

ORIGINAL ARTICLE

Airway Diseases

Aryl hydrocarbon receptor activation by diesel exhaust particles mediates epithelium-derived cytokines expression in severe allergic asthma

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Abstract

Background: Exposure to environmental pollutants promotes Th2 cell responses. Aryl hydrocarbon receptor (AhR) activation aggravates allergic responses. Epithelium-derived thymic stromal lymphopoietin (TSLP), interleukin (IL)-25, and IL-33 are implicated in the dysregulation of Th2 immune responses in severe allergic asthma.

Methods: Bronchial biopsies of 28 allergic severe asthma and 6 mild asthma subjects from highly polluted areas were analyzed for AhR nuclear translocation (NT), cytokine expression, and gene activation. Cultured primary epithelial cells were stimulated with diesel exhausted particles (DEP) to determine AhR-mediated IL-33, IL-25, and TSLP synthesis and release.

Results: Primary bronchial epithelial cells exposed to DEP showed upregulation of IL-33, IL-25, and TSLP. These effects were abolished by knockdown of AhR by siRNA. Increased AhR/ARNT binding to promoters of IL-33, IL-25, and TSLP was found using chromatin immunoprecipitation (ChIP) assay. Allergic severe asthma with high AhR NT had higher bronchial gene and protein expression of IL-33, IL-25, and TSLP. These patients derived clinical benefit from anti-IgE treatment.

Conclusion: Aryl hydrocarbon receptor activation by DEP mediates upregulation of IL-33, IL-25, and TSLP with Th2 activation, potentially linking environmental pollution and allergic severe asthma.

KEYWORDS

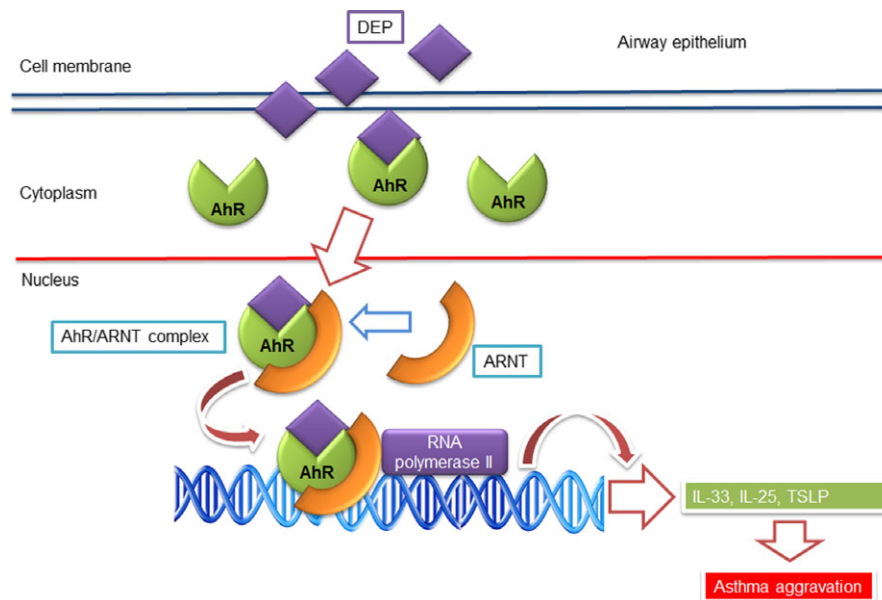
aryl hydrocarbon receptor, diesel exhaust particles, epithelium-derived cytokines, severe asthma

1 | INTRODUCTION

Severe asthma is a heterogeneous disease that affects 5%-10% of asthmatic patients and is defined as asthma which requires treatment with high-dose inhaled corticosteroids (ICS) plus a second

controller (and/or systemic corticosteroids) to prevent it from becoming "uncontrolled" or which remains "uncontrolled" despite this therapy.¹ A molecular phenotype of asthma has been described with expression of high Th2 genes characterized by eosinophilic inflammation, increased IgE production, and increased CD4⁺ Th2 cytokine expression of interleukin (IL)-4, IL-5, IL-9, and IL-13.² How patients with severe asthma may develop dysregulated

C.-M. Weng and C.-H. Wang contributed equally.



GRAPHICAL ABSTRACT

Environmental diesel exhaust particles (DEP) exposed to airway epithelium ligate cytoplasmic aryl hydrocarbon receptor (AhR), translocate to the nucleus with aryl hydrocarbon receptor nuclear translocator (ARNT), and then transactivate IL-33, IL-25, and TSLP gene expression. Patients with high AhR nucleus translocation overexpressed IL-33, IL-25, and TSLP cytokines compared to those with low. Patients with high AhR nucleus translocation are more response to anti-IgE therapy compared to those with low.

Th2-mediated immune responses in their airways is unclear but cytokines such as thymic stromal lymphopoietin (TSLP), IL-25, and IL-33, produced by airway epithelial cells, may stimulate the production of Th2-associated cytokines such as IL-4, IL-5, IL-9, and IL-13.³

Exposure to environmental pollutants has been associated as a cause of asthma as well as with causing exacerbations of asthma.^{4,5} Motor vehicle emissions and industrial emissions are a major source of airborne pollutants. Diesel exhaust particles (DEP) have been reported to enhance Th2 responses with upregulated IgE production⁶⁻⁸ and to induce IL-17A production that could exacerbate asthma severity.⁶ The generation of reactive oxygen species (ROS) with resulting oxidative stress has been identified as one of the major mechanisms for the adjuvant effects of DEP.⁹ Aryl hydrocarbon receptor (AhR) activation by stimulus, such as DEP, has been reported to mediate Th2 cell differentiation through upregulation of Notch signaling pathway.¹⁰ AhR activation by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the classical AhR ligand, has been demonstrated to induce airway inflammation, enhance IgE production, and can aggravate allergic diseases.^{11,12} After ligand binding, the response of gene induction is through the canonical AhR pathway to form a heterodimer with aryl hydrocarbon receptor nuclear translocator (ARNT) and is shuttled into the nucleus.¹³

In this study, we have demonstrated that AhR activation by DEP can directly induce the synthesis of IL-33, TSLP, and IL-25 in airway epithelial cells. We also found increased AhR/ARNT binding to the promoters of *IL-33*, *IL-25*, and *TSLP* in the airways of a subset of patients with severe asthma with AhR activation. This, in turn, was

associated with enhanced Th2-related responses. This mechanism may serve as a critical link between environmental exposure and the expression of allergic inflammation⁵ and could underlie a mechanism for the persistent airway inflammation observed in patients with severe allergic asthma.

2 | MATERIALS AND METHODS

2.1 | Study subjects

We recruited 28 patients with severe allergic asthma (56.9 ± 2.3 years old, 14 females) needing either continuous or near-continuous oral corticosteroids, high-dose inhaled corticosteroids, or both to achieve a level of mild-to-moderate persistent asthma, and with increased serum IgE levels¹ (Table 1). Six patients with allergic nonsevere asthma using inhaled beclomethasone (0-1000 $\mu\text{g}/\text{d}$ or equivalent) with control of their asthma symptoms were also recruited as control subjects for the patients with severe allergic asthma (Table 1). These patients lived in the northern Taiwan, including Taipei City, New Taipei City, and Taoyuan. The yearly average Particulate Matter_{2.5} (PM_{2.5}) concentrations in the ambient air of the three areas in 2014 and 2015 were in the range of 26.0-29.1 and 23.7-26.3 $\mu\text{g}/\text{m}^3$, respectively, all above the National Air Quality Standard of Taiwan (15 $\mu\text{g}/\text{m}^3$). All asthmatic patients were never-smokers or ex-smokers (abstained for >3 years) and have been stable for at least 3 months. The patients with severe asthma had higher serum levels of IgE, lower Asthma Control Test (ACT) scores, lower forced expiratory volume in one-second (FEV₁), and forced vital capacity (FVC) compared with

TABLE 1 Clinical characteristics of subjects

	Severe allergic asthma			Mild asthma with NPF (n = 6)
	Total (n = 28)	High AhR NT (n = 15)	Low AhR NT (n = 11)	
Age, years	56.2 ± 2.3	53.4 ± 2.9	62.3 ± 2.6	51.7 ± 5.1
Gender, F/M	14/14	11/4*	3/8	3/3
FVC, L	1.6 ± 0.1***	1.5 ± 0.1***	2.1 ± 0.2***	3.4 ± 0.23
FVC, %pred.	55.4 ± 3.4***	49.1 ± 3.2***	68.1 ± 5.7***	94.7 ± 4.4
FEV ₁ , L	0.9 ± 0.1***	0.85 ± 0.1***	1.2 ± 0.1***	2.3 ± 0.3
FEV ₁ , %pred.	38.7 ± 2.7***	35.5 ± 3.5***	45.7 ± 4.1***	79.5 ± 5.8
Reversibility, %	16.1 ± 2.8	15.5 ± 3.5	16.7 ± 2.3	21.8 ± 6.7
ACT scores	13.9 ± 0.5***	13.3 ± 0.5	14.5 ± 0.6***	23.2 ± 0.3
IgE, IU/mL	641.0 ± 104.5***	528.4 ± 126.9***	794.5 ± 172.3***	154.3 ± 25.1
Medication over previous year				
ICS alone, n	0	0	0	3
ICS + LABA, n	28	15	11	3
OCS (>50% of year), n	19	13	6	0
Eosinophilic, n	15	11***,***	3	1
Eosinophilic + Neutrophilic, n	3	3	0	0
Noneosinophilic, n	15	1	8	3
Paucigranulocytic, n	0	0	0	2

High AhR NT, nuclear location >50% of nucleated cells; ICS, inhaled corticosteroids; LABA, long-acting β_2 agonist; Low AhR NT, nuclear location <20% of nucleated cells; NPF, normal pulmonary function; OCS, oral corticosteroids.

