



Mono-n-butyl phthalate regulates nuclear factor erythroid 2-related factor 2 and nuclear factor kappa B pathway in an ovalbumin-induced asthma mouse model

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ABSTRACT

Recent studies have emphasized the role of endocrine-disrupting chemicals in asthma development, especially in eosinophilic asthma. However, the exact mechanism was unknown. Among all the endocrine-disrupting chemicals, mono-n-butyl phthalate (MnBP) was a chemical that was most frequently detected in human urine. Our study was performed with the aim of investigating the harmful effects of MnBP on airway epithelial cells (AECs), T cells, and eosinophils by using eosinophilic asthma mouse models. Mice that received OVA with MnBP had higher levels of airway hyperresponsiveness, total and eosinophil cell counts, as well as T cell proliferation and T helper 2 cytokine release than those which only received OVA. Moreover, MnBP contributed to directly enhancing the eosinophilic activation which was shown in. Long-term exposure MnBP activated AECs through the nuclear factor kappa B (NF-κB) pathway, decreased nuclear factor erythroid 2-related factor 2 (Nrf2) expression, and increased interleukin-33 expression. Additionally, MnBP can induce human eosinophil activation to release eosinophil extracellular traps (EETs). Taken together, our study suggested the roles of MnBP exposure increase the risk of asthma development and severity. Furthermore, vitamin E treatment (anti-inflammatory and antioxidant effects) can reduce MnBP-induced harmful effects through inhibiting EETs, restoring Nrf2, and suppressing the NF-κB pathway.

1. Introduction

The prevalence of asthma, which affected approximately 300 million people all over the world, has been raising with industrialization and urbanization (Saglan et al., 2020). A variety of environmental and genetic interplays have been suggested to be related to the increasing prevalence and development of asthma (Mattiuzzi and Lippi, 2020). As for environmental exposure, specific associations between endocrine disrupting-chemicals (EDCs) and asthma-related immune cells have been found in current studies (Nowak et al., 2019; Xie et al., 2016).

EDCs have been demonstrated as an exogenous substance or mixture that can change the function of the endocrine system, in which causes adverse health effects and is related to various diseases, including infertility, hormonal diseases, and even asthma (Nowak et al., 2019). EDCs originated mostly from man-made products such as pesticides,

metals, toys, cosmetics, additives in food, and personal care products (Monneret, 2017). Ingestion, inhalation, and skin contact are well-known as the major routes of exposure to EDCs in humans (Monneret, 2017). EDCs can bind to potential receptors such as steroid hormones, thyroid receptors, peroxisome proliferator-activated receptors, and aryl hydrocarbon receptors that exert their actions. Changes in mRNA, DNA methylation, and histone H3, H4 modification in the body can be influenced by these interactions (Monneret, 2017). Phthalates, one of the EDCs, originate from a variety of sources in the environment, particularly from consumer products, which are known to correlate with the high risk of asthma (Wang et al., 2019). However, the exact mechanisms of exposure to phthalates in asthma pathophysiology have not yet been clearly elucidated.

Eosinophilic asthma is the major subtype of asthma and T cells, eosinophils, and epitheliums are well-known pathologic cells in asthma development (Barnes, 2016). Recently, the role of eosinophil

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Abbreviation			
AECs	Airway epithelial cells	H&E	Haematoxylin and eosin
AHR	Airway hyperresponsiveness	IFN- γ	Interferon gamma
BALF	Bronchoalveolar lavage fluid	IL	Interleukin
DAPI	4',6-diamidino-2-phenylindole	ILC2s	Type 2 innate lymphoid cells
DnBP	Di-n-butyl phthalate	MnBP	Mono-n-butyl phthalate
dsDNA	Double-strand DNA	NF-kB	Nuclear factor kappa B
EDCs	Endocrine disrupting-chemicals	Nrf2	Nuclear factor erythroid 2-related factor 2
EDN	Eosinophil-derived neurotoxin	OVA	Ovalbumin
EETs	Eosinophil extracellular traps	PAS	Periodic acid schiff
ELISA	Enzyme-linked immunosorbent assay	PBS	Phosphate-buffered saline
EPx	Eosinophil protein X	ROS	Reactive oxygen species
EtOH	Ethanol	TSLP	Thymic stromal lymphopoietin
		VTE	Vitamin E

extracellular traps (EETs) has been emphasized because they can activate type 2 innate lymphoid cells (ILC2s) through stimulating airway epithelial cells (AECs) in severe asthma (Choi et al., 2020). Moreover, accumulating evidence revealed a close relationship between reactive oxygen species (ROS) and EDC in various acute and chronic inflammatory diseases by measurement of oxidative stress markers such as o, o-dityrosine (Neier et al., 2015). However, the inducers of EETs and the relationship between ROS and EDC is still not completely known, especially in asthma pathophysiology.

Mono-n-butyl phthalate (MnBP) is a white solid material that has both the butyl ester group and the carboxylic acid group. It is the major metabolite of di-n-butyl phthalate (DnBP) and a well-known EDC like many other phthalates. Since it is used as material for various products from children's toys to food containers, EU agency and Consumer Product Safety Improvement Act (CPSIA) restrict the concentration value of this phthalate to a maximum of 0.1% by weight in products. Despite these restrictions, MnBP was detected at the highest concentration than other phthalates when measured in the urine of healthy adults in a previous paper (Choi et al., 2017). In this study, we investigated whether ROS production, EET formation, and T-cell differentiation induced by MnBP can contribute to asthma development using eosinophilic asthma mouse models. We also provided novel evidence of vitamin E (VTE) treatment in an MnBP-induced asthma model.

2. Materials and methods

2.1. Animal groups and study protocol

A number of allergens have been used in animal models of asthma. In most studies, ovalbumin (OVA) has been used to sensitize and challenge host animals. OVA induced eosinophilic asthma is the classic mouse model to study the pathogenesis of allergic asthma. This model is relatively short-term and shows high reproducibility. The responses elicited include elevated levels of allergen-specific immunoglobulin E, eosinophilic airway inflammation, and airway hyper-responsiveness (AHR) (Shin et al., 2009).

Six-week BALB/c mice (Jackson Laboratory, Bar Harbor, ME, USA) were purchased and housed under specific pathogen- and OVA-free conditions. All animal experiments performed in this study were approved by the Institutional Animal Care and Use Committee (IACUC 2019-0037). MnBP and VTE were dissolved in ethanol (EtOH) and Kolliphor® EL for the experiments. Phosphate-buffered saline (PBS), OVA, MnBP, and VTE treatment protocol used in this study was described in Fig. 1 (Luu Quoc et al., 2021). Briefly, mice were divided into 5 groups (n = 10 for each group): (1) the OVA-sensitized group (OVA/PBS/PBS); (2) the MnBP-treated group (OVA/PBS/MnBP); (3) the OVA-sensitized and challenged group (OVA/OVA/EtOH); (4) the

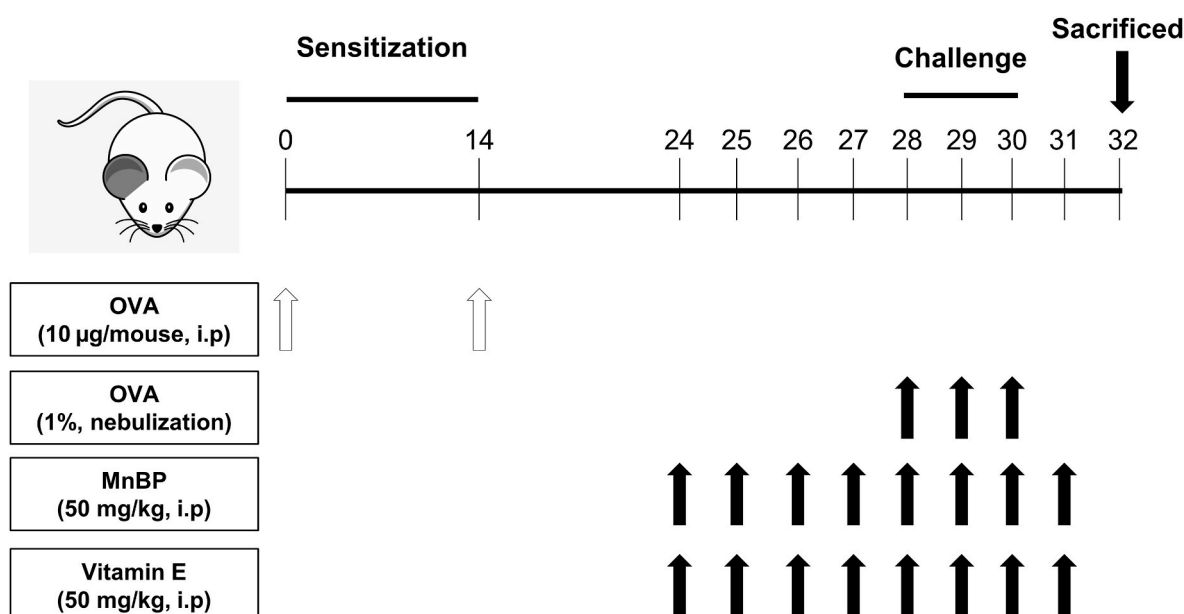


Fig. 1. Sensitization and exposure protocol. MnBP, mono-n-butyl phthalate; OVA, ovalbumin; i.p, intraperitoneal injection.

