



The impact of benzo[a]pyrene on murine allergic airway inflammation via epigenetic remodeling

Xihua Wang^{b,1}, Shuyuan Guan^{b,1}, Lingbin Sun^{a,b,*}, Zhongliang Dai^{a,**}

^a The Department of Anesthesiology, The Second Clinical Medical College (Shenzhen People's Hospital), Jinan University, Shenzhen, China

^b Department of Anesthesiology, Peking University Shenzhen Hospital, Shenzhen, China

ARTICLE INFO

Edited by Dr. Malcolm D. Tingle

Keywords:

Benzo[a]pyrene

Allergic airway inflammation

Epigenetic remodeling

ABSTRACT

Simultaneous exposure to both BaP and house dust mites (HDM) has been shown to exacerbate pulmonary inflammation and hyperresponsiveness in a murine asthma model. The mechanistic insight into epigenetic inheritance for this effect, however, remains to be clarified. As such, in this study, we explore the molecular basis for the enhancement of asthma. Female BAL/C mice were intranasally administered HDM (25 µg in 25 µL saline) and/or BaP (10 µg/kg) every other day for 9 weeks. RNA sequencing and DNA methylation assessment were used to explore the underlying mechanism. Following simultaneous exposure to HDM and BaP, mice exhibited pulmonary inflammation and the transcript level of IL4i1b, muc4 and IL22ra2 that were associated with altered DNA methylation, suggesting that there may be an epigenetic basis for BaP-induced asthma exacerbation. Our data suggest that DNA methylation is a major epigenetic modification that accompanies airway remodeling associated with changes in the allergic mice.

1. Introduction

Asthma is a chronic respiratory disease that affects anywhere from 1% to 18% of individuals in different populations (Zinellu et al., 2019). The symptoms of asthma are primarily mediated by abnormal type-2T helper cell (TH2) responses that cause aberrant pulmonary inflammation, mucus secretion, airway eosinophil proliferation, and airway hyperreactivity (AHR) in response to particular stimuli (Umetsu et al., 2002). Rates of asthma have risen rapidly in recent decades, and this change has been attributed to industrialization, environmental changes, and lifestyle alterations in many different populations (Huang et al., 2019). The increased prevalence of asthma has, for example, been suggested to be attributable to increased exposure to air pollution and tobacco smoke, given that population-level genetic changes occur too slowly to explain this pathological phenomenon (Eder et al., 2006).

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants that are produced when organic matter undergoes partial combustion, with PAH generation primarily being driven by combustion engine activity, industrial activity, and home heating (Phillips, 1999). Benzo[a]pyrene (BaP) resulting from natural processes

such as forest fires and volcanoes and anthropogenic activities including diesel vehicle exhaust, municipal and industrial wastewater effluents, oil spills and coal combustion, are widespread environmental pollutants (Rubin, 2001). Humans can be exposed to BaP by through the inhalation, dietary ingestion, and non-dietary ingestion pathways. This was explored by comparing a simulated hypothetical cigarette smoking regimen to a steady-state intake via dietary exposure using a daily dose of 3.36×10^{-3} µg/kg/day (EFSA, 2008). BaP is the best-studied PAH, and is commonly used as a prototypical carcinogenic PAH in experimental contexts. Both epidemiological and experimental evidence indicates that environmental pollutant exposure can result in respiratory deterioration and the increased severity of asthma and other respiratory diseases (Dick et al., 2014; Alessandrini et al., 2006; Bönisch et al., 2012). High concentrations of ambient B[a]P (6.3–8.5 ng/m³) were associated with 218% increase in the odds of the asthma diagnosis (95% CI of aOR, 1.77–5.71) (Choi et al., 2017). BaP alone stimulation upregulated the respiratory disorders associated-MUC5AC mRNA and protein levels and activated the ERK pathway in vitro (Sun et al., 2021). The effects of intratracheal BaP exposure can, at least in part, enhance allergic airway inflammation by facilitating Th2 responses and activating mediastinal

* Corresponding author at: The Department of Anesthesiology, The Second Clinical Medical College (Shenzhen People's Hospital), Jinan University, Shenzhen, China.

** Corresponding author.

E-mail addresses: slbaaa@163.com (L. Sun), daizhongliang@jnu.edu.cn (Z. Dai).

¹ The authors contributed equally to this work.

lymph node cells (Yanagisawa et al., 2016). Compared with Derf 1, 20 pmol BaP co-exposure with Derf 1 led to airway hyper-responsiveness and increased lung inflammation in mouse model of asthma (Wang et al., 2019). The epigenetic activation or silencing of immune-related genes has been shown to occur as a consequence of prenatal exposure to microbial infections, dietary factors, tobacco smoke, and other environmental pollutants, potentially shaping systemic immunity in both physiological and pathological contexts. Indeed, such epigenetic changes are thought to have a significant impact on health outcomes owing to altered downstream gene transcription, and altered patterns of CpG DNA methylation have previously been linked to a range of atopic diseases including asthma (Miller and Peden, 2014; DeVries and Verceci, 2015). Indeed, hundreds of CpG sites have been shown to exhibit patterns of differential methylation when comparing blood samples from adults with allergic and non-allergic asthma (Hoang et al., 2020). The development of a better understanding of these asthma-related epigenetic variations is thus essential.

Herein, we evaluated the association between BaP exposure and allergic asthma induced by house dust mite (HDM) extract exposure. We found that BaP and HDM co-exposure resulted in increased inflammation and AHR severity in these mice relative to HDM exposure alone. We then performed DNA methylation sequencing analyses in lung to identify patterns of differential methylation among mice in our HDM, BaP, and BaP-HDM treatment groups.

2. Material and methods

2.1. Chemicals

BaP (>96% pure) was purchased from Sigma-Aldrich (MO, USA) and was prepared at 0.1 mg/mL in methanol (Sigma-Aldrich). HDM extract was obtained from Greer Laboratories (Lenoir, NC) and was resuspended in normal saline.

2.2. Animals

The Institutional Animal Care and Use Committee of Jinan University approved all studies described herein. Combining results of higher antigen-specific serum IgE levels, higher numbers of eosinophils and more severe allergic inflammation, female BALB/c mice were chosen for study (Blacquiere et al., 2010). Female mice (BALB/c, 5 weeks old) were purchased from the Medical Laboratory Animal Centre of Guangdong Province (approval No. SCXK (Yue) 2008-0002), and were allowed to

acclimate to the climate-controlled laboratory setting (12-h light-dark cycle, 55 ± 5% humidity, 23 ± 1 °C) with free food and water access.

