxidase-conjugated secondary antibodies, the signals were visualized using a Millipore ECL kit (Millipore, Bedford, MA, USA) in accordance with the manufacturer's instructions.

2.5 | Cytokine assay

Lung tissues were homogenized in the presence of a lysis buffer (25 mM Tris-HCl, 2 mM EDTA, 1 mM benzamidine, 1 mM PMSF; pH 7.4) containing protease and phosphatase inhibitors (Thermo Fisher Scientific) and stored at -20°C prior to analysis. Cytokine levels were detected by enzyme-linked immunosorbent assay (ELISA) (for KC and eotaxin), which was performed according to the manufacturer's instructions, and western blotting (for interferon-γ (IFN-γ),

interleukin-4 (IL-4), IL-13, and IL-17). Standards were prepared from IFN-γ, IL-4, IL-13, IL-17, eotaxin, and KC (R&D systems, Minneapolis, MN, USA).

2.6 | Measurement of airway hyperresponsiveness by noninvasive whole-body plethysmography

Mice were placed in whole-body plethysmography chambers (BUXCO, USA) for 15 minutes. Baseline readings of airway resistance were collected every 2 seconds for 3 minutes. The baseline for the resistance index (RI) was determined using PBS as a control. A total of 500 µL each of varying concentrations of methacholine (MCh; 0, 3.125, 6.25, 12.5 mg/mL) was used for repeat nebulization for 2 minutes and an additional 6 minutes cycle. Airway resistance was determined by calculating the dimensionless parameter enhanced pause (Penh).²²

2.7 | OVA-specific antibody assay

Mice serum samples were collected on day 30 and the anti-OVA immunoglobulin (Ig)E, IgG1, and IgG2a antibody titers were determined by ELISA, as previously described.²⁴

2.8 | Flow cytometry and intracellular staining of lymph nodes CD4 T cells

Cells (1×10^5) were isolated from mediastinal lymph nodes as previously described.²⁴ The cells were stimulated with 50 ng/mL of phorbol 12-myristate 13-acetate (PMA; Sigma) and 500 ng/mL of ionomycin (Sigma) in the presence of GolgiPlug (BD Bioscience, San Jose, CA, USA) in RPMI 1640 medium contained 10% of FBS for 4 hours. Then, Fc blocker (Biolegend) was added and the cells incubated for 10 minutes after which they were surface-stained for CD4 using fluorescein isothiocyanate (FITC), permeabilized with Cytotfix/Cytoperm (BD Bioscience), washed, and then, intracellularly stained with phycoerythrin (PE)-labeled anti-IL4, allophycocyanin (APC)-IFN-γ, and peridinin-chlorophyll-protein (Percp)-labeled anti-IL-17 (Thermo Fisher Scientific, MA, USA). Flow cytometry was performed on a FACSCalibur instrument (BD Bioscience), and the results were analyzed using the CellQuest software (BD Bioscience).

2.9 | Analysis of AhR gene expression from the gene expression omnibus database

Data regarding the expression levels of AhR-related genes in asthmatics were obtained from the gene expression

omnibus (GEO) database, National Center for Biotechnology Information (NCBI), USA. Bronchial epithelial cells mRNA levels from normal control (healthy control), mild-moderate asthmatic (non-severe), and severe asthmatic (severe) patients were analyzed by microarray as previously described (GSE43696²⁶ and GSE63142²⁷).

2.10 | Real-time PCR

RNA was converted into cDNA, and then, measured by quantitative real-time PCR using Bio-Rad system (CFX96) (CA, USA). The PCR product was amplified using SYBR Green reagents (Bio-Rad 170-884AP). Relative gene expression was calculated by means of the comparative Ct method ($2^{-\Delta\Delta C_t}$) with the expression of GAPDH as an internal control. The primers used are listed below: GAPDH (5'-TGCAACCACTGCTTAGC-3', antisense, 5'-TCTTCTGGGTGGCAGTGATG-3'). AhR (sense, 5'-GGCTTGGAATTACAGGAATCC-3', antisense, 5'-CAGCC TCAGGATGTGAACTC-3').

2.11 | Cell migration assay

Cell migration was determined using the wound healing assay.²⁸ Briefly, cells were seeded in 12-well plate and incubated to confluence. A cell-free wound area was then created by scratching the cells with a pipette tip. Then, cells were allowed to migrate into the cell-free wound for 18 hours.

2.12 | Statistical analysis

Results are presented as means $\pm$ standard deviation (SD) with $N = 5$ (animal study) and $N \geq 3$ (cell study). The mRNA levels in bronchial epithelial cells were divided into normal controls (healthy control, $n = 47$), mild-moderate asthma patients (non-severe, $n = 122$), and severe asthma patients (severe, $n = 94$) groups. All data were compared using one-way ANOVA followed by Newman-Keuls post hoc test (GraphPad software). A value of $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Enhancement of inflammatory cells infiltration, mucus production, and airway hyperresponsiveness in the lungs of AhR^{-/-} mice

