Received: 17 September 2018 | Revised: 24 January 2019 | Accepted: 18 February 2019

DOI: 10.1111/all.13784

ORIGINAL ARTICLE

Experimental Allergy and Immunology

Allergy EUROPEAN JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY EAACI WILEY

Benzo(a)pyrene facilitates dermatophagoides group 1 (Der f 1)-induced epithelial cytokine release through aryl hydrocarbon receptor in asthma

Eryi Wang[1,2] | Xiaoyu Liu[2] | Wei Tu[1,2] | Danh C. Do[3] | Haiqiong Yu[1] | Liteng Yang[1] | Yufeng Zhou[4] | Damo Xu[5] | Shau-Ku Huang[1,3,6] | Pingchang Yang[1,2] | Pixin Ran[7] | Pei-Song Gao[3] | Zhigang Liu[1,2]

[1]The Affiliated Luohu Hospital of Shenzhen University, Shenzhen Luohu Hospital Group, Shenzhen, China

[2]The State Key Laboratory of Respiratory Disease for Allergy, Shenzhen University School of Medicine, Shenzhen University, Shenzhen, China

[3]Johns Hopkins Asthma and Allergy Center, Johns Hopkins University School of Medicine, Baltimore, Maryland

[4]Key Laboratory of Neonatal Disease, Ministry of Health, Children's Hospital and Institute of Biomedical Sciences, Fudan University, Shanghai, China

[5]Institute of Infection, Immunity and Inflammation, University of Glasgow, Glasgow, UK

[6]National Institute of Environmental Health Sciences, National Health Research Institutes, Miaoli, Taiwan

[7]Guangzhou Institute of Respiratory Disease, State Key Laboratory of Respiratory Diseases, The First Affiliated Hospital, Guangzhou Medical University, Guangzhou, China

Correspondence
Zhigang Liu, Institute of Allergy & Immunology, Medical College, Shenzhen University, Shenzhen 518060, Guang- dong, China.
Email: lzg@szu.edu.cn
Peisong Gao, Division of Allergy & Clinical Immunology, 5501 Hopkins Bayview Circle, Baltimore, MD 21224.
Email: pgao1@jhmi.edu
and
Pixin Ran, Guangzhou Institute of Respiratory Disease, State Key Laboratory of Respiratory Diseases, The First Affiliated Hospital, Guangzhou Medical University, Guangzhou 510120, China.
Email: rxran@vip.163.com.

Funding information
Shenzhen Peacock Plan Team Project, Grant/Award Number: No.201703313000321; Guangdong Province Science and Technology Planning Project, Grant/Award

Abstract

Background: Environmental pollutants, which coexist with allergens, have been associated with the exacerbation of asthma. However, the underlying molecular mechanisms remain elusive. We sought to determine whether benzo(a)pyrene (BaP) co-exposure with dermatophagoides group 1 allergen (Der f 1) can potentiate Der f 1-induced asthma and its underlying mechanisms.

Methods: The effect of BaP was investigated in Der f 1-induced mouse model of asthma, including airway hyper-responsiveness, allergic inflammation, and epithelial-derived cytokines. The impact of BaP on Der f 1-induced airway epithelial cell oxidative stress (ROS) and cytokine release was further analyzed. The role of aryl hydrocarbon receptor (AhR) signaling in BaP-promoted Der f 1-induced ROS, cytokine production, and allergic inflammation was also investigated.

Results: Compared with Der f 1, BaP co-exposure with Der f 1 led to airway hyper-responsiveness and increased lung inflammation in mouse model of asthma. Increased expression of TSLP, IL-33, and IL-25 was also found in the airways of these mice.

Abbreviations: 16HBEC, human bronchial epithelial cell; AHR, airway hyper-responsiveness; AhR, ryl hydrocarbon receptor; BALF, bronchoalveolar lavage fluid; BaP, benzo(a)pyrene; DEP, diesel exhaust particulates; Der f 1, dermatophagoides group 1 allergen; HDM, house dust mite; NAC, N-acetyl-L-cysteine; ROS, reactive oxygen species.

Wang, Liu and Tu are contributed equally to this work.

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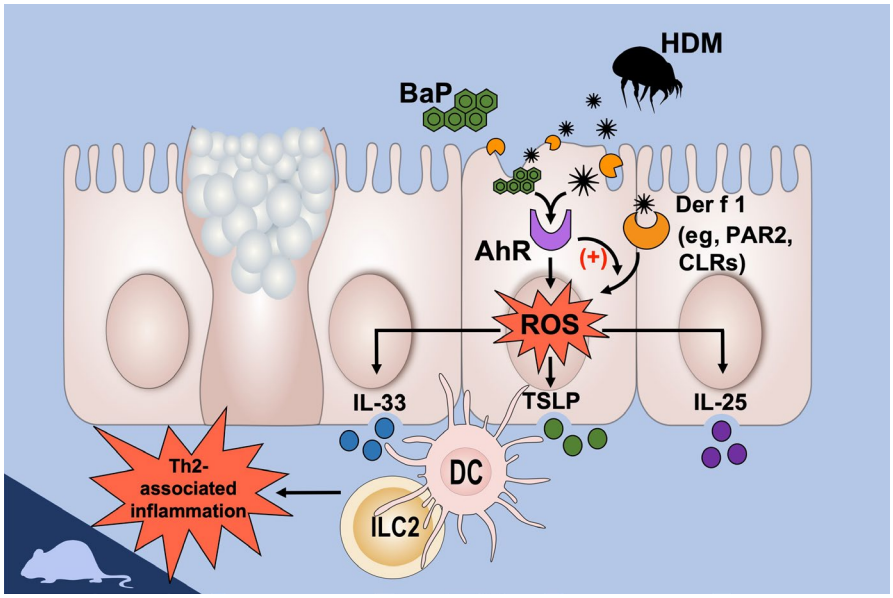
Number: No.2017B020226006; Shenzhen Health Planning Commission Scientific Research Project, Grant/Award Number: No. 201604130418; Shenzhen Scientific Technology Basic Research Projects, Grant/Award Number: No.JCYJ20160328144536436, JCYJ20170307162947583; National Natural Science Foundation of China, Grant/Award Number: No.81628001, 31770984; National Institutes of Health, Grant/Award Number: RO1ES021739, R21 AI121768, and R21 AI137547

Moreover, BaP co-exposure with Der f 1 activated AhR signaling with increased expression of AhR and CYP1A1 and promoted airway epithelial ROS generation and TSLP and IL-33, but not IL-25, expression. Interestingly, AhR antagonist CH223191 or cells with AhR knockdown abrogated the increased expression of ROS, TSLP, and IL-33. Furthermore, ROS inhibitor N-acetyl-L-cysteine (NAC) also suppressed BaP co-exposure-induced expression of epithelial TSLP, IL-33, and IL-25. Finally, AhR antagonist CH223191 and NAC inhibited BaP co-exposure with Der f 1-induced lung inflammation.

Conclusions: Our findings suggest that BaP facilitates Der f 1-induced epithelial cytokine release through the AhR-ROS axis.

KEYWORDS

Aryl hydrocarbon receptor, benzo(a)pyrene (BaP), dermatophagoides group 1 allergen (Der f 1), IL-25, IL-33, reactive oxygen species, TSLP



GRAPHICAL ABSTRACT

Compared with Der f 1 alone, BaP and Der f 1 co-exposure leads to airway hyperresponsiveness and increases Th2-associated lung inflammation in mouse model of asthma. AhR signaling plays a critical role in BaP and Der f 1 co-exposure-induced oxidative stress and cytokine expression in airway epithelial cells. The AhR-ROS axis regulates BaP and Der f 1 co-exposure-induced epithelial cytokine expression and allergic inflammation. AhR: Aryl hydrocarbon receptor; BaP: benzo(a)pyrene; CLRs: C-type lectin receptors; HDM: house dust mite; PAR2: protease-activated receptor 2; ROS: reactive oxygen species.

1 | INTRODUCTION

The prevalence of asthma has been increasing over the past few decades,[1] and the increase may be attributed to industrialization- and urbanization-generated environmental pollutants.[2-7] Environmental pollutants, which coexist with allergens, may contribute to the exacerbation of asthma. Particularly, environmental pollutant, di(2-ethylhexyl) phthalate (DEHP), which is commonly used as a plasticizer, has been shown to enhance cockroach allergen–induced airway inflammation.[8] Furthermore, prenatal exposure to either diesel exhaust particulates (DEP) or polycyclic aromatic hydrocarbons (PAHs), which are DEP-derived toxins, is associated with a greater risk of allergic sensitization, early childhood wheeze, and asthma.[9,10] Moreover, co-exposure to DEP and house dust mite (HDM) has been shown to exacerbate allergic sensitization and to induce key characteristics of a more severe asthma.[7,11-14] However, the key components in DEPs that are responsible for the aggravation of allergic response and the related underlying molecular mechanisms have not been revealed.

Benzo(a)pyrene (BaP) is a ubiquitous environmental pollutant, and carcinogenic polycyclic aromatic hydrocarbons (PAH) are generally

derived from automobile exhaust (eg, DEP), cigarette smoking, cooking, and industrial combustion.[15] BaP can induce expression of several pro-inflammatory cytokines/chemokines [eg, IL-1β, TNF-α, and CC chemokine ligand 1 (CCL1)] in macrophages.[16,17] Exposure to BaP alone can also induce oxidative stress, bronchial epithelium injury,[18,19] and pulmonary toxicity and inflammation.[20] Intriguingly, co-exposure to BaP with an artificial allergen ovalbumin (OVA) has been reported to enhance the production of allergen-specific IgE, systemic Th1/Th2 reaction, and airway inflammation in mice.[21-23] However, the causal relationship and underlying molecular mechanisms are poorly characterized.

Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that is activated by small molecules provided by the diet, microorganisms, metabolism, and pollutants.[24-29] Upon ligand binding, AhR translocates from cytosol to the nucleus, leading to changes in target gene transcription (eg, cytochrome P450 cyp1a1, cyp1b1) and immunotoxicological effects.[30-32] Furthermore, it has been established that AhR is critical in the adaptive[33] and innate immune regulation.[34,35] Specifically, AhR has a dominant effect on phenotype by controlling the expression of cytokines, including IL-10, type I interferons, IL-12, IL-17, and TGF-β1.[27,36-38] We have recently demonstrated that AhR plays an important role in mediating allergen-induced and ROS-dependent degranulation and IgE-mediated mast cell activation.[39-41] Additionally, growing evidence suggests that airway epithelial cells play a pivotal role in the initiation and propagation of asthma by producing pathogenic cytokines TSLP, IL-33, and IL-25, and inflammatory mediators in response to environmental stimuli.[42-45] However, it still remains poorly understood whether AhR signaling provides a molecular pathway that mediates the effects of co-exposure to BaP and common allergens on the epithelial release of cytokines and subsequently downstream immune responses in asthma.

In the present study, we investigated the immunomodulatory effects of BaP on Der f 1, a common allergen in human-induced airway hyper-responsiveness and lung inflammation. Furthermore, we focused on airway epithelial cells and investigated whether BaP co-exposure with Der f 1 can promote airway epithelial cell oxidative stress and cytokine release. More importantly, we explored whether the co-exposure can activate AhR signaling, which subsequently regulates the co-exposure-induced oxidative stress, cytokine release, and allergic inflammation.