2.3. Murine asthma model establishment

Our approach to BaP co-exposure in HDM-induced asthma model mice was achieved as detailed previously (Wang et al., 2019). Briefly, BALB/c mice (n = 12 per group) were intranasally instilled with 10 µg/kg BaP or 0.2% methanol (vehicle) under light isoflurane anesthesia every other day for 61 days beginning on day 1. In addition, mice were anesthetized and intranasally instilled with HDM extract (25 µg) in 25 µL of saline every other day (day 2 - day 60). On day 62, the hyper-responsivity of murine airways to methacholine (Mch) (Sigma-Aldrich) exposure was assessed as detailed below (Fig. 1A). On day 63, bronchoalveolar lavage fluids (BALF) samples were obtained from these mice, and a flow cytometer (LSR II; BD Biosciences) was used to profile the cells present therein. Blood samples were collected from euthanized mice via cardiac puncture with a 20-gauge cannula, and serum was isolated via centrifugation using serum tubes (Sarstedt, Helsingborg, Sweden) prior to storage at -20 °C for further analyses. The left lung lobe was fixed overnight with 4% formaldehyde (Histolab, Stockholm, Sweden), while the upper and middle lobes of the right lung from each mouse were snap-frozen and stored at -80 °C for further processing.

2.4. Measurement of AHR

Lung resistance was assessed via invasive plethysmography following methacholine (Mch) (Sigma-Aldrich) inhalation at 24 h following the final challenge using a Buxco RC system (Buxco Electronics) as detailed previously (Venkateshaiah et al., 2018). Briefly, mice were exposed to PBS or to 3.125, 6.25, 12.5 mg/mL Mch, and percentage curved for Penh values were plotted for these different Mch doses.

2.5. Quantification of HDM-specific IgG1 and IgE levels

Blood samples were spun at 3000 rpm for 6 min, after which supernatant plasma was collected and stored at -20 °C until used to analyze levels of total IgE, IgG1, and IgG2a therein via ELISA using mAb antibody pairs BD Biosciences.

Levels of HDM-specific antibodies in murine serum were assessed by coating plates overnight with HDM extract (100 µg/mL) at 4 °C in 0.1 mol Na₂HPO₄. Plates were then blocked for 2 h using 10% FBS in

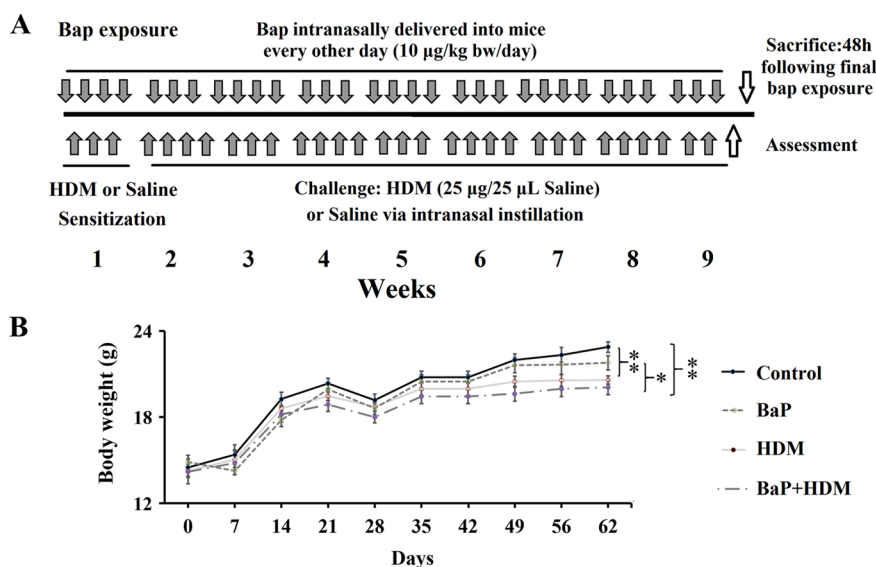


Fig. 1. Experimental process for intratracheal instillation of benzo[a]pyrene (BaP) and house dust mites (HDM) extract. (A) Experimental protocol for BaP and HDM exposure induced lung inflammation in mouse model. Six-week-old female mice were either exposed to BaP, HDM, BaP-HDM or 0.2% methanol for nine weeks. Lung sections were stained with H&E, periodic acid-Schiff (PAS), and Masson; Bronchoalveolar lavage fluid (BALF) was collected to assess the number of total cells, neutrophils, mononuclear cells, eosinophils, lymphocytes and cytokines (IL-4, IL-5, IL-17α); Serum was assayed for total IgE, HDM-Sp IgG1 by ELISA. (B) HDM and BaP-HDM-challenged mice exhibited decreased average body weight. All values are expressed as mean ± SEM (n = 9). *P < 0.05, **P < 0.01.

PBS at room temperature. Samples were then applied to individual wells in 10-fold serial dilutions (6 per sample, from 1:10 for 1:50 for IgG1). Following overnight incubation at 4 °C, plates were sequentially incubated with biotinylated detection antibodies (BD Bioscience) and Avidin Peroxidase, with Super Aqua Blue (eBioscience, CA, USA) being used for color development.

2.6. Cytokine measurement

BALF samples from mice were assessed to evaluate IL-1 α , TNF α , IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-17 α , IFN γ , LIX, and MIP2 concentrations using Milliplex assays (#MHSCTMAG-70K-milliplex, Merck Millipore, USA) based on provided instructions with a Luminex® 200™ System (Luminex Corporation, TX, USA) being used for detection.

2.7. Histological analyses

Following collection, lung samples were fixed for 24 h with 4% formalin, dehydrated with ethanol, and embedded in paraffin. Histopathological analyses were then conducted by staining samples with hematoxylin and eosin (H&E), PAS, and Masson's trichrome staining solutions.

2.8. qPCR

Total right lung RNA was isolated and cDNA was prepared with a Primescript 1st Strand cDNA Synthesis Kit (Takara Bio, Japan). All qPCR primers used herein and corresponding reaction details are shown in Table S1 (Supporting Information). Relative target gene expression was evaluated with the Relative Expression Software Tool (REST-MCS, version 2) (Pfaffl et al., 2002).

