

Diisononyl phthalate induces asthma via modulation of Th1/Th2 equilibrium



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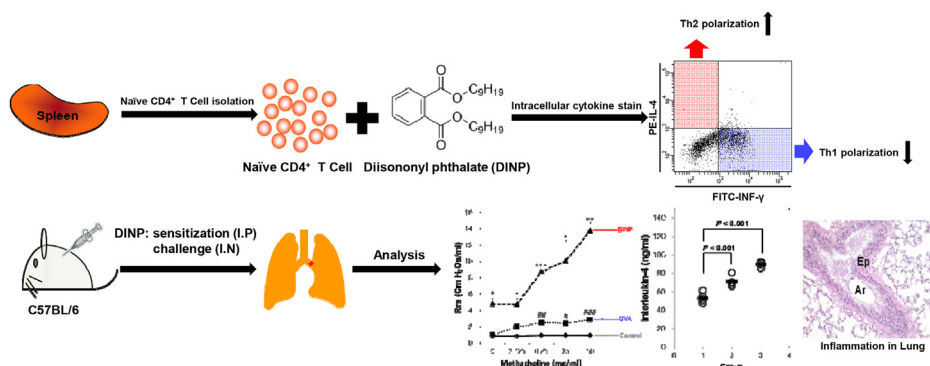
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HIGHLIGHTS

- DINP suppresses Th1 polarization and enhances Th2 polarization in vitro.
- DINP induces airway hyperresponsiveness in vivo.
- DINP induces Th2 mediated cytokine (IL-4, IL-5) and immunoglobulin (IgE, IgG1).
- DINP induces airway inflammation and enhancement of the goblet cells in lung.
- DINP induces expression of caspase-1 and caspase-3 in lung.

GRAPHICAL ABSTRACT



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ABSTRACT

Diisononyl phthalate (DINP), a member of the phthalate family, is used to plasticize polyvinyl chloride (PVC). This chemical is known to enhance airway inflammation in the OVA-induced asthma model (adjuvant effects) and aggravate allergic dermatitis. Moreover, DINP enhances the production of interleukin-4 in activated CD4⁺ T cells. However, the effect of DINP itself on the differentiation of naïve CD4⁺ T cells into T helper cells (Th1/Th2) in vitro and allergic asthma in vivo has not yet been studied. In this study, DINP was shown to suppress the polarization of Th1 and enhance the polarization of Th2 from naïve CD4⁺ T cells in vitro. Also, DINP induced allergic asthma via the production of IL-4, IL-5, IgE and IgG1 and the reduction of IFN- γ and IgG2a. Finally, we confirmed that exposure to DINP induces the infiltration of inflammatory cells and PAS positive cells and increases the expression of caspase-1 and caspase-3 in asthmatic mice. In conclusion, we suggest that DINP as an environmental pollutant or endocrine disruptor (ECD) induces asthma via the modulation of the Th1/Th2 equilibrium and production of Th2 mediated cytokines and immunoglobulin.

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1. Introduction

The prevalence of asthma as an allergic disease is increasing worldwide and its complexity and severity continue to increase in children and young adults (Pawankar, 2014; Beasley et al., 2000). Asthma is characterized by airway inflammation, airway remodeling and mucus hypersecretion, causing airway hyperresponsiveness

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(AHR) by increasing the numbers of inflammatory cells, such as eosinophils and lymphocytes, and infiltration of the mucosa and submucosa. For these reasons, asthma leads to clinical symptoms such as wheezing, breathlessness, chest tightness, and coughing. CD4⁺ T cells in asthma play a crucial role in controlling inflammation. Antigen-activated CD4⁺ T cells can differentiate into effector cells, viz. Th1 and Th2 cells (Ray and Cohn, 1999; Bateman et al., 2008). Th1 cells secrete IL-2, IFN- γ , lymphotoxin, whereas Th2 cells produce IL-4, IL-5, and IL-6 (Fiorentino et al., 1989). The Th2-polarized immune response plays an important role in the development of asthma, and the IL-4 and IL-5 secreted by Th2 cells are instrumental in initiating and sustaining the asthmatic response. On the other hand, IFN- γ produced by Th1 cells protects against allergic asthma (Hansen et al., 1999). Prolonged exposure of allergens such as viral infections, cigarette smoke, atmospheric pollution, stress, and environmental pollutants increase the risk of developing asthma (Pearce et al., 2000).

Phthalates, which have been deemed to be environmental pollutants, are used as materials for personal care products, food packaging, children's toys, pharmaceuticals, nutritional supplements, cleaning materials, lubricants, insecticides, solvents, adhesives, and paints (Chen et al., 2012). Several phthalates, such as diisononyl phthalate (DiNP), diisodecyl phthalate (DiDP), and di-2-ethylhexyl phthalate (DEHP), are used to plasticize polyvinyl chloride (PVC) (Jaakkola and Knight, 2008). Exposure to these phthalates is associated with the development of asthma and allergy in animals (Bornehag and Nanberg, 2010). Endocrine-disrupting chemicals (EDC) such as tributyltin chloride (TBT), benzophenone (BP) and p-octylphenol (OP) exacerbate airway inflammation via Th2 polarization (Kato et al., 2006). Phthalates such as EDC may be directly involved in airway inflammation (Dodson et al., 2012). Especially, DINP exposure induces allergic airway inflammation in rat pups (Chen et al., 2015) and has an adjuvant effect (Larsen et al., 2002). Moreover, long-term oral exposure to DINP aggravates allergic dermatitis by activating NF- κ B (Kang et al., 2016) and decreases the cognitive abilities in mice, while increasing their anxiety (Ma et al., 2015).

Lee MH et al. reported that DINP enhances the allergic response by enhancing IL-4 production in activated CD4⁺ T cells (Lee et al., 2004). However, the effect of DINP on the differentiation of naïve CD4⁺ T cells into Th1 and Th2 cells has not yet been studied. Furthermore, the adjuvant effect of DINP (OVA + DINP) is known, but the effect of DINP itself in allergic asthma is unknown. We hypothesized that DINP itself may induce asthma via Th2 polarization. In the present study, we confirmed the effects of DINP on the differentiation of naïve CD4⁺ T cells in vitro and the induction of asthma in vivo.

2. Materials and methods

2.1. Reagents

Ovalbumin (A5503) and diisononyl phthalate (376663) was purchased from Sigma-Aldrich (Louis, USA). Purified rat anti-mouse IgE (R35-72), purified rat anti-mouse IgG1 (A85-3), purified rat anti-mouse IgG2a (R11-89), purified rat anti-mouse IL-4, purified rat anti-mouse IL-5, purified rat anti-mouse IFN- γ , biotin rat anti-mouse IgE (R35-118), biotin rat anti-mouse IgG1 (A85-1), biotin rat anti-mouse IgG2a (19-5), biotin rat anti-mouse IL-4, biotin rat anti-mouse IL-5, and biotin rat anti-mouse IFN- γ were purchased from BD Biosciences (San Diego, USA).

2.2. Cells

CD4⁺CD62L⁺ T cells were prepared from the spleen of C57BL/6 or BALB/c mice and used as naïve CD4⁺ T cells. Naïve CD4⁺ T cells

were isolated by using a CD4⁺ CD62L⁺ T Cell Isolation Kit II (Order no. 130-093-227) and separation columns (MACS). Briefly, non-CD4⁺ T cells were indirectly magnetically labeled with a cocktail of biotin-conjugated monoclonal anti-mouse antibodies against CD8a, CD45R, CD11b, CD25, CD49b, TCR γ/δ , and Ter-119 and microbeads conjugated to monoclonal antibiotin antibody (isotype: mouse IgG1). The labeled cells are depleted by separation using a MACS[®] Column. CD4⁺CD62L⁺ T cells were labeled with microbeads conjugated to monoclonal antimouse CD62L (L-selectin; isotype: rat IgG2a) antibody and isolated by positive selection.