2 | MATERIALS AND METHODS

2.1 | Animals

Six- to eight-week-old C57BL/6 mice were purchased from experimental animal center of Guangdong province. Animals were maintained under specific pathogen-free conditions at the animal facility of Shenzhen University. The experimental protocols in this study were reviewed and approved by the Animal Care and Use Committee in Shenzhen University.

2.2 | Bap co-exposure with Der f 1-induced mouse model of asthma

Generation of the BaP co-exposure with Der f 1-induced asthma mouse model was established as previously described.[22,23] Briefly, mice were sensitized and challenged every other week for 6 weeks with intranasal administrations of 25 μg Der f 1 (Indoor biotechnologies) under light isoflurane anesthesia. To assess the role of BaP on Der f 1-induced allergic airway inflammation, BaP (Sigma-Aldrich) was dissolved in borate saline buffer (BBS, Sigma-Aldrich), and intranasally administered at a concentration of 1 or 20 pmol once every week during Der f 1 sensitization and challenge (Figure 1A). In some cases, mice were pretreated with CH223191 at a dose of 5 mg/kg or N-acetyl-L-cysteine (NAC) at a dose of 1 mmol/kg dissolved in buffered saline by means of intraperitoneal administration 1 hour before BaP treatment. Age- and gender-matched control mice were treated identically with BBS.

2.3 | Measurement of airway hyper-responsiveness

Airway hyper-responsiveness (AHR) to methacholine (Mch) (Sigma-Aldrich) was measured 24 hour after the last challenge with whole-body plethysmography (Buxco Europe Ltd, Winchester, UK) as previously described[46] and monitored by average enhanced pause (Penh).[47] Mice were exposed to increasing doses of methacholine (Mch) at 6.25, 12.5, 25, 50, and 100 mg/mL or PBS. The percentage curves for Penh values at different Mch doses were plotted, starting with PBS stimulation.

2.4 | Bronchoalveolar lavage and Lung histology

Mice were killed, and bronchoalveolar lavage (BAL) was performed by instillation of 0.8 mL of PBS through a tracheal cannula. BAL specimens were cytocentrifuged at 150 *g* for 5 minutes, placed on microscope slides, and stained with Wright-Giemsa. The total number of eosinophils, neutrophils, and macrophages was determined by counting 200 leukocytes in randomly selected areas of the slides under a light microscopy. Supernatants were collected and stored at −80°C. Lung tissues were fixed in 4% formalin for 24 hours and then embedded in paraffin wax after dehydration in alcohol. Lung sections were stained with H&E and PAS solution for histopathological analysis.

2.5 | ELISA

BALF were analyzed for IL-4, IL-13, IFNγ, IL-17A, TSLP, IL-33, and IL-25 by sandwich ELISA (Sizhengbo Inc, Beijing, China) according to the manufacturer's manual. Serum levels of Der f 1-specific IgE and IgG1 were analyzed by ELISA as previously described.[48,49]

2.6 | Immunofluorescence staining

For immunofluorescence staining, sectioned lung tissues were first blocked using 5% w/v BSA for 1 hour, followed by incubation with

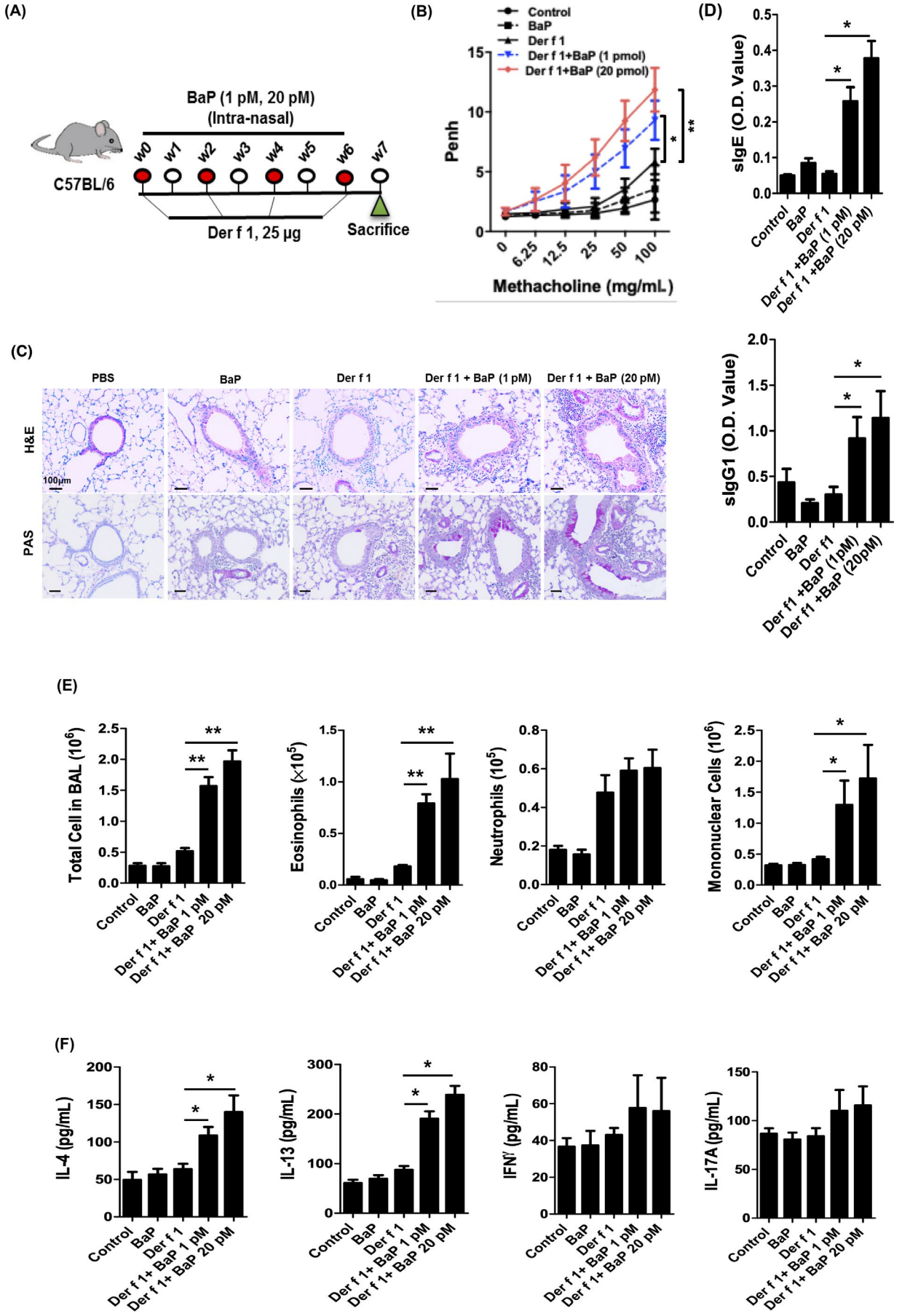

(A)
BaP (1 pM, 20 pM)
(Intra-nasal)
C57BL/6
w0
w1
w2
w3
w4
w5
w6
w7
Der f 1, 25 μg
Sacrifice
(B)
Control
BaP
Der f 1
Der f 1+BaP (1 pmol)
Der f 1+BaP (20 pmol)
Penh
Methacholine (mg/mL)
(D)
sIgE (O.D. Value)
Control
BaP
Der f 1
Der f 1 +BaP (1 pM)
Der f 1 +BaP (20 pM)
(C)
PBS
BaP
Der f 1
Der f 1 + BaP (1 pM)
Der f 1 + BaP (20 pM)
H&E
PAS
100μm
sIgG1 (O.D. Value)
Control
BaP
Der f1
Der f1 +BaP (1pM)
Der f1 +BaP (20pM)
(E)
Total Cell in BAL (10^6)
Eosinophils (×10^5)
Neutrophils (10^5)
Monoclear Cells (10^6)
Control
BaP
Der f 1
Der f 1+ BaP 1 pM
Der f 1+ BaP 20 pM
(F)
IL-4 (pg/mL)
IL-13 (pg/mL)
IFNγ (pg/mL)
IL-17A (pg/mL)
Control
BaP
Der f 1
Der f 1+ BaP 1 pM
Der f 1+ BaP 20 pM

FIGURE 1 BaP co-exposure exacerbates Der f 1-induced lung inflammation. A, Protocol for the generation of mouse model of asthma. W: week. Filled red circles represent the day for Der f 1 treatment. B, Effect of Der f 1 and BaP on airway hyper-responsiveness (AHR) was monitored by average Penh. Methacholine concentration was increased from 6.25 to 100 mg/mL). C, Paraffin tissue sections of lung were stained with H&E (upper panel) and periodic acid-Schiff (PAS, lower panel). D, ELISAs for serum levels of Der f 1-specific IgE and IgG1. E, Bronchoalveolar lavage (BAL) total inflammatory cells and eosinophil cells as assessed by Wright-Giemsa staining. F, ELISAs for BAL levels of Th1/Th2/Th17 cytokines. Data represent mean ± SEM (n = 6-8/group). *$P < 0.05$, **$P < 0.01$

antibodies against TSLP (GTX85059; GeneTex), IL-33 (AF3626; R&D Systems), IL-25 (207710; R&D Systems), and EpCAM (G8.8; ThermoFisher) overnight at 4℃. Sample sections were then incubated with secondary antibodies conjugated with Alexa Fluor dyes (ThermoFisher) at room temperature for 1 hour. Isotype-matched negative control antibodies (R&D Systems) were used under the same conditions. Nuclei were counterstained with 6-diamidino-2-phenylindole, dihydrochloride (DAPI, ThermoFisher). Sections were mounted with the ProLong Gold Anti-fade Kit (Molecular Probes) and observed with a Nikon Eclipse Ti-U microscope equipped with a DS-Fi2 camera (Nikon). The detection of intracellular superoxide in lung tissues was carried out using dihydroethidium (DHE) (ThermoFisher). To determine the fluorescence signal in tissue sections, fluorescent-positive cells in five different high-power fields from each slide were quantified using ImageJ v1.50e (NIH) and presented as mean fluorescence intensity per square micrometer. Two to three slides from each sample were used for analysis.

2.7 | Cell culture

Human bronchial epithelial cells (16HBECs) were purchased from Haoge biological company (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were maintained at 37˚C in a humidified atmosphere at 5% CO_2.

2.8 | RT-PCR

Total RNA was extracted using an RNeasy Plus Mini kit (Qiagen), and cDNA templates were synthesized with SuperScript II (Life Technologies). Real-time PCR (RT-PCR) was performed using C1000 Touch PCR machine (Bio-RAD). Data were analyzed using the $2^{-\Delta\Delta CT}$ method as described by Livak and Schmittgen[50] and expressed as fold change relative to untreated control. The mRNA levels were normalized to the internal control gene *β-actin*. Primer sequences used are included in the Online Repository (Table S1).