2.9. Western blotting

The middle lobes of the right lung proteins were isolated as detailed previously (Sun et al., 2019), combined with 4 $\times$ LDS sample buffer (NP0007, Invitrogen, USA), and boiled. Protein extracts (40 μ g/sample) were then separated using 4–20% Sure PAGE Bis-Tris gels (GenScript, Nanjing, China) and transferred to PVDF membranes (88585, Thermo Scientific, USA) that were subsequently blocked with 5% non-fat milk in TBST (pH 7.4) overnight at 4 °C, followed by incubation with primary antibodies specific for DNA methyltransferase 1 (DNMT1), DNA methyltransferase 3A (DNMT3A), DNA methyltransferase 3B (DNMT3B) (Abcam, UK), and β -actin (Cell Signaling Technology, USA). Blots were then probed with secondary antibodies at 37 °C, washed, and a chemiluminescence approach (Thermo Scientific) was used to detect protein bands with a ChemiDoc developer system (Bio-Rad). All densitometric measurements were conducted using ImageJ, with β -actin being used to normalize protein band intensity.

2.10. cDNA library construction and Illumina sequencing

Based on the previous method (Sun et al., 2012), 10 mg total RNA (a mixture of RNA from the BaP HDM and BaP-HDM group in equal ratios) was used to construct a cDNA library. Poly (A) mRNA was purified from total RNA using oligo (dT) magnetic beads. The cDNA library was sequenced on an Illumina HiSeq™ 2500 using paired-end technology in a single run. The differential gene expression in the lungs of mice exposed to BaP and HDM were analyzed for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. The quality of all steps was controlled in accordance with the recommendations of Illumina.

2.11. Quantification of global DNA methylation and hydroxymethylation

Levels of 5-methyl-cytosine (5-mC) and 5-hydroxy-methyl-cytosine

(5-hmC) were measured in gDNA samples (200 ng) prepared from lung tissue (Epigentek, Farmingdale, NY) (Shang et al., 2013).

2.12. DNA methylation status assessment

CpG islands within target gene promoter regions were identified using UCSC [GC percent > 50.0, observed/expected > 0.6] (Li and Dahiya, 2002). DNA samples were extracted from the lungs of mice, and a spectrophotometer was used to ensure that samples were sufficiently concentrated and pure (≥ 20 ng μ L⁻¹, OD260/280 = 1.7–2.0, OD260/230 ≥ 1.8). MethylTarget was then used to assess promoter methylation for target genes of interest using an Illumina next-generation sequencing approach in combination with bisulfite-based DNA methylation mapping by Genesky Biotechnologies Inc. (Shanghai, China). Bisulfite conversion of DNA was achieved with an EZ DNA Methylation-Gold Kit (ZYMO, CA, USA) followed by PCR (HotStart Taq polymerase, TaKaRa, Dalian, China), purification, and PCR product recovery (TIANGEN Gel Extraction kit, TIANGEN, Beijing, China). Samples were then sequenced.

2.13. Data processing

Data are mean $\pm$ SEM and were analyzed using two-way ANOVA followed by a Sidak's multiple comparisons test were performed using GRAPHPAD PRISM V7.00 software. $P < 0.05$ was the significance threshold for this study.

3. Results

3.1. Co-exposure to BaP and HDM is associated with reduced murine body weight

We began by evaluating the impact of BaP exposure on the severity of HDM-induced asthma in mice by intranasally exposing 6-week-old female BALB/c mice to BaP and HDM for 9 weeks. Murine body weight was monitored over time, and while low-level exposure to BaP alone failed to alter body weight, exposure to HDM and BaP-HDM was associated with significant decreases in body weight relative to control animals (Fig. 1B).

3.2. BaP co-exposure enhanced the severity of murine HDM-induced allergic airway inflammation

Histological analyses of H&E-stained lung tissue sections from mice exposed to BaP and HDM were next conducted, revealing that animals in the control group exhibited intact terminal bronchioles with no evidence of inflammation of the alveolar epithelial cells (Fig. 2A). Following exposure to low BaP concentrations, mice exhibited thickening of the bronchial wall with slight inflammatory cell infiltration. In line with prior results, mice in the HDM group exhibited evidence of abnormal pulmonary pathology including bronchial lumen narrowing, thickening of the basal layer (Fig. 2A), increased luminal mucus secretion, goblet cell hyperplasia (Fig. 2B), apparent fibrosis (Fig. 2C), and pronounced peribronchial inflammatory cell infiltration relative to the control group (Fig. 2A). Lung tissue samples from mice in the BaP-HDM group exhibited exacerbated pathological changes compare with the HDM-induced asthma severity (Fig. 2A and Fig. S1, Supporting Information).

We then collected BALF samples from these asthmatic model mice, revealing that more total cells, neutrophils and eosinophils were present in the lungs of mice in the BaP-HDM treatment group relative to the HDM group ($P < 0.05$) (Fig. 2 D, E, G). In addition, BaP-HDM group mice exhibited a trend towards increased mononuclear cells in their BALF, although the difference was not significant (Fig. 2F). Furthermore, HDM-BaP mice also exhibited increased airway resistance following methacholine exposure relative to HDM model animals (Fig. 2I).