OVA-sensitized, challenged, and MnBP-treated group (OVA/OVA/MnBP); and (5) the OVA-sensitized, challenged, MnBP-, and VTE-treated group (OVA/OVA/VTE). Mice were sensitized with intraperitoneal injections with 10 µg of OVA in 1 mg of alum on days 0 and 14. On days 28, 29, and 30, mice were subjected to allergen challenges by nebulization with 1% OVA for 30 min, using an ultrasonic nebulizer (NE-Y2, Omron, Kyoto, Japan). Mice were given MnBP (50 mg/kg) via the intraperitoneal route with or without VTE (50 mg/kg) 30 min before the OVA challenge from days 24 to days 31. Twenty-four hours after the last MnBP treatment, mice were sacrificed for further analysis.

2.2. Evaluation of airway resistance, cytokines in BALF, and lung histology

Mice were anesthetized with pentobarbital sodium. AHR to acetyl-β-methyl choline chloride was evaluated at increasing doses of methacholine (0, 1.56, 3.12, 6.25, 12.5, and 25 mg/mL) and measured by the FlexiVent System (SCIREQ, Montreal, Canada). BALF was collected through washing with 1x PBS plus 1% bovine serum albumin through a cannula and centrifuged at the program 1200 rpm, 5 min, 4 °C. Supernatants were stored at −70 °C until the measurement of cytokine levels. Cell pellets were used to count the differential and total cells and measure ROS. The fixed tissues were sectioned at 5-µm thickness. Lung tissues were stained with Haematoxylin and Eosin (H&E) for the evaluation of inflammatory cell infiltration, and Periodic Acid Schiff (PAS) for the evaluation of mucus production. Cells isolated from lung tissues were used to measure ROS levels.

2.3. T-cell activation and differentiation protocol

Mononuclear cells were collected from the spleen of 5 groups (OVA/PBS/PBS; OVA/PBS/MnBP; OVA/OVA/EtOH; OVA/OVA/MnBP; and OVA/OVA/VTE). CD4⁺ T cells were isolated by using a CD4⁺ T cell isolation kit; 2 × 10⁵ cells were seeded on CD3- and CD28-coated 24-well plates.

2.4. Measurement of intracellular ROS

Intracellular ROS were measured as described previously (Luu Quoc et al., 2021). BALF cell pellets, lung tissue-derived cells, and human peripheral eosinophils were washed with 1x PBS. Then, 2 × 10⁵ cells/100 µl was incubated with 20 µM H2DCFDA for 30 min. The signals of H2DCFDA-labeled ROS were measured at excitation and emission wavelengths of 485 and 528 nm, respectively, with a fluorescence microplate reader (Synergy HT; BioTek Instrument, Inc., Winooski, VT, USA).

2.5. Analysis of protein expressions by immunofluorescence in lung tissues

Immunofluorescence staining was performed to analyze the immunofluorescence of nuclear factor erythroid 2-related factor 2 and nuclear factor-κB (NF-κB) p65 to evaluate pathways of MnBP; and eosinophil protein X (EPx) and eosinophil-derived neurotoxin (EDN) in eosinophils in formalin-fixed lungs. The lungs from unstained slices were deparaffinized in xylene and rehydrated in ethanol in a concentration-dependent manner (Luu Quoc et al., 2021). The slices were permeabilized with 0.1% Triton X for 10 min and blocked with 5% bovine serum albumin in 10% normal donkey serum at room temperature for 1 h. The sections were incubated overnight with appropriate primary antibodies.

2.6. Evaluation of EPx and dsDNA activity

The detection of EPx in BALF was based on the oxidation of O-phenylenediamine (OPD) in the presence of hydrogen peroxide which develops color (yellow color). Double-strand DNA (dsDNA) in the presence

of ssDNA, RNA, and free nucleotides was measured by using a Quant-IT Pico-green dsDNA kit.

BALF supernatant was transferred onto a 96-well plate. Working reagent (0.1 mM OPD, 0.05 M Tris pH 8.0, Triton X-100, and 1 mM hydrogen peroxide) was added for EPx measurement. After incubation for 1 h, the reaction was stopped with 50 µl of 1.0 M sulfuric acid and absorbance was measured at 492 nm by using a spectrophotometer. For dsDNA measurement, the mixture of PicoGreen was added to dsDNA and measured at excitation and emission wavelengths of 485 and 528 nm, respectively, with a fluorescence microplate reader (Synergy HT).

2.7. Epithelial cell culture and treatment protocol

The human lung carcinoma cell line (A549) was purchased from the American Type Culture Collection. A549 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin G sodium, and 100 µg/mL streptomycin sulfate at 37 °C with 5% CO₂ in humidified air. The optimal doses for VTE and MnBP treatment were based on the evaluation of CCK8 and ROS formation as shown in [Supplementary Figs. 5 and 6](#). Cells received 0.2 µg/mL MnBP in a time-dependent manner (24, 48, and 72 h). In some experiments, cells were incubated with 1 µM VTE for 1 h and treated with MnBP for 48 h for Nrf2 expression evaluation and immunofluorescent assay.

For real-time PCR (quantitative PCR, qPCR), TRIZOL® was used to extract RNA. Then, cDNA was synthesized from RNA by using the SuperScript™ First Strand Synthesis System. Gene expression analysis was conducted using the KAPA SYBR® FAST qPCR Master Mix (2x) kit. All data were normalized to GAPDH expression. The annealing temperature was 60.6 °C for all genes. Nrf2, IL-33, thymic stromal lymphopoietin (TSLP), and GAPDH cDNAs were amplified using the following primers: human Nrf2, human IL-33; TSLP; GAPDH ([Supplementary Table S2](#)). The relative mRNA amount in each sample was calculated based on its threshold cycle (Ct) in comparison to the threshold cycle of housekeeping gene GAPDH.

2.8. Peripheral blood eosinophil isolation and EET detection

Peripheral blood eosinophils were isolated from healthy donors. The procedure for eosinophil isolation was followed as described previously (Luu Quoc et al., 2021). Eosinophils were separated from neutrophils by immunomagnetic cell sorting (Miltenyi Biotec Inc.) according to the manufacturer's recommendations. These cells were pretreated with VTE for 1 h; then, they were treated with MnBP for 1 h (for ROS assay), 6 h (for immunofluorescent assay), and 24 h (for western blot analysis, intracellular staining with EDN or EPx, and ELISA).

For ICC assay, 2 × 10⁵ cells were seeded on 24 well plates containing poly-L-lysine-coated-glass for 30 min after isolation. After treatment procedure, eosinophils were stained with EPx, EDN, and 4',6-diamidino-2-phenylindole (DAPI) for localization of EET formation in the extracellular environment.

2.9. Confocal microscopy

After primary antibody incubation overnight, the sections or slides were stained with Alexa Fluor 488, and/or donkey anti-rabbit, and/or 633 donkey anti-goat for 1 h. The slices were incubated with DAPI (1:1.000) for 5 min and dropped by Biomedica Mounting solution. Fluorescent images were acquired using confocal laser scanning microscopy (LSM710; Carl Zeiss Microscopy GmbH, Jena, Germany) at the Three-Dimensional Immune System Imaging Core Facility.