2.9 | Western blotting

Cells were collected and lysed in RIPA buffer (Sigma-Aldrich) containing Protease and Phosphatase Inhibitor Cocktails (Roche). Protein concentration was measured using a BCA™ Protein Assay kit (Pierce). Aliquots of 30-50 μg protein samples were separated by electrophoresis on 10% SDS-polyacrylamide gels and then electrophoretically transferred to a methanol-pretreated PVDF membrane (Invitrogen). After blocking with 5% nonfat milk in TBST, the membrane was incubated with anti-AhR (Abcam, ab23672), anti-CYP1A1 (Abcam, ab23672), anti-TSLP (GeneTex, 85059), anti-33 (Santa Cruz, 4E9), anti-IL-25 (Abcam, Q9H293), and anti-β-actin antibodies. Detailed information is included in the Online Repository (Table S2). Blots were visualized with an HRP-conjugated secondary antibody (Santa Cruz) and Western blotting analysis system Multiskan Go (Thermo scientific). Relative protein expression was determined by densitometric analysis using ImageJ (NIH).

2.10 | Detection of intracellular reactive oxygen species

Intracellular reactive oxygen species were quantified with Reactive Oxygen Species Assay Kit (Beyotime Institute of Biotechnology) as previously described.[48,51] Briefly, cells were cultured with dihydrodichlorofluorescein diacetate (DCF-DA) in serum-free medium at 37˚C for 20 minutes and then added BaP (1.0 μmol/L) or/and Der f 1 (30 μg) in the presence or absence of NAC (1.0 μmol/L) for 1 hours. The DCF fluorescence was quantitated with a Multiskan Go plate reader (Thermo), with an excitation wavelength of 488 nm and an emission wavelength of 525 nm, and normalized with the total protein content.

2.11 | Statistical analysis

Data are expressed as means ± SEM. Statistical significance for normally distributed samples was assessed using an independent two-tailed Student's *t* test or with ANOVA. Non-normally distributed samples were analyzed by Wilcoxon rank-sum test. All analyses were performed with GraphPad Prism version 5.1 software (GraphPad Software). A *P*-value < 0.05 was considered statistically significant for all analyses.

3 | RESULTS

3.1 | BaP co-exposure exacerbates Der f 1-induced lung inflammation

To determine whether BaP co-exposure can exacerbate Der f 1-induced lung inflammation, we generated a mouse model of asthma as illustrated in Figure 1A. AHR to inhaled Mch was analyzed to evaluate changes in airway resistance in response to allergen exposure and challenge. Although both BaP and Der f 1 alone failed to induce airway hyper-responsiveness (AHR), BaP co-exposure with Der f 1 induced a strong and significant increase in airway reactivity to inhaled Mch as compared to Der f 1 (Figure 1B). Next, we found

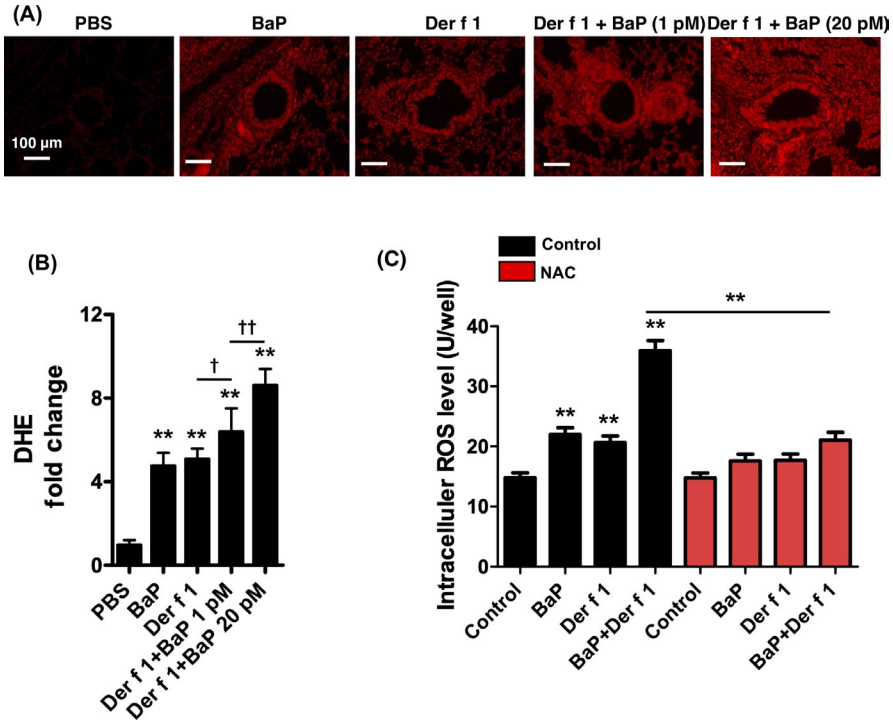


FIGURE 2 BaP exacerbates Der f 1-induced oxidative stress. A, Representative images of dihydroethidium-stained (DHE-stained) lung tissues of BaP and Der f 1-treated mice. Scale bar: 100 μm. B, Quantitative data for ROS expression as expressed by DHE fold change. Data represent mean ± SEM (n = 6-8/group), $^{**}P < 0.01$ (compared to PBS control), $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ (Compared to Der f 1). C, Detection of intracellular ROS production with Reactive Oxygen Species Assay Kit in BaP and Der f 1-treated 16HBECs in the absence (black bar) or presence of NAC (red bar) (n = 3). The dihydrodichlorofluorescein diacetate (DCF-DA) fluorescence was quantitated with a Multiskan Go plate reader and normalized with the total protein content. Data represent mean ± SEM. $^{**}P < 0.01$

increased recruitment of inflammatory cells to the lungs, with dense peri-bronchial cell infiltrates and goblet cell hyperplasia on histological examination after BaP co-exposure with Der f 1 (Figure 1C, H&E and PAS) as compared with Der f 1-treated mice. Moreover, mice exposed to BaP and Der f 1 also showed increased serum titers of Der f 1-specific IgE and IgG1 (Figure 1D). Total and differential cell counts of BAL fluid samples indicated increased number of total inflammatory cells with a significant eosinophil recruitment into the lungs (Figure 1E). In addition, BAL fluids from mice exposed to BaP and Der f 1 showed higher levels of IL-4 and IL-13, but no changes for IFNγ and IL-17A, when compared with these from Der f 1-treated mice (Figure 1F). Collectively, these results suggest that BaP co-exposure exacerbates Der f 1-induced airway hyper-responsiveness and Th2-associated lung inflammation.

3.2 | BaP exacerbates Der f 1-induced oxidative stress

Our recent studies have demonstrated that cockroach allergen can induce ROS production in mouse model of asthma and airway epithelial cells, leading to increased COX-2 expression.[48] To extend our findings, we examined whether Der f 1 can induce ROS generation, and whether BaP co-exposure can enhance Der f 1-induced ROS expression in the mouse model of asthma. Both BaP and Der f 1 alone can induce ROS generation in the mouse lung tissues (Figure 2A, B), but BaP co-exposure can further enhance the Der f 1-induced ROS response. This finding was also confirmed by the direct detection of ROS expression in airway epithelial cells (16HBECs). As expected, ROS production was increased in 16HBECs after treatment with either BaP or Der f 1 (Figure 2C), and Der f 1-induced ROS generation was further enhanced by BaP co-exposure. As expected, no increased ROS was observed in cells pretreated with ROS inhibitor NAC (N-acetyl-L-cysteine). Taken together, these results suggest that Der f 1 can induce ROS generation, which can be further enhanced by BaP co-exposure.

3.3 | BaP promotes Der f 1-induced epithelial cytokine release

Next, we investigated whether BaP or Der f 1 exposure can induce epithelial cytokine production, and whether BaP co-exposure can further enhance this response. No significant changes for TSLP, IL-33, and IL-25 were observed in either BaP- or Der f 1-treated mice, but significant increases were found in the airway epithelial cells of mice after exposure to the combination of BaP and Der f 1 as assessed by co-immunofluorescent staining with EpiCaM, a marker for epithelium (Figure 3A, B). These findings were further supported by the analyses of TSLP and IL-25 levels in BAL fluids of mice exposed to BaP and Der f 1 as compared to Der f 1 alone (Figure 3C). IL-33 showed the same trend, but statistically no significance. To further confirm BaP-induced cytokine secretion in airway epithelial cells, 16HBECs were treated with BaP alone (0.01 to 1.0 μmol/L). We found a dose-dependent response for TSLP and IL-33, but not for IL-25 and the inflammatory cytokine IL-1β, and GM-CSF, in BaP-treated 16HBECs (Figure 4A). Furthermore, we found that Der f 1 (30 μg/mL) alone induced TSLP, IL-33, IL-25, IL-1β, and GM-CSF secretion release from 16HBECs, and BaP co-exposure (1.0 μmol/L) promoted Der f 1-induced TSLP and IL-33, but not other tested cytokines (Figure 4B). To further validate these findings, we cultured 16HBECs with an air-liquid interface (ALI) culture method as previously described.[52] Similarly, either BaP alone or co-exposure can induce TSLP and IL-33, but not IL-25 expression, in the ALI cultured