2.3. In vitro priming of naïve CD4⁺ t cells

The priming of the naïve CD4⁺ T cells (1×10^5 cells/2 mL/culture) was conducted using 2 μ g/mL of anti-CD3 (145-2C11) and anti-CD28 (37.51) in a 24-well plate. The cultures received only medium or 1–1000 μ M of DINP. The cultures in the Th1 condition were supplemented with 5 mg/mL of anti-IL-4 (11B11) plus 1000 U/mL of rmlL-12 (BD Biosciences; San Diego, USA) or 5 mg/mL of anti-IL-12 (C17.8) plus 1000 U/mL of rmlL-4 (R&D System). At 4 days, the CD4⁺ T cells were expanded into four wells with fresh medium containing 2.5 ng/mL of rhIL-2 (BD Biosciences; San Diego, USA) and cultured for another 2 days. The supernatant was stored at -80°C for ELISA. These primed T cells (1×10^6 cell/ml) were re-stimulated with PMA (50 μ g/mL) and ionomycin (1 μ M) for 5 h in the presence of brefeldin A (5 mg/mL) for the last 3 h, after which they were assayed for intracellular cytokines by FACS.

2.4. Intracellular staining

The primed CD4⁺ T cells were blocked for FcR by staining with anti-FcR (2.4G2), followed by staining with biotin-anti-CD4 in the presence of streptavidin-cychrome. The cells were then fixed with 4% paraformaldehyde and permeabilized with 0.1% saponin, followed by staining with FITC-anti-IFN- γ and PE-anti-IL-4 (BD Biosciences; San Diego, USA). The CD4⁺ T cells were gated and analyzed on a FACScanto II (BD Biosciences).

2.5. Animals and ethics statement

Female C57BL/6 mice and BALB/c mice (7–8 weeks old) were bred and maintained under specific pathogen-free conditions at ORIENT BIO (Seongnam, Korea). The animals were housed at a controlled temperature of $22 \pm 2^\circ\text{C}$ and at $50 \pm 5\%$ relative humidity. The mice were housed in polycarbonate cages and fed a standard animal diet with water. All mice were treated in strict accordance with the Suncheon National University Institutional Animal Care and Use Committee's (SCNU IACUC) guidelines for the care and use of laboratory animals. All procedures were approved by the SCNU IACUC (permit number: SCNU IACUC-2016-10). All experiments were performed under zoletil/rumpun anesthesia, and all effort was made to minimize suffering.

2.6. Sensitization and provocation of airway inflammation with OVA or DINP

C57BL/6 mice were primary sensitized by the intraperitoneal injection of 100 μ g/mL of OVA or 50 mg/kg of DINP in 0.2 mL of saline on day 0. On days 3 and 10, 100 μ g/mL of OVA or 50 mg/kg of DINP dissolved in corn oil were secondary sensitized by intraperitoneal injection. The mice were challenged with PBS or 10 μ g of OVA or 50 mg/kg of DINP dissolved in 50 μ L of saline (intranasal injection) under anesthesia on days 19, 21, and 23 (Fig. 4). 24 h after the last airway challenge, blood was collected in a retro orbital plexus. After centrifugation (5000 rpm, 4°C , 5 min),

the serum was stored at -20°C until assayed for immunoglobulins by ELISA. The animals were sacrificed by cervical dislocation. Bronchoalveolar lavage (BAL) of the mice was performed four times each with 0.5 mL of saline. After centrifugation (1200 rpm, 4°C , 5 min), the supernatant of BAL obtained from 2 mL of instilled saline was stored at -20°C until assayed for cytokines by ELISA. The red blood cells in BAL were removed by tris-buffered ammonium chloride. The total cells were counted using a hemacytometer.

2.7. Assessment of airway hyperresponsiveness in OVA or DINP-induced asthmatic mice

After the final challenge, the mice were weighed and anesthetized with by injecting Zoletil and Rumpun. Once the anesthesia was effective, the mouse was tracheostomized using an 18G metal cannula. The mice was then placed in a flow-type body plethysmograph and connected by the endotracheal cannula to a small-animal ventilator (FlexiVent, SCIREQ Inc., Montreal, Canada). Doses of methacholine (MCh) were administered using a nebulizer (Aeroneb) and progressively doubled concentrations ranging from 0 to 50 mg/mL. The respiratory system resistance (Rrs) and respiratory system elastance (Ers) were determined before each challenge and after each dose of MCh. The corresponding parameters, airway resistance (Rn), tissue damping (G), and tissue dynamic elastance (H), were computed by multiple linear regression. The aeroneb (FINE MIST ANP-1100) specifications: particle size (VMD-Volume Median Diameter; $3.5\ \mu\text{m}$), nominal nebulization output rate ($>0.1\ \text{mL/min}$), residual volume ($<0.2\ \text{mL}$), mass median aerodynamic diameter (MMAD; $\sim 1.8\ \mu\text{m}$), and geometric standard deviation (GSD; $2.0\ \mu\text{m}$).

2.8. Measurement of inflammatory cytokine and immunoglobulin production in supernatant and OVA or DINP-induced asthmatic mice

The levels of the various cytokines, viz. IL-4, IL-5, and IFN- γ , in supernatant or BAL and immunoglobulins (Ig's), viz. the total IgE, IgG1, and IgG2a, in the serum were measured by enzyme-linked immunosorbent assay (ELISA).

2.9. Histological analysis of lung tissue induced by OVA or DINP

After BAL, the left lungs of the mice were harvested and paraffin embedded after fixation in 4% formalin. $4\ \mu\text{m}$ sections were cut and stained with Hematoxylin & Eosin (H&E) and Periodic acid-Schiff (PAS). Images of the lung tissue sections stained with H&E and PAS were acquired with a microscope equipped with a $\times 20$ or $\times 40$ objective lens. For immunohistochemistry, the paraffin-embedded sections were deparaffinized. Slides were washed at room temperature and hydrated. The endogenous peroxidase activity was then quenched with 3% hydrogen peroxidase. The sections were then blocked and the endogenous avidin and biotin were blocked, following the manufacturer's instructions. The samples were then stained with an antibody against caspase-1 or caspase-3 (1:200; Abcam). Biotinylated secondary antibodies ($2\ \mu\text{g/mL}$) were used and were detected with horseradish peroxidase, using a Vectastain Elite ABC (Vector Laboratories). Inflammatory cells/epithelium, PAS positive cells, and expression of caspase were analyzed by Image J program.

2.10. Statistical analysis

The results are presented as means $\pm$ SDs. Statistical analyses were performed using the SPSS program (SPSS, Chicago, IL). The Student's *t*-test was used to determine the significances of the differences between the groups. P values of $p < 0.001$, $p < 0.01$, or $p < 0.05$ were considered to be statistically significant, as indicated.

3. Results

3.1. DINP is not toxic to naïve CD4^+ T cell

To confirm the effect of DINP on their survival, naïve CD4^+ T cells were isolated from the spleens. We identified that 77.3% of the isolated T cells were CD4^+ CD62L^+ T cells (naïve CD4^+ T cells) (Fig. 1A) and DINP at 1–1000 μM had no cytotoxic effect on the naïve CD4^+ T cells (Fig. 1B). Therefore, we determined the concentrations of DINP (1, 10, 100, 1000 μM) to be used in the in vitro experiments.