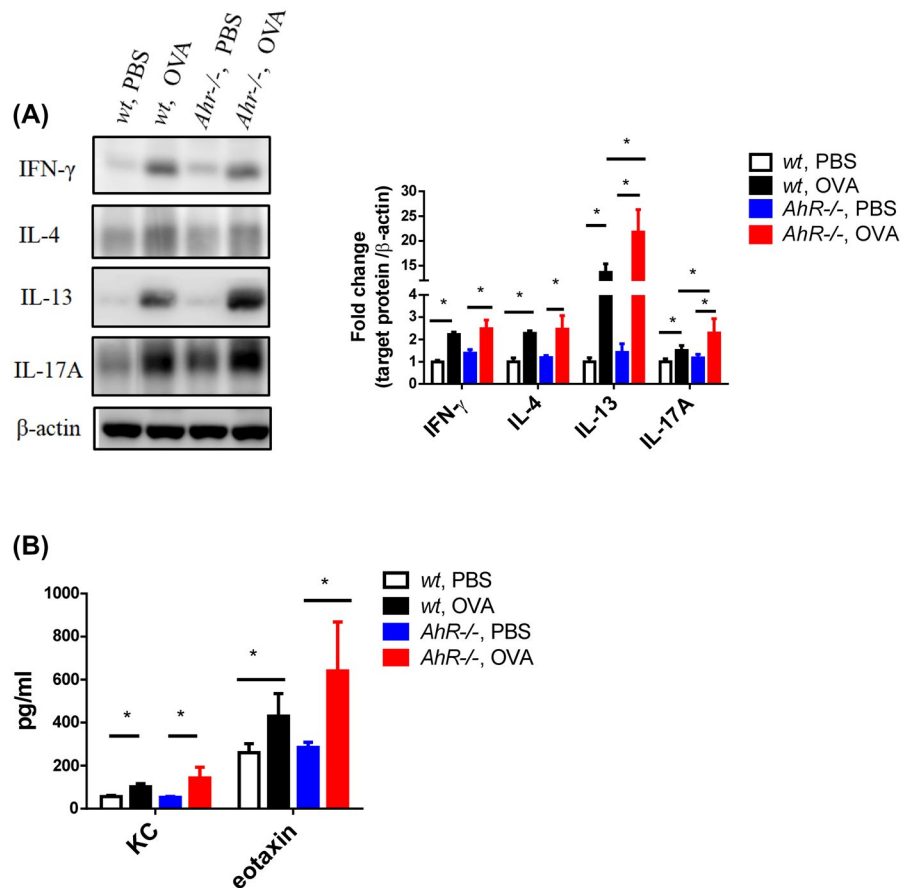
In order to create a chronic allergic asthma mouse model, OVA-immunized mice were sensitized and challenged with

OVA (Figure 1A). These mice exhibited obvious airway inflammation and mucus production. In AhR^{-/-} mice, we confirmed the knockdown of AhR expression in the lung tissue, as shown in Figure 1B. After OVA immunization, AhR^{-/-} mice exhibited a significant increase in airway inflammation and mucus production when compared with wild-type. Without OVA immunization, wild-type and AhR^{-/-} mice did not exhibit obvious infiltration of inflammatory cells and mucus hypersecretion in the lungs (Figure 1C). Analysis of inflammatory cells profile in bronchial alveolar lavage fluid (BALF) revealed an increase in the number of macrophages, eosinophils, neutrophils, and lymphocytes in OVA-immunized and AhR^{-/-} mice, as compared to wild-type mice (Figure 1D). Moreover, lung function was analyzed by quantifying airway hyperresponsiveness. Our results show that exposure of mice to methacholine induces bronchial contractions, as shown in Figure 1E,F. OVA immunization-induced airway hyperresponsiveness in both wild-type and AhR^{-/-} mice; however, AhR^{-/-} mice exhibited an enhancement of airway hyperresponsiveness after OVA immunization. Therefore, AhR^{-/-} mice showed an enhancement of asthma symptoms after OVA immunization.

3.2 | AhR^{-/-} mice showed an increase in Th2- and Th17-related cytokine levels in the lungs

In order to investigate which T helper cells-related cytokine activation is involved in the enhancement effects of airway inflammation in AhR^{-/-} mice, we detected the cytokines profile in lung tissue. As shown in Figure 2, in OVA-immunized mice, Th2-related cytokines (IL-4, IL-13, and eotaxin), Th1-related cytokines (IFN- γ), and Th17-related cytokines (IL-17A and KC) were all increased in lung tissue homogenates when compared to wild-type mice. Among these T cells differential cytokines, Th17-related cytokines, IL-17A, and KC, as well as Th2-related cytokines IL-13 and eotaxin were shown to correlate with enhanced airway inflammation. However, Th1-related cytokine IFN- γ expression levels in AhR^{-/-} mice had no effect on airway inflammation. Moreover, the examination of Th cell profiles in mediastinal lymph nodes to confirm Th cells differentiation revealed that OVA-immunization-induced Th1, Th2, and Th17 cells differentiation (Figure 3). After OVA immunization, total cell numbers and CD4⁺ T cell numbers revealed a significantly increase in mediastinal lymph node (Figure 3A,B). Among different Th cells, Th2 cells are the predominant differentiated cells in mediastinal lymph nodes (Figure 3E,F). AhR^{-/-} mice did not exhibit an obviously enhanced Th1 and Th2 cells profile for either OVA-immunized or naïve mice. However, AhR^{-/-} mice showed significantly increased activation of Th17 cells in OVA-immunized mice.

FIGURE 2 Detection of cytokine profiles in the lungs. Cytokines expression in the lungs was analyzed by western blotting (A) and ELISA (B). Data are expressed as mean $\pm$ SD (n = 5). * P < .05



3.3 | Extreme airway inflammation-induced epithelial-mesenchymal transition in AhR^{-/-} mice

In chronic asthma, inflammatory cytokines, such as TGF- β and HMGB1, induce epithelial to mesenchymal transition (EMT). This is a crucial feature of airway remodeling, which can impair airway function. In our previous study, we found that knockdown of AhR caused lung cancer cells to undergo EMT, leading to a more aggressive phenotype due to increased migration and invasion. As such, it is interesting to note the effect of EMT activation in AhR^{-/-} mice. As shown in Figure 4A, OVA-immunization led to airway inflammation, which caused downregulation of E-cadherin and upregulation of vimentin protein expression, thereby inducing EMT activation in the lungs. Furthermore, we used IHC to verify whether airway epithelial cells undergo EMT. Our results showed that E-cadherin expression was indeed decreased (Figure 4B), while vimentin expression increased in bronchial epithelial cells in OVA-immunized mice. Airway inflammation-induced EMT was shown to worsen in AhR^{-/-} mice. In addition, we observed that infiltrated inflammatory cells also expressed low levels of E-cadherin and high levels of vimentin. From our previous study, we found that overexpression of AhR in a lung cancer cell line- (H1299) induced Smad4 degradation, thereby reducing cancer invasiveness.²⁹