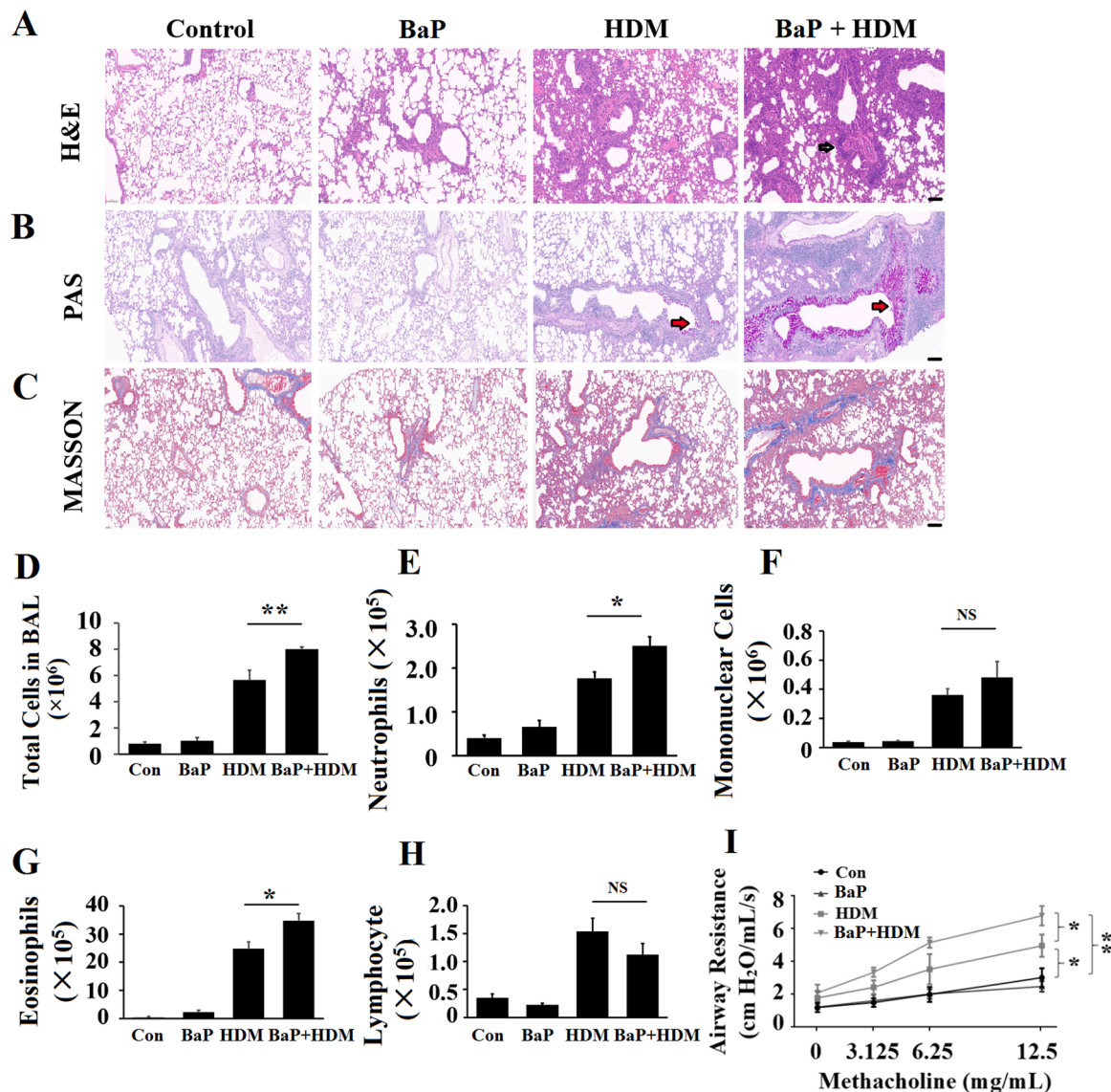


Fig. 2. BaP co-exposure exacerbates HDM-induced lung inflammation. (A–C) Histological changes of lung stained with H&E (upper panel), periodic acid-Schiff (PAS, middle panel), and Masson (lower panel) in male mice exposed to BaP and HDM for 9 weeks. Bar = 100 μ m; Black arrow: inflammatory cell infiltration; Red arrow: mucus secretion. Bronchoalveolar lavage fluid (BALF) was collected to assess the number of total cells (D) and neutrophils (E), mononuclear cells (F), eosinophils (G), and lymphocytes (H). (I) Effect of HDM and BaP on airway hyper-responsiveness (AHR) was monitored by average Penh. Methacholine concentration was increased from 3.125 to 12.5 mg/mL. Data are presented as mean $\pm$ SEM ($n = 5$). Asterisks (*) indicate that the values are significantly different from that of HDM group at $P < 0.05$ vs. HDM group as assessed using two-way ANOVA.

3.3. BaP co-exposure alters epithelial cytokine release

Next, we assessed differential chemokine and cytokine expression in the lungs of our HDM- and BaP-treated mice. Relative to control mice, those in the BaP group exhibited no significant changes in analyzed Th1 or Th2 cytokine levels in BALF samples, whereas IL-4, IL-5, and IL-17 α levels were significantly elevated in HDM and BaP-HDM mice relative to controls (Fig. 3A–C). As we found IL-5 levels to be significantly higher in the BALF of BaP-HDM mice relative to HDM model animals, our data suggested that BaP was able to exacerbate HDM-induced asthma in mice. The levels of IL-2, IL-6, IL-7, IL-1 α , TNF α , IFN γ , LIX and IL-10 did not reach statistical significance (Fig. S2, Supporting Information).

3.4. BaP exposure enhanced IgE production

Total IgE levels were significantly higher in the serum of BaP-HDM mice relative to serum from HDM model animals (Fig. 3D). Relative to

control mice, HDM-specific IgG1 levels were also significantly increased in the serum of mice from the HDM and HDM-BaP groups, and these levels differ significantly between these latter two groups ($P < 0.05$) (Fig. 3E).

3.5. Assessment of BaP co-exposure-associated changes in gene expression and DNMT1 and DNMT3 activity

How BaP and HDM impact DNA methylation and hydroxymethylation in the context of lung inflammation remains poorly understood. Therefore, we conducted western blotting assays to assess any potential changes in DNA methyltransferase expression in our different murine treatment groups. We found that BaP-treated mice exhibited significantly decreased pulmonary DNMT1 levels relative to controls, although in HDM and BaP-HDM mice these levels were comparable to those in controls (Fig. 4A, B). DNMT3a levels were also significantly altered in HDM groups (Fig. 4A, B). Pulmonary DNMT3b levels were

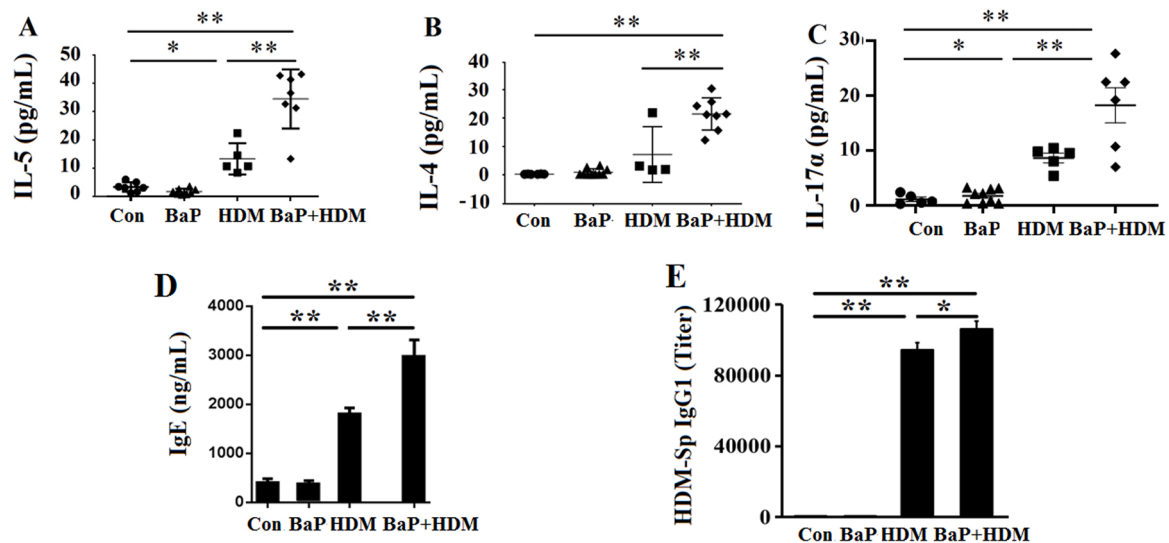


Fig. 3. Mice exhibit increased T helper type 2 (Th2) cytokines following BaP-HDM challenge. BALB/c mice were sensitized and challenged with BaP and HDM as described, and cytokines (A–C) in bronchoalveolar lavage (BAL) fluid were measured by multiplex assay as described in the Materials and methods. Results shown are mean $\pm$ SEM (n = 5) for four samples per group. * P < 0.05, ** P < 0.01. Increased total IgE and HDM-Sp IgG1 production in BaP and HDM-exposed mice. Serum isolated at times of sacrifice was assayed for total IgE (D), HDM-Sp IgG1 (E) by ELISA. Sp, specific. The data are expressed as mean $\pm$ SEM (n = 5). * P < 0.05, ** P < 0.01.