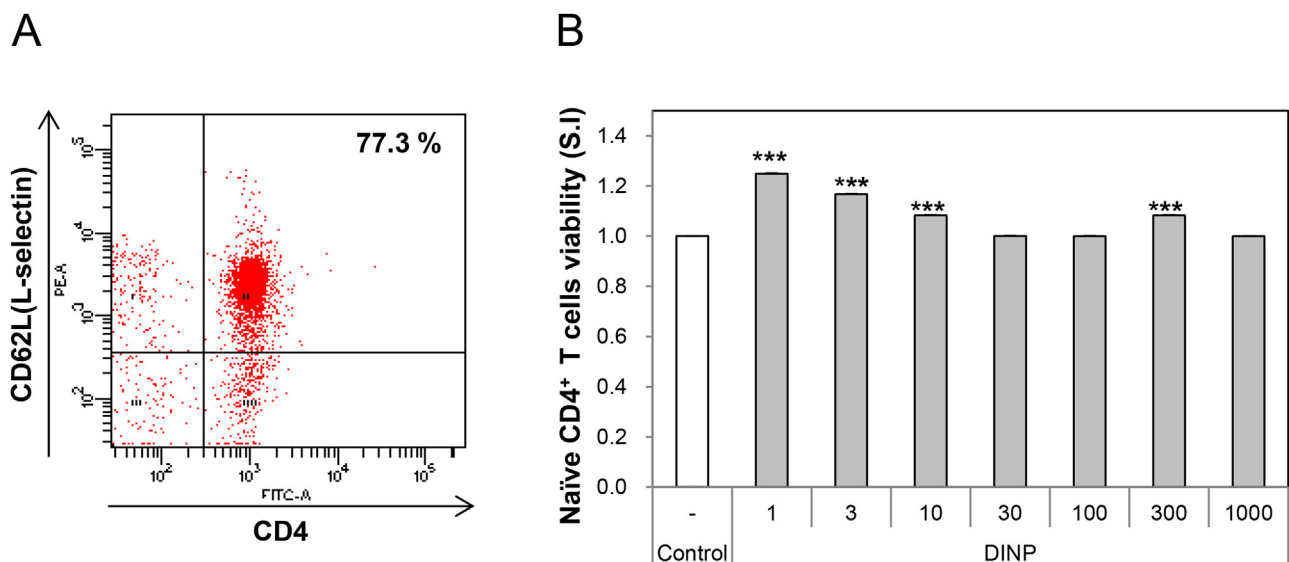


Fig. 1. Percentage of naïve CD4^+ T cells isolated from spleen cells and effect of diisononyl phthalate (DINP) on these naïve CD4^+ T cells. (A) The percentage of naïve CD4^+ T cells was analyzed by flow cytometry through double staining of FITC-conjugated CD4 and PE-conjugated CD62L. (B) The viability of the naïve CD4^+ T cells in the presence of environmental pollutants was confirmed through trypan blue staining. ### $p < 0.001$ control vs DINP. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. DINP promotes Th2 polarization and suppresses Th1 polarization in vitro

To determine whether DINP affects Th1 or Th2 polarization, naïve CD4⁺ T cells were primed with 2 µg/mL anti-CD3 and anti-CD28 in the absence and presence of 1–1000 µM DINP. The phenotype of the helper T cells was assayed by intracellular cytokine staining of IFN-γ (Th1) and IL-4 (Th2) after restimulation with PMA plus ionomycin. In the Th1 condition, the population of Th1 cells (47.6 ± 1.13) was significantly increased in comparison with the control group (9.7 ± 0.28). In the Th2 condition, the population of Th2 cells (2.1 ± 0.28) was increased in comparison with the control group (0.5 ± 0.0). The inclusion of DINP at a concentration of 10 µM during priming augmented the Th1 development of naïve CD4⁺ T cells from the C57Bl/6 mice. However, DINP at 1000 µM suppressed the Th1 development of naïve CD4⁺ T cells (Fig. 2A). Furthermore, the presence of DINP during priming augmented the Th2 development of naïve CD4⁺ T

cells from the C57Bl/6 mice (Fig. 2B). Finally, the ratio of Th2/Th1 was increased by the treatment with DINP (Fig. 2C). Culture supernatants were collected at day 6 and assayed for cytokines by ELISA. In the supernatant treated with DINP, IFN-γ was not detected when compared to control group (Fig. 2D). In contrast, IL-4 and IL-4/IFN-γ ratio were increased compared to control group (Fig. 2E, F). Therefore, these results suggest that DINP suppresses the polarization of Th1 and promotes the polarization of Th2 from naïve CD4⁺ T cells.

3.3. DINP suppresses Th1 polarization in Th1 condition

In order to clarify the inhibitory effect of DINP on Th1 polarization, we confirmed the inhibition of Th1 polarization in the Th1 condition. Naïve CD4⁺ T cells isolated from BALB/c mice were treated with DINP in the Th1 differentiation state. As a result, DINP significantly suppressed the population of Th1 cells (Fig. 3A). Furthermore, IFN-γ of the primed CD4⁺ T cells in Th1 condition

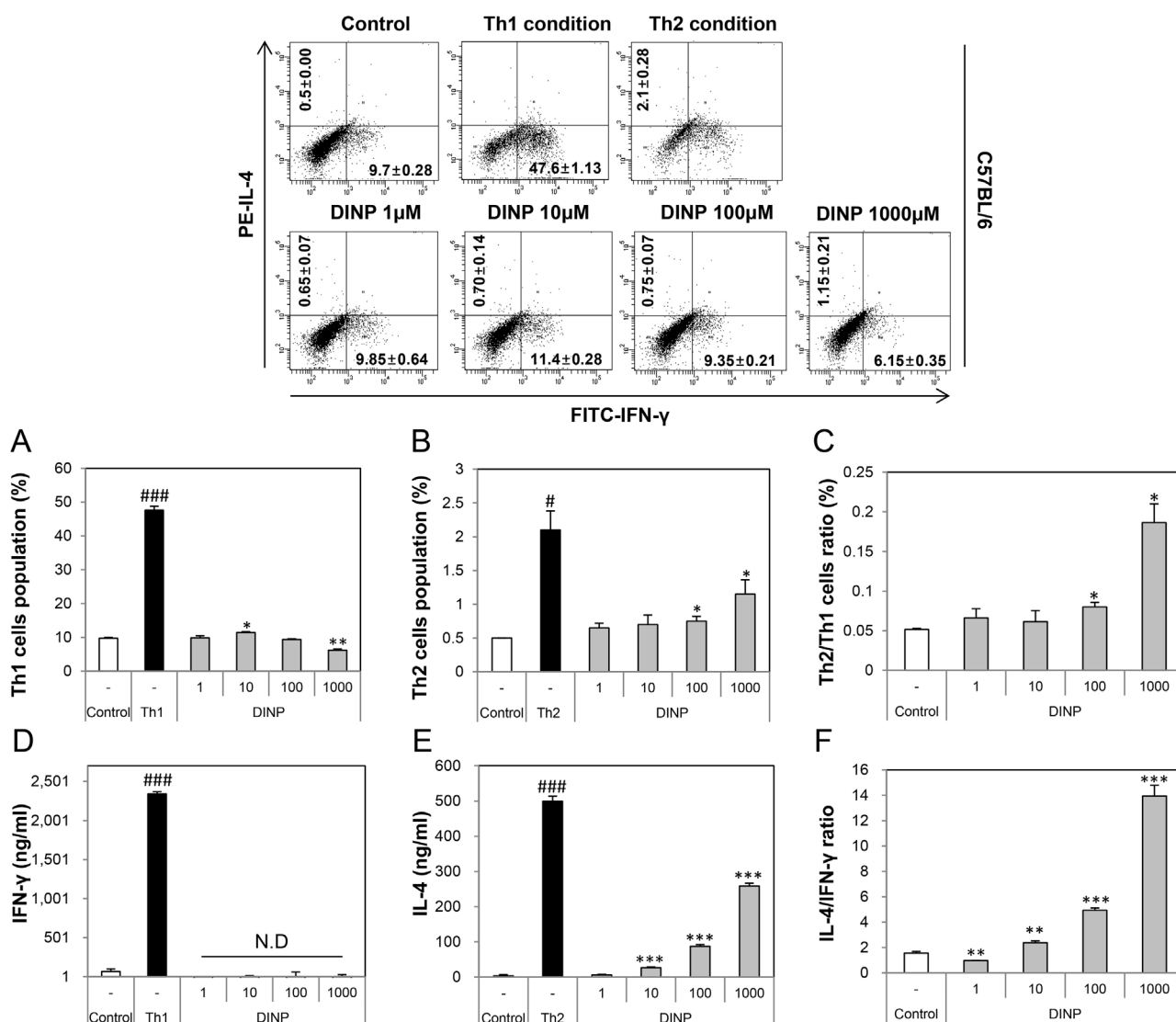


Fig. 2. Cytokine production profiles of T cells in the presence of diisononyl phthalate (DINP). Flow cytometric analysis of intracellular (A) IFN-γ and (B) IL-4 staining in CD4⁺ T cells primed with anti-CD3 plus anti-CD28 with the indicated concentrations of environmental pollutants. (C) Th2/Th1 cell population ratio. (D) IFN-γ and (E) IL-4 in supernatant of the primed CD4⁺ T cell were measured by ELISA. (F) IL-4/IFN-γ ratio. # $p < 0.05$ and ### $p < 0.001$ control vs Th1 or Th2 condition. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ Th1 or Th2 condition vs DINP (A, B, D, E), * $p < 0.05$ control vs DINP (C, F).