* $P < .05$; ** $P < .01$ compared with low AhR NT group; *** $P < .01$ compared with mild asthma with NPF.

those of nonsevere asthma. All asthmatic patients underwent fiberoptic bronchoscopy to obtain bronchial biopsy.¹⁴ All subjects provided informed consent to participate in this study, which was approved by the local ethics committee. Finally, those patients with severe allergic asthma who met the criteria for treatment with the anti-IgE antibody treatment, omalizumab, and who met reimbursement criteria received a trial for 4 months.

2.2 | Analysis of bronchial biopsies

Fiberoptic bronchoscopy was performed in asthma subjects under sedation with midazolam, and bronchial biopsies were obtained. The inflammatory state was assessed as described previously.¹⁵ Eosinophils and neutrophils in bronchial tissues were counted by staining with anti-EG2 and antineutrophil elastase antibodies, respectively. The presence of eosinophilic inflammation was defined by eosinophil counts >10/mm², while mixed eosinophilic and neutrophilic phenotype was defined by neutrophil counts >2/mm² and eosinophil counts >10/mm².

2.3 | Immunofluorescence staining for confocal microscopy

The paraffin-embedded airway tissue sections from asthmatic patients were obtained from Chang Gung Medical Foundation, Linkou Chang Gung Memorial Hospital Tissue Bank. The tissues were cut, placed onto glass slides, dewaxed in xylene and alcohol, and

then washed twice with phosphate-buffered saline. AhR was immunostained using mouse monoclonal anti-human AhR (Santa Cruz, CA) antibodies, followed by an Alexa 555- or FITC-conjugated secondary antibody (Invitrogen, Carlsbad, CA). The images were acquired with a confocal microscope (Leica, Exton, PA), as described previously.¹⁴ Hoechst dye was used as a nuclear stain. The presence of AhR in the nuclei of the airway epithelium was randomly counted per 100 epithelial cells by two observers unaware of patients' status.

2.4 | Immunohistochemical staining

The paraffin-embedded airway tissue sections from asthmatic patients were immunostained using mouse monoclonal anti-human IL-33 (Santa Cruz, CA), rabbit polyclonal anti-human IL-25 (Abcam, MA), rabbit polyclonal anti-human TSLP (Millipore, CA), and nonimmune IgG primary antibodies, respectively, and followed by IHC select HRP/DAB (an Immunoperoxidase Secondary Detection System, Millipore, CA). The images were acquired with an optical microscope (Olympus, USA), as described previously.¹⁶ The presence of IL-33, IL-25, and TSLP in the airway epithelium was randomly observed in five HPF in patients' airway tissue.

2.5 | In vitro airway epithelial cell studies

Bronchial epithelial cells were obtained by bronchial brushing of right lower bronchus in 6 mild asthmatic patients with normal pulmonary function (NPF). Primary epithelial cells were

separated as previously described¹⁷ and expanded in growth factor-supplemented medium (BEGM, Lonza); the experiments were performed in passage 3-7. After reaching confluence, primary epithelial cells were stimulated with DEP (0.1-10 $\mu\text{g/mL}$) or vehicle as indicated. Pharmacological inhibition with an AhR selective antagonist (CH223191) (1-10 $\mu\text{mol/L}$) (Selleckchem, TX, USA) was also assessed the effect on DEP-induced cytokine release and synthesis.

Well-characterized DEPs consisting of 85% of these particles with a mean diameter of 380 nm and 14% in the ultrafine range of ≤ 100 nm as assessed by dynamic light scattering were kindly supplied by Dr. Masaru Sagai, Aomori University of Health and Welfare, Japan, and suspended in phosphate-buffered saline and 0.05% tween 80, and sonicated by an ultrasonic disrupter.^{18,19}

2.6 | siRNA transfection

Primary epithelial cells (1×10^5 cells per well) were transfected with siRNA against AhR (siAhR) or scramble siRNA (negative control for AhR siRNA) (Dharmacon, UK) using DharmaFect 1 transfection reagent for 6 hours, and cells were then stimulated with DEP at different concentrations and time intervals. Cell viability determined by MTT assay was more than 98% (Figure S1).

2.7 | RNA extraction, cDNA preparation, and Real time-PCR

Total RNA was isolated from human bronchial tissues derived from bronchial biopsy or cultured primary epithelial cells using

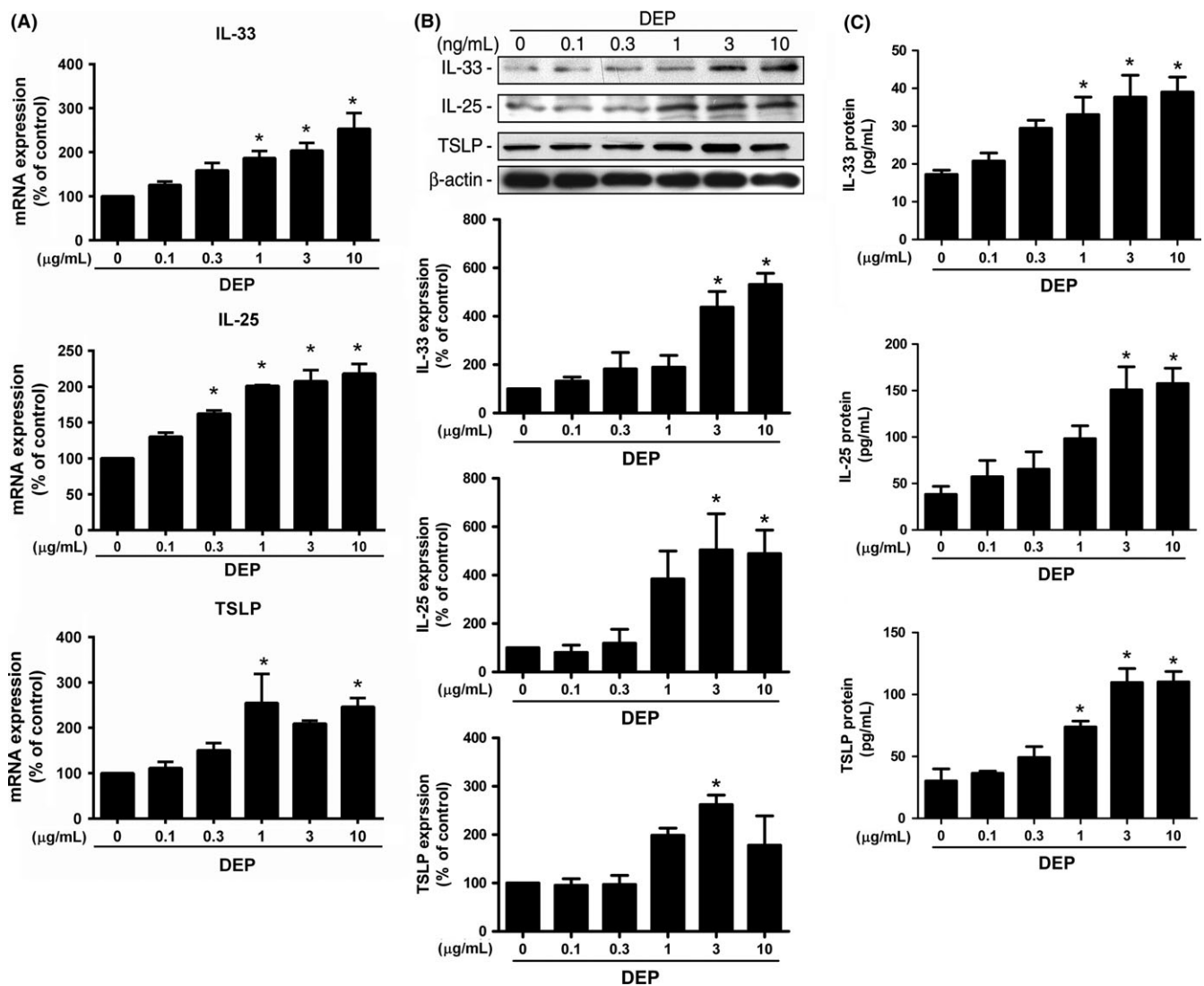


FIGURE 1 Diesel exhaust particles (DEP)-induced interleukin (IL)-33, IL-25, and thymic stromal lymphopoietin (TSLP) upregulation in primary bronchial asthmatic epithelial cells. A, Cells were harvested after stimulation with DEP (0.1-10 $\mu\text{g/mL}$) for 2 h, and then transcript levels were assessed by quantitative PCR ($n = 4$). B, Cells were stimulated with DEP (0.1-10 $\mu\text{g/mL}$) for 6 h, and then protein levels were detected by immunoblot analysis ($n = 3$). C, Cells were stimulated with DEP (0.1-10 $\mu\text{g/mL}$) for 24 h, and the levels of IL-33, IL-25, and TSLP were determined by ELISA ($n = 3$). Data were shown as mean $\pm$ SEM from four (A) or three (B-C) independent experiments. * $P < .05$ compared to the corresponding vehicle control as the 100% reference

the RNeasy Mini Kit (Qiagen, MD) and quantified using a Nano-Drop 1000 spectrophotometer (Thermo Scientific, MA). Single-stranded cDNA was then synthesized from 0.5 μ g of total RNA using iScript cDNA synthesis kit (Bio-Rad, CA). cDNA was quantified by means of RT-PCR (Rotor-Gene 3000, Corbett Research) using Tools SuperFast SYBR qPCR Reagent (Biotools Co., Ltd, Taiwan). Data were analyzed through Rotor-Gene 6 software (Corbett Research). Quantification of cytokine gene expression of bronchial tissues was initially normalized to GAPDH expression.

Relative cytokine expression of bronchial tissues among asthmatic patients was normalized to a reference gene expression of one normal subject to compensate inter-PCR variation to standardize each reaction run.