2.10. Measurement of inflammatory cytokines by ELISA

The levels of cytokines in the BALF were measured by ELISA according to the manufacturer's protocol.

2.11. Western blot and antibodies

Cells were lysed with lysis buffer containing protease/phosphatase inhibitor cocktail. Protein concentrations were measured by BCA Protein Assay.

2.12. Statistical analysis

We performed the D'Agostino-Pearson omnibus test to evaluate the normal distribution. Differences between the two groups were analyzed by Mann-Whitney *U* test for non-normally distributed variables and Pearson chi-square test for categorical variables. Data are presented as Geometric mean with 95% CI. Comparisons of data from multiple groups were made by using one-way ANOVA, followed by Bonferroni's *post hoc*

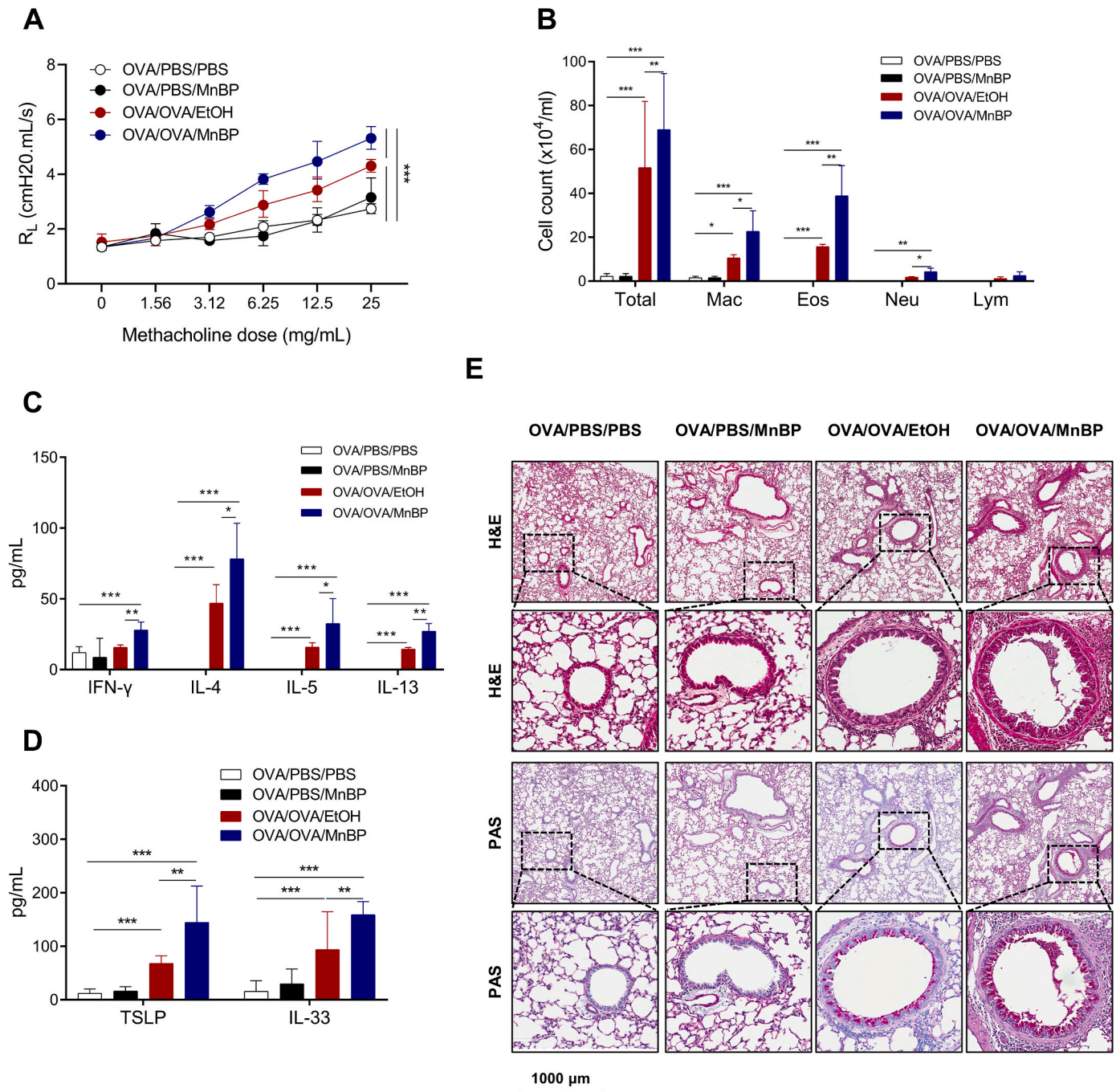


Fig. 2. MnBP enhances airway inflammation in an eosinophilic asthma mouse model. (A) Airway hyperresponsiveness, (B) Total and differential cell counts. (C) MnBP enhanced the concentrations of Th1 cytokines (IFN- γ) as well as Th2 cytokines (IL-4, IL-5, and IL-13) in BALF. (D) The levels of TSLP and IL-33 were increased in the BALF of the OVA/OVA/MnBP. The assays were performed in independent experiments ($n = 10$). (E) The lung tissues were stained with hematoxylin and eosin (H&E) and periodic acid–schiff (PAS) for histological examination. Scale bar, 1000 μ m. Data are presented as Geometric mean with 95% CI. * $P < 0.050$, ** $P < 0.010$, and *** $P < 0.001$ were obtained using one-way ANOVA with Bonferroni's *post hoc* test. BALF, bronchoalveolar lavage fluid; Eos, eosinophils; EtOH, ethanol; IL, interleukin; IFN- γ , interferon gamma; Lym, lymphocytes; MnBP, mono-n-butyl phthalate; Mac, macrophages; Neu, neutrophils; OVA, ovalbumin; PBS, phosphate buffer saline; R_L , total pulmonary resistance; TSLP, thymic stromal lymphopoietin.

test for comparison of data from multiple groups if the data were nonparametric. All data were analyzed using SPSS version 22.0 (SPSS Inc, Chicago, IL, USA), and graphed by using GraphPad Prism 7 software (GraphPad Software, La Jolla, Calif), and a *P*-value of less than 0.050 was considered statistically significant.

3. Results

3.1. Effects of MnBP on AHR, differential cell counts, and cytokine release

AHR and larger numbers of total cells, eosinophils, and neutrophils were observed in the OVA/OVA/MnBP group than in the OVA/OVA/EtOH group ($P < 0.001$, Fig. 2A and B). The levels of Th2 cytokines (IL-4, IL-5, and IL-13) and even Th1 cytokine (interferon gamma [IFN- γ]) were significantly higher in the OVA/OVA/MnBP group than in the OVA/PBS/PBS and OVA/OVA/EtOH groups ($P < 0.001$ for each, Fig. 2C). In addition, MnBP increased the levels of AEC-derived cytokines (TSLP and IL-33) ($P < 0.01$, Fig. 2D). H&E and PAS staining showed increased inflammatory cell infiltration and mucus production in the airways of the OVA/OVA/EtOH and OVA/OVA/MnBP groups (Fig. 2E and Supplementary Fig. 2). There were no changes in AHR and airway inflammatory markers in the OVA/PBS/MnBP group.

3.2. Ex vivo effects of MnBP on T-cell differentiation

CD4⁺ T cells from the spleens of the OVA sensitized mice were isolated. CD4⁺ T cells isolated from OVA/OVA/MnBP released more the concentrations of IFN- γ , IL-5, and IL-13 compared to those from OVA/OVA/EtOH ($P < 0.050$; Fig. 3A–C).

3.3. Effects of MnBP in the oxidant-antioxidant system of the lungs

8-hydroxydeoxyguanosine in BALF and ROS production in lung tissue were more significantly increased in the OVA/OVA/MnBP group ($P < 0.001$ for all, Fig. 4A and B). Furthermore, the endogenous levels of VTE were more significantly decreased in the OVA/OVA/MnBP group than in the OVA/OVA/EtOH group ($P < 0.050$, respectively, Fig. 4C). The endogenous antioxidants (SOD and GSH) were also reduced in the OVA/OVA/MnBP group in Supplementary Fig. 3 ($P < 0.001$). The signals for Nrf2 and NF- κ B in the lung tissues were more considerably

increased in the OVA/OVA/MnBP group than in the OVA/OVA/EtOH group (Fig. 4D and E and Supplementary Fig. 4).