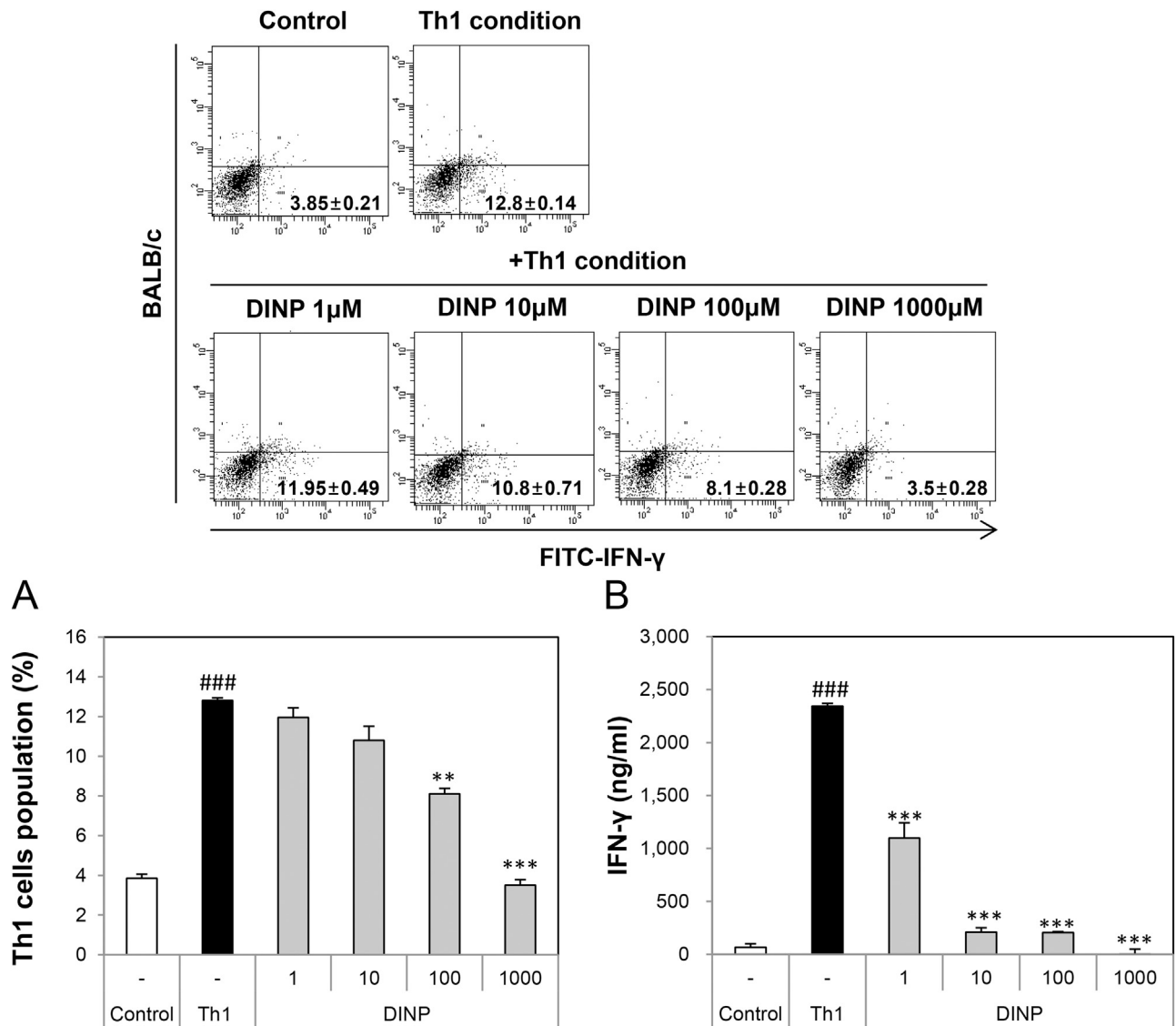
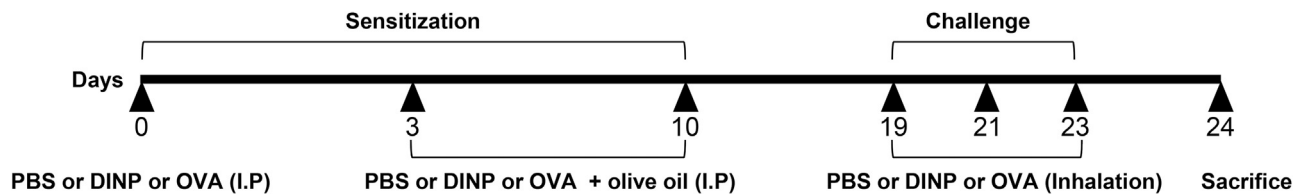


Fig. 3. Effect on Th1 cell differentiation of diisononyl phthalate (DINP) in Th1 condition. (A) Flow cytometric analysis of intracellular IFN-γ staining or (B) level of supernatant IFN-γ in CD4⁺ T cells primed with anti-CD3 plus anti-CD28 with the indicated concentration of DINP. ### $p < 0.001$ control vs Th1 condition. ** $p < 0.01$ and *** $p < 0.001$ Th1 condition vs DINP.



	0day	3day	10day	19day	21day	23day
Control	PBS (I.P)	PBS + Corn oil (I.P)		PBS (I.N)		
OVA	OVA (I.P)	OVA + Corn oil (I.P)		OVA (I.N)		
DINP	DINP (I.P)	DINP + Corn oil (I.P)		DINP (I.N)		

Fig. 4. Experimental protocol for induction of airway inflammation along with treatment scheme.

(supernatant) was suppressed by DINP (Fig. 3B). Thank you for your good comments. These results indicate that DINP inhibits Th1 polarization in the Th1 condition.

3.4. Assessment of airway hyperresponsiveness (AHR) to methacholine

To determine the effect of OVA or DINP on their airway function, the mice were exposed to MCh aerosols. The total respiratory system resistance (Rrs) and elastance (Ers) to MCh in the OVA group were significantly higher than those in the control group. The mice exposed to DINP showed higher levels than the control group (Fig. 5A, B). Moreover, the Newtonian resistance (Rn), tissue damping (G), and tissue elastance (H) in the DINP induced asthmatic mice model were significantly higher than those in the control group. The levels of R, G, and H in the asthmatic mice induced by DINP exposure were increased in comparison with those in the control group (Fig. 5C, 5D, 5E). These results suggest that DINP contributes to airway hyperresponsiveness.

3.5. Assessments of cytokines and BAL total cells in OVA or DINP induced asthmatic mice

To confirm the expression of the Th1 or Th2 mediated cytokines, we measured the numbers of IL-4, IL-5, IFN- γ , and total bronchoalveolar lavage fluid (BALF) cells. The total number of BALF cells in the OVA group was increased in comparison with that in the control group. The total cell number of the asthmatic mice group induced by DINP exposure was significantly higher than that

in the control group (Fig. 6A). The level of IL-4 in the BALF of the OVA group was increased in comparison with that in the control group. The level of IL-4 in the DINP group was significantly higher than that in the control group (Fig. 6B). The IL-5 levels did differ between the control group and OVA group. Also, exposure to DINP significantly increased the IL-5 level compared to the control group (Fig. 6C). The level of IFN- γ as a Th1 mediated cytokine was not different between the control group and OVA or DINP group (Fig. 6D). These results indicate that DINP induced the production of Th2 mediated cytokines.

