Di-(2-ethylhexyl) phthalate enhances cytokine release from group 2 innate lymphoid cells in the presence of interleukin-33

Short Running Title: DEHP enhances cytokine release in ILC2

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Epidemiological and experimental studies have shown that di-(2-ethylhexyl) phthalate (DEHP), a plasticizer, can aggravate allergic diseases. DEHP promotes adaptive immune responses; however, its effect on the innate immune system remains largely unknown. The present study investigated the effects of DEHP on group 2 innate lymphoid cells (ILC2) that produce Th2 cytokines in response to epithelial cell-derived cytokines, such as interleukin (IL)-33. ILC2 (lineage-negative, CD45.2$^+$, Sca1$^+$, KLRG1$^+$) were isolated from the lungs of C57BL/6J mice. Co-exposure to DEHP and IL-33 significantly increased IL-5 release from ILC2, whose level was higher than that of the vehicle and IL-33 alone. The effects of DEHP in the presence of IL-33 showed an inverted-U dose-response. The present is the first report showing that DEHP exacerbates allergy through the innate immune system.

Key words: DEHP, ILC2, innate immune system, allergy

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Declaration of competing interest
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Authors' contribution statement
Akiko Honda: Formal analysis, Investigation, Writing - Original Draft, Project administration, **Megumi Nagao:** Formal analysis, Investigation, Visualization, **Michitaka Tanaka**: Investigation, **Wang Zaoshi**: Investigation, **Hirohisa Takano:** Conceptualization, Writing - Review & Editing, Supervision, Funding acquisition

1. Introduction

A large number of epidemiological and experimental studies have reported that environmental pollutants aggravate allergic diseases (Rosário Filho et al., 2021). Adaptive immunity is believed to play a critical role in allergic responses; however, recognition of the importance of innate immunity in the pathogenesis of allergic diseases is emerging (Caminati et al., 2018). Moro et al. (2010) discovered natural helper cells in the peritoneal cavity adipose tissues; these cells produce large amounts of Th2 cytokines, including IL-5, when stimulated by epithelial cell-derived cytokines such as IL-33. Later, these natural helper cells were named group 2 innate lymphoid cells (ILC2), and have since been detected in the lungs and bronchoalveolar lavage fluid (BALF) (Moro et al., 2015). Several studies suggested that air pollutants, particulate matter, and fibers activate ILC2 in the innate and adaptive immune systems (Estrella et al., 2019; Lee et al., 2019).

However, the impact of environmental chemicals on innate immunity remains to be elucidated.

Di-(2-ethylhexyl) phthalate (DEHP), a widely used plasticizer present in vinyl film, building materials, and consumer products, is a representative allergic disease-exacerbating chemical (Wang et al., 2019). Epidemiologically, indoor DEHP levels correlate positively with the number of pediatric asthma cases (Bornehag et al., 2004). Experimentally, *in vivo* studies indicate that DEHP enhances total IgE level, eosinophil recruitment, and histological changes characteristic of allergic disease in a murine model of asthma. Similarly, DEHP increases IL-5 levels in BALF, associated with eosinophil-mediated inflammation (You et al., 2016). *In vitro* studies have reported that DEHP promotes dendritic cell maturation and T-cell receptor (TCR) expression on splenocytes (Koike et al., 2009). It, therefore, appears that DEHP modulates adaptive immune responses via promoting the release of T helper 2 (Th2) cytokines such as IL-5. However, the effects of DEHP and other environmental chemicals on innate immune system functioning have not yet been clarified. Therefore, we investigated the effects of DEHP on ILC2 to determine whether this may be one mechanism by which DEHP exacerbates allergic diseases.