3.4. MnBP induces eosinophilic activation and eosinophil extracellular trap production

Eosinophilic activation was evaluated by measuring EPx and EDN as well as by observing EET formation in BALF and lung tissues. Eosinophilic activation-related proteins, EDN and EPx, were more significantly expressed in the BALF of the OVA/OVA/MnBP group than in that of the OVA/OVA/EtOH group ($P < 0.050$, Fig. 5A and B), and EPx, EDN, and histone H3 were more significantly expressed in the lung tissue of the OVA/OVA/MnBP group (Fig. 5D). Moreover, the dsDNA levels were more considerably elevated in the OVA/OVA/MnBP group than in the OVA/OVA/EtOH group (Fig. 5C). Co-expression of DAPI, EPx, and EDN were also more significantly expressed in the OVA/OVA/MnBP group than in the OVA/PBS/PBS and OVA/OVA/EtOH groups (Fig. 5E).

3.5. The restoration of MnBP-induced lung tissue damage by VTE treatment

VTE was administered to evaluate the anti-oxidant effects in the OVA/OVA/MnBP group based on the finding that MnBP more significantly induced ROS elevation and VTE consumption compared to the OVA/OVA group in Fig. 6. Lower AHR and smaller numbers of total cells eosinophils were observed in the OVA/OVA/VTE group than in the OVA/OVA/MnBP group ($P < 0.010$, Fig. 6A and B). VTE also reduced the levels of Th1 cytokine (IFN- γ), Th2 cytokines (IL-4, IL-5, IL-13), and AEC-derived cytokines (TSLP, IL-33) ($P < 0.050$, Fig. 6C). The concentration of IL-5 released from CD4⁺ T cells isolated from OVA/OVA/MnBP was diminished after VTE administration (Fig. 6D). VTE also decreased ROS release ($P < 0.050$ for BALF, $P < 0.010$ for lung tissue) and down-regulated the increase in dsDNA and eosinophilic activation induced by MnBP stimulation ($P < 0.010$, Fig. 6E–G). Finally, VTE can reduce inflammatory cell infiltration and mucus production in H&E staining and PAS staining (Fig. 6H).

3.6. Effects of MnBP and VTE in airway epithelium

Next, the effects of MnBP were evaluated in airway epithelium using the A549 cell-line. The A549 cells were treated with MnBP in a time-

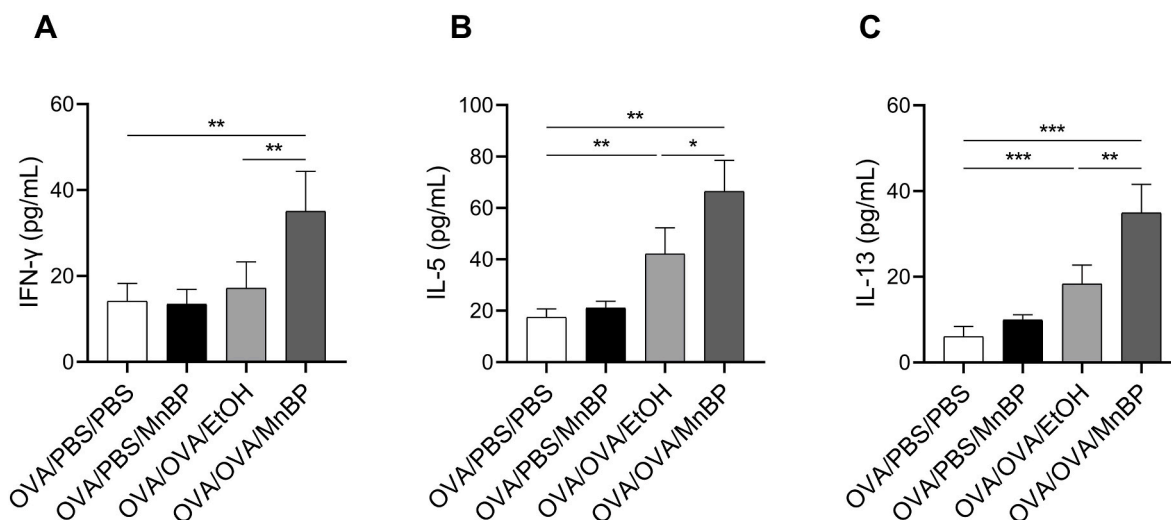


Fig. 3. Cytokine production by CD4⁺ T cells in the presence of MnBP. CD4⁺ T-cells were isolated by using a CD4⁺ T-cell isolation kit. Amounts of (A) IFN- γ , (B) IL-5, and (C) IL-13 of plate-bound anti-CD3 and anti-CD28 CD4⁺ T cells in the mice. The assays were performed in independent experiments ($n = 10$). Data are presented as Geometric mean with 95% CI. * $P < 0.050$, ** $P < 0.010$, and *** $P < 0.001$ were obtained using one-way ANOVA with Bonferroni's *post hoc* test. EtOH, ethanol; IL, interleukin; IFN- γ , interferon gamma; MnBP, mono-n-butyl phthalate; OVA, ovalbumin.