Therefore, we examined Smad4 expression in the lung tissue of wild-type and AhR^{-/-} mice after OVA immunization. As shown in Figure 4A, Smad4 was highly expressed in OVA-immunized mice and its expression was enhanced in AhR^{-/-} mice. In naïve AhR^{-/-} mice, Smad4 expression in the lungs was significantly increased when compared with wild-type mice. Further, we investigated the effects of EMT in bronchial epithelial cell in vitro after decrease of AhR expression. After knockdown of AhR expression by shRNA (Figure 5A), bronchial epithelial cells showed a decrease of E-cadherin and increase of vimentin expression (Figure 5B) as well as acceleration of cell migration (Figure 5C). Therefore, downregulation of AhR expression in vivo or in vitro might lead bronchial epithelial cells dysfunction to induce EMT, especially under airway inflammation.

3.4 | Enhancement of airway remodeling factors expression in the lungs of OVA-immunized AhR^{-/-} mice

Airway remodeling, which has been implicated in corticosteroid resistance, hampers drug delivery to the parenchymal lung tissue in severe asthma and chronic obstructive pulmonary diseases.^{30,31} As shown in Figure 1C, we discovered that airway remodeling by detection of collagen deposition was

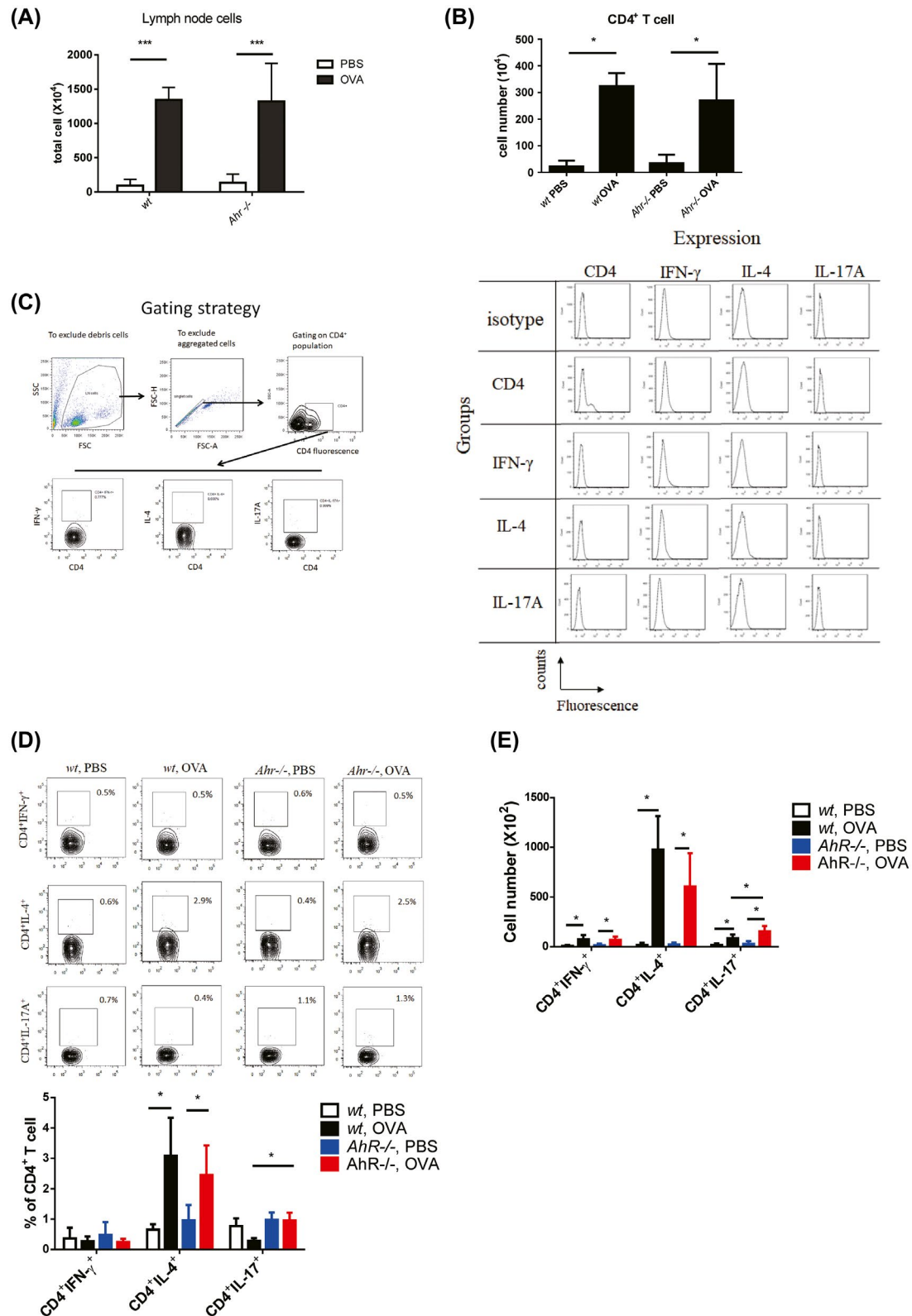


FIGURE 3 Detection of CD4⁺ T cells profiles in lungs draining lymph nodes. A, Total cell numbers (B) Total CD4⁺ T cells number in mediastinal lymph node. C, Gating strategy of flow cytometry. In FSC and SSC panel, gated the region without cells debris. In FSC-A and FSC-H, gated the region without cells aggregation. Representative data of PBS group. Compensation controls including isotype Abs controls (isotype) and signal Ab staining controls (CD4, IFN-γ, IL-4, and IL-17A). D, Representative figure for expression in Th1, Th2, and Th17 cells in lymph nodes and quantification graph. E, Th1, Th2, and Th17 cells cell numbers. Data are expressed as mean ± SD (n = 5). **P* < .05; ****P* < .001