2. Material and methods

2.1. Preparation of ILC2

ILC2 were obtained using a modified version of a previously reported protocol (Moro et al., 2015). Sixteen-week-old SPF C57BL/6J male mice (n = 15) were purchased from CLEA Japan, Inc. (Tokyo, Japan). Mice were housed in an animal facility, maintained at 24–26 °C and a 12 h light/dark cycle, under conventional conditions. All animal experiments were approved by the Animal Research Committee at Kyoto University. The mice were sacrificed by an overdose of pentobarbital. The lung ILC2 was identified as lineage-negative ($CD3\varepsilon^-$, $CD4^-$, $CD8\alpha^-$, $CD11c^-$, $Fc\varepsilon Ri\alpha^-$, $NK1.1^-$, $CD19^-$, $TER119^-$, $CD5^-$, $F4/80^-$, $LyG6/Ly6c^-$), $CD45.2^+$, $Sca1^+$, $KLRG1^+$ cell. Flow cytometric analysis was performed using a BD FACS Melody Flow Cytometer and analyzed with FlowJo Software (BD Biosciences, San Jose, CA, USA).

2.2. DEHP exposure

The isolated ILC2 (approximately 1000 cells/well) were exposed to DEHP (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) at the concentrations of 0, 0.012, 0.06, 0.3 μM for 2, 5, and 7 days in the presence or absence of 0.5 ng/mL IL-33 (R&D Systems, Minneapolis, MN, USA). DEHP and IL-33 were dissolved in dimethyl

sulfoxide (DMSO; Sigma) and phosphate-buffered saline (PBS), respectively. The doses of DEHP were selected based on a prior report (Koike et al., 2009). Of note, the serum DEHP concentration of healthy volunteers is reportedly less than 1 μg/ml (2.56 μM) (Luisi et al., 2006).

2.3.Quantitation of IL-5 and cell viability

IL-5 concentration and cell viability were evaluated using enzyme-linked immunosorbent assay (ELISA; Thermo Fisher Scientific) and Cell Counting Kit-F (Dojindo Laboratories, Kumamoto, Japan), respectively. The cell counting kit produces a highly sensitive fluorescent dye upon enzymatic hydrolysis by esterases in living cells. The amount of the fluorescent dye is directly proportional to the number of viable cells in a culture medium. The results were expressed as the percentage of viable cells compared with vehicle (0 μg/mL).

2.4. Statistical analysis

Data are presented as mean ± standard error of the mean (S.E.M.) for each experimental group (n = 4). The differences were analyzed using Two-way repeated measure ANOVA and Tukey's HSD (Excel Statistics 2012, Social Survey Research Information Co. Ltd., Tokyo, Japan).

3. Results

Flow cytometry data demonstrated that ILC2 represent ~1 % of total cells present in pulmonary tissue. Furthermore, IL-33 increased IL-5 release from ILC2 at five days of exposure compared to vehicle. The addition of DEHP resulted in a more significant increase in IL-5 release than IL-33 alone. DEHP-induced enhancement showed maximal activity at a dose of 0.012 μM and then decreased at 0.06 and 0.3 μM. However, DEHP alone did not increase IL-5 release (Figure 1). Finally, IL-33 alone or combined with DEHP increased ILC2 viability at seven days of exposure, while DEHP alone did not (Figure 2).

4. Discussion

To the best of our knowledge, this is the first study demonstrating that DEHP enhances IL-5 release from ILC2 in the presence of IL-33. Moreover, even at the low concentration of 0.012 μM, DEHP exhibits a remarkable ILC2-activating effect. Previous studies have reported that multi-walled carbon nanotubes, O_3, diesel exhaust particles, and cigarette smoke (Estrella et al., 2019; Lee et al., 2019) activate ILC2 of pulmonary or BALF in the presence or absence of allergens. We have demonstrated that, in addition to

such particulate, fibrous, or gaseous air pollutants, DEHP, one of the most common environmental chemicals, can contribute to ILC2 activation.

Phthalates, such as DEHP and di(isononyl) phthalate (DINP), reportedly enhance IL-5 or IL-33 levels in murine allergy models such as asthma, atopic dermatitis, and rhinitis (Zou et al., 2020; Qin et al., 2020). Interestingly, the present study indicates that DEHP alone is insufficient to induce IL-5 release from ILC2 but enhances ILC2 cytokine release in the presence of IL-33, which may contribute to the aggravation of allergic symptoms.