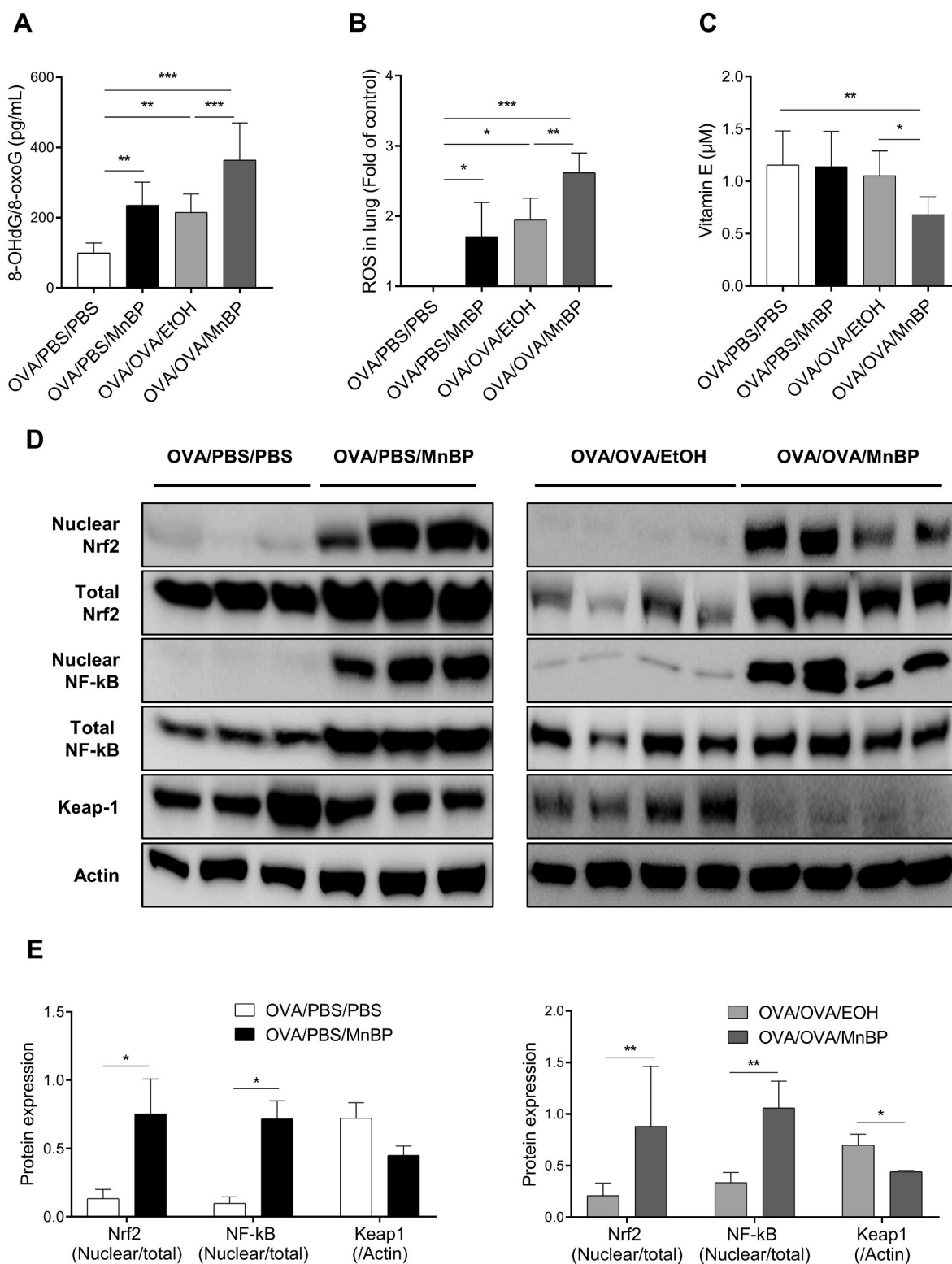


Fig. 4. MnBP induces the dysfunction of antioxidant defense in the eosinophilic asthma mouse model. Concentrations of (A) 8-ohdG/8-oxoG in BALF, (B) ROS in lung tissue, and (C) vitamin E in serum. The assays were performed in independent experiments ($n = 10$). (D) The expressions of Nrf2, NF-kB, and Keap-1 were detected in lung tissue. (E) Representative data of western blot results in the comparisons between OVA/PBS/PBS and OVA/PBS/MnBP ($n = 3$) as well as OVA/OVA/EtOH and OVA/OVA/MnBP ($n = 4$). Data are presented as Geometric mean with 95% CI. * $P < 0.050$, ** $P < 0.010$, and *** $P < 0.001$ were obtained using Mann-Whitney or one-way ANOVA with Bonferroni's *post hoc* test. 8-oxoG, 8-oxoguanine; 8-OHdG, 8-hydroxy-2'-deoxyguanine; EtOH, ethanol; Keap-1, kelch-like ECH-associated protein 1; MnBP, mono-n-butyl phthalate; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; Nrf2, nuclear factor erythroid 2-related factor 2; OVA, ovalbumin; PBS, phosphate buffer saline; ROS, reactive oxygen species.

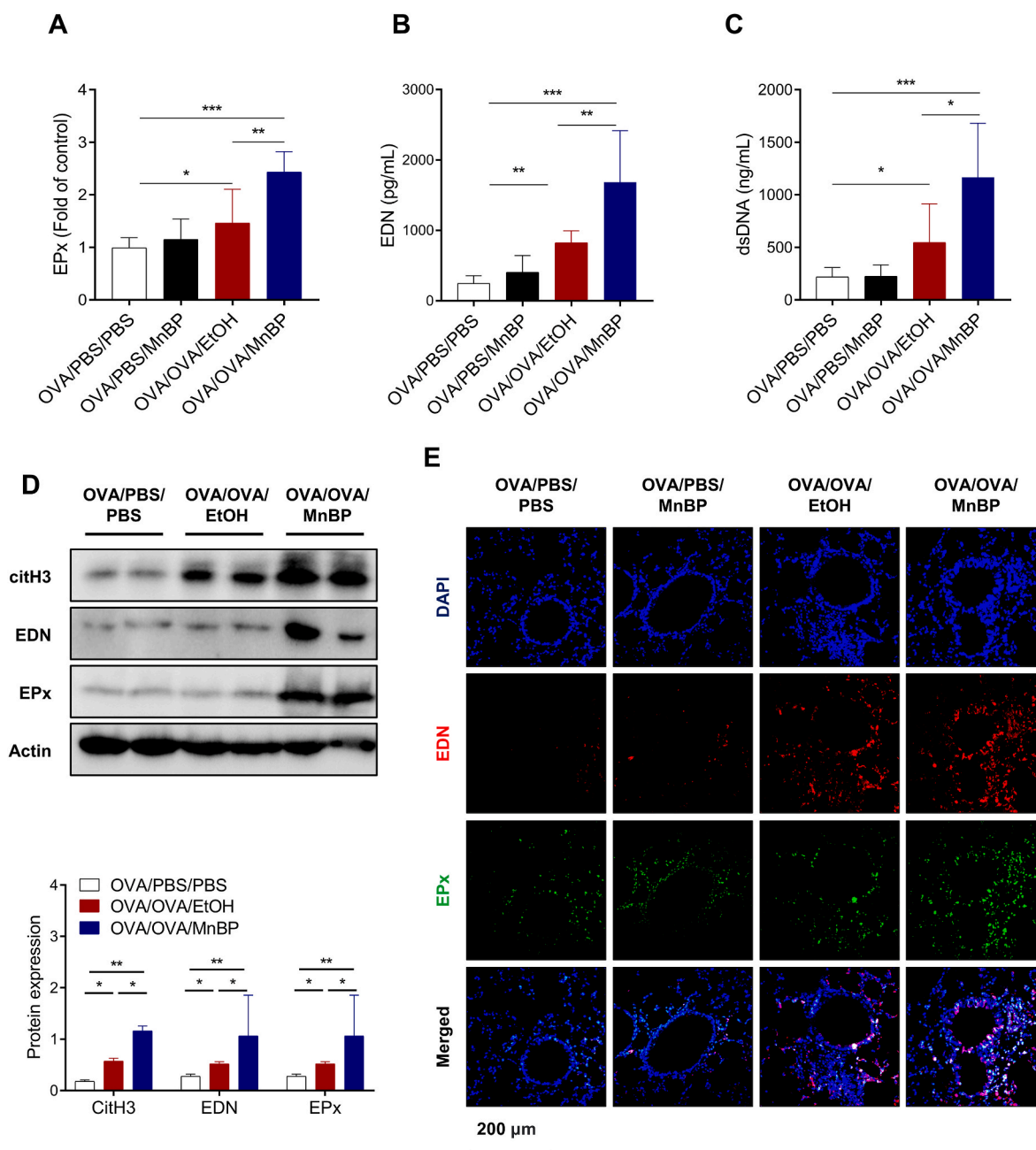


Fig. 5. MnBP induces eosinophil extracellular trap (EET) formation in the lung tissue. Concentrations of MnBP-induced (A) EPx and (B) EDN measured in BALF. (C) The levels of dsDNA in BALF. The assays were performed in independent experiments ($n = 10$). (D) MnBP increased the expression of eosinophil granule proteins (EPx and EDN) and histone H3 in the lung tissue. (E) The lung tissues were stained for EDN expression (red), EPx expression (green), and DAPI (nuclear, blue) for EET colocalization. Scale bar, 200 μm. Data are presented as Geometric mean with 95% CI. * $P < 0.050$, ** $P < 0.010$, and *** $P < 0.001$ were obtained using one-way ANOVA with Bonferroni's *post hoc* test. BALF, bronchoalveolar lavage; DAPI, 4',6-diamidino-2-phenylindole; EDN, eosinophil-derived neurotoxin; EPx, eosinophil protein X; EtOH, ethanol; MnBP, mono-n-butyl phthalate; OVA, ovalbumin; PBS, phosphate buffer saline.

dependent manner. The translocation from cytoplasmic to nuclear and mRNA expression of IL-33, TSLP, NF-κB, and Nrf2 were increased after MnBP stimulation in a time-dependent manner (Fig. 7A–D). VTE treatment restored the increased translocation of NF-κB, and IL-33, and the depleted Nrf2 expression (Fig. 7E and F).

3.7. Effects of MnBP and VTE in human peripheral eosinophils

MnBP stimulation up-regulated the protein expressions of Nrf2, phosphorylated NF-κB, ECP, and EPx in human peripheral eosinophils (Fig. 8A), and there was a significant increase in the levels of EPx and EDN in the MnBP group than in the negative control group (from 10.8 to

21.9 and from 13.1 to 20.5, respectively) by using flow cytometry (Supplementary Fig. 7). Additionally, MnBP induced the EET formation as assessed by the double-staining of EPx and EDN (Fig. 8B). There were significant increases in the levels of dsDNA, ROS, and EDN released from these cells after MnBP stimulation ($P < 0.001$, Fig. 8C–E). All of the harmful effects of MnBP on human peripheral eosinophils were reduced by VTE treatment (Fig. 8).