2.8 | ELISA assay

The concentrations of IL-33, IL-25, and TSLP in the conditioned media were measured using human IL-33, TSLP Quantikine ELISA kit (R&D

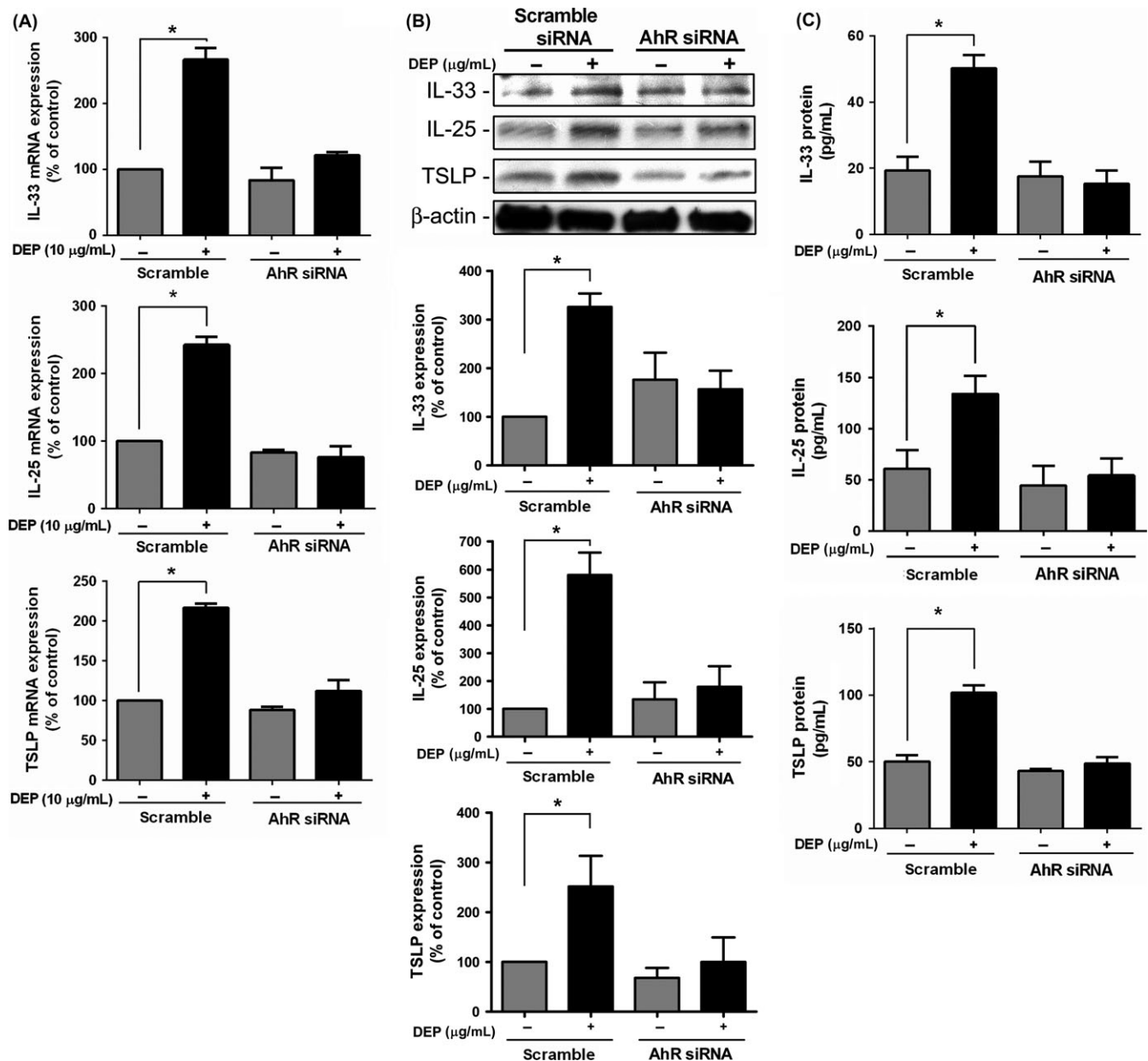


FIGURE 2 Aryl hydrocarbon receptor (AhR) mediation of diesel exhaust particles (DEP)-induced interleukin (IL)-33, IL-25, and thymic stromal lymphopoietin (TSLP) upregulation in primary bronchial asthmatic epithelial cells. A, Cells were transfected with AhR SiRNA for 6 h and then stimulated with DEP (10 μ g/mL) for 2 h, and then transcript levels were assessed by quantitative PCR ($n = 4$). B, After transfected with AhR SiRNA for 6 h, cells were stimulated with DEP (10 μ g/mL) for 6 h, and then protein levels were detected by immunoblot analysis ($n = 3$). C, Cells were transfected with AhR SiRNA for 6 h and then stimulated with DEP (10 μ g/mL) for 24 h, and the levels of IL-33, IL-25, and TSLP were determined by ELISA ($n = 3$). Data represent the mean $\pm$ SEM from four (A) or three separate experiments (B-C). * $P < .05$ compared to as compared to scramble control as the 100% reference

Systems, Minneapolis, MN), and human IL-25 ELISA kit (Abnova, TW), respectively, according to the manufacturer's instructions.

2.9 | Western blotting

Protein extract (30 µg per lane) was fractionated by means of SDS-PAGE on a 4%-15% TGX bis-Tris precast gel (Bio-Rad, CA) and then transferred to a PVDF membrane (Bio-Rad, CA), which was incubated with mouse monoclonal anti-human IL-33 (Santa Cruz, CA), rabbit polyclonal anti-human IL-25 (Abcam, MA), rabbit polyclonal anti-human TSLP (Millipore, CA) primary antibodies, or antibodies against mouse monoclonal AhR (Santa Cruz, CA) or with anti-β-actin (Abcam, MA), and then incubated with HRP-conjugated secondary antibodies. The immunoreactivity was detected using Tools Sensitive

ECL-HRP substrate (TR-ECL250, Biotools Co., Ltd, Taiwan) following the manufacturer's instructions. Relevant band intensities were quantified by densitometric analysis normalized to β-actin.

2.10 | ChIP assay

Chromatin immunoprecipitation (ChIP) assay was performed using the ChIP-IT Express assay Kit (Active Motif, CA) according to the manufacturer's instructions. Soluble cross-linked chromatin was immunoprecipitated with mouse monoclonal anti-AhR (Santa Cruz, CA), rabbit polyclonal anti-ARNT (cell signaling, Danvers, MA), or nonimmune IgG antibodies for overnight. The purified DNA was analyzed by quantitative PCR. Sample DNA was normalized to input DNA. The primer sequences are listed in Table S1.

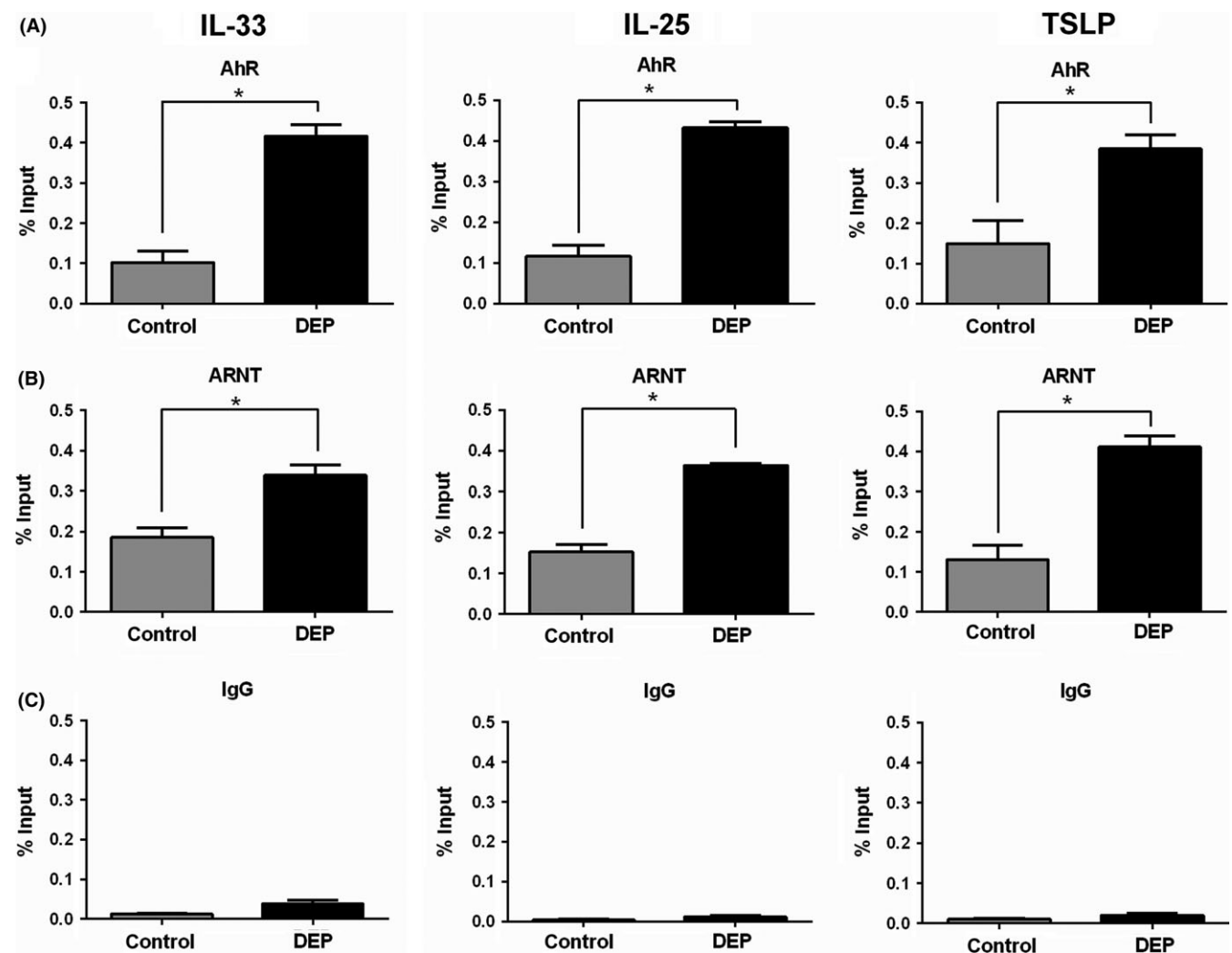


FIGURE 3 Diesel exhaust particles (DEP)-induced recruitment of Aryl hydrocarbon receptor (AhR)/aryl hydrocarbon receptor nuclear translocator (ARNT) and increased transcriptional activities of interleukin (IL)-33, IL-25, and thymic stromal lymphopoietin (TSLP) promoter regions. A-C, After incubated with DEP (10 µg/mL) for 1 h, the cells were harvested and analysis by chromatin immunoprecipitation (ChIP) assay for the recruitment of AhR and ARNT to the IL-33, IL-25, and TSLP promoters. Nonimmune IgG (IgG) was used as a negative control. D-F, Transcriptional activities were performed by the level of acetyl-histone H3 and recruitment of RNA polymerase II on promoter regions of IL-33, IL-25, and TSLP. After incubated with DEP (10 µg/mL) for 1 h, the cells were harvested and analysis by ChIP assay for the level of acetyl-histone H3 in IL-33, IL-25, and TSLP promoter regions, and recruitment of RNA polymerase II on IL-33, IL-25, and TSLP promoters. IgG was used as a negative control. Data represent the % of input ± SEM from three independent experiments. **P* < .05 as compared to the vehicle control