A previous study has also shown that exposure to DEHP at 1 and 10 nM for 72 h significantly enhanced antigen-stimulated proliferation of splenocytes compared with control. The DEHP-induced enhancement showed maximal activity at these concentrations and then decreased at 100 and 1000 nM (Koike et al., 2009). Besides, previous *in vivo* reports have also shown an inverted-U dose-response of DEHP using the allergic model (Takano et al., 2006). These results are in accordance with the present study, which may indicate that DEHP affects immune cells, including ILC2, via mechanisms similar to those of endocrine-disrupting chemicals.

We assessed cellular viability using the fluorescent intensity of calcein (produced by esterase-mediated hydrolysis), which is directly proportional to the number of viable

cells. Consistent with our findings, Bartemes et al. (2012) demonstrated that IL-33 increases the number of ILC2 for up to 10 days *in vitro*. In the presence of IL-33, DEHP did not alter the number of viable ILC2, whereas it did increase IL-5 release. Therefore, it is likely that the increase in IL-5 release following DEHP exposure in the presence of IL-33 is not attributable to an increase in the number of ILC2.

In conclusion, our results demonstrate that DEHP enhances IL-5 release from ILC2 in the presence of IL-33, revealing for the first time a novel mechanism by which DEHP, an abundant environmental chemical, exacerbates allergy via interaction with the innate immune system.

Reference

Bartemes, K.R., Iijima, K., Kobayashi, T., Kephart, G.M., McKenzie, A.N., Kita, H. 2012. IL-33-responsive lineage- CD25+ CD44(hi) lymphoid cells mediate innate type 2 immunity and allergic inflammation in the lungs. J. Immunol. 188, 1503–1513. doi: 10.4049/jimmunol.1102832.

Bornehag, C.G., Sundell, J., Weschler, C.J., Sigsgaard, T., Lundgren, B., Hasselgren, M., Hägerhed-Engman, L. 2004. The association between asthma and allergic symptoms in children and phthalates in house dust: a nested case-control

study. Environ. Health. Perspect. 112, 1393–1397. doi:10.1289/ehp.7187.

Caminati, M., Pham, D.L., Bagnasco, D., Canonica, G.W. 2018. Type 2 immunity in asthma. World Allergy Organ. J. 11, 13.

Estrella, B., Naumova, E.N., Cepeda, M., Voortman, T., Katsikis, P.D., Drexhage, H.A. 2019. Effects of Air Pollution on Lung Innate Lymphoid Cells: Review of In Vitro and In Vivo Experimental Studies. Int. J. Environ. Res. Public Health 16, 2347.

Koike, E., Inoue, K., Yanagisawa, R., Takano, H. 2009. Di-(2-ethylhexyl) phthalate affects immune cells from atopic prone mice in vitro. Toxicology. 259, 54–60. doi:10.1016/j.tox.2009.02.002.

Lee, H.S., Park, D.E., Lee, J.W., Kim, H.N., Song, W.J., Park, H.W., Cho, S/H. 2019. Critical role of interleukin-23 in development of asthma promoted by cigarette smoke. J. Mol. Med. (Berl) 97, 937–949. doi: 10.1007/s00109-019-01768-y.

Luisi, S., Latini, G., de Felice, C., Sanseverino, F., di Pasquale, D., Mazzeo, P., Petraglia, F. 2006. Low serum concentrations of di-(2-ethylhexyl)phthalate in women with uterine fibromatosis. Gynecol. Endocrinol. 22, 92–95.

Moro, K., Yamada, T., Tanabe, M., Takeuchi, T., Ikawa, T., Kawamoto, H., Furusawa, J., Ohtani, M., Fujii, H., Koyasu, S. 2010. Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. Nature 463, 540–544.

doi: 10.1038/nature08636.