4. Discussion

This is the first study indicating the evidence of MnBP exposure, asthma development, and its potential mechanisms, especially in

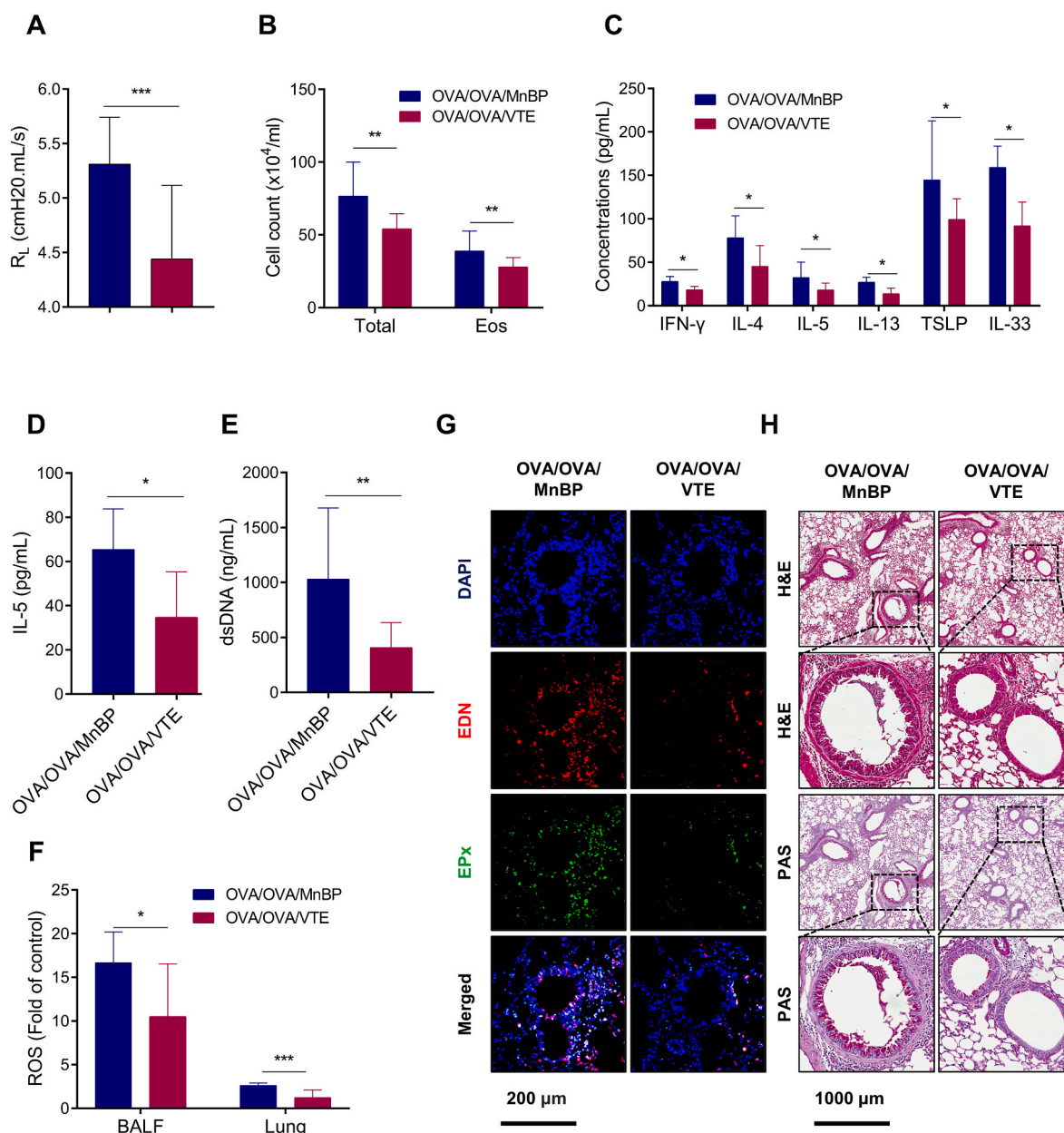


Fig. 6. Vitamin E inhibits the damage of MnBP in the lung tissue through reactive oxygen species (ROS)- and EET-dependent pathways. (A) Airway hyperresponsiveness. (B) Total and eosinophil counts. (C) Proinflammatory cytokines. (D) The concentration of IL-5 released from CD4⁺ T cells. (E) dsDNA concentration measured in BALF. (F) ROS production evaluated in BALF and lung tissue. The assays were performed in independent experiments (n = 10). The lung tissues were stained for (G) EPx expression, EDN expression, and DAPI for EET localization (scale bar, 200 μ m) and (H) with PAS for mucus production, and with H&E for cell infiltration (scale bar, 1000 μ m). Data are presented as Geometric mean with 95% CI. * P < 0.050, ** P < 0.010, and *** P < 0.001 were obtained using Mann-Whitney or one-way ANOVA with Bonferroni's *post hoc* test. BALF, bronchoalveolar lavage; DAPI, 4',6-diamidino-2-phenylindole; H&E, hematoxylin and eosin; PAS, periodic acid-Schiff; IL, interleukin; IFN- γ , Interferon gamma; EDN, eosinophil-derived neurotoxin; EPx, eosinophil protein X; MnBP, mono-n-butyl phthalate; OVA, ovalbumin; ROS, reactive oxygen species; R_L , total pulmonary resistance; VTE, vitamin E; TSLP, thymic stromal lymphopoietin.

eosinophilic asthma. MnBP contributing to the breakdown of the balance between antioxidants and oxidants activates Nrf2 and NF- κ B pathways in a mouse model. With the ROS inducer function, MnBP induced eosinophil activation to release proinflammatory cytokines (EPx and EDN) and EET formation. Additionally, it played the adjuvant role in enhancing Th2 cytokines released from CD4⁺ T cells and Th0 differentiation to Th2. In AECs, long-term exposure to MnBP inhibited the expression of Nrf2 which caused cytokine enhancement. VTE administration inhibited MnBP-induced AHR and inflammatory responses in asthma through the restoration of Nrf2 and the inhibition of EET formation. These findings suggest that MnBP may play critical role in developing and maintaining eosinophilic airway inflammation in

eosinophilic asthma.

4.1. Corstalk between EDCs and eosinophilic asthma

The endocrine system is the organs or glands with the functions of producing and releasing hormones into the bloodstream in order to directly come to the targeted tissues in the body, and this system has meaningful functions in the regulations of growth, development, metabolism, and reproduction. The natural or man-made chemicals which cause the breakdown and synthesis disruption, alteration, and interference of the endocrine system are called EDCs (Kumar et al., 2020). The EDC-induced disequilibrium between immune and