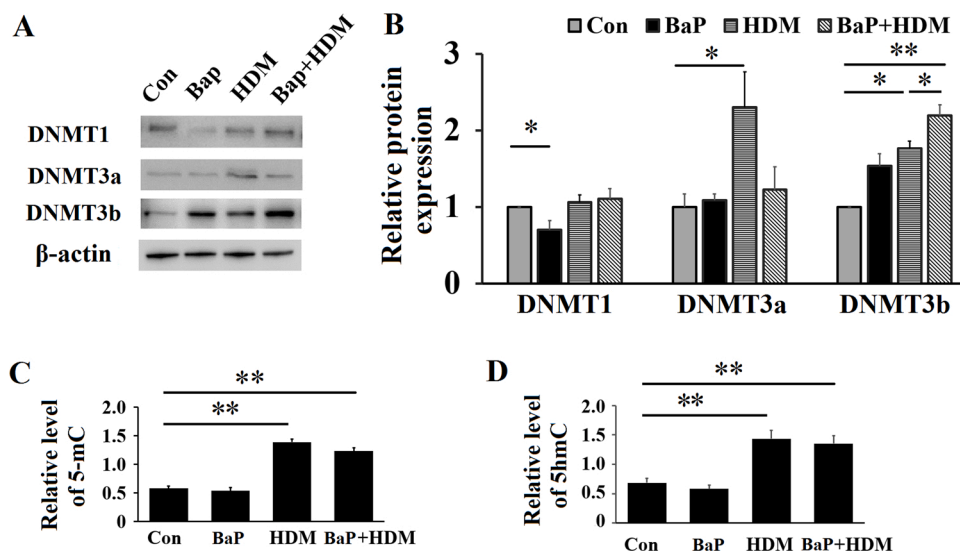


Fig. 4. BaP co-exposure with HDM induced DNA methylation through regulation of DNMT. (A) DNMT (DNMT1, DNMT3A, and DNMT3B) expression in BaP and HDM exposed mice analyzed by Western blot. (B) Expression was defined by fold change in relative to control. Band intensity was quantified by ImageJ software, and the values of target proteins were normalized to that of β -actin. BaP and BaP-HDM-exposed mice showed increase in level of both 5-methyl-cytosine (5-mC) DNA (C) and 5-hydroxy-methyl-cytosine (5-hmC) DNA (D) in lung. Results were expressed as mean $\pm$ SEM (n = 4). * P < 0.05, ** P < 0.01.

significantly higher in the BaP, HDM, and BaP-HDM mice relative to controls, with these levels being significantly higher in BaP-HDM mice relative to HDM mice, thus suggesting that BaP can promote DNMT3b expression and thereby alter DNA methylation in the context of asthma (Fig. 4A, B).

3.6. BaP alters global hypermethylation to promote asthma

Global methylation (mC) and hydroxymethylation (hmC) levels were next assessed to understand how BaP influences HDM-induced asthma pathogenesis, with hmC representing an intermediate in the context of the DNA demethylation that contributed to overall methylation dynamics. We found that both 5-mC and 5-hmC levels were significantly increased in the lung tissue of HDM and BaP-HDM mice relative to controls, consistent with altered pulmonary methylation (Fig. 4C, D). However, there were no significant differences between the HDM and BaP-HDM groups (Fig. 4C, D).

3.7. Assessment of differential gene expression in the lungs of mice exposed to BaP and HDM

To better understand how BaP co-exposure exacerbates HDM-induced allergic airway inflammation, RNA-Seq was next conducted to evaluate whole-genome transcriptomic changes in these mice. We identified 194 differentially expressed genes (DEGs) in pulmonary samples from BaP-HDM mice (178 upregulated, 16 downregulated) (Fig. 5A, B). The 194 DEGs in BaP-HDM group are shown in Table S2. The putative functions of DEGs from BaP-HDM group were analyzed according to gene ontology (GO). In the libraries, most of the corresponding biological process genes were involved in cell adhesion, ion transport and cell differentiation. Most of the cellular component genes encoded proteins associated with membrane, extracellular region and plasma membrane; and most of the molecular function genes were associated with protein binding and calcium ion binding (Fig. 5C). KEGG analyses revealed these DEGs to be associated with pathways including