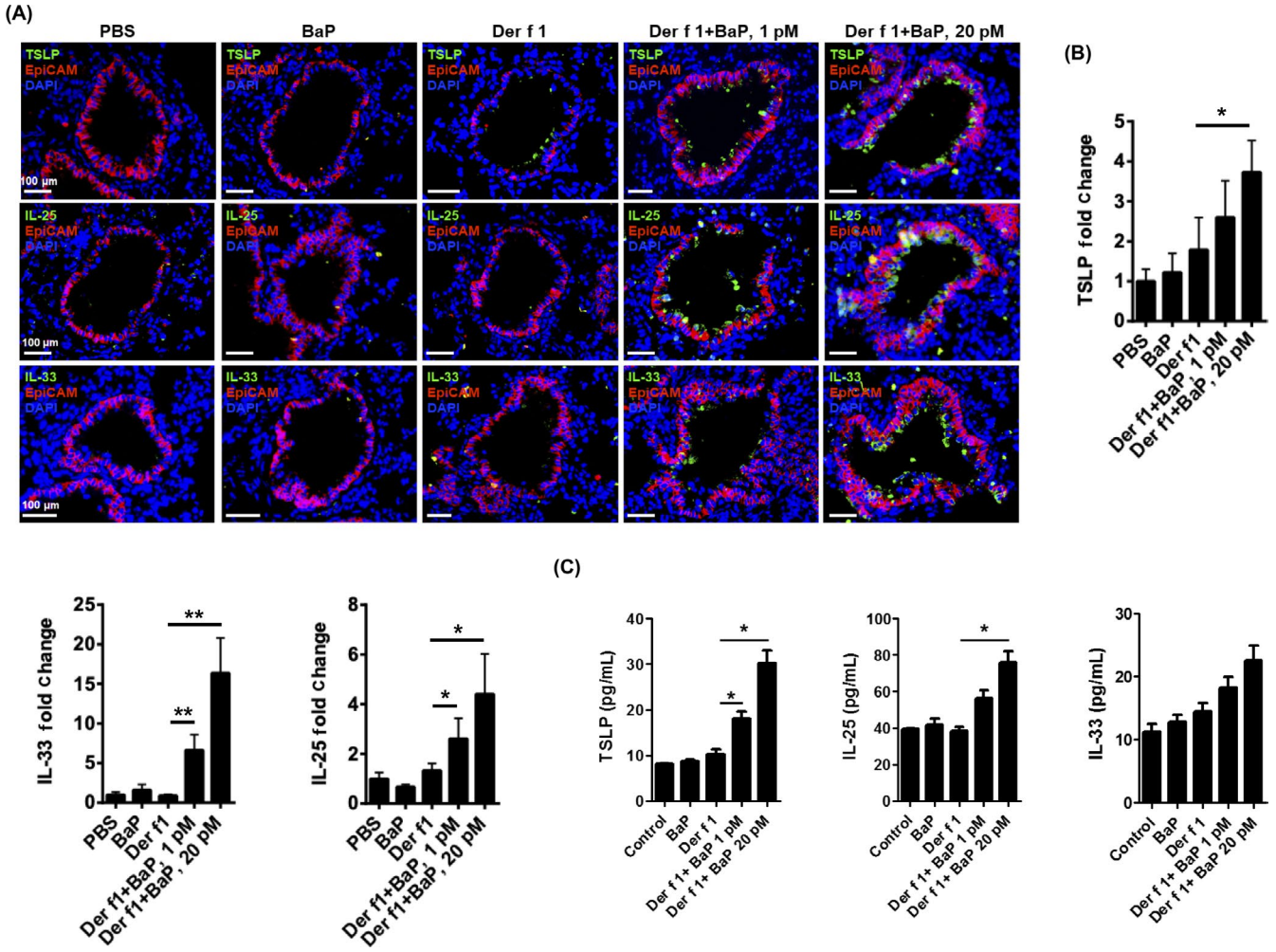


FIGURE 3 BaP promotes Der f 1-induced epithelial cytokine release. A, Representative image of double immunofluorescence staining with the primary antibodies against TSLP (green, upper), IL-25 (green, middle), and IL-33 (green, lower) with EpiCAM (red) in the mouse lung tissues. Nuclei were counterstained with DAPI, n = 6/group. Scale bar: 100 μm. B, Quantification data for cytokine expression. C, ELISAs for BAL levels of epithelial-derived cytokines (TSLP, IL-25, and IL-33). Data represent mean ± SEM (n = 6-8/group). *$P < 0.05$, **$P < 0.01$

16HBECs (Figure S1). Collectively, these results suggest that BaP co-exposure can potentiate Der f 1-induced epithelial cytokine production, particularly TSLP and IL-33.

3.4 | Aryl hydrocarbon receptor activation by BaP mediates cytokine expression

AhR activation has been recently shown to be critical in mediating DEP-induced production of IL-33, IL-25, and TSLP in allergic severe asthma.[45] To determine whether AhR signaling can also be activated by BaP and plays a role in mediating BaP co-exposure-induced upregulation of TSLP and IL-33 expression, we investigated the BaP co-exposure-induced activation of AhR signaling. 16HBECs were treated with BaP, and expression of AhR and its major downstream gene CYP1A1 was assessed. As expected, BaP induced significantly increased AhR with the strongest expression at 1 hour (Figure S2A). Similar results were observed for CYP1A1 with the strongest expression at 24 hours (Figure S2B). The BaP-induced expression of AhR and CYP1A1 was further confirmed by Western blotting (Figure S2C, D). Next, we investigated whether BaP co-exposure can potentiate Der f 1-induced activation of AhR signaling. Intriguingly, we found that Der f 1 alone, similar to BaP, activated AhR signaling as defined by RT-PCR analyses of AhR and CYP1A1 expression, and BaP co-exposure potentiated Der f 1-induced activation of AhR signaling (Figure 5A). The co-exposure-induced activation was antagonized by CH223191 (0.1 μmol/L), a selective AhR antagonist. The Western blotting showed the same pattern as RT-PCR (Figure 5B). To determine whether AhR is involved in BaP co-exposure-induced epithelial cytokine expression, 16HBECs were pretreated with AhR antagonist CH223191 and then exposed to BaP and Der f 1. Expression of TSLP, IL-33, and IL-25 was analyzed by Western blotting (Figure 5C). Similar to the previous results, BaP co-exposure promoted the expression of epithelial TSLP (Figure 5D) and IL-33 (Figure 5E) as compared to Der f 1 alone, and the increased expression was almost completely blocked by CH223191 treatment. However, no further increase was observed for IL-25 (Figure 5F). To further validate the role of AhR in mediating BaP co-exposure-induced increased TSLP and IL-33 expression, AhR in 16HBECs was knocked down by siRNA and confirmed by Western blotting (Figure 6A). As

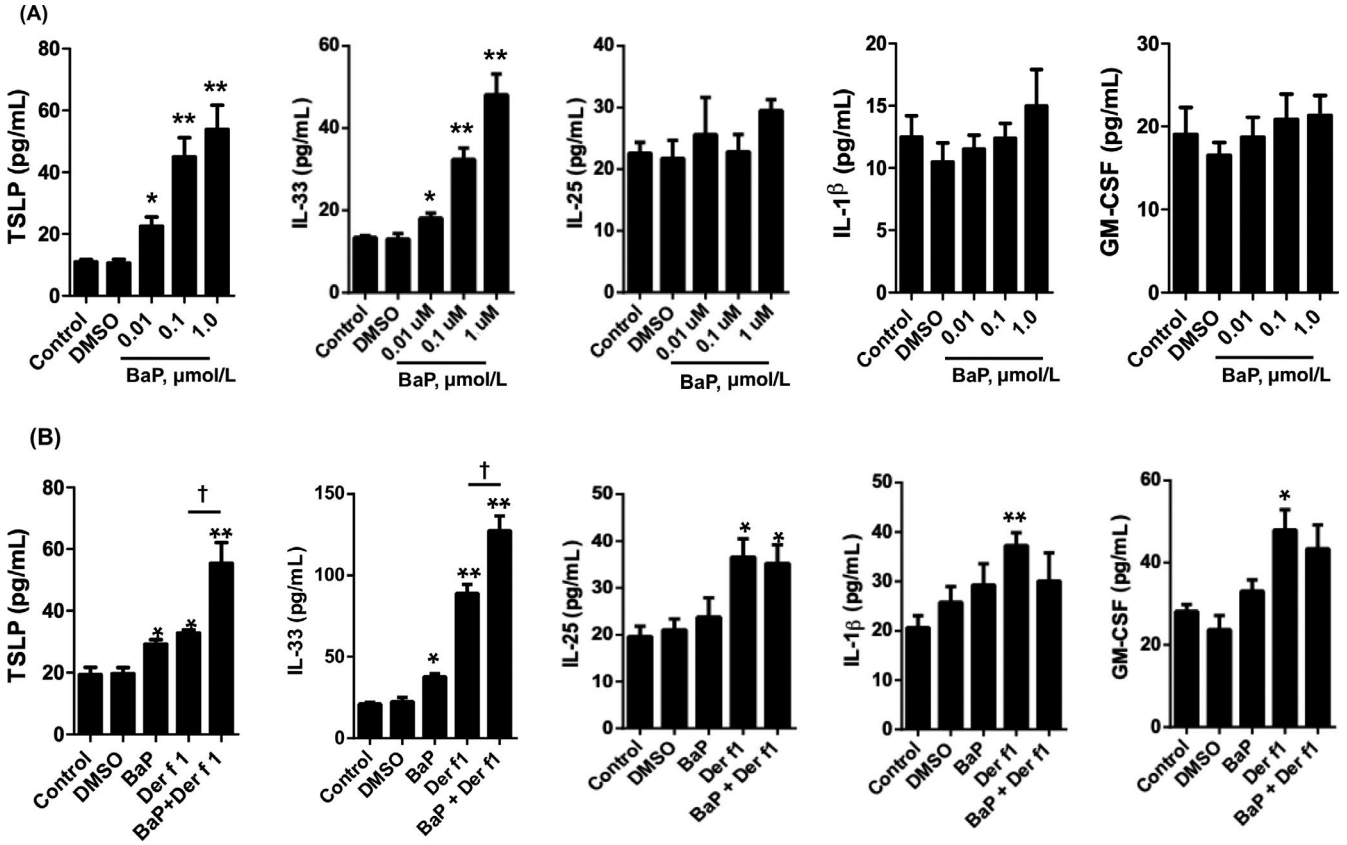


FIGURE 4 In vitro analysis of epithelial cell cytokine release in BaP- and Der f 1-treated 16HBECs. A, Levels of TSLP, IL-33, IL-25, IL-1β, and GM-CSF in culture supernatants of BaP-treated 16HBECs as detected by ELISA (n = 3). B, Levels of TSLP, IL-33, IL-25, IL-1β, and GM-CSF in culture supernatants of either BaP or Der f 1 alone or combination-treated 16HBECs by ELISA (n = 3). Data represent mean ± SEM. *$P < 0.05$, **$P < 0.01$ (compared to control). †$P < 0.05$ (compared to Der f 1)

expected, AhR knockdown significantly blocked the BaP-induced TSLP (Figure 6B) and IL-33 (Figure 6C) expression as detected by RT-PCR, but no change was noted for IL-25 (Figure 6D). Furthermore, the Western blotting illustrated that 16HBECs with AhR knockdown showed reduced TSLP expression after exposure to BaP alone or co-exposure (Figure 6E, F). The same trend was observed for IL-33, but no statistical significance (Figure 6E, G). Similarly, AhR knockdown showed no change for IL-25 (Figure 6E, H). These results imply that AhR activation may play a pivotal role in mediating BaP-induced production of airway epithelial TSLP and IL-33 in asthma.

3.5 | Aryl hydrocarbon receptor mediates BaP-induced cytokine expression through ROS

To explore the underlying mechanism by which AhR mediates BaP-promoted epithelial cytokine production, we investigated the regulation of AhR on ROS generation, and then examined whether ROS plays a role in the exacerbation of cytokine expression. To determine whether AhR regulates ROS generation, 16HBECs were treated with BaP or BaP co-exposure in the presence or absence of CH223191. The intracellular ROS was measured with the reactive oxygen species assay. Similar to our previous findings, BaP- and Der f 1-induced ROS generation, which was further enhanced by BaP co-exposure. Interestingly, the increased ROS generation was significantly inhibited by CH223191 (Figure 7A). These findings were further supported by AhR knockdown in 16HBECs (Figure 7B). The BaP co-exposure-induced ROS generation was almost completely abolished in AhR knockdown 16HBECs. Finally, to examine whether ROS is involved in BaP-induced exacerbation of epithelial cytokine expression, 16HBECs were pretreated with NAC (1.0 μmol/L) and then treated with BaP, Der f 1, or co-exposure. We found that BaP, Der f 1, and co-exposure induced TSLP expression, which was remarkably suppressed by NAC (Figure 7C, D). Furthermore, the BaP co-exposure-induced expressions of IL-33 (Figure 7D) and IL-25 (Figure 7 E) were also inhibited by NAC. These results imply that AhR signaling regulates ROS generation, which is critical in BaP co-exposure-induced increased expression of TSLP, IL-33, and IL-25 in the airway epithelial cells.