3.6. Assessment of immunoglobulins in OVA or DINP induced asthmatic mice

The total IgE, IgG1, and IgG1/IgG2a ratio in the OVA group were significantly increased compared with the control group. Furthermore, the total IgE, IgG1, and IgG1/IgG2a ratio in the DINP exposure group were significantly increased compared with the control group (Fig. 7A–C). On the other hand, the total IgG2a in the OVA and DINP groups was lower than that in the control group (Fig. 7D). These results suggest that DINP increases the levels of the IgG's (IgE and IgG1) associated with the Th2 response and reduces the level of IgG2a associated with the Th1 response.

3.7. Effect of DINP on histopathological analysis

The histopathological changes in the lungs of the mice treated with OVA or DINP were examined to estimate the degree of inflammation, using H&E and PAS staining. The airways of the

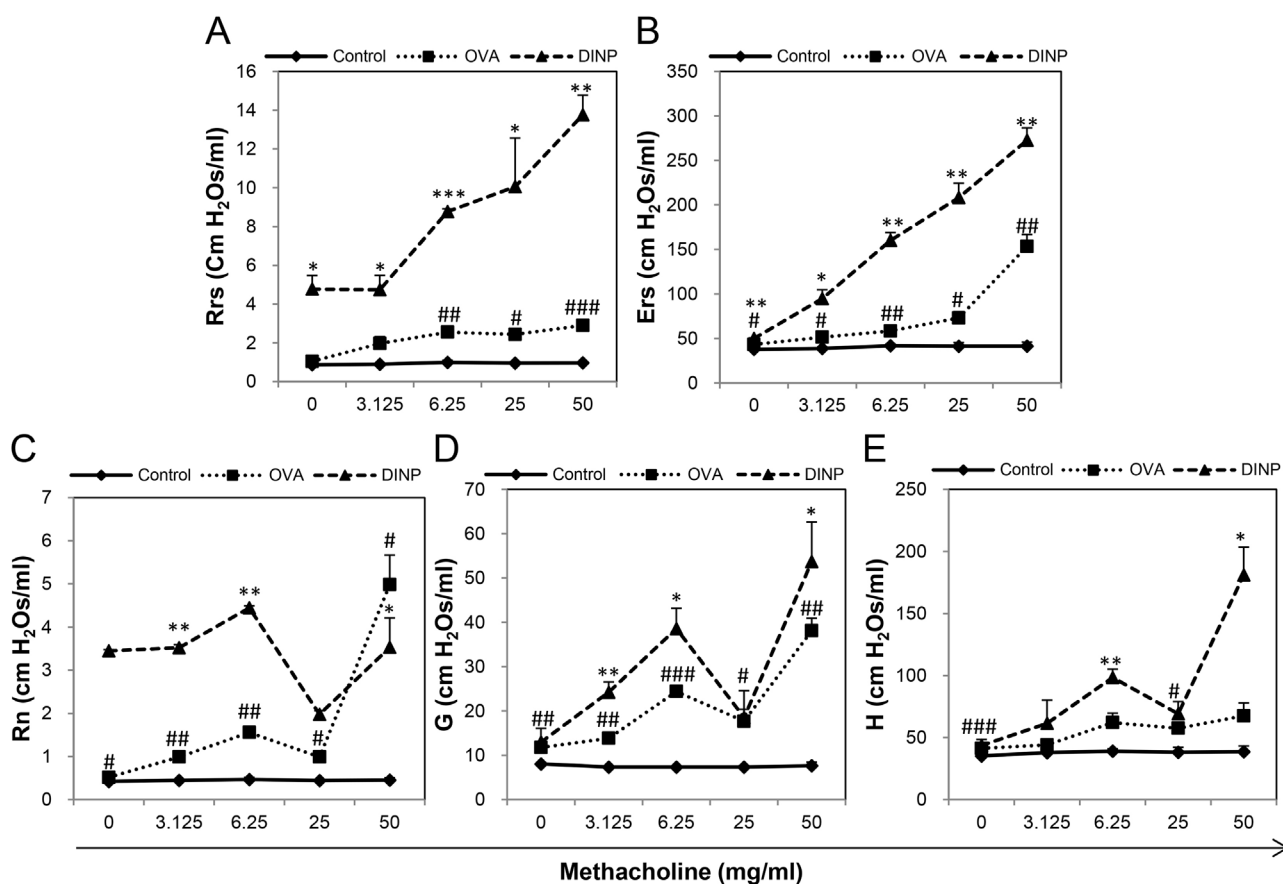


Fig. 5. Assessment of allergen-induced airway hyperresponsiveness by the forced oscillation technique. (A) Respiratory system resistance (Rrs), (B) elastance (Ers), (C) Newtonian resistance (Rn), (D) tissue damping (G), and (E) tissue elastance (H) were determined by OVA or DINP-induced asthma micel model. All data were expressed as means $\pm$ SD (n = 2). # p < 0.05, ## p < 0.01, and ### p < 0.001 control vs OVA. * p < 0.05, ** p < 0.01, and *** p < 0.001 control vs DINP.