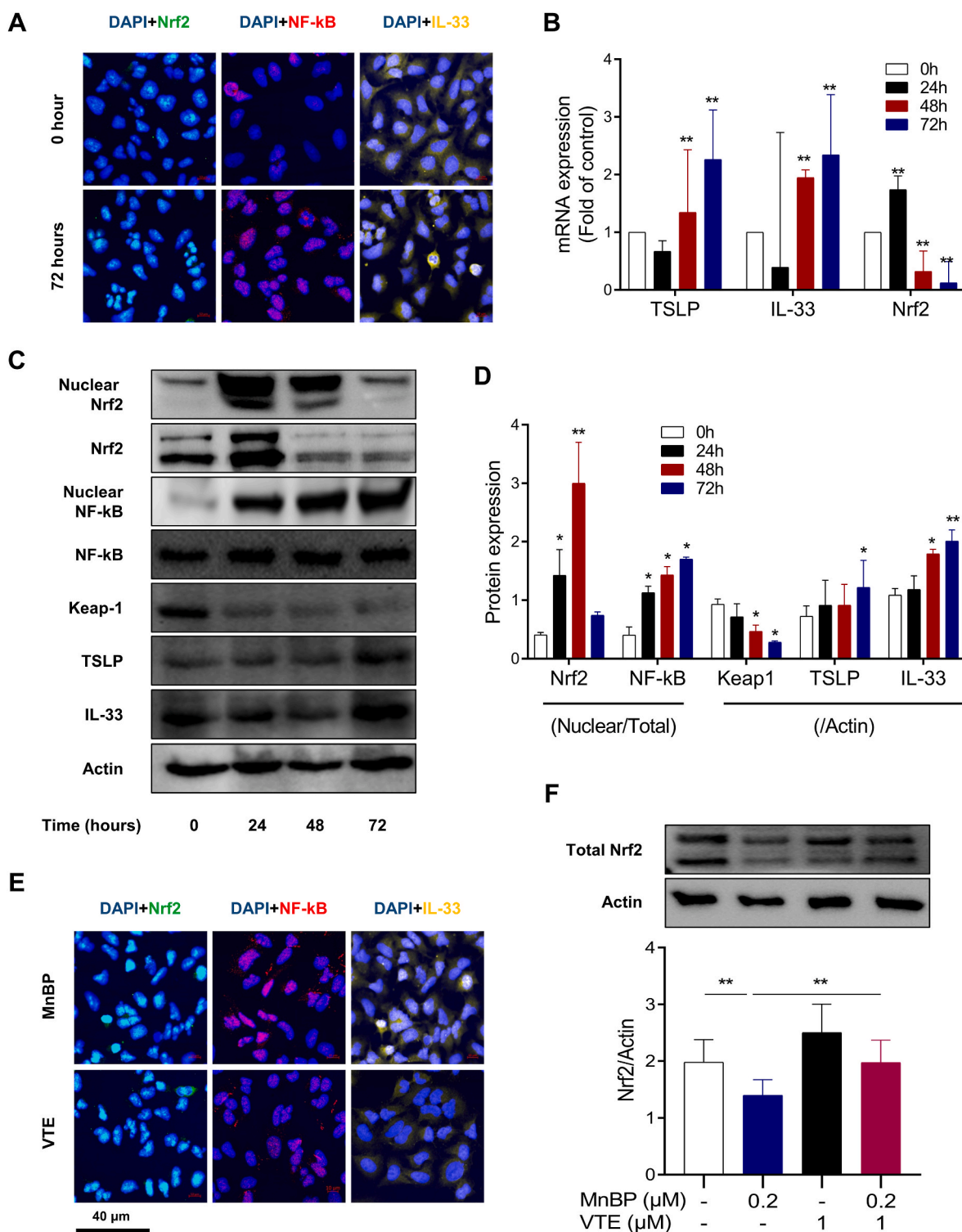


Fig. 7. MnBP induces airway inflammation and remodeling and increases the expressions of IL-33, Nrf2, and NF-kB. (A) Antibodies against DAPI (blue) and Nrf2 (green), NF-kB (red), and IL-33 (yellow) were assessed using immunofluorescence analysis. (B) The changes in TSLP, IL-33, and Nrf2 mRNA expression after MnBP stimulation. The assays were performed in double in three independent experiments ($n = 6$). (C) Their expressions were quantified by western blot analysis. (D) Represent data of protein expression. The assays were performed in double in two independent experiments ($n = 4$). (E) Antibodies against DAPI (blue) and Nrf2 (green), NF-kB (red), IL-33 (yellow) were assessed using immunofluorescent analysis. The image of MnBP-treated A549 at the time-point of 48 h was used for the positive control group. (F) Western blot analysis to assess MnBP-induced Nrf2 down-regulation. Data are presented as Geometric mean with 95% CI. $*P < 0.050$ and $**P < 0.010$ were obtained using one-way ANOVA with Bonferroni's *post hoc* test. DAPI, 4',6-diamidino-2-phenylindole; IL, interleukin; MnBP, mono-n-butyl phthalate; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; Nrf2, nuclear factor erythroid 2-related factor 2; VTE, vitamin E; TSLP, thymic stromal lymphopoietin.

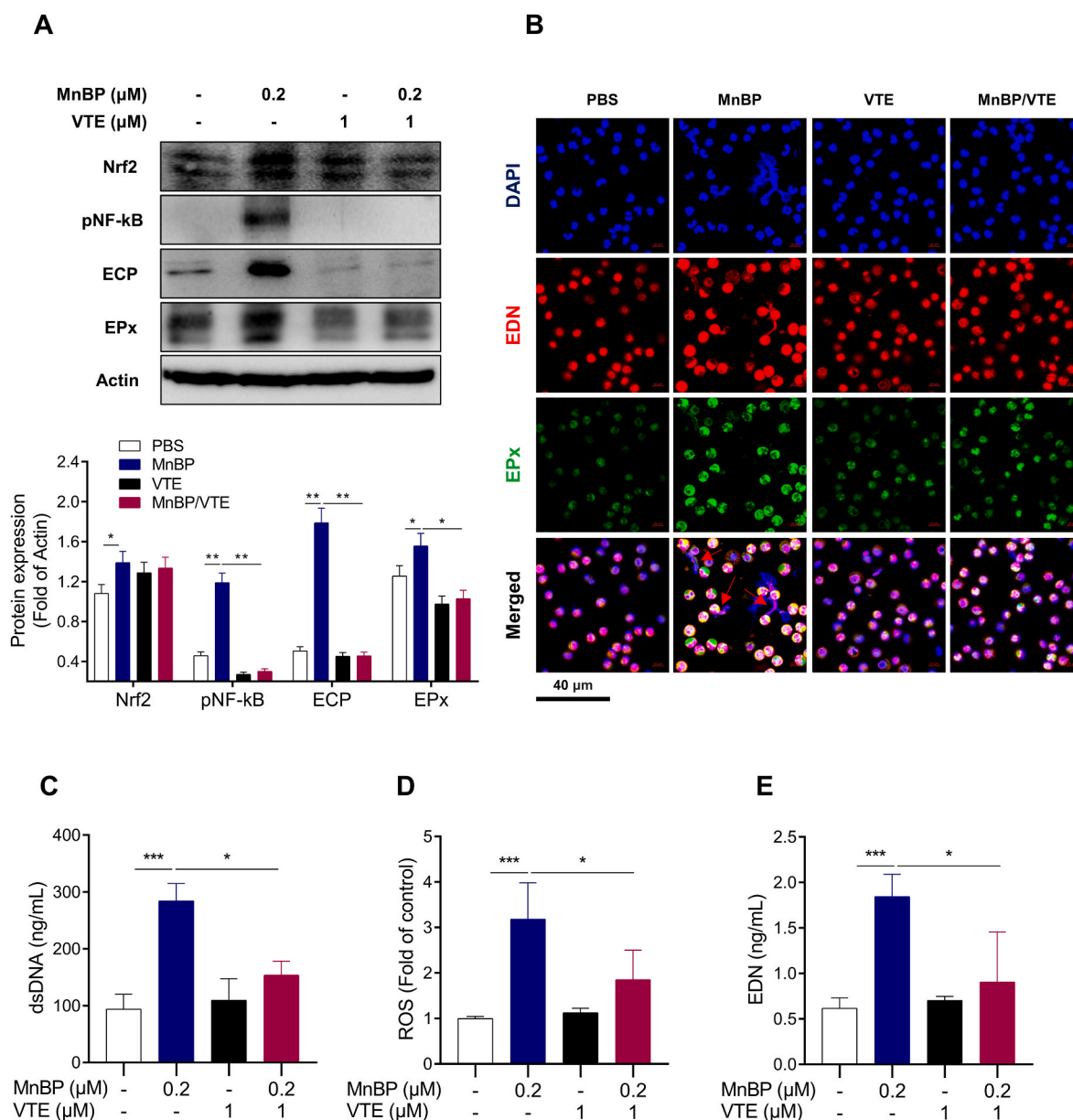


Fig. 8. MnBP induces human peripheral eosinophil activation and releases EETs. (A) Protein expressions were detected by western blot analysis (above panel). Represent data of protein expression (below panel). The assays were performed in double in two independent experiments ($n = 4$). (B) EET formation as observed by using confocal microscopy. Scale bar, 40 μm . (C) The EET levels and extracellular DNA concentrations were measured using the PicoGreen assay. (D) The level of ROS released from eosinophils after MnBP stimulation. (E) The levels of EDN released from human eosinophils. The assays were performed in double in five independent experiments ($n = 10$). Data are presented as Geometric mean with 95% CI. * $P < 0.050$, ** $P < 0.010$, and *** $P < 0.001$ were obtained using one-way ANOVA with Bonferroni's post hoc test. DAPI, 4',6-diamidino-2-phenylindole; ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; EPx, eosinophil protein X; MnBP, Mono-n-butyl phthalate; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; Nrf2, nuclear factor erythroid 2-related factor 2; ROS, reactive oxygen species; VTE, vitamin E.