Moro, K., Ealey, K.N., Kabata, H., Koyasu, S. 2015. Isolation and analysis of group 2 innate lymphoid cells in mice. Nat Protoc, 10, 792–806. doi: 10.1038/nprot.2015.047.

Qin, W., Duan, J., Xie, X., Kang, J., Deng, T., Chen, M. 2020. Exposure to diisononyl phthalate promotes atopic march by activating of NF-κB and p38 MAPK. Toxicol. Appl. Pharmacol. 395, 114981. doi: 10.1016/j.taap.2020.114981.

Rosário Filho, N.A., Urrutia-Pereira, M., D'Amato, G., Cecchi, L., Ansotegui, I.J., Galán, C., Pomés, A., Murrieta-Aguttes, M., Caraballo, L., Rouadi, P., Chong-Neto, H.J., Peden, D.B. 2021. Air pollution and indoor settings. World Allergy Organ, J. 14, 100499.

Takano, H., Yanagisawa, R., Inoue, K., Ichinose, T., Sadakane, K., Yoshikawa, T. 2006. Di-(2-ethylhexyl) phthalate enhances atopic dermatitis-like skin lesions in mice. Environ. Health Perspect. 114, 1266–1269. doi: 10.1289/ehp.8985.

You, H., Li, R., Wei, C., Chen, S., Mao, L., Zhang, Z., Yang, X. 2016. Thymic stromal lymphopoietin neutralization inhibits the immune adjuvant effect of di-(2-ethylhexyl) phthalate in Balb/c mouse asthma model. PLoS One 11:e0159479. doi: 10.1371/journal.pone.0159479.

Wang, Y., Zhu, H., Kannan, K. 2019. A review of biomonitoring of phthalate exposures. Toxics. 7, 21.

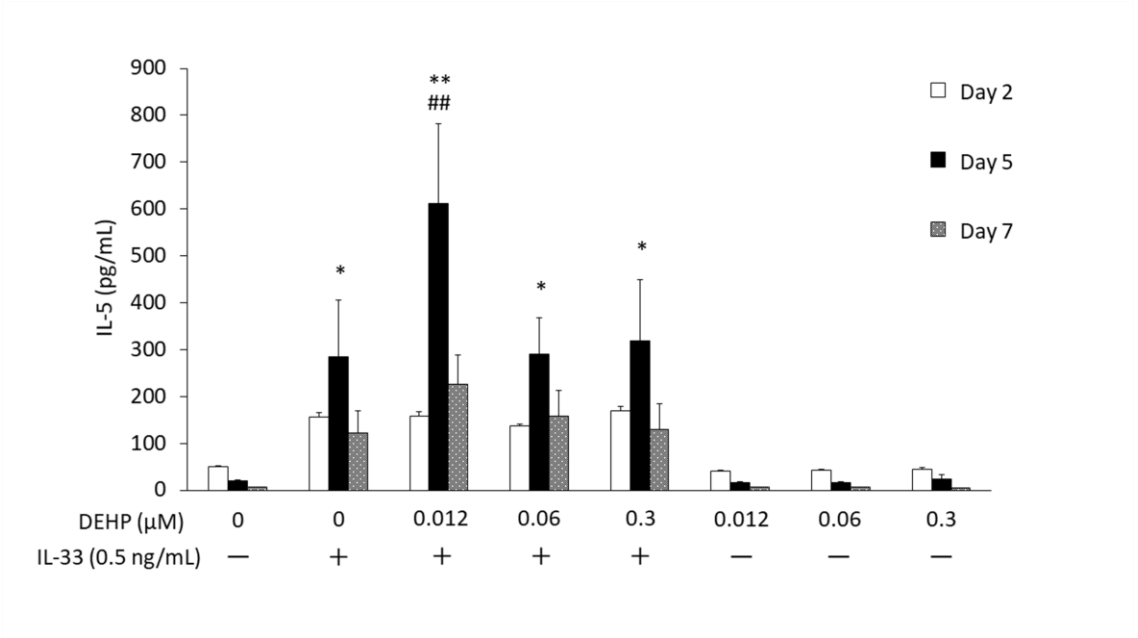
Zou, Q.Y., Hong, S.L., Kang, H.Y., Ke, X., Wang, X.Q., Li, J., Shen, Y. 2020. Effect of di-(2-ethylhexyl) phthalate (DEHP) on allergic rhinitis. Sci. Rep. 10, 14625. doi: 10.1038/s41598-020-71517-6.