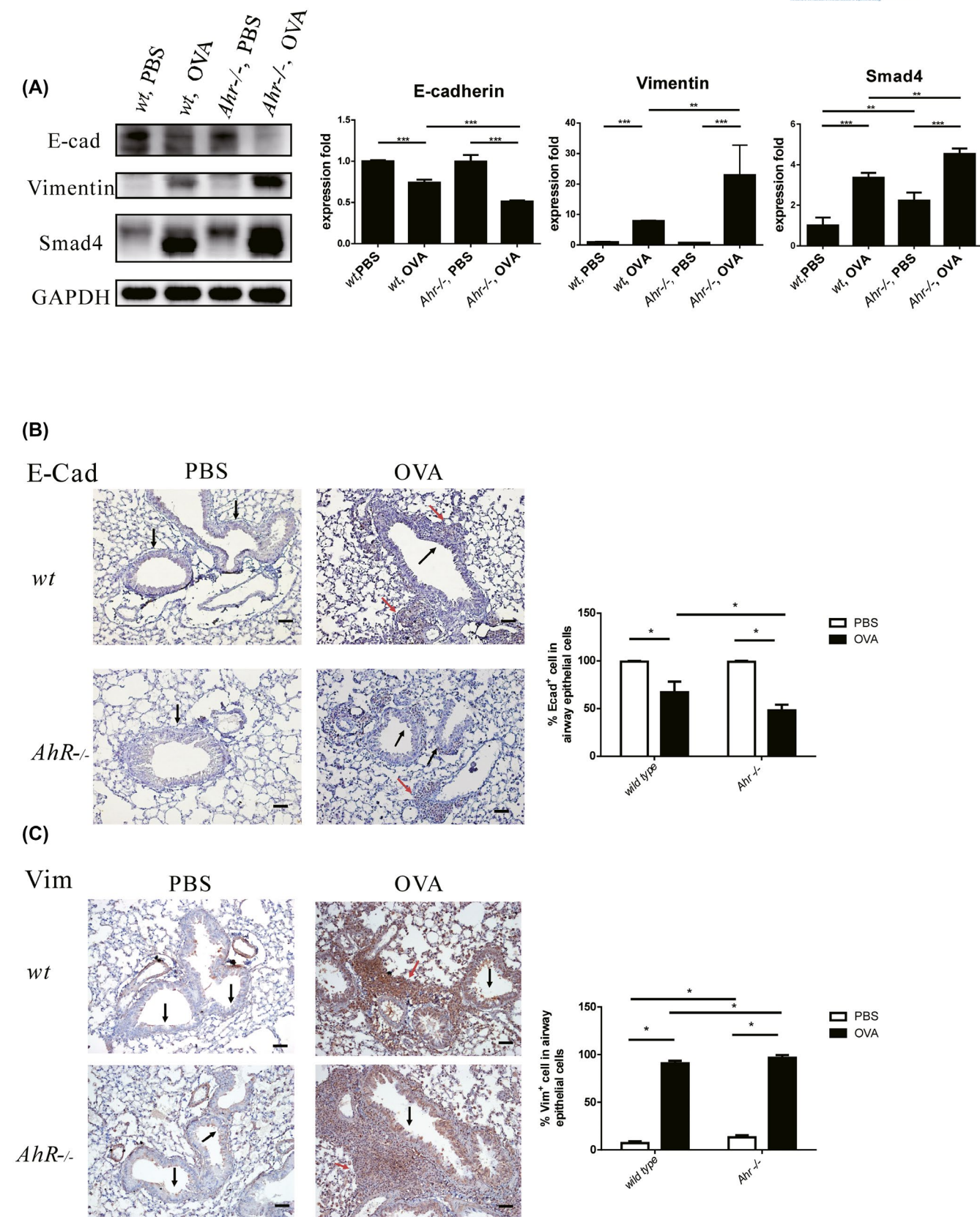


FIGURE 4 EMT-related factors expression in lung tissue. A, Detection of E-cadherin, vimentin, and Smad4 expression in lung tissue was detected by western blot and quantified using the ImageJ software. B, Immunohistochemistry of E-cadherin expression in lung tissue. C, Immunohistochemistry of vimentin expression in lung tissue. Quantification of percentage of E-cadherin and vimentin positive cells around bronchioles. Black arrow indicated bronchial epithelium and red arrow indicated inflammatory cells. Data are expressed as mean $\pm$ SD ($n = 5$). * $P < .05$, ** $P < .01$, *** $P < .001$