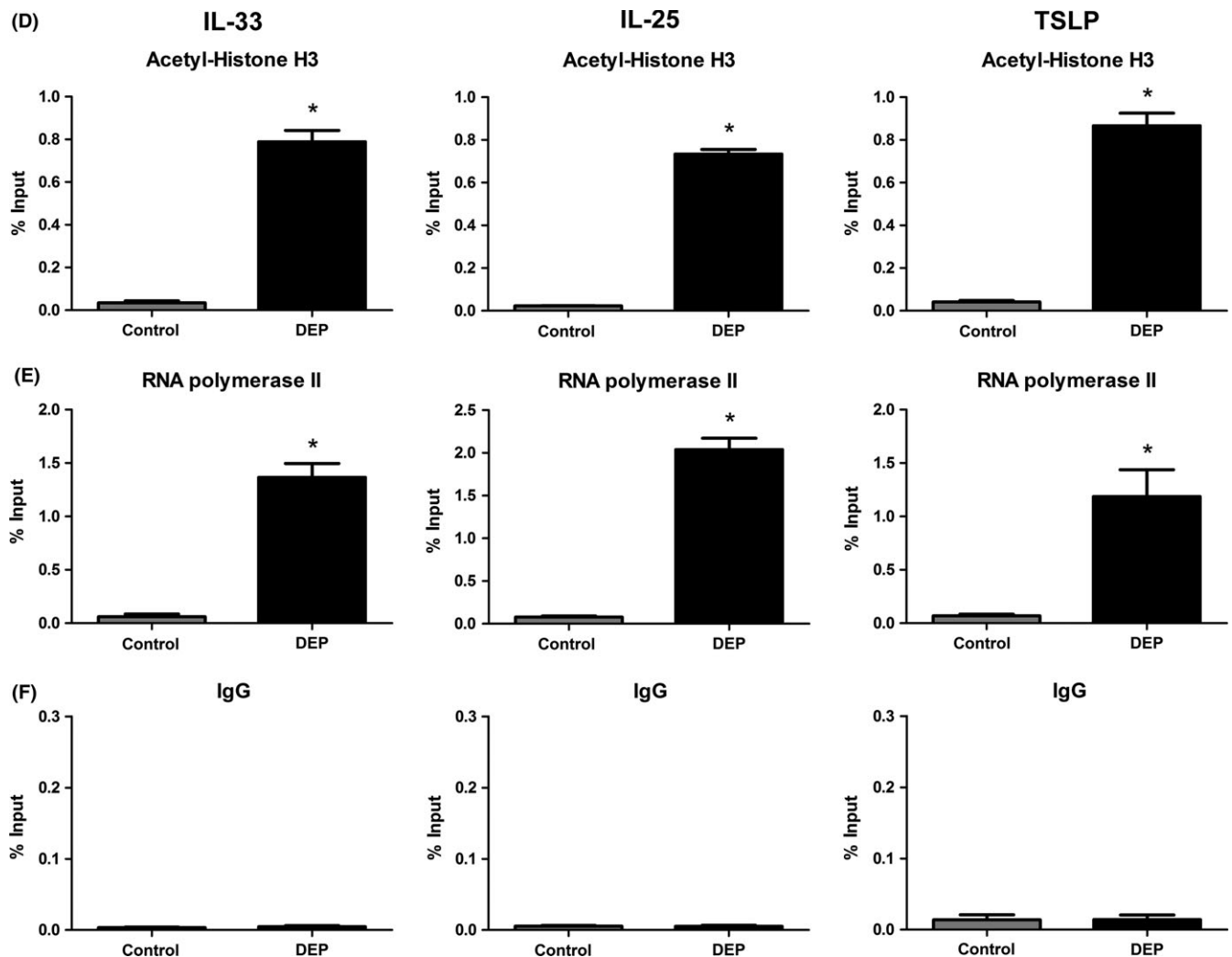


FIGURE 3 Continued

2.11 | Transcriptional activity

Transcriptional activity analysis was performed using specific ChIP assay for acetyl-histone H3 and RNA polymerase II (Millipore, CA) or nonimmune IgG antibodies according to the manufacturer's instruction (Millipore, CA). The purified DNA was analyzed by quantitative PCR. Sample DNA was normalized to input DNA. The primer sequences are listed in Table S1.

2.12 | Statistical analysis

We used Mann-Whitney test and Kruskal-Wallis analysis to determine the statistical significance of differences between groups, and Wilcoxon matched pairs test was used to determine within-group differences. The proportion of AhR nuclear translocation was analyzed using the chi-square test. One-way analysis of variance (ANOVA) followed by Dunnett's test, where appropriate, was used to determine the statistical significance of the difference between means for the results of in vitro cell line studies. $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | AhR mediates DEP-induced IL-33, IL-25, and TSLP expression

Diesel exhausted particles concentration-dependently increased IL-33, IL-25, and TSLP mRNA (Figure 1A), and protein levels (Figure 1B) in cultured primary human epithelial cells. We also found similar results with DEP-induced IL-33, IL-25, and TSLP release (Figure 1C). To determine whether AhR involved in DEP-induced IL-33, IL-25, and TSLP expression, cells were transfected with SiAhR. SiAhR transfection almost completely inhibited DEP (10 $\mu\text{g/mL}$)-induced IL-33, IL-25, and TSLP mRNA expression (Figure 2A). This inhibitory effect of SiAhR transfection was accompanied by similar degree of inhibition of protein secretion (Figure 2B,C). Pretreatment with a selective AhR antagonist (CH223191) (1–10 $\mu\text{mol/L}$) also significantly inhibited DEP-induced IL-33, IL-25, and TSLP mRNA as well as protein expression (Figure S2).