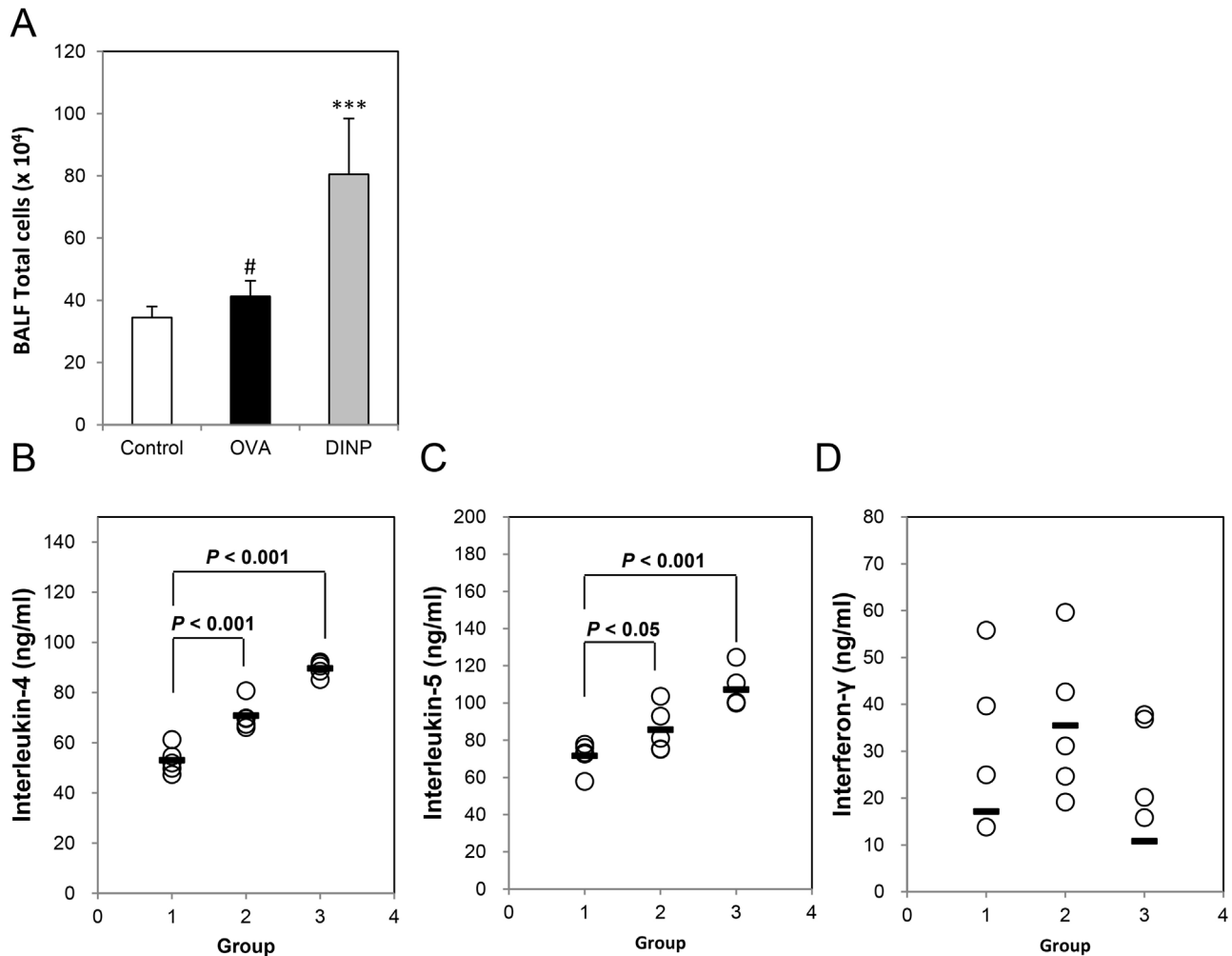


Fig. 6. Effects on total cells and Th2-mediated cytokines of DNP in BALF. C57BL/6 mice were sensitized and challenged with DNP. In these mice, BALF were collected 24 h after the last challenge. The total BALF cells were counted by trypan blue staining (A). The levels of IL-4 (B), IL-5 (C), and IFN- γ (D) in BALF were measured by ELISA. The data represent five mice per group. " indicates the mean of five mice. All data were expressed as means $\pm$ SD. # $p < 0.05$ control vs OVA. *** $p < 0.001$ control vs DNP. Group numbers (1 = control, 2 = OVA, 3 = DNP).

asthmatic mice exposed to OVA or DNP were narrower than those in the control group. The infiltration of inflammatory cells in the airway epithelium of the mice exposed to OVA or DNP was increased in comparison with that of the control group (Fig. 8A, C). To observe the interstitial goblet cell hyperplasia, we performed PAS staining. The numbers of PAS positive cells in the OVA and DNP treated groups were higher than that in the vehicle group (Fig. 8B, D). Further evidence of airway inflammation was obtained by caspase-1 and caspase-3 immunohistochemistry. The levels of caspase-1 and caspase-3 expression of the OVA and DNP treated groups were increased in comparison with those of the control group (Fig. 9). These results suggest that DNP induces airway inflammation by enhancing the infiltration of inflammatory cells, goblet cell hyperplasia, and expression of caspase-1, 3.

4. Discussion

The occurrence of asthma and allergic disorders may be increasing in industrialized countries due to exposure to various environmental pollutants (Andrae et al., 1988). Major changes in the building materials and consumer products used indoors have occurred over the past half-century. Materials such as composite-

wood, polymeric flooring, and plastic items have become ubiquitous. Concern about the effect on human health of Di-2-ethylhexyl phthalate (DEHP), which is used as a plasticizer for PVC flooring, has increased. For this reason, DEHP has been replaced by diisononyl phthalate (DINP). Exposure to the various chemicals found in the indoor environment which act as indoor pollutants may be increasing in humans, because indoor air is not well ventilated (Charles and Weschlara, 2009). For example, DEHP has an adjuvant effect on airway inflammation in combination with ovalbumin (OVA) through oxidative stress in Balb/c mice (You et al., 2016; You et al., 2014). Moreover, di-n-butyl phthalate (DnBP), di-n-octyl phthalate (DnOP), di-iso-nonyl phthalate (DINP), and di-iso-decyl phthalate (DIDP) have adjuvant effects by increasing the levels of IgE and IgG1 (Larsen et al., 2002).

Naïve CD4⁺ T cells can differentiate into helper T cells (Th) such as Th1, Th2, Th9, Th17, and Th22 cells, and the inflammatory response by Th2 cells plays an important role in the asthmatic airway (Robinson, 2010). DINP has been shown to induce hepatic and renal tissue injury through the accumulation of ROS (Ma et al., 2014). However, the concentrations of DINP used in this study were not cytotoxic toward the naïve CD4⁺ T cells. The naïve CD4⁺ T cells primed by anti-CD3 and anti-CD28 can develop into IL-4 producing

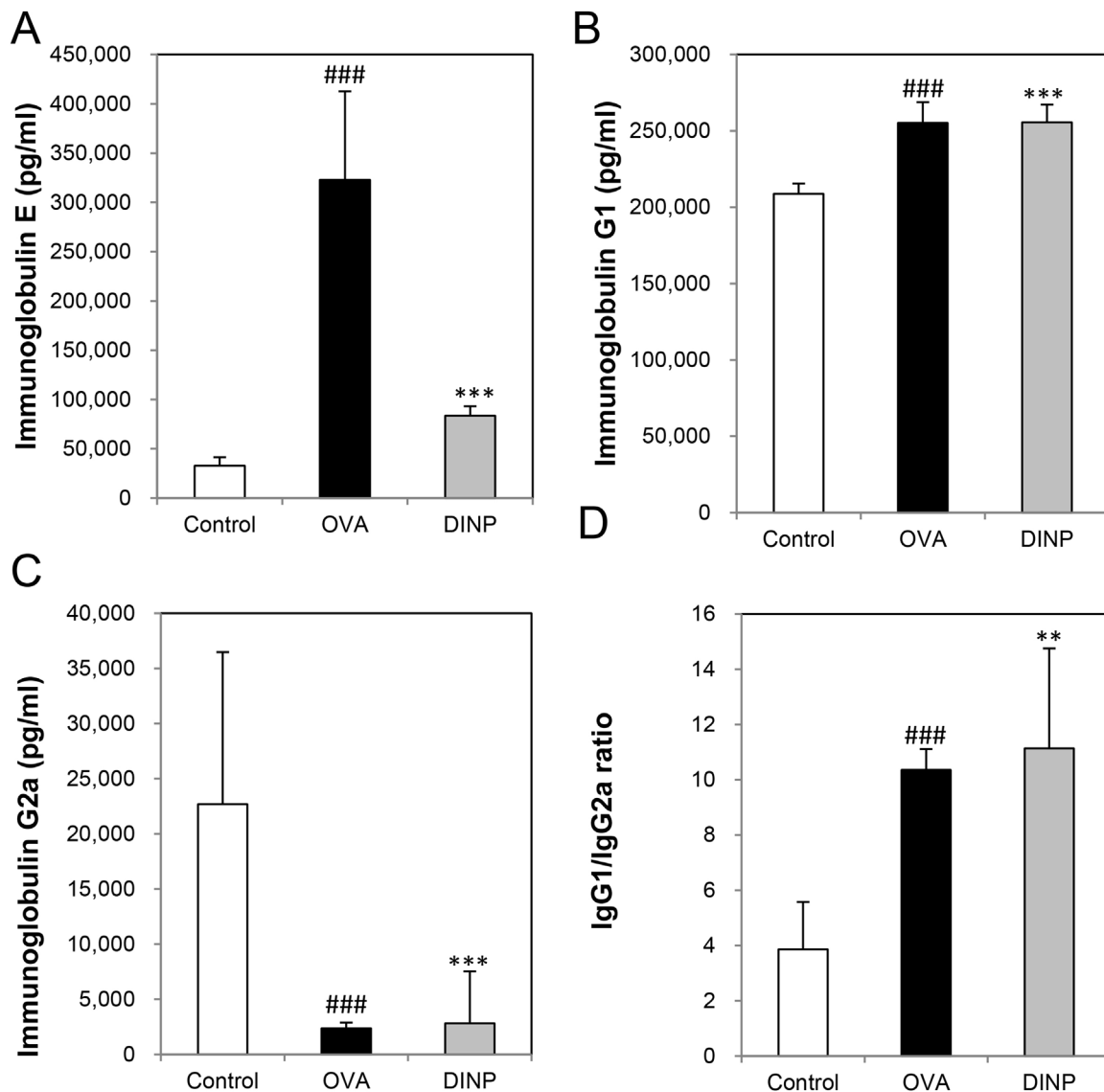


Fig. 7. Effects on immunoglobulin of DINP in serum. C57BL/6 mice were sensitized and challenged with DINP. In these mice, serum was collected 24 h after the last challenge. The levels of IgE (A), IgG1 (B), IgG2a (C), and IgG1/IgG2a ratio (D) in BALF were measured by ELISA. The data represent five mice per group. All data were expressed as means $\pm$ SD. ### $p < 0.001$ control vs OVA. ** $p < 0.01$, and *** $p < 0.001$ control vs DINP.