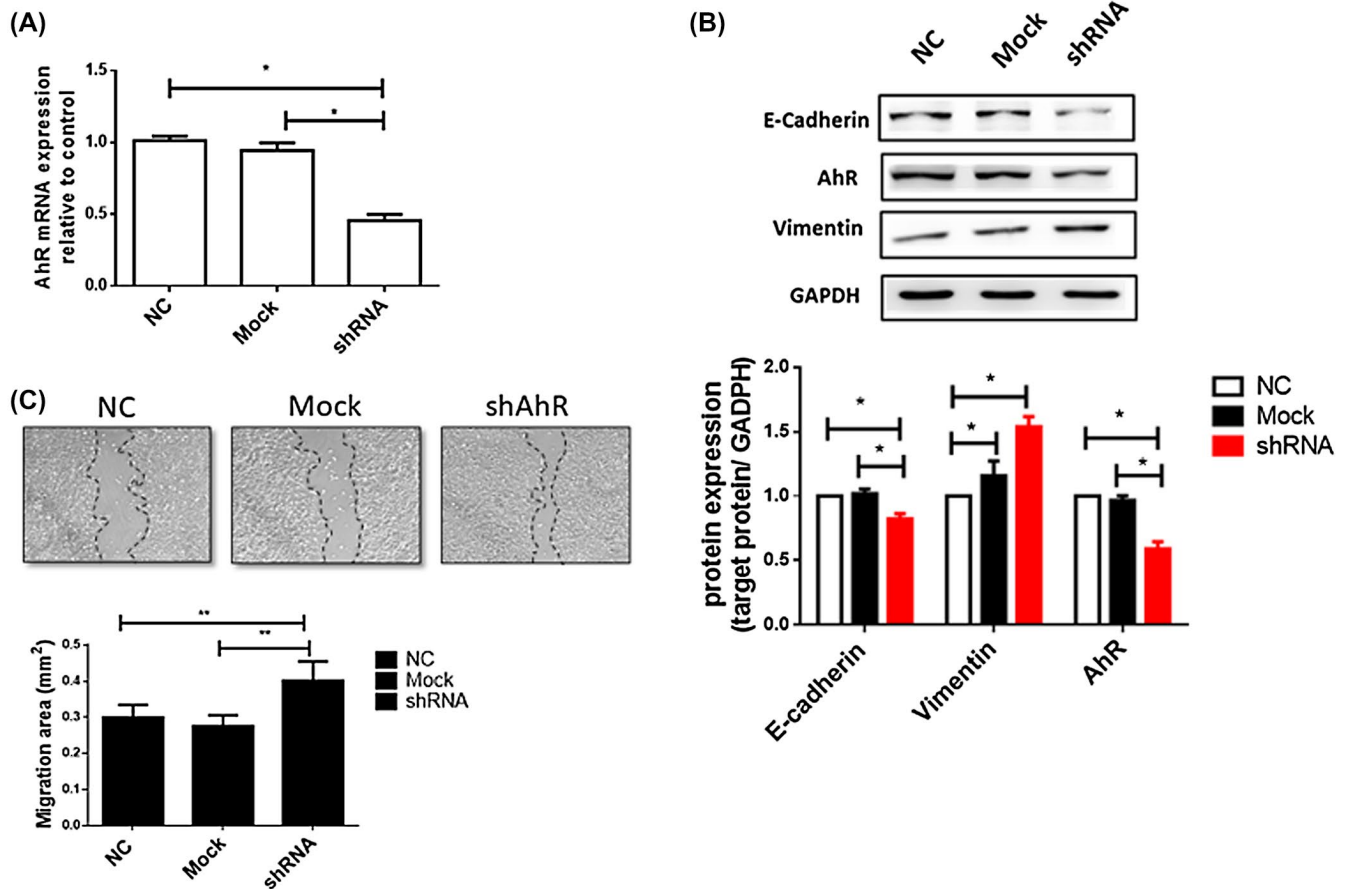


FIGURE 5 Epithelial-mesenchymal transition in human bronchial epithelial cells after knockdown of AhR expression. Transfection of AhR shRNA lentiviral vector and vector only (mock group) in human bronchial epithelial cells using puromycin selection to get stable clone. A, Detection of AhR mRNA expression by real-time PCR. B, Detection of E-Cadherin, AhR, and vimentin protein expression by western blotting and quantification, using the ImageJ software. C, Detection of cell migration by wound healing assay. Data are expressed as mean $\pm$ SEM ($n \geq 3$). * $P < .05$, ** $P < .01$

significantly enhanced in AhR^{-/-} mice. Therefore, we further analyzed airway remodeling-related factors, including 3-nitrotyrosine (3-NT), TGF- β , and VEGF expression in the lungs. As shown in Figure 6, the expression of 3-NT, TGF- β , and VEGF in the lungs was significantly increased in OVA-immunized mice, and 3-NT, TGF- β , and VEGF expression was also shown to be enhanced in AhR^{-/-} mice. In AhR^{-/-} naïve mice, 3-NT expression was shown to be increased, suggesting a loss of phase II antioxidant enzyme activation without AhR expression.

3.5 | OVA-specific IgE was dramatically increased in OVA-immunized AhR^{-/-} mice

Next, we analyzed the expression of OVA-specific antibodies. As shown in Figure 7, OVA-specific IgE, IgG1, and IgG2a were all increased in OVA-immunized mice. In AhR^{-/-} mice, OVA-specific IgE showed a significant increase but OVA-specific IgG2a and IgG1 did not exhibit

significant differences when compared with wild-type AhR mice with OVA immunization. This might indicate that OVA-immunized AhR^{-/-} mice tend to enhance Th2 and Th17 responses.

3.6 | AhR and ARNT expression were also decreased in bronchial epithelial cells of severe asthmatics

In order to confirm AhR expression in asthmatics, we analyzed the data from two cohort studies from GEO from the National Center for Biotechnology Information (NCBI) website. As shown in Figure 8, data from the microarray analysis of bronchial epithelial cells from healthy controls, non-severe asthmatics, and severe asthmatics showed that AhR and the co-transcription factor, aryl hydrocarbon receptor nuclear translocator (ARNT), were significantly decreased in severe asthma as compared with healthy control and non-severe asthma groups. The repressor of AhR (AhRR)³² did