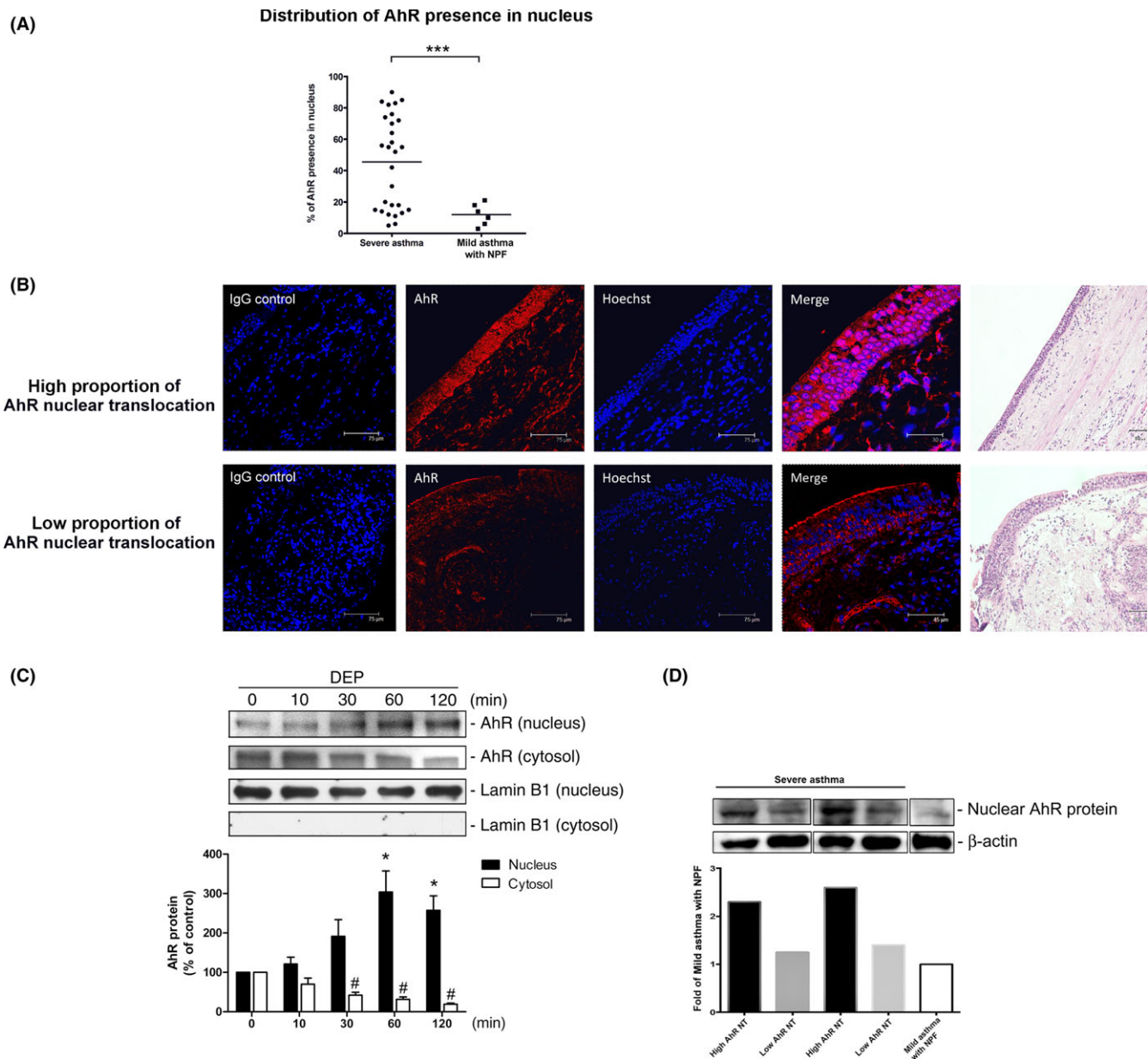


FIGURE 4 Aryl hydrocarbon receptor (AhR) nuclear localization in the airways of patients with allergic asthma. A, Paraffin sections of airway tissue from severe asthmatic patients ($n = 28$) and mild asthma with normal pulmonary function (NPF) ($n = 6$) were immunostained and analyzed under fluorescent confocal microscopy. Each dot represents an individual patient, and horizontal lines indicate mean values. $***P < .05$ compared with mild asthma with NPF group. B, Illustrative photomicrograph pictures showing high (upper panel) or low (lower panel) AhR nuclear localization, defined by the presence of AhR in the nuclei of airway epithelial cells of more than 50%, or $<20\%$, respectively. The upper and lower right panel shows H&E stain of high or low AhR nuclear localization. C, Primary bronchial asthmatic epithelial cells were stimulated with diesel exhausted particles (DEP) ($10 \mu\text{g/mL}$) for indicated time intervals, and then cells were harvested and separated for nuclear and cytosol fraction. The AhR protein levels were detected by immunoblot analysis ($n = 3$). Lamin B1 was used as an internal control. Data were shown as mean $\pm$ SEM from three independent experiments. $*P < .05$ and $\#P < .05$ compared to the corresponding control as the 100% reference. D, the lysates from tissue of high AhR NT ($n = 2$), low AhR nuclear translocation (NT) ($n = 2$), and mild asthma with NPF ($n = 1$) were separated for nuclear fraction, and the AhR level was detected by immunoblot analysis. β -actin was used as an internal control. E, chromatin immunoprecipitation assays of bronchial tissues from patients with allergic severe asthma. Patient with high AhR nuclear translocation (NT) had increased AhR and ARNT binding to IL-33, IL-25, and TSLP promoters ($n = 5$), when compared with patients with low AhR NT ($n = 6$). Nonimmune IgG (IgG) was used as a negative control. Data represent the mean % input $\pm$ SEM. $**P < .05$ compared with high AhR NT group

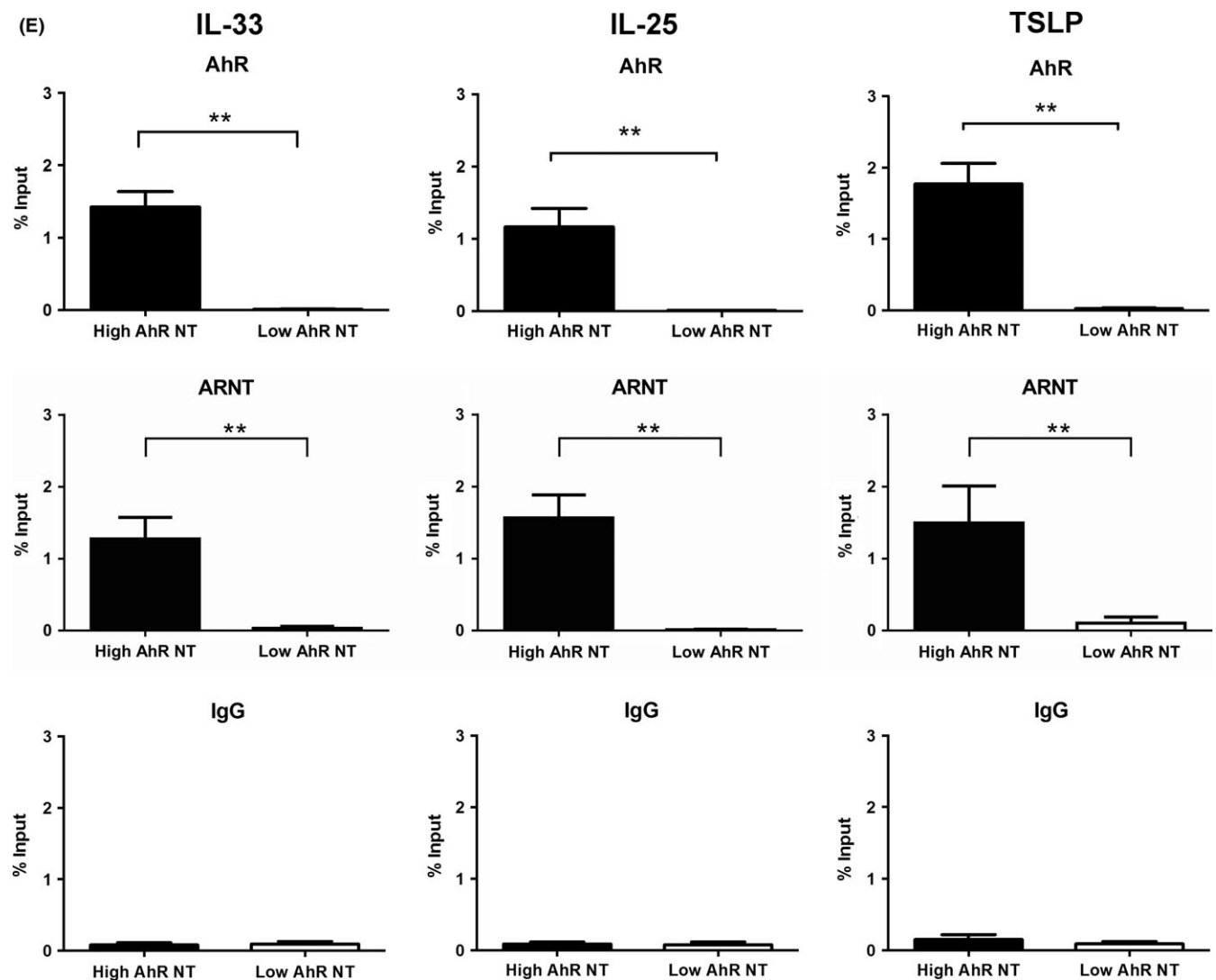


FIGURE 4 Continued

3.2 | DEP-induced AhR binding to the promoter sites of IL-33, IL-25, and TSLP

Chromatin immunoprecipitation assay revealed that DEP (10 $\mu\text{g/mL}$) induced a prominent increase in AhR/ARNT complex binding to IL-33, IL-25, and TSLP promoter sites in cultured primary human epithelial cells (Figure 3A,B). We also found increased level of acetyl-Histone H3 and binding of RNA polymerase II on IL-33, IL-25, and TSLP promoters (Figure 3C,D) under DEP treatment, which indicates that DEP increased the transcriptional activity of target genes.

3.3 | AhR nuclear translocation in biopsies

Aryl hydrocarbon receptor nuclear translocation (NT) was found in most asthmatic patients to a variable extent (Figure 4A). The proportion of bronchial tissue cells with AhR NT was significantly higher in allergic severe asthma ($45.5 \pm 5.6\%$, $n = 28$, $P = .0048$) compared with that of mild asthma with normal pulmonary function (NPF) ($12.0 \pm 2.8\%$, $n = 6$) (Figure 4A). AhR nuclear localization was not

limited to the airway epithelium, but was also seen in subepithelial cells including inflammatory cells, smooth muscle cells, endothelial cells, as well as fibroblasts (Figure 4B). To determine whether DEP induces AhR translocation to nucleus, the primary cultured epithelial cells were stimulated with DEP for different time intervals. AhR protein levels in primary cultured epithelial cells gradually decreased in the cytosol 10 minutes after DEP (10 $\mu\text{g/mL}$) stimulation with nuclear translocation, most prominent at 60 minutes (Figure 4C). It was confirmed that higher expression of AhR protein in the nuclear extracts of bronchial tissues in patients with high AhR NT (Figure 4D).

Chromatin immunoprecipitation assay of bronchial tissues also revealed increased AhR/ARNT complex binding to IL-33, IL-25, and TSLP promoter sites in patients with proportion of AhR NT more than 50% of nuclei present (defined as high AhR NT), compared with those with <20% of nuclei (defined as low AhR NT) (Figure 4E). There was a small binding of AhR to those promoters in 2 patients with proportion of AhR NT between 20% and 50%. Their results were not included into analysis.