endocrine systems contributed to various diseases, including reproductive, metabolic, and malignant diseases as well as immune cell dysfunction-related diseases such as asthma (Nowak et al., 2019; Quirós Alcalá et al., 2021). Phthalates are a specific group of EDCs that can be divided into low- and high-molecular-weight subgroups (Hsieh et al., 2012; Schettler, 2006). While the majority of studies focused on the high-molecular-weight subgroups of phthalates, their counterpart (low-molecular-weight) is still unclear the functions and roles in allergic diseases (Kang et al., 2018; Qin et al., 2018). DnBP is classified as a low-molecular-weight phthalate group that is normally found in building and finishing materials, coatings, and adhesives for the joints of food packaging (Liao et al., 2018). By measurement of biomarkers for phthalate compounds and their metabolism in human fluids, scientists

indicate 4 routes that phthalates can come inside the body: (1) ingestion of phthalate-contaminated food and water related to environmental pollution in lakes, rivers, and sea; (2) inhalation of contaminated air and dust in the domestic environment; (3) direct dermal exposure; and (4) transfer from mothers to infants through the placenta and breast milk (Högberg et al., 2008; Huang et al., 2009; Wittassek et al., 2011). When DnBP enters the body, it will undergo a 2-phase process before release in urine or feces, including hydrolysis and hydroxylation, oxidation (Koch et al., 2012). MnBP can be used as the biomarkers for presenting DnBP because it can be excreted in urine within 24 h of intake (Koch et al., 2012). Some human biomonitoring studies have reported that urinary MnBP can be mostly detected among Taiwanese adults aged 21–67 years and Chinese children aged 5–10 years (Chen et al., 2008; Shi et al.,

2018). Additionally, the concentrations of urinary DnBP metabolites are higher in the Korean population than in the US and other populations (Kim et al., 2018).

Eosinophilic asthma is characterized by airway inflammation and remodeling with the participation of key factors, including CD4⁺ T cells, ILC2, and eosinophils. Particularly, when allergens enter the lung, AECs released the alarmins, such as IL-33 and TSLP, to adapt to allergens. Additionally, the co-stimulatory interactions between dendritic cells and T-cells induced T cell differentiation into Th2 type T cells and produce Th2 cytokines (IL-4, IL-5, and IL-13). As a consequence, eosinophils were migrated into the lungs and activated to form EETs. Recently, the role of ILC2 has been emphasized because it can activate eosinophils besides the roles of CD4⁺ T cells. Interestingly, EETs are recognized as an inducer for ILC2 activation in eosinophilic asthma (Choi et al., 2020; Holgate et al., 2015). The crosstalk between MnBP exposure and asthma pathophysiology is currently very limited to the immuno-toxicological effects.

4.2. MnBP exposure increased the risk of asthma development and severity

Mouse experiments normally evaluated the role of MnBP by measuring the levels of IgG1 and total IgE in serum (Larsen et al., 2002; Thor Larsen et al., 2001). However, the results are still controversial because a variety of administration routes, exposure time, and sensitization protocols used in those studies made it difficult to compare between them and draw a conclusion. Chen et al. (2015) found that giving mice 10 mg/kg DnBP, of which 84 percent was metabolized into MnBP, had no effect on oxidative status or asthma-like symptoms (Chen et al., 2015; Koch et al., 2012). The dose and diverse roles of DnBP metabolites in mice's bodies were most likely the main factors. To rule out the effects of other DnBP side chain oxidized metabolites, we administered MnBP directly intraperitoneally to mice in a dose-dependent manner in the current investigation (10 mg/kg, 30 mg/kg, 50 mg/kg, 100 mg/kg; Supplementary Fig. 1). The maximum dose of MnBP was accorded on the reports from Scientific Committee on Toxicity, Ecotoxicity and the Environment, the no-observed-adverse-effect level (NOAEL) of DnBP is 150 mg/kg body weight per day (CSTEE, 1998). AHR, inflammatory cell recruitment, and cytokine production were all affected by the medium dose of MnBP. Furthermore, we discovered that at the appropriate dose, accumulative ROS generation and Nrf2/NF- κ B pathway activation were dramatically increased which was consistent with the previous study in the high-molecule weight studies (Kang et al., 2018; Qin et al., 2018).

In vitro and *ex vivo* experiments using the monocytic cell line THP-1 and peripheral blood mononuclear cells from allergic and non-allergic subjects suggested that MnBP increased the levels of IL-4 and IFN- γ released from PBMCs in allergic asthmatics compared to the control group (Glue et al., 2002). According to our study comprehensively evaluated the adjuvant effects of MnBP in asthma mouse models, human eosinophils, and CD4⁺ T cells, the administrations of MnBP up-regulated the differentiation of CD4⁺ T cells into Th2 and enhanced the production of AEC- (TSLP, IL-33) and CD4⁺ T cell-derived cytokines (IL-4, IL-5, IL-13) measured in BALF. Additionally, MnBP activated eosinophils to form EETs in lung tissue, BALF, and human eosinophils, contributing to the feedback loop in the axis MnBP-AECs -EETs-ILC2.

4.3. VTE treatment reduced MnBP-induced negative effects

ROS, which are mainly derived from macrophages, neutrophils, and eosinophils, function as a protector against pathogen invasion (Finkel, 2011). In the normal condition, ROS were balanced with the support of antioxidant systems, ranging from endogenous antioxidants (glutathione [GSH], catalase [CAT], and superoxide dismutase [SOD]) to exogenous antioxidants (vitamin C and VTE) (Zuo et al., 2013). ROS and antioxidant systems are regulated by the transcription factors which are called NF- κ B and Nrf2 (Finkel, 2011). The disequilibrium of these

systems can induce the overproduction of ROS and decrease Nrf2, which further causes negative effects on various processes, including cell division, inflammation, immune function, autophagy, stress response, and even EET formation (Finkel, 2011; Silveira et al., 2019). The interactions between ROS, MnBP, and asthma are still unclear. In this study, we found that MnBP can induce ROS production in human peripheral eosinophils as well as in mouse lung tissue and BALF. In the same fashion, both endogenous and exogenous antioxidants were diminished after MnBP administration. Hence, MnBP caused the translocation of Nrf2 to the nucleus and transcript of various antioxidant genes in the response to ROS and endogenous antioxidant malfunction. However, the long-time stimulation of MnBP (48 and 72 h) suppressed the expression of Nrf2 along with the productions of AEC-derived cytokines (TSLP and IL-33). These findings suggested that MnBP contributed to airway inflammation through the Nrf2 pathway. Additionally, our previous study reported the decrease in Nrf2 caused uncontrolled ROS production and eosinophil activation, which was inhibited by VTE treatment (Luu Quoc et al., 2021). In other words, EET formation seemed to be mediated through ROS formation. VTE can be a potential therapeutic to control diseases related to the Nrf2-ROS-EET pathway by the inhibition of ROS.

There were some limitations in our study. First, the route or dose of MnBP administered in this study did not fully reflect phthalate contacts in actual human life. Secondly, our study employed a short-term exposure protocol (32 days) which is difficult to mirror the pattern of actual environmental exposure in humans. Thirdly, we did not measure the level of MnBP from asthmatic patients which can support our results. Despite these limitations, we found the adjuvant effects of MnBP in allergic inflammation, immunomodulatory effects, and the suppressive effects of VTE in the ROS-Nrf2-EET pathway.

Taken together, the results of this study suggest that MnBP may play a pivotal role in eosinophilic asthma via enhancing eosinophil activation, EET formation, and CD4⁺ T-cell differentiation. In addition, VTE may be a potential therapeutic of eosinophilic airway inflammation induced by MnBP.

Author contributions

Yoo Seob Shin, Min Sook Ryu, and Hae-Sim Park conceived and designed the study. Quang Luu Quoc, Tra Cao Thi Bich, and Seo-Hee Kim carried out the experiments, and analyzed the data. All authors reviewed, edited and approved the final 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fct.2022.113171>.

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