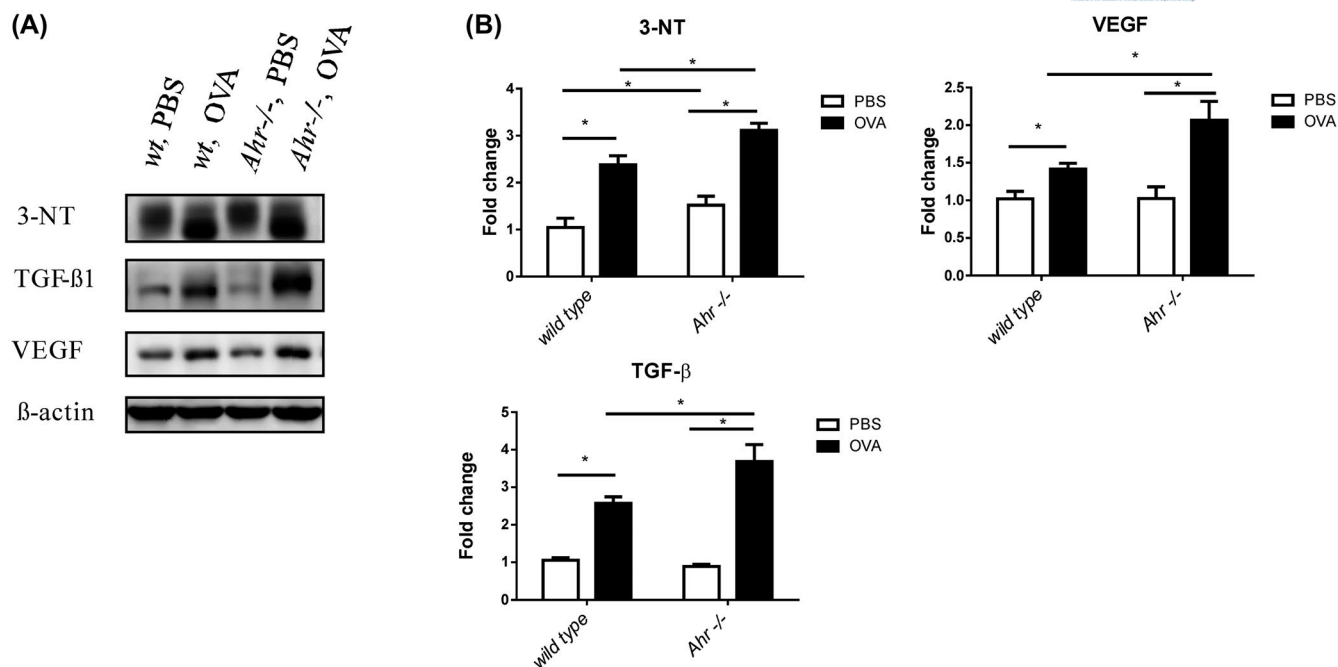


FIGURE 6 Detection of airway remodeling factors expression in lung tissue and antigen-specific antibodies expression in serum. A, Detection of 3-NT, VEGF, and TGF-β expression in lung tissue was done by western blot and quantified by using the ImageJ software (B). Data are expressed as mean $\pm$ SD ($n = 5$). $*P < .05$

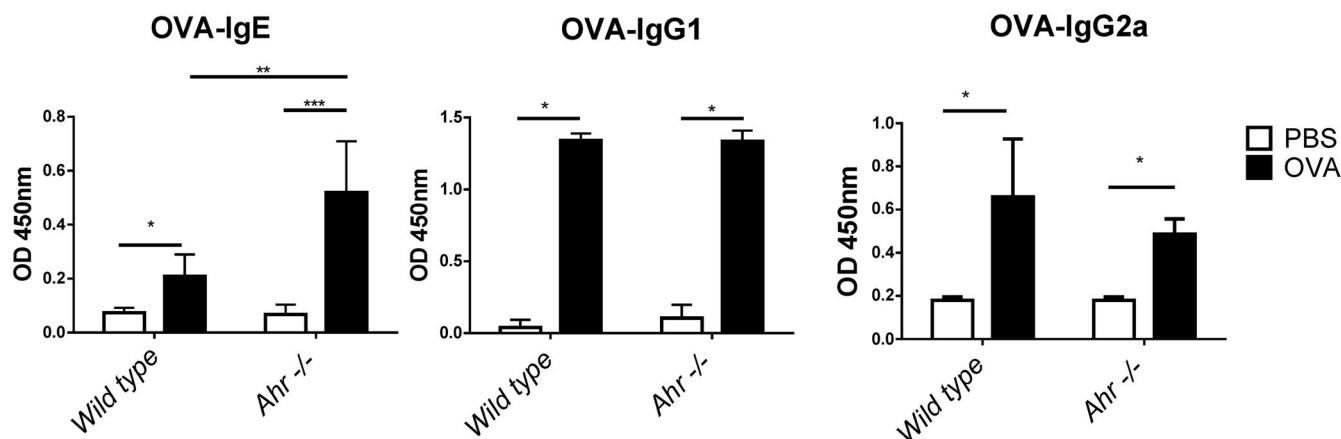


FIGURE 7 Detection of OVA-specific Abs production in serum. Analysis of OVA-specific IgE, IgG1, and IgG2a expression in serum was done by ELISA. Data are expressed as mean $\pm$ SD ($n = 5$). $*P < .05$

not show significant changes in expression between healthy control, non-severe asthmatics, and severe asthmatics. We also examined two important genes, aryl hydrocarbon receptor nuclear translocator-like protein 1 and 2 (ARNTL1 and ARNTL2),³³ which are negatively regulated by AhR and are involved in the control of circadian and metabolic rhythmicity. Severe asthmatics showed a decrease in AhR gene expression but had the highest levels of ARNTL1 and ARNTL2 expression. Therefore, the results of the microarray analysis of bronchial epithelial cells in a human cohort suggest that a decrease in AhR expression might be associated with disease severity.