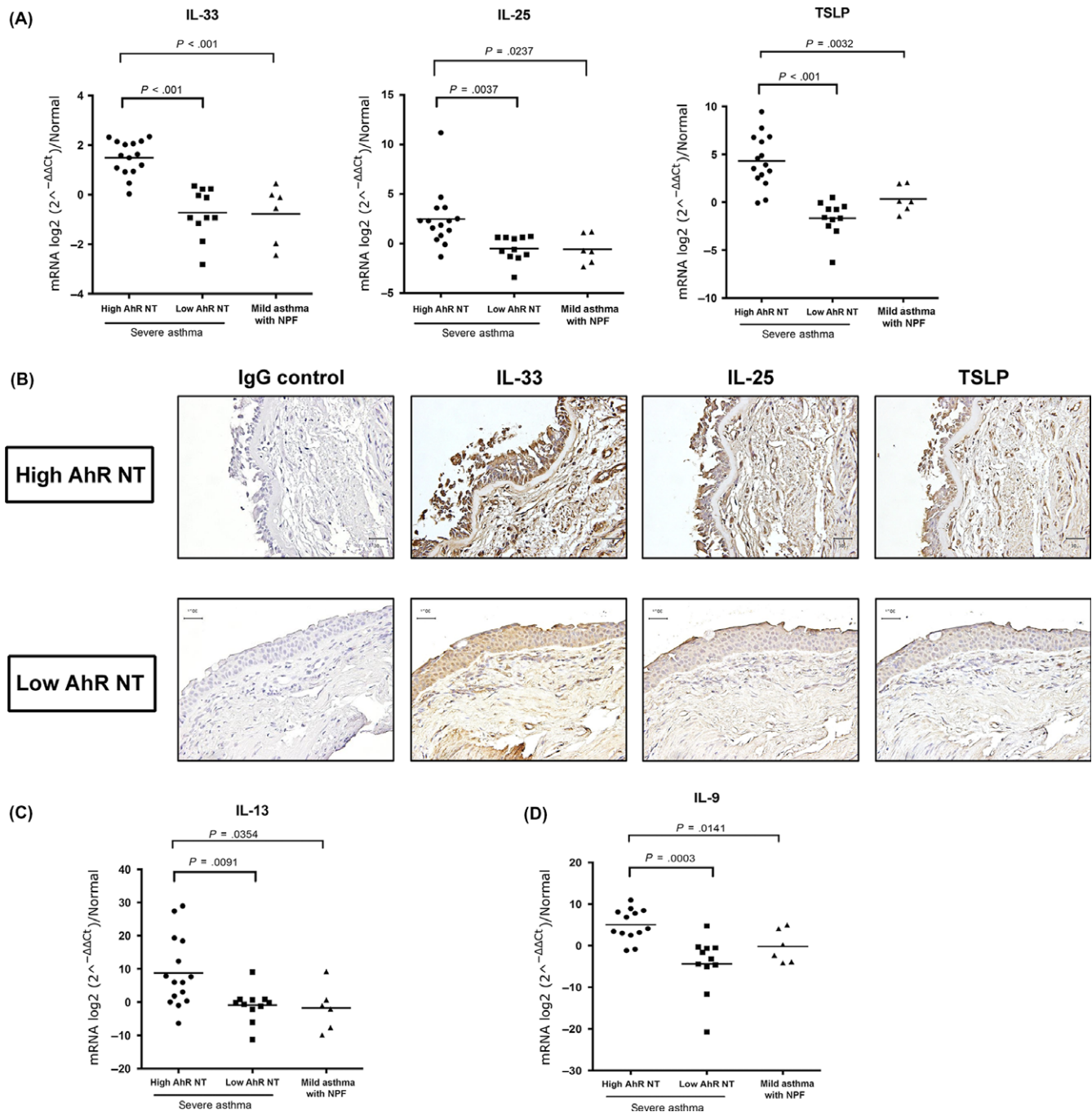


FIGURE 5 Upregulation of interleukin (IL)-33, IL-25, thymic stromal lymphopoietin (TSLP), IL-13, and IL-9 mRNA in the biopsied bronchial tissues of allergic severe asthma. A, IL-33, IL-25, and TSLP mRNA of tissues from patients with high AhR nuclear translocation (high aryl hydrocarbon receptor [AhR] nuclear translocation [NT], $n = 15$), low AhR nuclear translocation (low AhR NT, $n = 11$), or mild asthma with normal pulmonary function (NPF) ($n = 6$) were analyzed by quantitative PCR. B, Immunohistochemical staining for IL-33, IL-25, and TSLP proteins in the airway of patients with allergic severe asthma with high or low AhR NT. C-D, IL-13 and IL-9 mRNA of tissues from patients with high AhR NT ($n = 15$), low AhR NT ($n = 11$), or mild asthma with NPF ($n = 6$). The data were normalized with respect to the control group. Each dot represents an individual patient, and horizontal lines indicate mean values

3.4 | Cytokine expression in bronchial tissues

Quantitative PCR analysis of bronchial tissues revealed higher mRNA expression level of IL-33, IL-25, and TSLP in patients with allergic severe asthma with high AhR NT, compared with those with low

AhR NT or in mild asthma with NPF (Figure 5A). Immunohistochemical analysis confirmed the upregulation of IL-33, IL-25, and TSLP proteins in bronchial tissues in patients with high AhR NT (Figure 5B). The upregulation of IL-33, IL-25, and TSLP in patients with high AhR NT was also accompanied by upregulation of Th2

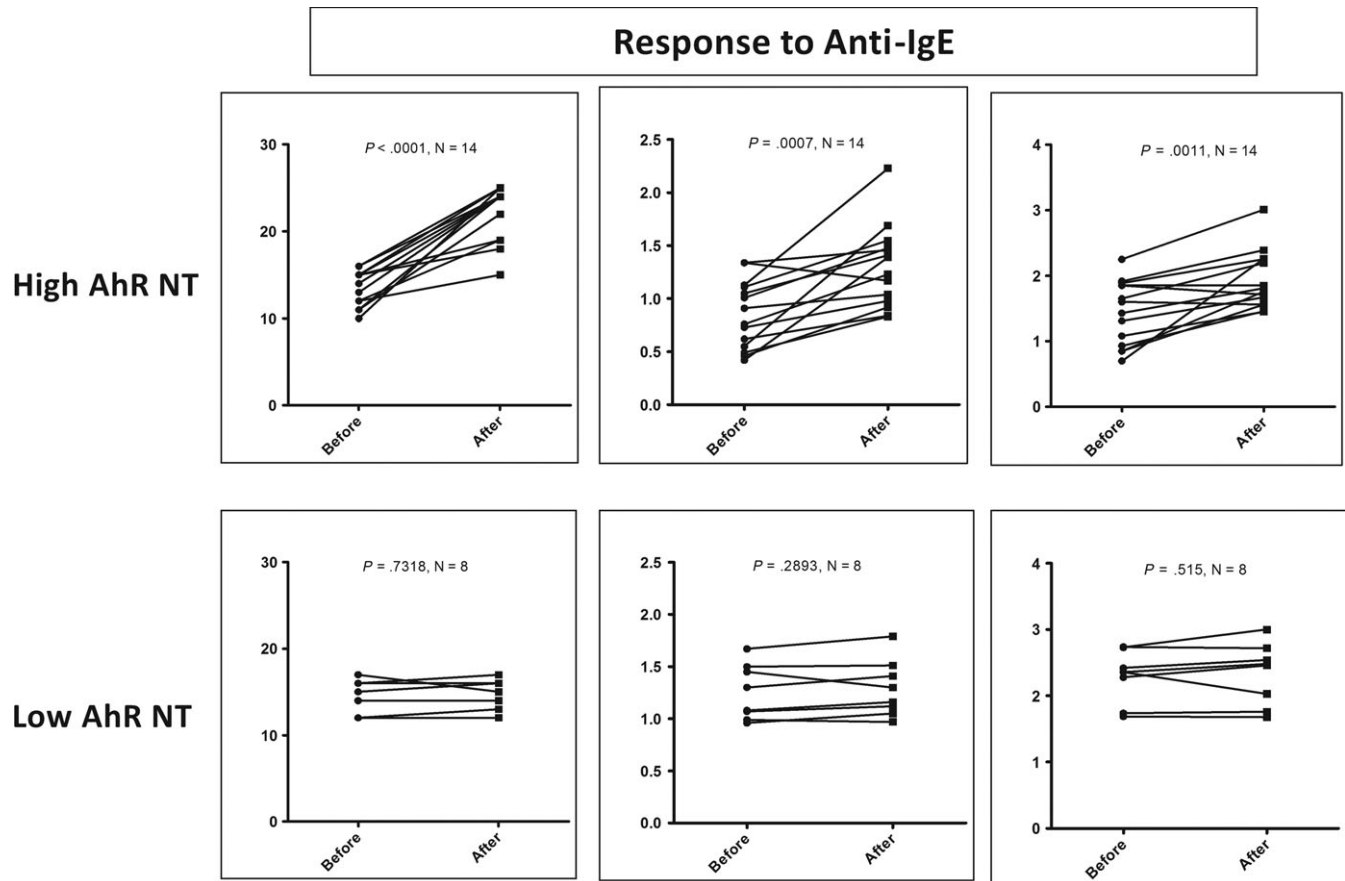


FIGURE 6 Patients with high aryl hydrocarbon receptor (AhR) nuclear translocation were responsive to anti-IgE therapy. Fourteen patients with high AhR nuclear translocation (NT) (upper panel) or eight patients with low AhR NT (lower panel) were studied with measurements of the Asthma Control Test score, forced expiratory volume in one-second, and forced vital capacity, before and after anti-IgE therapy for 4 mo. Each dot represents an individual patient

cytokines, IL-13, and IL-9 mRNA in the bronchial tissues compared with those with low AhR NT (Figure 5C,D). The expression of IL-33, IL-25, and TSLP mRNA in the bronchial tissues was well correlated with the expression of IL-9 and IL-13 mRNA (Figure S3). All the patients with high AhR NT were Th2-high phenotype. In contrast, only 4 of 11 patients with low AhR NT were Th2-high phenotype.

Most of the patients with high AhR NT (14/15) had increased eosinophil infiltration in the lamina propria ($>10/\text{mm}^2$), and 3 of them had a concomitant increase in neutrophil counts (Table 1).

3.5 | Clinical response to anti-IgE therapy

Fourteen patients with high AhR NT and eight patients with low AhR NT met the criteria of reimbursement and received anti-IgE therapy. Patients with high AhR NT who were responsive to anti-IgE therapy had a significant increase in the ACT scores (from 13.4 ± 0.5 to 22.3 ± 0.8 , $n = 14$, $P < .001$), FEV₁ (from 0.85 ± 0.1 to 1.3 ± 0.1 L, $n = 14$, $P < .001$), and FVC (from 1.4 ± 0.1 to 1.9 ± 0.1 L, $n = 14$, $P < .01$) at 4 months after therapy, respectively (Figure 6). In contrast, uncontrolled patients with allergic severe asthma with low AhR NT were not responsive to anti-IgE

therapy in terms of improvement in ACT scores, FEV₁, or FVC (Figure 6).