Th2 cells and IFN- γ producing Th1 cells, respectively (Bix et al., 1998). The phenotype of the Th cells generated was assayed by the intracellular staining of IFN- γ and IL-4 after re-stimulation with ionomycin and PMA. DINP at 1000 μ M suppressed the development of the Th1 cells and augmented the polarization of the Th2 cells, and the Th2/Th1 ratio was increased by exposure to DINP. Furthermore, the naïve CD4 $^{+}$ T cells primed under the Th1-polarizing condition (anti-IL-4 plus IL-12) were inhibited from developing into Th1 cells by DINP.

Airway hyperresponsiveness (AHR) is a key pathophysiological feature of asthma. Asthma, as a chronic inflammatory disorder, is associated with AHR, which leads to clinical symptoms (Brannan and Loughheed, 2012). Exposure to DINP enhanced the respiratory system resistance (Rrs), respiratory system elastance (Ers), central airway resistance (Rn), lung tissue damping (G), and lung tissue elastance (G) as much as in the OVA-induced asthmatic mice. Therefore, we demonstrated that DINP itself induces AHR.

The IL-4 produced by Th2 cells is a key cytokine in the development of allergic inflammation and is associated with the

secretion of IgE by B lymphocytes (Coffman et al., 1986). The IgE-mediated allergic immune response is increased by IL-4 through the up-regulation of the high-affinity IgE receptor (Fc ϵ RI) on mast cells and basophils (Pawankar et al., 1997). IgE bound to Fc ϵ RI induces the release of mediators such as histamine, PGD2 and TNF maturation form stimulated mast cell degranulation, which promote the recruitment and migration of Th2 cells (Galli and Tsai, 2012). Histamine contributes to airway inflammation through the stimulation of smooth muscle cell contraction and mucus secretion (Hart, 2001). DINP increased the total cell number and IL-4 level in the bronchoalveolar lavage fluid (BALF) and the level of IgE in the serum. Furthermore, we confirmed that effect of DINP itself on the release of histamine from mast cells. DINP was shown to induce the release of histamine from the human mast cell line, HMC-1 (Supplementary Fig. 1). IL-5 as a Th2-mediated cytokine acts as a mediator of eosinophils, influencing their adhesion and causing membrane receptor expression (Tomasiak-Łozowska et al., 2010). We confirmed that IL-5 was increased in the BALF of the DINP exposed mice. In contrast, the IFN- γ secreted by the Th1 cells

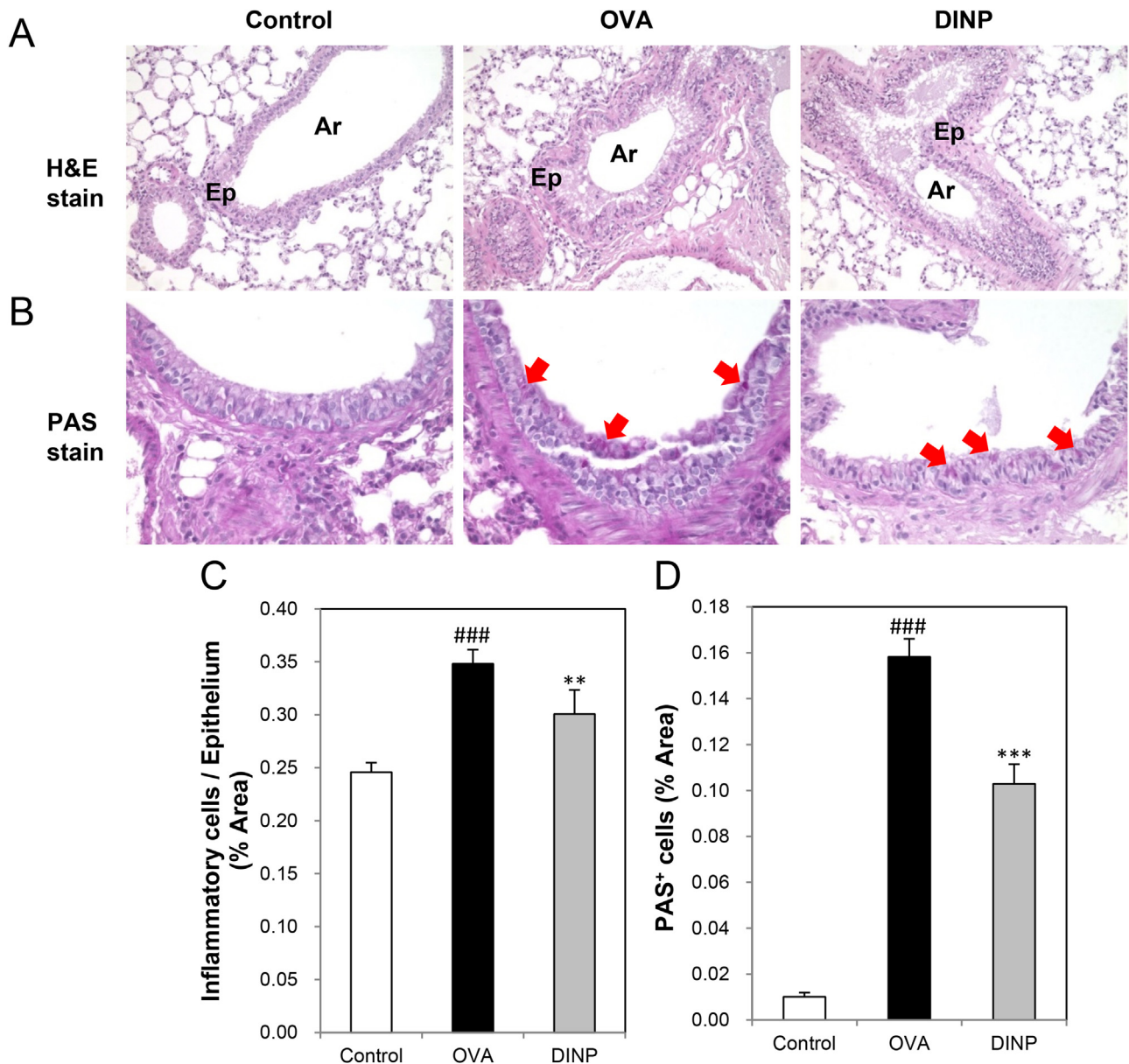


Fig. 8. Effect of OVA or DINP on histology of lung tissue in OVA or DINP-induced murine model of asthma. The C57BL/6 mice were sensitized and challenged with OVA or DINP for 23 days for asthma induction. At the end of the experiment, the mice lungs were removed. The lungs were stained by (A) H&E (×200) and (B) PAS (×400). (C) Quantitative analyses of airway inflammation in lung sections via H&E staining. (D) The percentage of PAS-positive cells in the lung sections were measured via PAS staining. The data represent five mice per group. All data were expressed as means ± SD. ### p < 0.001 control vs OVA. ** p < 0.01 and *** p < 0.001 control vs DINP. H&E: hematoxylin-eosin staining, PAS: Periodic acid-Schiff staining.

suppresses Th2 cell differentiation, eosinophilia, mucus goblet cell hyperplasia and bronchial hyper-responsiveness (Chung, 2001). The IFN- γ level of the DINP exposed mice was not significantly decreased compared with the control group. However, the IFN- γ level (0.1153 ± 0.01) of the DINP exposed mice was slightly reduced compared with the control group (0.1172 ± 0.01). IgG1 was switched by IL-4 and IFN- γ is known to be an important switch factor for IgG2a (Bergstedt-Lindqvist et al., 1988; Van Oosterhout et al., 1998). In this study, DINP increased the IgG1 level and suppressed IgG2a.