Figure Legends

Figure 1. IL-5 release from ILC2 in response to DEHP in the presence and absence of IL-33. IL-5 levels in the culture supernatant were measured by ELISA. Data are presented as mean ± S.E.M. of four individual cultures. **$p < 0.01$, *$p < 0.05$ versus vehicle on the same day. $^{\#\#}p < 0.01$ versus IL-33 alone on the same day.

Figure 2. Effects of DEHP on the viability of ILC2. Cell viability was assessed by Cell Counting Kit-F. The amount of the fluorescent dye, calcein, produced by hydrolysis by esterases in cells is directly proportional to the number of viable cells in a culture medium. Cells were treated with DEHP in the presence of IL-33 for 7 days. Data are presented as mean ± S.E.M. of four individual cultures. **$p < 0.01$, *$p < 0.05$ versus vehicle.

Fig.1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IL-5 (pg/mL)
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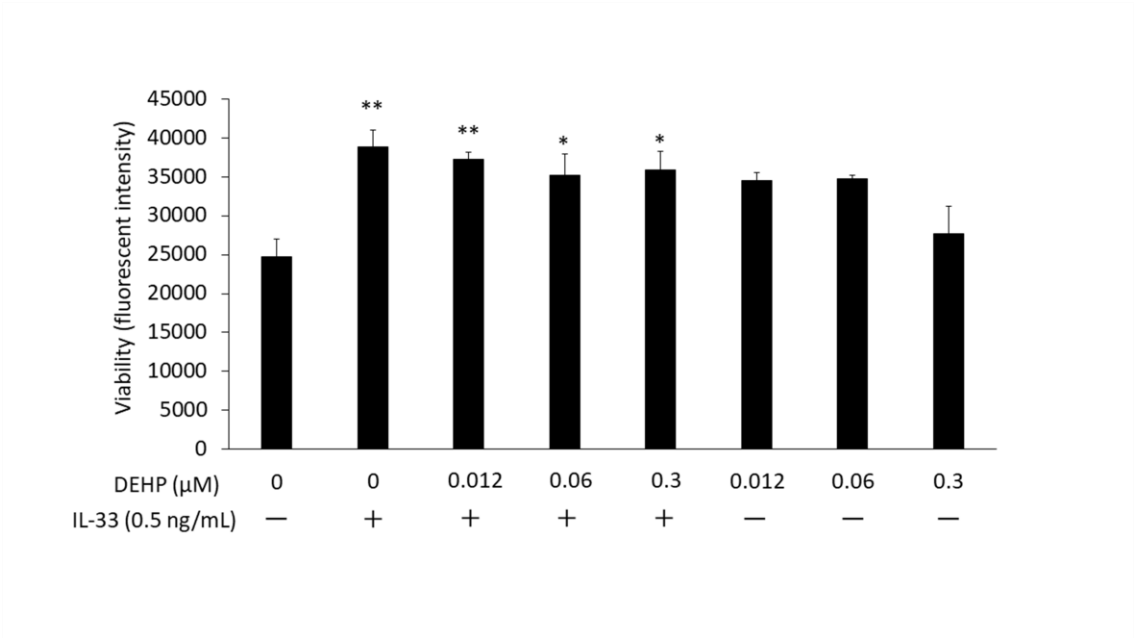
Fig.2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IL-33 (0.5 ng/mL) — + + + + — — —