4 | DISCUSSION

We have demonstrated that the major constituent of ambient pollution, DEP, can directly increase IL-33, IL-25, and TSLP mRNA and protein expression in airway epithelial cells. Transfection with siRNA against AhR abolished DEP-induced cytokine release, suggesting that these responses were mediated via AhR. AhR upon ligand interaction translocates from cytosol into nucleus and binds to the specific regulatory DNA sequences located within the promoters of target genes.²⁰ ChIP assay revealed an increase in AhR and ARNT binding to IL-33, IL-25, and TSLP promoters in airway epithelial cells after stimulation with DEP. AhR nuclear translocation (AhR NT) in the bronchial tissues was found in most of our patients with severe allergic asthma to a variable extent. Patients with the higher proportion of cells with AhR NT also had increased AhR/ARNT binding to the promoters of IL-33, IL-25, and TSLP, with higher expression of these cytokines.

There is evidence indicating that IL-33, IL-25, and TSLP enhance Th2-type airway inflammation.²¹ These cytokines also activate type 2 innate lymphoid cells (ILC2), which directly secrete Th2 cytokines.²² IL-33 also directly acts on CD4⁺ T cells, mast cells, and eosinophils to aggravate adaptive phase of type 2 immune responses.³ In this study, we have demonstrated that the increased expression of IL-33, IL-25, and TSLP in patients with allergic severe asthma was accompanied by comparably higher gene expression of Th2 cytokines, IL-13, and IL-9 in the bronchial tissues. All of the patients with high AhR NT were Th2-high phenotype. Thus, the AhR pathway may be implicated in promoting Th2 cytokine-mediated airway inflammation in a subgroup of allergic severe asthma through the epithelial release of IL-33, IL-25, and TSLP.

Aryl hydrocarbon receptor can mediate various immunotoxic responses, including accumulation of lymphocytes and the development of lymphoid organs,^{23,24} T-cell differentiation, promotion of Treg and inhibition of Th17 cells,^{24,25} and synthesis of TNF- α and IL-6.²⁶ However, short-term stimulation by biodegradable AhR ligands, such as 6-formylindolo [2] carbazole (FICZ), results in promotion of Th17 differentiation.⁶ Thus, activation of AhR may have dual roles in regulation of Th17 response. A recent report indicates that ambient air pollutants, particulate matter (PM such as DEP), induce the Notch pathway, an unbiased amplifier of Th cell differentiation,²⁷ via activation of AhR.¹⁰ In the context of pro-Th2 genetic element, DEP upregulates Th2 cytokines from CD4⁺ T cells in vitro and promotes allergic airway inflammation in vivo.¹⁰ ILC2 cells by responding to epithelium-derived cytokines IL-25, IL-33, and TSLP represents another way of generating Th2 cytokines (IL-13, IL-5, and IL-9) without involvement of the adaptive immune system, thus contributing to tissue eosinophilia and to airway hyperresponsiveness in severe asthma. The enhanced Th2 cytokine expression in patients with high AhR NT can be attributed to AhR-mediated PM interaction with prevailing pro-Th2 asthmatic genetic factors to promote Th2 cytokine expression and asthma inflammation. However, AhR-mediated DEP-enhanced IL-33, IL-25, and TSLP synthesis may provide another pathway contributing to traffic-related PM enhancement of Th2 cytokine-mediated inflammation.

The clinical significance of our study is that it provides a potential link between exposure to environmental pollutants and the development or worsening of allergic asthma in those patients with asthma defined as having severe allergic asthma. This group of high AhR nuclear translocation was associated with eosinophilic inflammation as assessed in bronchial biopsies, with a minority associated with concomitant neutrophilic inflammation. In addition, these patients with high AhR nuclear translocation responded clinically to a 4-month trial of anti-IgE antibody treatment with omalizumab, while those with a low AhR nuclear translocation did not respond. Thus, we have been able to delineate a subset of severe allergic asthma associated with Th2-high expression and airway wall eosinophilia who benefit from omalizumab treatment. Our data are compatible with previous results showing that only a proportion of patients with severe allergic asthma respond to omalizumab treatment.²⁸ Of course, it would not be pragmatic to perform bronchial biopsies in such patients to do

ex vivo studies on these biopsies and bronchial epithelial cells. Therefore, an alternative approach to detecting the response of the airway epithelium to environmental pollutants using noninvasive biomarkers need to be explored. One weakness of our study is that we do not have personal exposure data of these asthmatics to environmental pollution but, in Taiwan, where these patients reside, the level of yearly average PM_{2.5} concentrations in 2014 and 2015 was 27.3 and 24.6 $\mu\text{g}/\text{m}^3$, respectively. These levels are above the permissible level of 15 $\mu\text{g}/\text{m}^3$ by the National Air Quality Standard of Taiwan. The response to environmental pollution may be genetically influenced by factors such as nuclear factor erythroid-derived 2-like 2 (NFE2L2, also known as Nrf2) and its negative regulator protein Kelch-like ECH-associated protein 1 (KEAP1).^{29,30} These genes have been reported to be involved in the regulation of antioxidant oxidative stress, airway inflammation, as well as Th2 cytokine release.^{29,30}

The compositions of DEP used in this study were elemental carbon and organic compounds¹⁹ such as polyaromatic hydrocarbons (PAHs), nitroarene, and dioxine derivatives, and it also contains SO₄²⁻, NO₃⁻, and their ammonium salts. Large amounts of light metals and trace amount of heavy metals are also contained in DEP. Concentrations of the light metals in our DEP were 2710, 944, 374, 228, 204, and 173 ppm for Na, Ca, K, Zn, Mg, and Al, respectively. Those of the heavy metals were 126, 12, and 6 ppm for iron, copper, and chromium, respectively. Further studies on the effects of major constituents of DEPs, including carbon particles, are needed in the future.

In conclusion, DEP induces airway epithelial cells to increase the synthesis of IL-33, IL-25, and TSLP via AhR activation, thus linking AhR-ligand axis with environmental exposure and the enhanced Th2 cytokine-mediated airway inflammatory responses in patients with allergic severe asthma. Our studies provide a potential link between environmental pollution and the development of severe allergic asthma.

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CONFLICTS OF INTEREST

H-P Kuo is a consultant for Novartis, Taiwan, a speaker for GSK, Novartis, Eli Lilly, Pfizer, Roche, Boehringer Ingelheim, Astra-Zeneca, Sanofi-Aventis, and MSD, and has received research grants from Eli Lilly, Sanofi-Aventis, Roche, Astra-Zeneca, and GSK. K F Chung has consulted for Gilead, has been on Advisory Boards of Merck and GSK, and has received grants from UK Medical Research Council, Asthma UK, and the Wellcome Trust. The other authors have no financial disclosures.

AUTHOR CONTRIBUTIONS

C-M Weng, C-H Wang, and H-P. Kuo conceived and designed the study. C-H Wang and H-P Kuo supervised the research. C-M Weng

and M-J Lee performed the experiments including quality control. C-M Weng, C-H Wang, and H-P Kuo drafted the manuscript. All authors contributed to analyses and interpretation, and read and approved the final manuscript.