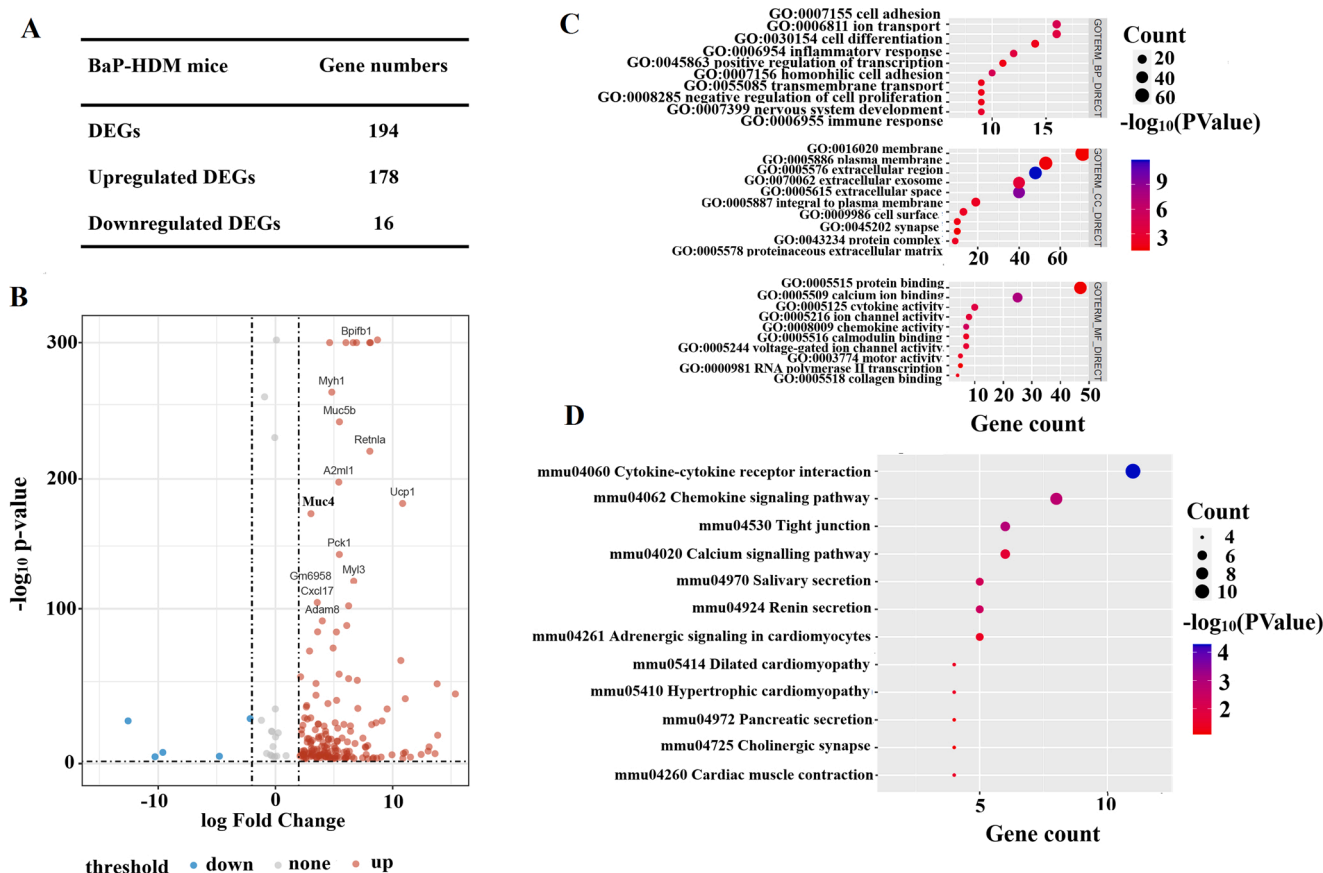


Fig. 5. Assessment of differential gene expression in the lungs of mice exposed to BaP and HDM. (A) Summary of the number of up- and down-regulated genes in the BaP-HDM-exposed lungs vs control. $n = 3$, Fold change ≥ 2 and adjusted P value ≤ 0.001 were used as the threshold to judge the significance of gene expression difference. (B) Volcano plot analysis shows the number of different and overlapped DEGs from BaP-HDM group. Blue represents down-regulated. DEGs, red represents up-regulated DEGs. (C) Functional annotation of 194 differentially expressed genes (DEGs) using STRING database. (D) Advanced bubble chart shows the top 12 significantly enriched KEGG pathways of differentially expressed genes in the lungs of mice administered the combination exposure of BaP and HDM. Y-axis label represents pathway, and X-axis label represents gene count. Size and color of the bubble represent amount of DEGs enriched in pathway and enrichment significance, respectively.

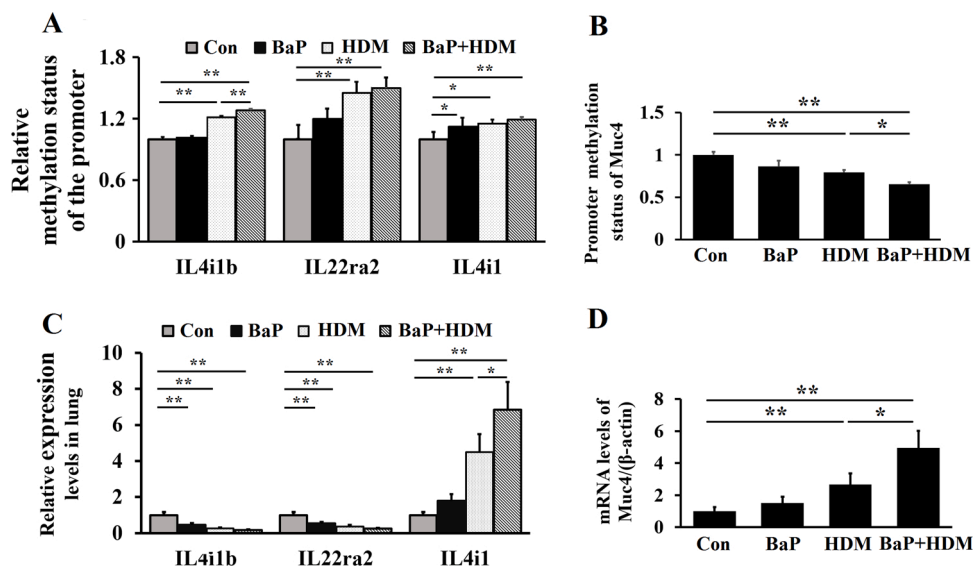


Fig. 6. BaP co-exposure with HDM led to the methylation percentage of IL4i1b, IL22ra2, IL4i1 (A) and Muc4 (B) and on the gene expression of IL4i1b, IL22ra2, IL4i1 (C) and Muc4 (D). Fold change of genes expression was normalized to β -actin expression and relative to controls ($n = 5$; $*P < 0.05$, $**P < 0.01$). All values are expressed as mean $\pm$ SEM.

cytokine-cytokine receptor interactions, chemokine signaling pathways, and tight junctions, in addition to other metabolic pathways including the calcium signaling pathway, salivary secretion, and renin secretion (Fig. 5D).

3.8. BaP treatment alters *IL4ilb*, *IL22ra2*, and *IL4i1* expression and methylation

We next evaluated the methylation status of three candidate genes associated with cytokine-cytokine receptor signaling and that were differentially expressed between BaP-HDM and control animals. When we assessed these three DEGs in lung samples from BaP-HDM mice, we found that *IL4ilb* and *IL22ra2* were downregulated while *IL4i1* was upregulated in these BaP-HDM animals (Fig. 6C). We further found that the CpG islands in all three of these genes were hypermethylated in lung samples from BaP-HDM-challenged mice. The methylation of *IL4ilb* level to be significantly higher in the lung of BaP-HDM mice relative to HDM model animals (Fig. 6A). Together, these data suggest that co-exposure to BaP and HDM may alter the epigenetic regulation of *IL4ilb*, *IL-4-induced gene 1 (IL4i1)*, and *IL22ra2* in murine lungs, thereby impacting the expression of these genes.