4 | DISCUSSION

In this study, we investigated the role of AhR in chronic airway inflammation in a mouse model. In AhR knockout mice, we found that asthma symptoms, including airway inflammation, mucus production, airway hyperresponsiveness, and airway remodeling were all enhanced. To elucidate the mechanisms, we first analyzed cytokines profiles in the lungs and Th cells activation in lung draining lymph nodes. We found that Th2-specific cytokines, IL-13 and eotaxin, and Th17-specific cytokines, IL-17A and KC, were all enhanced in the lungs of OVA-immunized AhR knockout mice. However,

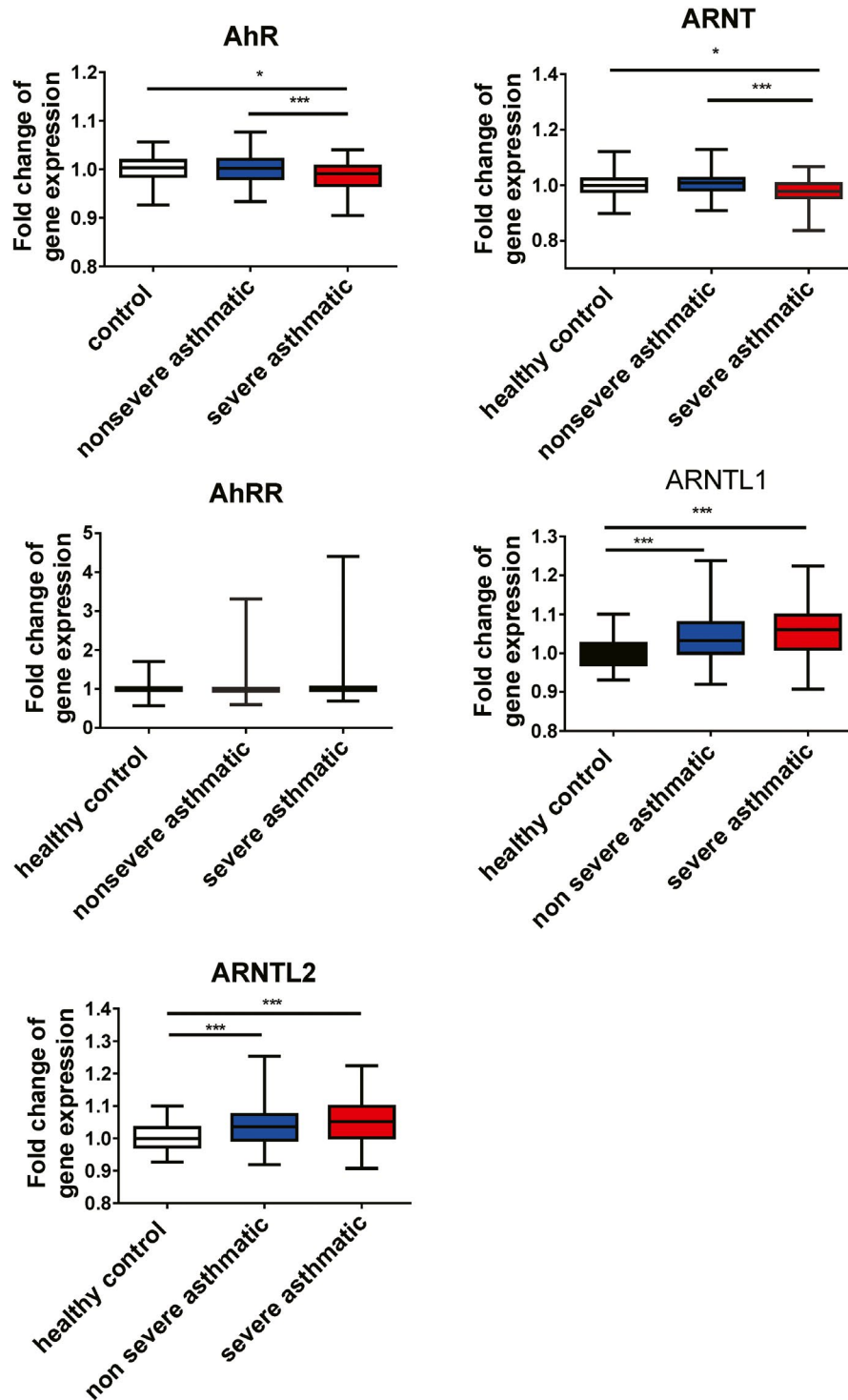


FIGURE 8 Analysis of AhR-related gene expression in a human cohort study. Analysis of AhR, ARNT, AhRR, ARNTL1, and ARNTL2 gene expression in human bronchial epithelial cells from GEO database. Data are expressed as mean $\pm$ SD * P < .05, *** P < .001 Analysis of AhR, ARNT, AhRR, ARNTL1, and ARNTL2 gene expression in human bronchial epithelial cells obtained from GEO database. Data are expressed as mean $\pm$ SD Bronchial epithelial cell mRNA levels from normal controls (healthy control, n = 47), mild-moderate asthma patients (non-severe, n = 122), and severe asthma patients (severe, n = 94). * P < .05, *** P < .001

there was no obvious increase in IFN- γ production in OVA-immunized AhR knockout mice. The analysis of the types of CD4⁺ T cells in mediastinal lymph nodes showed that there was no significant increase in Th1 and Th2 cells, but

there was an increase in Th17 cells differentiation in OVA-immunized AhR knockout mice. Therefore, the induction of Th17 cells differentiation and activation played a crucial role in the enhancement of airway inflammation in AhR knockout

mice in our chronic asthma mouse model. Previous studies have found that AhR knockout mice showed increased Th2 cells activation in acute OVA-immunized asthma mouse models. Likewise, an ex vivo study where AHR^{-/-} lung DCs were isolated from mice stimulated with OVA and LPS, and were cocultured with T cells harvested from peripheral lymph nodes of OT-II mice (AHR^{+/+}), revealed induction of Th2 and Th17 cells differentiation.¹⁹ Therefore, AHR^{-/-} mice might show an increase in allergic inflammation via enhancement of Th2 and Th17-related responses.