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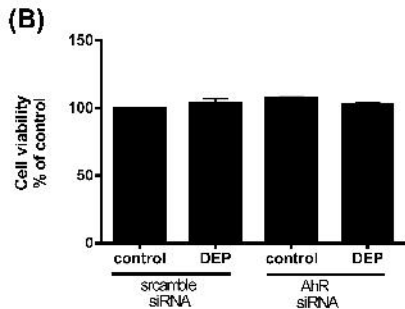
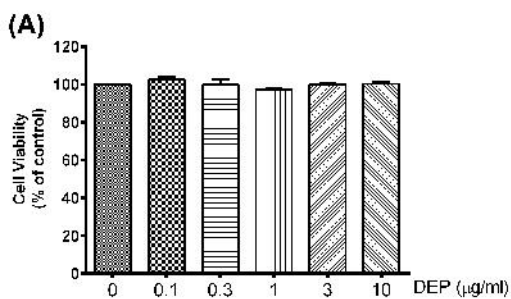
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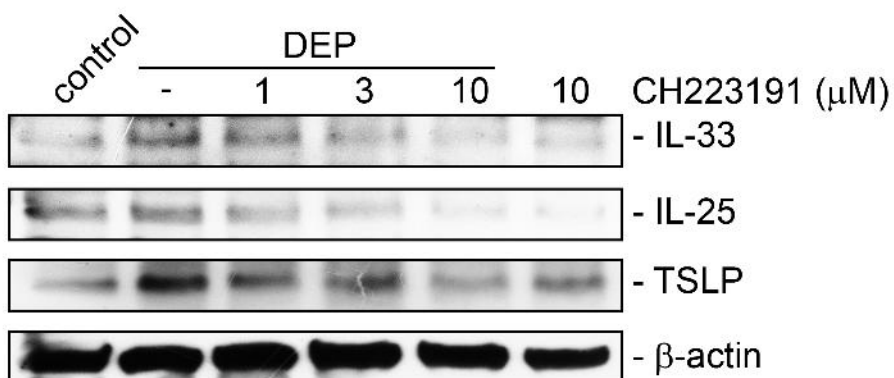
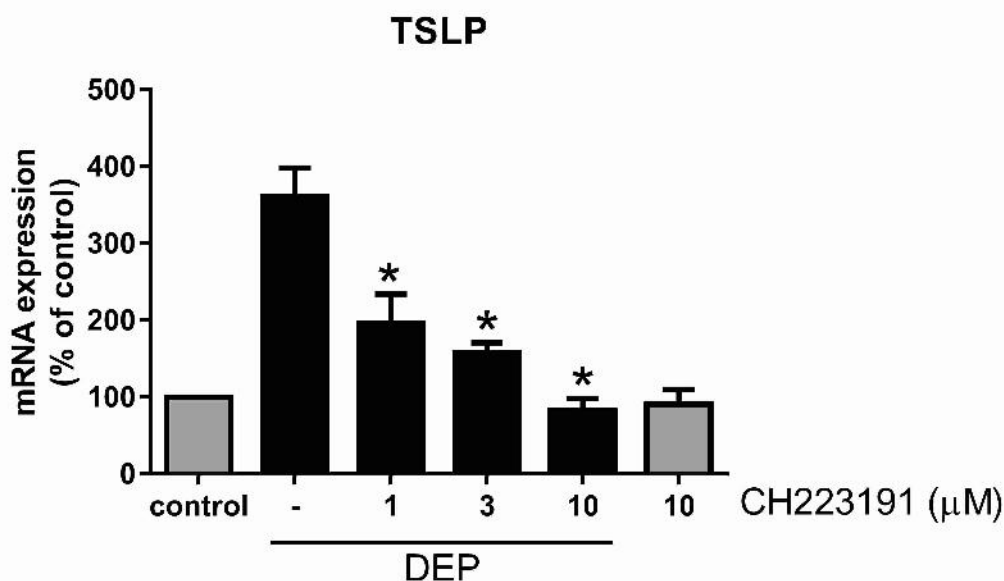
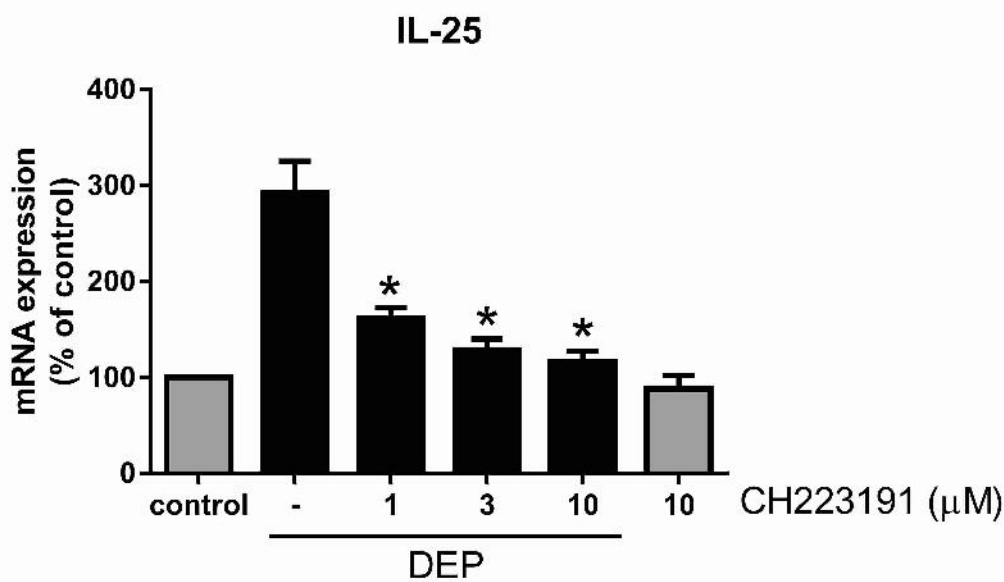
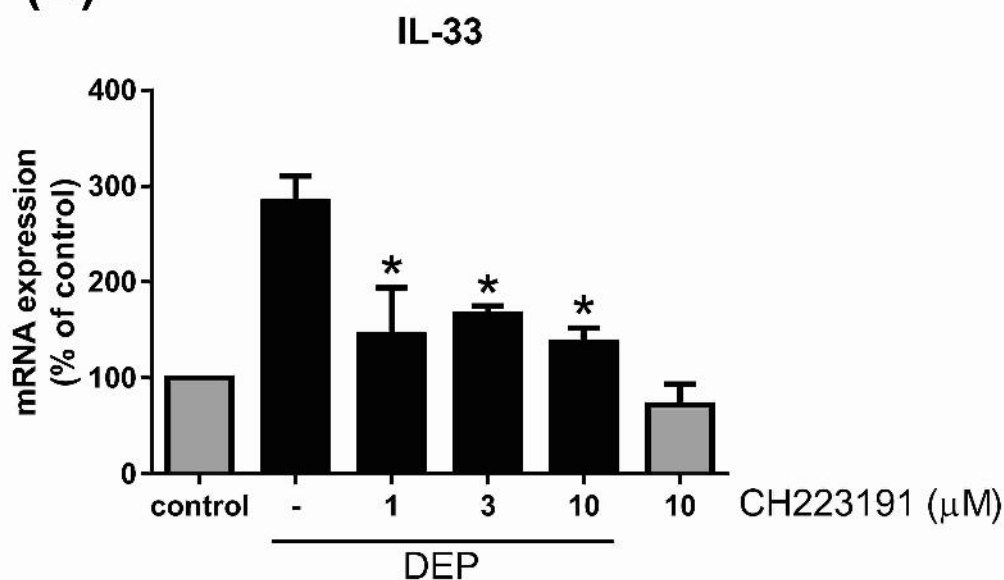
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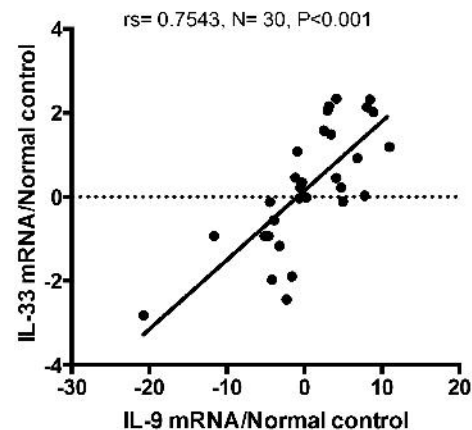
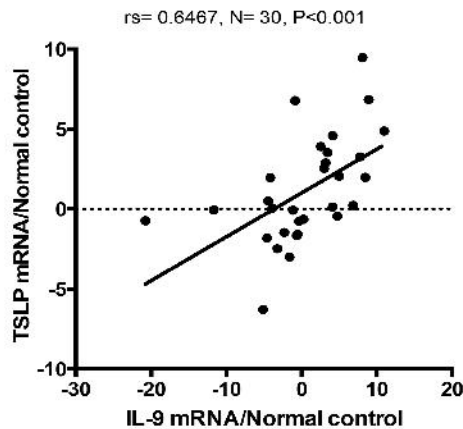
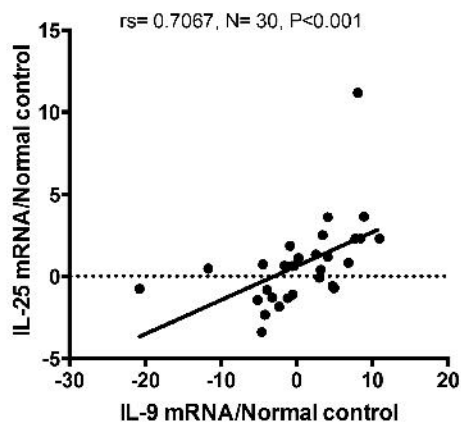
Additional supporting information may be found online in the Supporting Information section at the end of the article.

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(A)**(B)**



Correlation	Spearman r	P value
IL-13 mRNA vs IL-33 mRNA (N=32)	0.5299	0.0018
IL-13 mRNA vs IL-25 mRNA (N=32)	0.4495	0.0099
IL-13 mRNA vs TSLP mRNA (N=32)	0.4989	0.0037

**Aryl Hydrocarbon Receptor activation mediates epithelium-derived
cytokines expression in severe allergic asthma**

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Online Data Supplement

Materials and Methods

Cell viability assay

Primary bronchial asthmatic epithelial cells (2×10^3 cells) were cultured in 96-well plates. After overnight incubation, the medium was changed and transfected with scramble or AhR siRNA for 6 h, and then stimulated with DEP (0.1-10 $\mu\text{g/ml}$) for 24 h. Cell viability was determined by an MTT assay (Promega, Madison, WI) according to the manufacturer's instructions.

Table S1

IL-33 promoter Forward	5' GCAAAGCTCTGCTAATGGAG 3'
IL-33 promoter Reverse	5' CCAGAGCAATCATCTGCTAC 3'
IL-25 promoter Forward	5' CGAAGGTGGATGATCAGAGG 3'
IL-25 promoter Reverse	5' ATGGAAGTTGTGATGACCATC 3'
TSLP promoter Forward	5' GCACTAGTTGAAAGTGGCCAC 3'
TSLP promoter Reverse	5' GCACTAGTTGAAAGTGGCCAC 3'
GAPDH Forward	5' AGGGCTGCTTTTAACTCTGGT 3'
GAPDH Reverse	5' CCCCACCTTGATTTTGGAGGGA 3'

Figure legend supplement

Figure S1 Primary bronchial asthmatic epithelial cells were stimulated with DEP (0.1-10 $\mu\text{g/ml}$) for 24 h (A) or were transfected with scramble or AhR siRNA for 6 h before stimulation with DEP (10 $\mu\text{g/ml}$) for another 24 h (B). After incubation with MTT for 1 h, cells were harvested, and read absorbance at 570 nm. Data represent the mean $\pm$ SEM from three separate experiments.

Figure S2 A, Primary bronchial asthmatic epithelial cells were pretreated with an AhR antagonist, CH223191, (1-10 μM ,) for 30 min, and then stimulated with DEP (10 $\mu\text{g/ml}$) for another 6 h. The protein levels were detected by immunoblot analysis. B, Primary bronchial asthmatic epithelial cells were pretreated with CH223191 (1-10 μM) for 30 min, and then stimulated with DEP (10 $\mu\text{g/ml}$) for another 2 h, then transcript levels were assessed by quantitative PCR (n=4). Data were shown as mean $\pm$ SEM from four (B) independent experiments. * $p < 0.05$ compared to the corresponding vehicle control as the 100% reference.

Figure S3 Relationship between the IL-13 (n=32), IL-9 (n=30) and IL-33, IL-25, TSLP mRNA expression in tissues from patients with high AhR nuclear translocation.