3.6 | Functional role of the aryl hydrocarbon receptor-ROS axis in BaP co-exposure-induced lung inflammation

Given the significance of the AhR-ROS axis in mediating BaP co-exposure-induced cytokine production in airway epithelial cells, we further investigated whether the AhR-ROS axis is also important

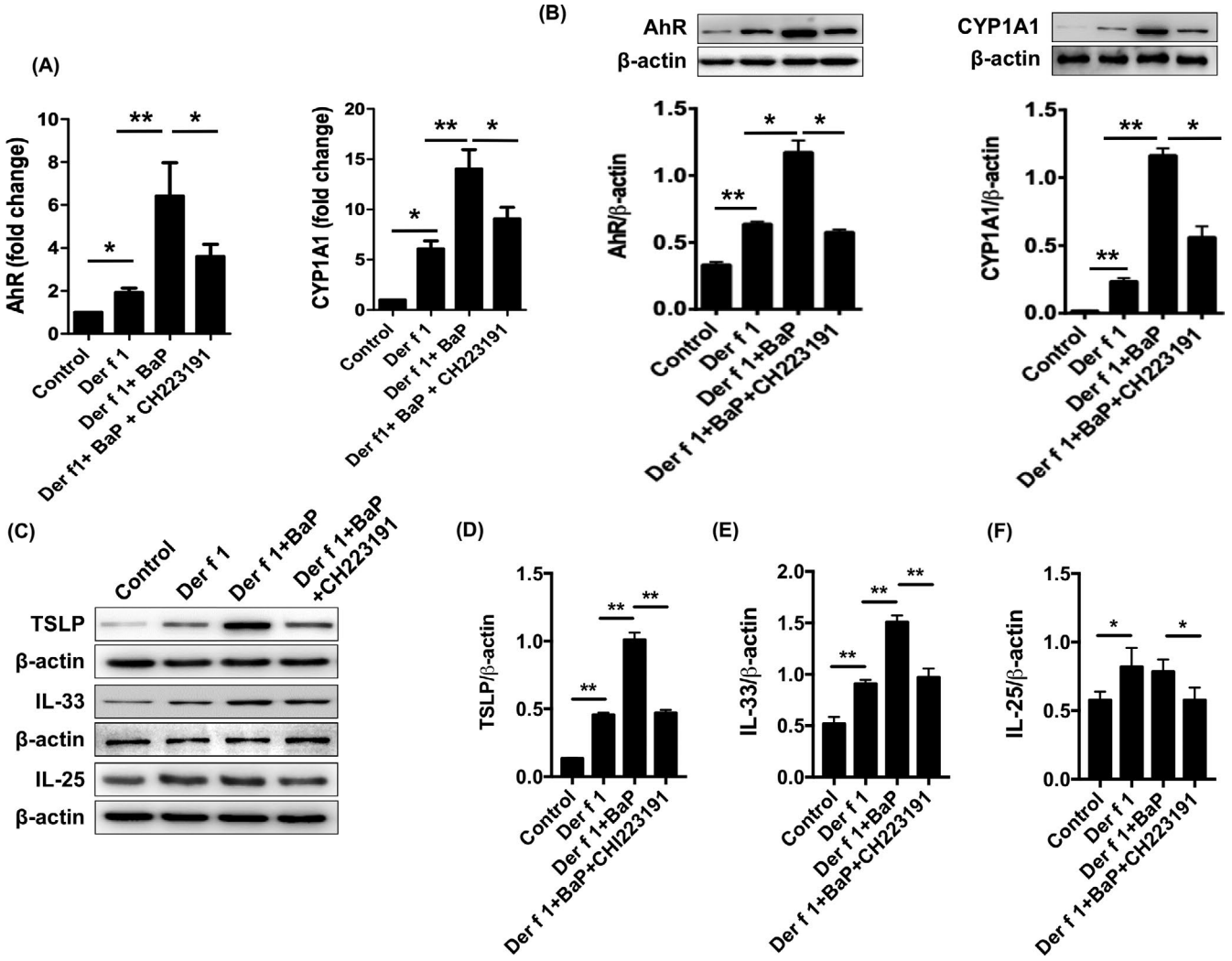


FIGURE 5 BaP co-exposure with Der f 1-induced aryl hydrocarbon receptor activation mediates TSLP and IL-33 expression. A, BaP co-exposure-induced activation of AhR signal was assessed with the expression of AhR and CYP1A1 by qRT-PCR. Expression was defined by fold change in relative to control. N = 3. B-C, Representative immunoblots for BaP co-exposure-induced expression of AhR, CRP1A1 (B), and epithelial cytokines (C) in 16HBECs. Quantitative data were obtained by densitometric analysis for the expression of AhR and CRP1A1 (B), and TSLP (D), IL-33 (E), and IL-25 (F) relative to expression of β-actin. Data represent mean ± SEMs of at least two independent experiments. *$P < 0.05$, **$P < 0.01$

in BaP co-exposure-induced lung inflammation in a mouse model of asthma. The asthma mouse model was generated as illustrated in Figure 1A; AhR antagonist CH223191 or ROS inhibitor NAC was administered by means of intraperitoneal injection 1 hour before BaP treatment. We found that treatment with either CH223191 or NAC before BaP co-exposure significantly suppressed AHR (Figure 8A) and lung inflammation, including recruitment of inflammatory cells to the lungs with goblet cell hyperplasia on histologic examination (Figure 8B, H&E and PAS). Similar effects were observed for serum levels of Der f 1-specific IgE and IgG1 (Figure 8C) and number of total inflammatory cells, specifically eosinophils (Figure 8D), and levels of IL-4 and IL-13 in BAL fluid (Figure 8E). Furthermore, treatment with CH223191 significantly suppressed increased lung tissue ROS caused by BaP co-exposure (Figure 8F, G). Taken together, the results suggest that the AhR-ROS axis may play a critical role in BaP co-exposure-induced lung inflammation.

4 | DISCUSSION

Here, we provide evidence that environmental pollutants, which coexist with allergens, contribute to the exacerbation of asthma. Particularly, we investigated whether BaP can enhance Der f 1-induced airway hyper-responsiveness, lung inflammation, and oxidative stress. Moreover, we explored the underlying molecular mechanisms by studying the role of AhR signaling in mediating BaP and Der f 1 co-exposure-induced oxidative stress and epithelial cytokine release. Furthermore, we examined the functional significance of the AhR-ROS axis in BaP and Der f 1 co-exposure-induced lung inflammation. Our study suggests for the first time that AhR signaling regulates BaP co-exposure-induced epithelial cytokine release, particularly TSLP and IL-33, through regulation of ROS generation.

Allergic asthma has been associated with the expression of Th2 genes characterized by increased eosinophilic inflammation,

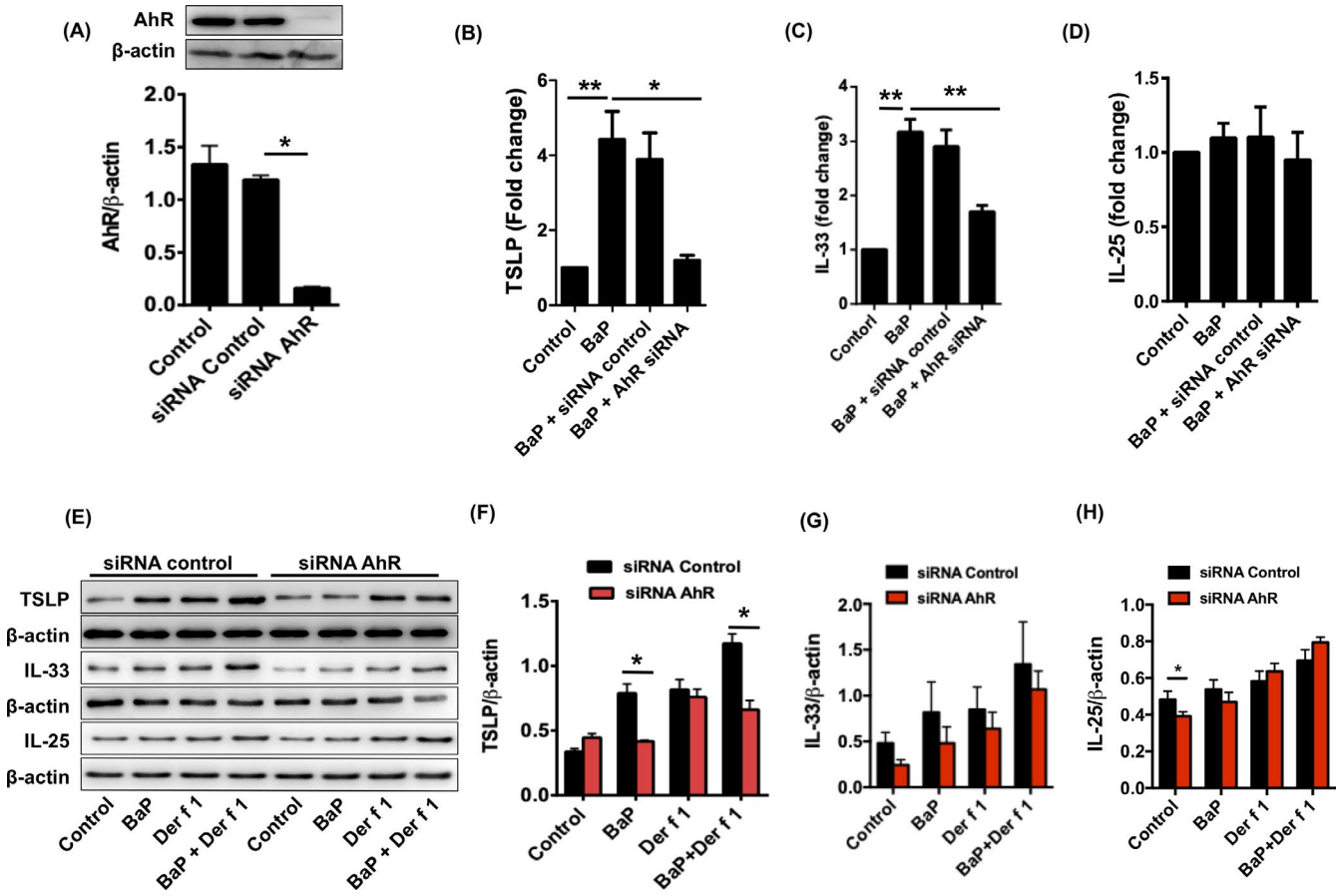


FIGURE 6 Aryl hydrocarbon receptor activation mediates BaP-promoted Der f 1-induced epithelial cytokine expression. A, AhR knockdown was confirmed by Western blotting and quantified by densitometric analysis for AhR. B-D, RT-PCR analysis for the expression of TSLP (B), IL-33 (C), and IL-25 (D) in BaP-treated 16HBECs with or without AhR knockdown. E, Representative immunoblots for BaP co-exposure with Der f 1-induced cytokine expression in 16HBECs with or without AhR knockdown. F-H, Quantitative data were obtained by densitometric analysis for the expression of TSLP (F), IL-33 (G), and IL-25 (H) relative to β-actin. Data represent mean ± SEMs of two independent experiments. *$P < 0.05$, **$P < 0.01$ [Color figure can be viewed at wileyonlinelibrary.com]