3.9. BaP treatment alters the methylation and expression of *Muc4*

Mucin family proteins include *Muc2*, *Muc4*, *Muc5ac*, and *Muc5b*. *Muc4* is expressed at high levels by goblet cells within the airway epithelium, making it an important focus of differential methylation analyses in the context of persistent childhood asthma (Frey et al., 2020). We observed *Muc4* hypomethylation in our analyses, consistent with the fact that it was upregulated at the mRNA level (Fig. 6B, D). Our research supported the previous results that *Muc4* mRNA levels of airway epithelium samples from patients with asthma have significantly upregulated relative to healthy control individuals (Ordoñez et al., 2001). The methylation of *Muc4* suggested that there may be an epigenetic basis for BaP-induced *Muc4* hypomethylation of HDM group (Fig. 6B).

4. Discussion

Several studies have highlighted a link between air pollution and increased asthma or allergy severity (Huang et al., 2015). The ability of BaP to alter different cell types in pathological contexts has been a focus of increasing research interest in recent years, but the mechanisms whereby such environmental pollutants can increase asthma severity remain unclear. Environment-induced changes in the epigenetic regulation of genes have been linked to the severity of a range of disease types (Ho, 2010). Exposure to high levels of BaP has been associated with a significant increase in asthma risk in children with susceptible *CTLA4*, *STAT4*, and *CYP2E1* genotypes (Choi et al., 2017), but the exact mechanistic basis for this effect is uncertain. Herein, we determined that BaP was able to increase airway hyperresponsiveness and pulmonary inflammation in an HDM-induced model of murine asthma. Our data suggest that such disease exacerbation may be linked to epigenetic remodeling, indicating that targeting such epigenetic regulatory pathways may be a novel approach to asthma treatment. We, therefore, conducted methylomic and transcriptomic analyses to better understand BaP-HDM co-exposure-induced epigenetic remodeling in our model mice, revealing that BaP exposure was linked to more severe allergic responses including IgE accumulation, increased AHR, and eosinophilia through mechanisms associated with cytokine-cytokine receptor and chemokine signaling pathways.

Allergic asthma is linked to the increased production of Th2 cytokines, mucus secretion, and AHR upon exposure to particular allergens, with symptoms arising through the effects of these different allergen-induced inflammatory mediators on local fibroblasts, smooth muscle tissue, and epithelial cells (Kuruvilla et al., 2019). BaP is a common

environmental pollutant and carcinogen that is often found in particulate matter $\leq 2.5 \mu\text{m}$ (PM_{2.5}) in size. Exposure to high BaP levels can enhance the dermatophagoides group 1 (Derf 1)-induced production of Th2 cytokines, although the epigenetic basis for such enhancement has yet to be clarified given that neither BaP nor Derf 1 in isolation have any impact on AHR or pulmonary inflammation, whereas exposure to both results in mucus production, Th2 cytokine production, and eosinophil infiltration in the lungs (Wang et al., 2019). For example, IL-5 is known to be a key regulator of eosinophil proliferation, adhesion molecule expression, and the consequent regulation of eosinophil degranulation and chronic airway inflammation in the context of bronchial asthma. Our study presented that BaP exposure aggravated HDM-induced Th2 cytokines in mice. While useful in some contexts, *in vitro* pulmonary cell lines are of limited value as a means of assessing epigenetic changes in the context of complex multicellular diseases such as asthma. Even so, data derived from prior *in vitro* studies suggest that PAH-induced environmental diseases are associated with fetal epigenetic reprogramming (Tang et al., 2012). We found that low-dose BaP exposure increased the severity of HDM-induced Th2 inflammation in mice through a mechanism correlated with changes in DNA methylation, suggesting that while BaP alone does not alter Th1 or Th2 responses, BaP+HDM co-exposure may alter the methylation status of genes associated with lung inflammation, thereby inducing a Th2 response. As such, targeting these pathways to facilitate epigenetic reprogramming may represent a viable approach to alleviating BaP-HDM induced airway remodeling and disease.

Our data suggest that altered *IL4ilb*, *IL4i1*, *IL22ra2*, and *Muc4* methylation may be associated with BaP-HDM-induced changes in allergen-induced AHR. *IL4ilb* is in *IL-4-induced L-amino acid oxidase* protein that is secreted from cells and that can catabolize L-phenylalanine and L-arginine. *IL4i1* is an immunosuppressive lysosomal protein secreted by dendritic cells and macrophages that is reported to be important in the context of antigen presentation, M2 macrophage differentiation, and the suppression of Th1, Th2, and Th17 responses (Santarasci et al., 2012). Prior studies have explored the role of *IL4i1* in the context of AHR and tumorigenesis, but its role in the context of chronic respiratory disease has yet to be elucidated (Copie-Bergman et al., 2003). We observed *IL4i1* overexpression and hypomethylation in pulmonary tissue samples from BaP-HDM-co-exposed mice. However, BaP+HDM group showed that the elevated *IL4i1*-mediated immunosuppression and exacerbated allergic asthma. We inferred that *IL4i1* may participate in the negative feedback regulation of allergic asthma. As such, more research is required to elucidate the role of *IL4i1/IL4ilb* in the context of asthma pathogenesis.

Muc4 encodes a mucin protein that is expressed in epithelial cells within the nasal and bronchial pathways in healthy individuals (Ma et al., 2018). We found that the *Muc4* promoter exhibited a 34% reduction in methylation in pulmonary tissues from mice exposed to BaP+HDM relative to control mice, and that this coincided with an increase in *Muc4* expression. *Muc4* hypomethylation has been linked to BaP-induced *Muc4* overexpression in HDM-induced mice. The gene encoded by *Muc4* consists of the *Muc4 α* and *Muc4 β* subunits, with the former being a hyperglycosylated extracellular subunit and the latter being a transmembrane subunit that contains three epidermal growth factor (EGF)-like domains and a short CT fragment that may facilitate interactions between proteins (Jonckheere and Van Seuningen, 2008; Milara et al., 2017). *Muc4* has previously been found to be upregulated in inflammatory contexts following fungal, bacterial, or rhinovirus-induced injury (Kim et al., 2011), and it is also overexpressed in the bronchial epithelium of patients with severe uncontrolled asthma relative to healthy controls or to those with well-controlled disease (Hattori et al., 2013). *Muc4* can also impair the anti-inflammatory impact of corticosteroids in individuals suffering from chronic rhinosinusitis with nasal polyps (Milara et al., 2017). The *IL-4/Muc4* axis is thought to be a key determinant of chronic AHR in individuals with allergic asthma (Damera et al., 2006).