Generally, the airways in asthma are infiltrated by inflammatory cells, such as activated lymphocytes and eosinophils, which induce goblet cell hyperplasia, leading to phenotypic changes in the airway epithelium (Jeffery, 1992; Blyth et al., 1996). Furthermore, caspase-1, as an interleukin (IL)-1-converting enzyme,

cleaves the cytokine pro-interleukin (IL)-1 β to form an active secreted molecule in monocytes and macrophages (Denes et al., 2012). Caspase-3 was increased in the epithelium and submucosa of human bronchial biopsies compared with that in subjects without asthma (Benayoun et al., 2001). Airway inflammation in the lungs of the DINP induced asthmatic mice was induced through the infiltration of inflammatory cells and enhancement of the goblet cells. Furthermore, caspase-1 and caspase-3 were expressed in the airway epithelium of the DINP exposed mice.

5. Conclusion

It is well known that asthma usually occurs due to the presence of a larger number of Th2 cells than Th1 cells. DINP is an EDC and several EDCs have been reported to cause naive CD4⁺ T cells to

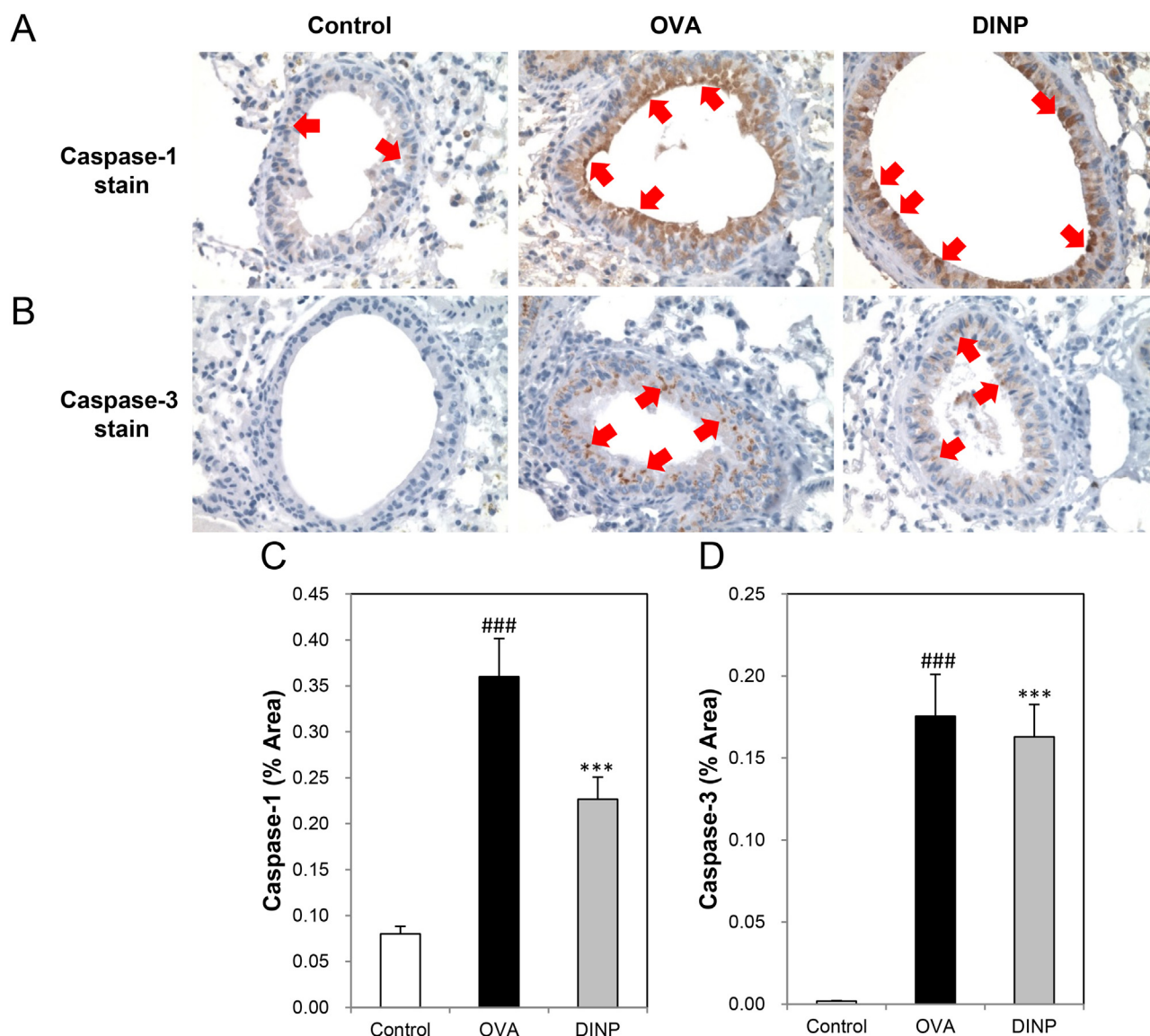


Fig. 9. Effect of OVA or DINP on immunohistochemistry in lung tissue of OVA or DINP-induced murine model of asthma. C57BL/6 mice were sensitized and challenged with OVA or DINP for 23 days for asthma induction. At the end of the experiment, the mice lungs were removed. The lungs were stained by (A) caspase-1 ($\times 200$) and (B) caspase-3 ($\times 200$) immunohistochemistry. The percentages of (C) caspase 1 or (D) caspase 3-positive cells in the lung sections measured via caspase-1 or caspase-3 immunohistochemistry staining. All data were expressed as means $\pm$ SD. The data represent five mice per group. ### $p < 0.001$ control vs OVA. *** $p < 0.001$ control vs DINP.

differentiate into Th2 cells. In this study, we demonstrated that DINP suppresses Th1 differentiation and promotes Th2 polarization from naïve CD4⁺ T cells in vitro. Airway hyperresponsiveness in the respiratory system of animals sensitized and challenged with DINP was induced in vivo through the enhancement of the Th2 mediated cytokines or immunoglobulins in the BALF and serum, respectively. Moreover, the enhancement of inflammation and caspase-1, 3 was observed in the lungs of the asthmatic mice exposed to DINP. Therefore, exposure to DINP, which is used as a PVC material, may cause asthma in humans.

Conflict of interests

The authors declare that they have no conflicts of interest.

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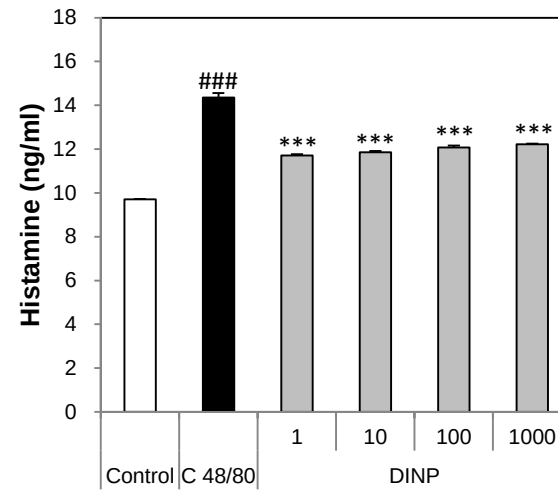
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.toxlet.2017.03.014>.

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Supplement Figure 1. Effect of DNP on histamine release in HMC-1. HMC-1 cells (3×10^6 cells) were cultured with or without DNP for 30 min, and then stimulated with compound 48/80 (300 $\mu\text{g/ml}$) for 20 min at 37°C. After stimulation, the histamine contents were quantified using an automated fluorometric method.