IgE production, and Th2 cytokine expression (eg, IL-4, IL-5, and IL-13).[48,53] Air pollution has been considered as one of the most important environmental factors for asthma.[54] Exposure to traffic pollution particulate matter, predominantly DEPs, has been shown to increase the risk of asthma.[11,55] However, experimental studies suggest that DEP alone had no effect on the major phenotypes of allergic asthma.[56] By contrast, DEP co-exposure with HDM exacerbated allergic responses, including airway hyper-responsiveness, increased levels of allergen-specific IgE, and accumulations of eosinophils and Th2/Th17 cells.[7,11,12,56] These findings were well supported by our findings in this study. Specifically, we used BaP, a major DEP component, and Der f 1, the highly related and major natural allergen purified from HDM,[57] but not heterogeneous mixture of DEP or HDM. By using BaP and Der f 1, we found very similar results that neither BaP nor Der f 1 alone had any effect on airway hyper-responsiveness and lung inflammation, but BaP co-exposure with Der f 1-induced airway hyper-responsiveness, accumulation of cell infiltrates, particularly eosinophils, and increased mucus production and BAL Th2 cytokines. Furthermore, it has been suggested that low-dose BaP (≤1 pM) can enhance allergic airway inflammation by facilitating Th2 responses, and a high BaP dose (20 pM) may contribute to activating both Th1 and Th2 responses.[23] Thus, both low dose (1 pM) and high dose (20 pM) were applied to our mouse model. We found that both low dose and high dose of BaP co-exposure enhanced Der f 1-induced Th2, but not Th1 and Th17 responses, in a dose-dependent manner. These findings raise the possibility that BaP alone may induce either Th1 or Th2 responses depending on the levels of BaP exposure, but BaP co-exposure with Der f 1 induces a Th2 immune response and Th2-associated lung inflammation irresponsive of levels of exposure.

The significance of our study is that it provides a potential link between exposure to environmental pollutants and allergens and the development or worsening of allergic asthma. The airway epithelium is the lung's first line of defense against inhaled allergens and pollutants and plays an important role in the innate immune responses.[58] It is recognized that epithelial-derived cytokines IL-33, IL-25, and TSLP enhance Th2-associated lung inflammation, airway remodeling, and pathological changes that are associated with asthma.[42-44] A recent study suggested that these cytokines can activate type 2 innate lymphoid cells (ILC2), which directly secrete Th2 cytokines.[59]

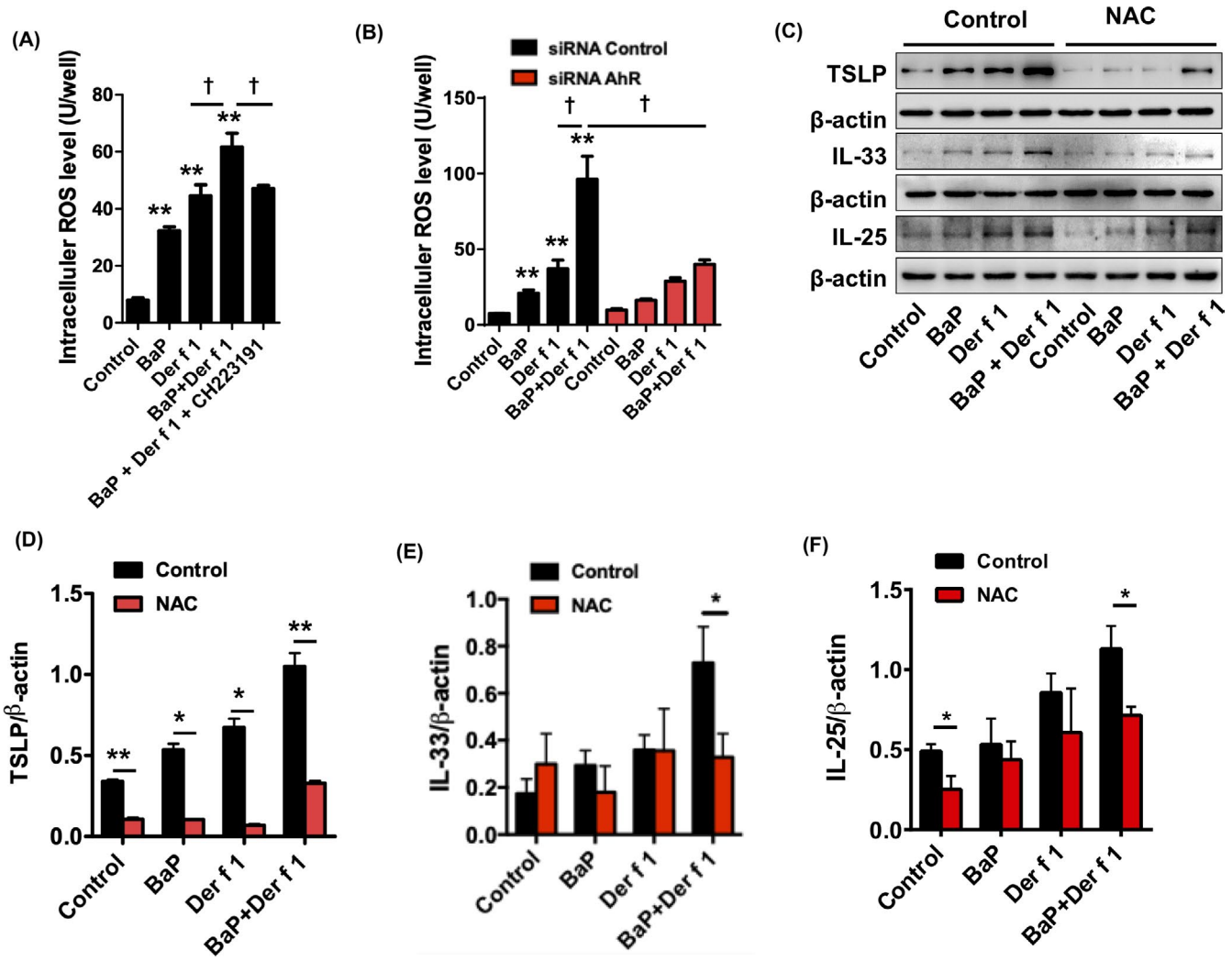


FIGURE 7 Aryl hydrocarbon receptor mediates BaP-induced ROS generation that is critical in BaP co-exposure-induced epithelial cytokine expression. A-B, Detection of intracellular ROS production with Reactive Oxygen Species Assay Kit in BaP and Der f 1-treated 16HBECs in the absence or presence of CH223191 (A) or 16HBECs with or without AhR knockdown (B). n = 3. $^{**}P < 0.01$ (compared to control). $^{\dagger}P < 0.05$. C, Representative immunoblots for BaP co-exposure-induced expression of epithelial cytokine expression in 16HBECs with or without NAC. D-F, Quantitative data were obtained by densitometric analysis for the expression of TSLP (D), IL-33 (E), and IL-25 (F) relative to β-actin. Data represent mean ± SEMs of two independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ [Color figure can be viewed at wileyonlinelibrary.com]

Thus, it is tempting to speculate that BaP co-exposure may challenge airway epithelial cells to release cytokines, leading to the activation of ILC2 that contributes to tissue eosinophilia, airway hyper-responsiveness, and Th2-associated lung inflammation. Indeed, we found increased expression of TSLP, IL-33, and IL-25 in mice, particularly in the airway epithelial layer, after exposure to BaP and Der f 1. These findings were well supported by several previous reports, including the upregulation of TSLP, IL-33, and IL-25 in DEP-treated primary bronchial epithelial cells,[45] and in asthmatic epithelium.[60-62] A very recent study reported that allergen challenge highly increased expression of IL-25, IL-33, and TSLP in the bronchial epithelium and submucosa that correlate with the extent of the late phase of airway obstruction.[63] Thus, these molecules may be considered as potential molecular targets for the inhibition of allergen-induced airway inflammation and obstruction. Indeed, neutralization of TSLP with an anti-TSLP mAb has been shown to reverse airway inflammation, prevent structural alterations, and decrease airway hyper-responsiveness and TGF-β1 level.[64] Taken together, these findings demonstrate that these epithelial-derived cytokines may play a pivotal role in the initiation of Th2-associated lung inflammation in BaP co-exposure-induced allergic asthma.

One of the central goals of this study is to explore the molecular mechanisms underlying the BaP co-exposure-induced epithelial cytokine release. AhR is a receptor and transcription factor that is critical in xenobiotic metabolism[65] and has a key function in immunity. Our previous studies suggest a critical role for AhR in mediating allergen-induced and ROS-dependent degranulation and IgE-mediated mast cell activation.[39-41] Our data in this study provide evidence that AhR is a key molecular regulator for BaP and allergen co-exposure-induced TSLP and IL-33 expression. In particular, we found that BaP co-exposure exacerbated Der f 1-induced the activation of AhR signaling as determined by expression of AhR and