We speculated that the development of AHR may be linked to epigenetic changes that modulate gene expression patterns within the airways of HDM-treated mice. Thus, we assessed patterns of gene expression in lung samples from mice in our different samples groups, although these experiments are inherently limited by the cell-type heterogeneity within overall pulmonary tissues. We additionally assessed promoter methylation status in these tissue samples. Additional research will be necessary to examine the cell-type-specific changes in gene expression and methylation in the lungs of BaP+HDM co-exposed mice.

5. Conclusion

In summary, in the present study, we conducted an analysis of the epigenetic basis for BaP-HDM-induced allergic asthma. We determined that chronic BaP exposure can exacerbate HDM-induced allergic asthma associated with epigenetic changes affecting genes related to cytokine-cytokine receptor interactions and airway mucus hypersecretion, including IL-4, IL-5, Muc4 and IL-17 α , which are important drivers of pathogenic Th2-associated lung inflammation in this model of allergic asthma. While we did not evaluate chronic airway wall thickening or ASM hyperplasia in our chronic model of murine asthma, we did find that multiple genes associated with AHR and airway inflammation exhibited epigenetic reprogramming in mice following BaP+HDM exposure. Further research will be necessary to establish whether these modifications can be suppressed by the pharmacological inhibition of methylation. In addition, *in vitro* and *in vivo* analyses of transgenic mice will be essential to characterize the functional roles of IL4i1b, IL4i1, IL22ra2, and Muc4 in the context of BaP-HDM-induced AHR and lung inflammation.

CRedit authorship contribution statement

Xihua Wang designed the study, performed and analyzed all experiments, and wrote the manuscript. Shuyuan Guan performed the immunoblots and qRT-PCR. Lingbin Sun and Zhongliang Dai supervised the study and edited the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China 81703263 (L.S.), Shenzhen Science and Technology Foundation JCYJ20170307095506831 (L.S.), JCYJ20190807155005597 (L.S.).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.etap.2021.103782](https://doi.org/10.1016/j.etap.2021.103782).

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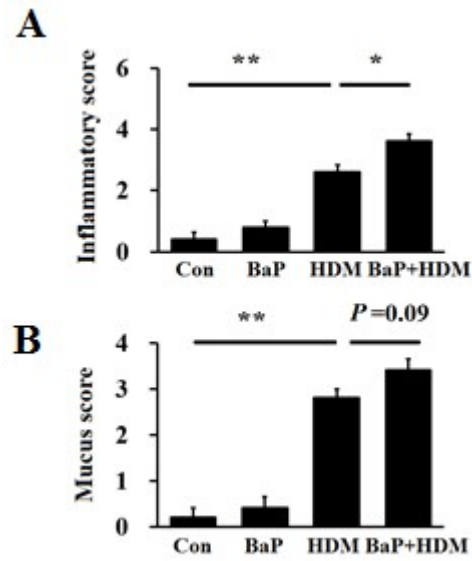


Fig. S1. Quantitative analyses of inflammatory cell infiltration (A) and mucus production (B) in lung sections were performed. Results shown are mean $\pm$ SEM (n = 4). * P < 0.05, ** P < 0.01.

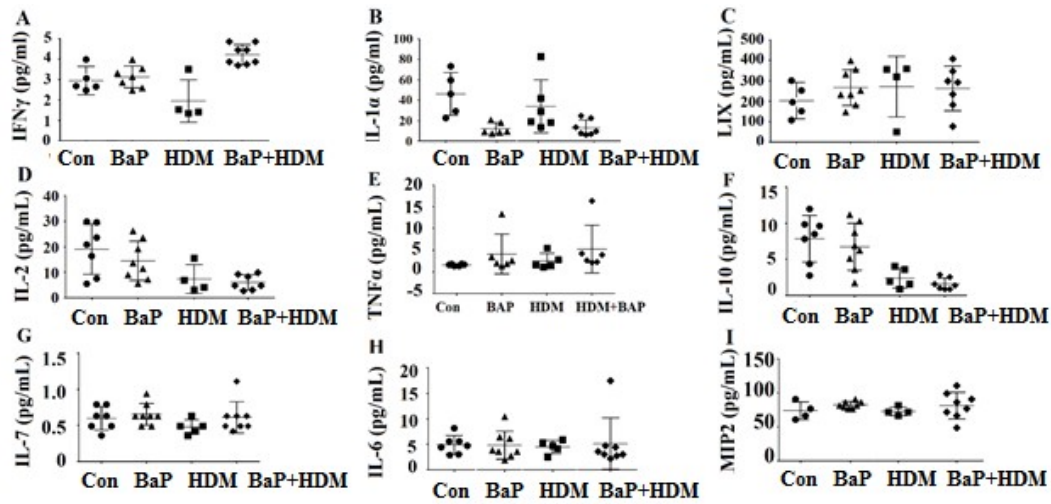


Fig. S2. Mice exhibit cytokines following BaP-HDM challenge. C57BL/6 mice were sensitized and challenged with BaP and HDM as described, and cytokines (A-I) in bronchoalveolar lavage (BAL) fluid were measured by multiplex assay as described in the Materials and methods. Results shown are mean $\pm$ SEM ($n = 5-8$). * $P < 0.05$, ** $P < 0.01$.

Table S1. Sequences of forward and reverse primers used for real-time RT-PCR.

Target	Sequence	Genbank number
IL4i1b	F: 5'- TTGGGCCTCAATCGGACTTC -3' R: 5'- CCGTCCACGTGTTCTCATCA -3'	NM_001171024.1
IL22ra2	F: 5'- AGCTCGTGTATCTCTGACGC -3' R: 5'- GTCCCCCAGCAGTCAACTTT- 3'	NM_178258.5
IL4i1	F: 5'- GCATACGCTTCTGGAATACCT -3' R: 5'- AAGCCCTCCTCGGACAT -3'	NM_010215.3
MUC4	F: 5'- CCCCCATCTTTCTGTCTCAA -3' R: 5'- AGGATGGAATTGGTGTTTG -3'	NM_080457.4
AhR	F: 5'- GACAGCAGAATCCCACATCCGCA -3' R: 5'-TCTCGTCCTTCTTCATCCGTCAGT -3'	AY662081.1
CYP1A1	F: 5'- CAAGAGCTGCTCAGCATAGTC -3' R: 5'-GCTCAATGAGGCTGTCTGTG -3'	NM_009992.4
β -actin	F: 5'- CCGTGAAAAGATGACCCAGA-3' R: 5'- TACGACCAGAGGCATACAG-3'	NM_007393.5

(F): forward primer; (R): reverse primer.