As airway remodeling impedes penetration into parenchymal tissue of drugs that lead to steroid resistance, it is important to know whether AhR^{-/-} affects airway remodeling and related mechanisms. We found that airway remodeling was enhanced in OVA-immunized AhR^{-/-} mice, including an increase in collagen deposition around the airway, as well as increased mucus production and epithelial dysfunction. This indicates that knockout of AhR not only increases Th2 and Th17-related inflammatory responses, but also activates structural cells, such as fibroblasts, goblet cells, and bronchial epithelial cells. In our previous study, we found that knockdown of AhR in human non-small cell lung carcinoma cell lines (H1299) enhanced the migration and invasiveness of tumor cells via activation of EMT and increased Smad4 expression.²⁹ Importantly, in the OVA-induced asthma model, we also found that knockdown of AhR-induced EMT activation and Smad4 expression in the bronchial epithelium of the lungs. Moreover, Smad4 expression was also shown to be increased in the lungs of naïve AhR^{-/-} mice. Therefore, AhR might control Smad4 homeostasis in the lungs. Although the mechanism by which AhR controls Smad4 expression is still unclear, one important pathway was identified. Previous reports have shown that AhR is an E3 ligase and our study has found that Smad4 was a target for AhR, which induced Smad4 ubiquitination and subsequent degradation.²⁹ However, whether enhancement of Smad4 expression in AhR knockout mice was due to a decrease of Smad4 ubiquitination and subsequent degradation requires further investigation.

Oxidative stress is an important factor that can damage the lung parenchymal tissue and cause danger signals that accelerate inflammatory responses, especially in severe asthma.³⁴ During airway inflammation, NADPH oxidase and iNOS are activated to release H₂O₂ and NO, which form 3-nitrotyrosine (3-NT) in airway epithelial and inflammatory cells, including macrophages, eosinophils, and neutrophils.³⁵ 3-NT is a stable oxidative product and has shown a positive correlation with the severity of asthma.³⁶ Among different types of Th cells, Th17 cells were found to be directly associated with iNOS activation, as IL-17A directly induces iNOS expression in airway epithelial cells. In our study, IL-17A production in the lungs was also shown to be increased in AHR knockout mice with OVA-immunization, which might be important for 3-NT formation.

In addition, increase of Smad4 and 3-NT expression was seen in the non-OVA-immunized AhR^{-/-} mice. From previous study, this might be because AhR involved in regulation of Smad4 and 3-NT expression. In our previous study,²⁹ AhR contributes to Smad4 ubiquitination and degradation in cancer cells. Therefore, knockout AhR might increase Smad4 expression by decreasing Smad4 proteasome degradation in lung tissue of AHR knockout mice. AhR-deficient mice are more susceptible to hyperoxic lung injury due to decrease of antioxidant enzymes such as phase I (cytochrome P450, CYP1A) and phase II (NAD(P)H quinone reductase-1, NQO1, and microsomal glutathione S-transferase, GST) as well as increase in inflammation.³⁷ ROS is important for induction of 3-NT formation in inflammatory diseases. The increased 3-NT basal condition in AhR knockout mice might result from increase in ROS amount through downregulation of antioxidant enzymes in lungs.

In order to further explore the role of AhR in asthma, we analyzed the data for an asthmatic cohort from GEO database, NCBI, USA. The mRNA expression in bronchial epithelial cells analyzed by microarray showed that the expression of AhR and its coactivator ARNT was significantly decreased in severe asthmatics. In contrast, the expression of two AhR inhibitors, ARNTL1 and ARNTL2, was shown to be significantly increased in both asthmatic groups. The results of the human cohort study also showed that a dysregulation of AhR and related proteins in bronchial epithelium might associate with the pathogenesis of severe asthma.

Sex difference in asthma has been found both in human clinical study and murine model of asthma. In human study, sex affects asthma prevalence and incidence that showed difference outcome across the life course. The prevalence of asthma is higher in males than in females before adolescence, the onset of puberty reverses the trend.³⁸ The reason might strongly suggestive of a protective action of male sex hormones.^{38,39} From OVA-induced Th2-dominant asthma mouse model, most studies found that asthma symptoms including airway eosinophilia, serum IgE production, Th2 cytokines production in lung tissues were elevated in female than male. However, the estrogen and androgens as critical mediators of sex differences in asthma are still controversy.⁴⁰ The sex difference in AhR deficiency in asthma is an important question; however, there is no study to explore. Previous study found that AhR can modulate sex hormone synthesis and signaling through regulate cytochrome P450 (CYP) 1A1, 1B1, 19A1.⁴¹ Although the role of estrogen in asthma is still complex, studies found estrogen may polarize T cells to Th2 cells differentiation and degranulation of mast cells that might directly cause deleterious effects in allergic asthma.^{42,43} The 17 β -estradiol (E2) and estrone (E1) can be metabolized by CYP 1A1 and 1B1 in lungs and the estrogen signaling might show

deleterious effects on asthma. Whereas the role of sex difference in AhR knockout mice in asthma pathological mechanisms is still unknown and complex.

In conclusion, we found a novel role of AhR in chronic airway inflammation. By analyzing the data from a human cohort study, we revealed the dysregulation of AhR-related gene expression in human bronchial epithelium of severe asthmatics. To dissect the underlying mechanism, we used a chronic asthmatic mouse model and revealed that AhR knockout mice exhibited enhancement of asthma clinical features via an increase in Th2 and Th17 cells-specific cytokines, airway remodeling-related factors, and oxidative stress in the lungs. Further studies to clarify the pathological effects and mechanism of action of AhR in chronic airway inflammatory diseases will increase our understanding of how AhR regulates airway inflammation and remodeling, as well as its potential as a therapeutic target for severe asthma and chronic airway inflammatory diseases.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

YD Chang performed experiments and analyzed data. CH Lee and CH Tasi, performed experiments and data analysis. YWC provided intellectual input and experimental support. JJK designed the project and experimental support. CCL designed the project and wrote the manuscript.

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