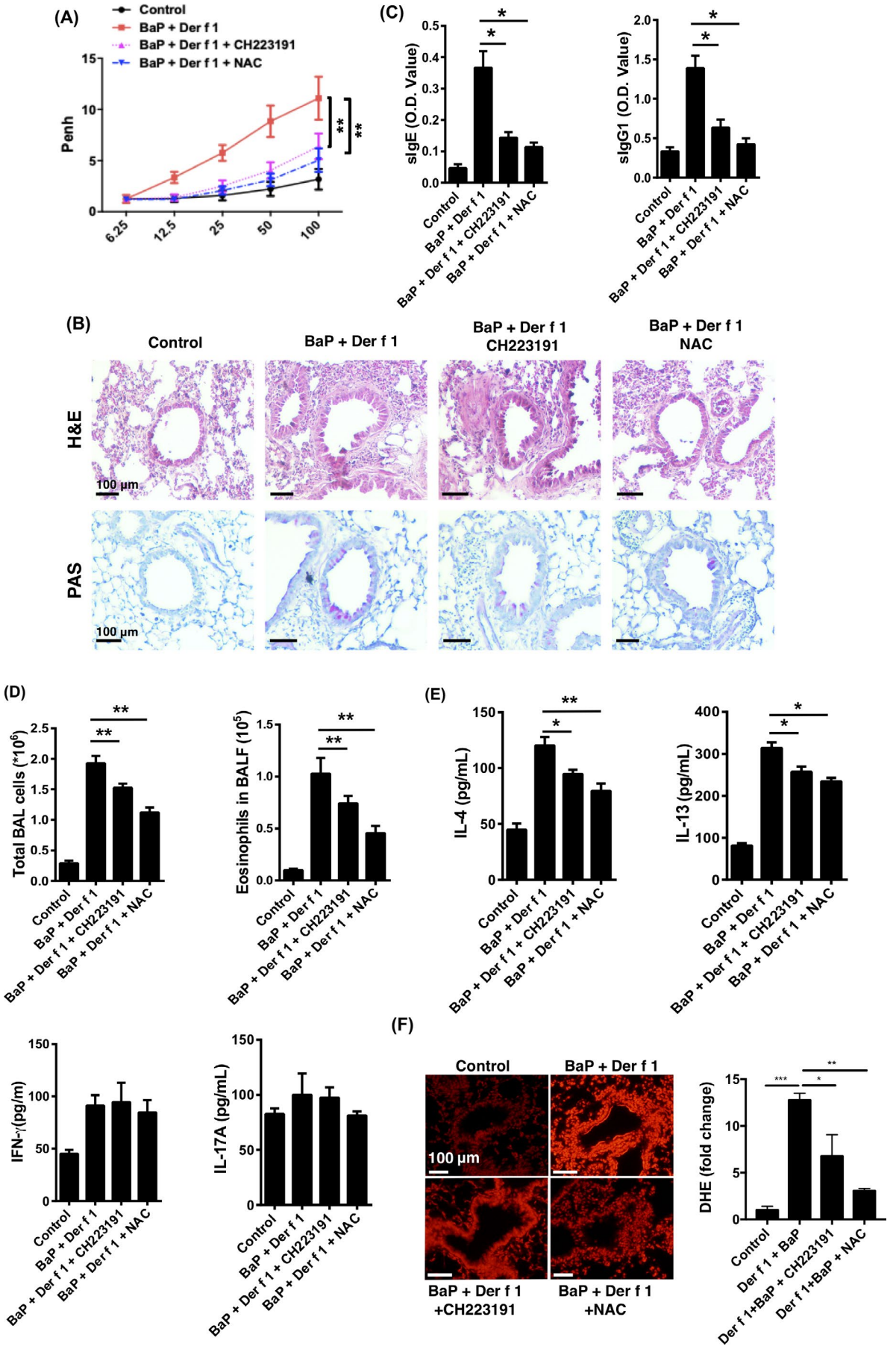

(A)
Control
BaP + Der f 1
BaP + Der f 1 + CH223191
BaP + Der f 1 + NAC
Penh
6.25
12.5
25
50
100
(B)
Control
BaP + Der f 1
BaP + Der f 1 CH223191
BaP + Der f 1 NAC
H&E
PAS
100 μm
(C)
sIgE (O.D. Value)
sIgG1 (O.D. Value)
(D)
Total BAL cells (*10^6)
Eosinophils in BALF (10^5)
(E)
IL-4 (pg/mL)
IL-13 (pg/mL)
IFN-γ(pg/m)
IL-17A (pg/mL)
(F)
BaP + Der f 1 +CH223191
BaP + Der f 1 +NAC
DHE (fold change)
Der f 1 + BaP
Der f 1+BaP + CH223191
Der f 1+BaP + NAC

FIGURE 8 Aryl hydrocarbon receptor antagonist or ROS inhibitor suppresses BaP co-exposure-induced lung inflammation. A, Effect of CH223191 or NAC on BaP co-exposure-induced airway hyper-responsiveness (AHR) as detected by average Penh. B, Paraffin tissue sections of lung were stained with H&E (upper panel) and periodic acid-Schiff (PAS, lower panel). C, ELISAs for serum levels of Der f 1-specific IgE and IgG1. D, Bronchoalveolar lavage (BAL) total inflammatory cells and eosinophil cells as assessed by Wright-Giemsa staining. E, ELISA analyses for BAL levels of Th1/Th2/Th17 cytokines. F, Representative images of dihydroethidium-stained (DHE-stained) mouse lung tissues and quantitative data for ROS expression as expressed by DHE fold change. Data represent mean ± SEM (n = 6/group), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

CYP1A1. Furthermore, BaP co-exposure also enhanced Der f 1-induced TSLP and IL-33 expression in airway epithelial cells, and intriguingly, the increased expression was significantly inhibited by AhR antagonist CH223191 or impaired in cells with AhR knockdown. These data indicate that AhR activation may be essential in mediating BaP-promoted Der f 1-induced TSLP and IL-33 expression in the airway epithelial cells. Interestingly, although BaP co-exposure potentiated Der f 1-induced IL-25 expression in the airway epithelial cells of asthma mouse model, this was not found by in vitro analysis. Particularly, there was no dose-dependent induction in BaP-treated 16HBECs, and also no potentiation for BaP co-exposure with Der f 1 for IL-25. However, we found that Der f 1 alone can induce IL-25 expression, representing that there may be distinct mechanisms for the regulation of TSLP, IL-33, and IL-25 production in BaP or Der f 1-treated airway epithelial cells.

It is well documented that exposure to environmental pollutants (eg, PM2.5) can induce an oxidative stress and impair lung function in asthmatic patients.[66-70] ROS are important mediators that may contribute to oxidative damage and chronic inflammation in allergic diseases. [71,72] The airways of asthma patients and allergen-challenged mice contain high levels of ROS that appear to contribute to asthma.[73,74] Intriguingly, we found that environmental allergens (eg, cockroach allergen) can induce ROS production, particularly mitochondrial-derived ROS, by inducing COX-2 expression in airway epithelial cells.[48] In this study, we provide not only supporting evidence that both BaP and Der f 1 alone can induce ROS generation, but also a novel evidence that BaP co-exposure further enhanced Der f 1-induced ROS production in our mouse model or airway epithelial cells in vitro, further supporting that ROS may be key mediators in mediating BaP co-exposure-induced epithelial TSLP, IL-33, and IL-25 release and lung inflammation. However, the source of ROS still remains unknown. Mitochondrial ROS is important for promoting asthma because mitochondrial targeted antioxidant mitoTEMPO showed protection from allergen-induced asthma.[48,73,75,76] Additionally, NADPH oxidase may also be important.[73] $p40^{phox}$, a NADPH oxidase subunit, has been recently identified to be a novel transcriptional target of AhR.[77] Thus, future studies are needed to determine how and where ROS is generated and possible mechanisms in response to environmental pollutants or allergens. Additionally, we found that both BaP and Der f1 alone induced remarkable ROS generation, but did not see the apparent release of TSLP, IL-33, and IL-25 in lung. Although the reason is not clear, we postulate that either BaP- or Der f 1-induced ROS are not sufficient to trigger cytokine production, but exposure of airway epithelial cells to combined BaP and Der f 1 can produce larger amounts of ROS that cause oxidative damages in DNA, lipids, proteins, and other cellular components, leading to the production of TSLP, IL-33, and IL-25. Thus, it would be of interest to confirm the hypothesis and investigate the underlying mechanisms regarding the ROS-induced expression of epithelial TSLP, IL-33, and IL-25 in the future.

Most importantly, we tested our hypothesis that AhR signaling as an upstream may control ROS generation, and the increased ROS may subsequently regulate downstream immune responses. As hypothesized, we found that BaP co-exposure-induced increased ROS generation was significantly inhibited in the presence of AhR antagonist CH223191 or cells with AhR knockdown. Furthermore, we demonstrated that ROS is involved in the upregulation of BaP co-exposure-induced increased expression of TSLP, IL-33, and IL-25. These studies support our novel hypothesis that the AhR-ROS axis may be critical in regulating BaP and Der f 1 co-exposure-induced epithelial cytokine expression and subsequently Th2-associated lung inflammation. Indeed, our asthma mouse model demonstrated that treatment with either CH223191 or NAC before BaP co-exposure significantly suppressed AHR and lung inflammation with reduced levels of Th2 cytokines. However, a recent study demonstrated that AhR can bind to the promoter sites of IL-33, IL-25, and TSLP in cultured primary human epithelial cells using chromatin immunoprecipitation assay,[45] implying that AhR may directly regulate IL-33, IL-25, and TSLP. Thus, while we believe that the AhR-ROS axis may be a key pathway in the regulation of TSLP and IL-33 expression, other mechanisms are also possible for the AhR-involved regulation of epithelial cytokine release.

In summary, our findings provide evidence that BaP can enhance Der f 1-induced airway hyper-responsiveness, lung inflammation, and oxidative stress. Specifically, we identified TSLP, IL-33, and IL-25 as major epithelial-derived cytokines that play a pivotal role in the initiation of Th2-associated lung inflammation in BaP co-exposure-induced allergic asthma. Furthermore, we suggest a novel mechanism by which BaP co-exposure with Der f 1 can activate AhR signaling, which may control ROS generation and TSLP and IL-33 expression. Alternatively, Der f 1, through its receptor (s) [eg, PAR2, C-lectin receptors (CLRs)], may promote the BaP-induced ROS through AhR signaling and subsequently IL-25. These increased expressions of TSLP, IL-33, and IL-25 will lead to Th2-associated inflammation through either ILC2 or DCs (See graphic summary). Taken together, our findings provide supporting evidence that BaP facilitates Der f 1-induced epithelial cytokine release through the AhR-ROS axis. Our data open the door to future studies targeting the AhR-ROS axis, which may yield therapeutic approaches for the treatment of allergic inflammation and asthma.

ACKNOWLEDGEMENTS

This study was supported by grants from The National Natural Science Fund (No. 81628001, 31770984), Guangdong Province Science and Technology Planning Project (No. 2017B020226006), Shenzhen Scientific Technology Basic Research Projects (No. JCYJ20160328144536436, JCYJ20170307162947583), Shenzhen Peacock Plan Team Project (No. 201703313000321), and Shenzhen Health Planning Commission Scientific Research Project (No. 201604130418). This work was supported by grants from the US National Institutes of Health (NIH) (RO1ES021739, R21 AI109062, R21 AI121768, and R21 AI137547). We also thank Karan for his proofreading.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

EW, XL, WT, and DD performed experiments, analyzed data, and review the manuscript. EW and PG wrote the manuscript. EW, PR, Z. L., and PG designed and supervised the study, and wrote the manuscript. HY, LY, DX, YZ, SH, and PY provided intellectual input and aided in the experimental design. All authors read and approved the final version of the manuscript.

ORCID

Pei-Song Gao https://orcid.org/0000-0002-7651-1339

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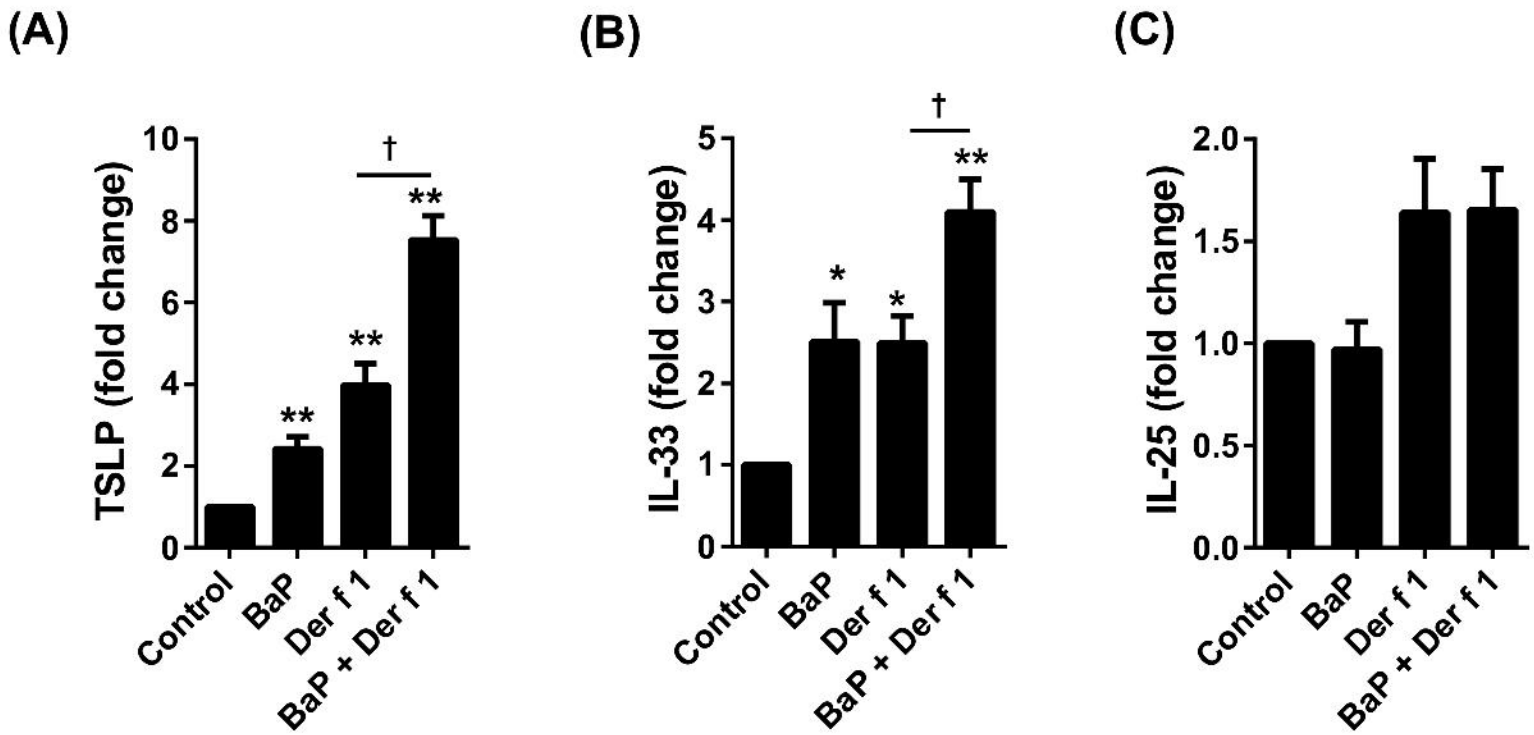
SUPPORTING INFORMATION

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

How to cite this article: Wang E, Liu X, Tu W, et al. Benzo(a) pyrene facilitates dermatophagoides group 1 (Der f 1)-induced epithelial cytokine release through aryl hydrocarbon receptor in asthma. *Allergy*. 2019;74:1675–1690. https://doi.org/10.1111/all.13784

S 1.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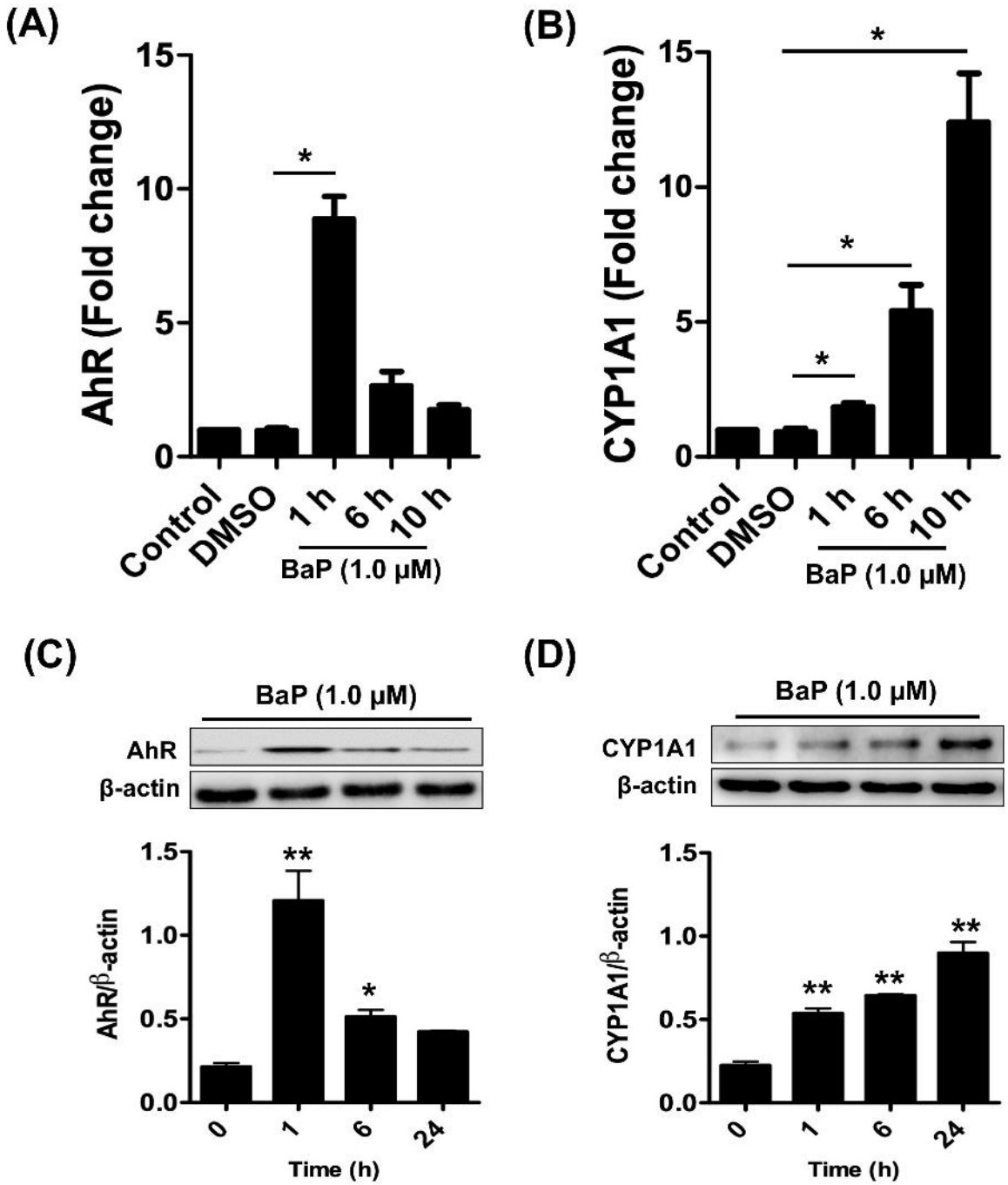
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β-actin
CYP1A1/β-actin
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0.5
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Time (h)

Online Repository

Benzo(a)pyrene Facilitates Dermatophagoides Group 1 (Der f 1)-Induced Epithelial Cytokine release through Aryl Hydrocarbon Receptor in Asthma

E. Wang[1,2#], X. Liu[2#], W. Tu[1,2#], D. C. Do[3], H. Yu[1], L. Yang[1], Y. Zhou[4], D. Xu[5], S. K. Huang[1,3,6], P. Yang[1,2], P. Ran[7*], P. Gao[3*], Z. Liu[1,2*]

[1]The Affiliated Luohu Hospital of Shenzhen University, Shenzhen Luohu Hospital Group, Shenzhen, China

[2]The State Key Laboratory of Respiratory Disease for Allergy at Shenzhen University, Shenzhen University School of Medicine, Shenzhen, China

[3]Johns Hopkins Asthma and Allergy Center, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA.

[4]Children's Hospital and Institute of Biomedical Sciences, Fudan University. Key Laboratory of Neonatal Disease, Ministry of Health, Shanghai, China.

[5]Institute of Infection, Immunity and Inflammation, University of Glasgow, Glasgow, UK.

[6]National Institute of Environmental Health Sciences, National Health Research Institutes, Miaoli, Taiwan.

[7]Guangzhou Institute of Respiratory Disease, State Key Laboratory of Respiratory Diseases. The First Affiliated Hospital, Guangzhou Medical University, Guangzhou, China.

METHODS

Air–liquid interface culture

Air–liquid interface (ALI) is an improved respiratory physiology model, which is used widely in *in vitro* to study recapitulate airway epithelial biology morphologically and transcriptionally.[1] 16HBE cells were grown at the ALI as described previously.[2,3] Briefly, 16HBE cells were cultured on collagen gel-coated Transwell chamber (Transwell-Clear, 24-mm diameter, 0.4-μM pore size; Corning3450). Cells with the number 10^5 were seeded in the upper compartment incubated in 1 ml Airway Epithelial Cell Basal Medium (BEGM, ATCC), and 2 ml medium was filled in the lower compartment. Cells were used after 21 days to fully differentiate into mature epithelial cells, as manifested by the formation of more mucociliary epithelium[4]. These mature cells were then treated with BaP (1.0 μM), Der f 1 (30μg/ml) or combined, and harvested at various time points. Total RNA was extracted for RT-PCR analysis.

REFERENCE

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ONLINE FIGURE LEGENDS

FIGURE S1. *In vitro* analysis of epithelial cell cytokine release in BaP co-exposure with Der f 1-treated Air Liquid Interface cultured (ALI) 16HBECs. A-C, RT-PCR analysis for the expression of TSLP (A), IL-33 (B), and IL-25 (C). Data represent mean ± SEMs of two independent experiments. *$P<0.05$, **$P<0.01$ (compared to control). $^{\dagger}P<0.05$ (compared to Der f 1).

FIGURE S2. BaP exposure induces the activation of AhR signaling in 16HBECs. A-B, RT-PCR analysis for the expression of AhR (A) and CYP1A1 (B) in BaP-treated 16HBECs at varying time points. C-D, Representative Immunoblots for BaP-induced expression of AhR (C) and CYP1A1 (D) in 16HBECs. Quantitative data was obtained by densitometric analysis for AhR (C) and CYP1A1 (D) expression relative to expression of β-actin. Data represent mean ± SEMs of two independent experiments. *$P<0.05$, **$P<0.01$.

TABLE S1: Primers used for RT-PCR assay

Gene	GenBank Accession	Primer Sequence	Amplicon Size	PrimerBank ID*
Ahr	NM_00121	Fwd:5'CAAATCCTTCCAAGCGGCATA Rev: 5'CGCTGAGCCTAAGAACTGAAAG	123	229577137c2
Cyp1a1	NM_000499	Fwd:5'TCGGCCACGGAGTTTCTTC Rev:5' GGTCAGCATGTGCCCAATCA	141	189339226c1
TSLP	NM_138551	Fwd:5' ATGTTCGCCATGAAAACTAAGGC Rev: 5' GCGACGCCACAATCCTTGTA	162	372466598c1
IL-33	NM_001199641	Fwd: 5'GTGACGGTGTTGATGGTAAGAT Rev: 5'AGCTCCACAGAGTGTTCCTTG	94	314122336c1
IL-25	NM_022789	Fwd:5'CAGGTGGTTGCATTCTTGGC Rev:5'GAGCCGGTTCAAGTCTCTGT	249	291045205c1

Primer identification number given by PrimerBank, a PCR primer database for quantitative gene expression analysis (https://pga.mgh.harvard.edu/primerbank/).

TABLE S2: Antibodies used for western blotting and immunostaining

Antibody	Clone	Specie	Application	Source
AhR	D5S6H	Rabbit	WB (1: 1000)	Cell Signaling Technology
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