

Acute and Subacute Oral Toxicity of Deoxynivalenol Exposure in a *Dermatophagoides farinae*-Induced Murine Asthma Model

Toa Ookawara,* Ryota Aihara,* Ai Morimoto,* Naoki Iwashita*,†
Keigo Kurata,‡ Yoshiichi Takagi,*§ Atsushi Miyasaka,|| Masayo Kushiro,||
Shiro Miyake,|||| and Tomoki Fukuyama *,1

*Laboratory of Veterinary Pharmacology, School of Veterinary Medicine, Azabu University, Sagamihara-shi, Kanagawa 252-5201, Japan †Bioalchemis, Fuchu-shi, Tokyo 183-0032, Japan ‡Institute of Tokyo Environmental Allergy, ITEA Inc, Bunkyo-ku, Tokyo 113-0034, Japan §Japan SLC, Inc, Hamamatsu-shi, Shizuoka 433-8102, Japan ||Kyushu Okinawa Agricultural Research Center, National Agriculture and Food Research Organization (NARO), Koshi, Kumamoto 861-1192, Japan ||Food Research Institute, NARO, Tsukuba, Ibaraki 305-8642, Japan; and ||||Laboratory of Food and Hygiene, School of Food and Life Science, Azabu University, Sagamihara-shi, Kanagawa 252-5201, Japan

¹To whom correspondence should be addressed at Laboratory of Veterinary Pharmacology, School of Veterinary Medicine, Azabu University, 1-17-71 Fuchinobe, Chuo-ku, Sagamihara-shi, Kanagawa 252-5201 Japan. E-mail: t-fukuyama@azabu-u.ac.jp.

ABSTRACT

Previously, researchers have demonstrated that mycotoxin deoxynivalenol (DON) significantly enhances immunocyte activation. However, the interaction between DON exposure and immune disorders remains unclear. In this study, we aimed to investigate whether acute and subacute oral exposure to DON exacerbates the development of respiratory allergy using a mite allergen (*Dermatophagoides farinae*, Derf)-induced mouse model of asthma. The direct relationship between DON exposure and asthma development was examined following acute oral DON administration (0, 0.1, or 0.3 mg/kg body weight), immediately before the final mite allergen challenge. Simultaneously, the influence of subacute oral exposure via low dose DON contaminated wheat (0.33 ppm) was evaluated using the same settings. To detect the proinflammatory effects of DON exposure, we examined the total and Derf-specific serum IgE levels, histology, number of immunocytes, and cytokine and chemokine secretion. Acute oral DON significantly enhanced the inflammatory responses, including cellular infiltration into bronchoalveolar lavage fluid, infiltration of immunocytes and cytokine production in local lymph nodes, and cytokine levels in lung tissues. Corresponding proinflammatory responses were observed in a mouse group exposed to subacute oral DON. *In vivo* results were validated by *in vitro* experiments using the human bronchial epithelial (BEAS-2B) and human eosinophilic leukemia (EoL-1) cell lines. Following exposure to DON, the secretion of interleukin (IL)-1 β , IL-6, IL-8, and/or tumor necrosis factor- α in BEAS-2B cells, as well as EoL-1 cells, increased significantly. Our findings indicate that DON exposure is significantly involved in the proinflammatory response observed in respiratory allergy.

Key words: mycotoxin; deoxynivalenol; inflammation; asthma; *Dermatophagoides farinae*; IL-33; TSLP; BALB/c mouse; eosinophil; bronchial epithelial cell.

Contamination with mycotoxins such as type B trichothecenes (eg, deoxynivalenol, DON) is known to exert toxic effects on farm animals, causing distress and reduced productivity (Gruber-Dorminger et al., 2019). Furthermore, some mycotoxins may transfer into livestock products, including meat, eggs, and milk (Becker-Algeri et al., 2016), thereby compromising the safety of human consumers. DON is one of the most prevalent mycotoxins produced by groups of *Fusarium* fungi, and grains (family of wheat) are often exposed to DON. According to the recent international survey by Becker-Algeri et al. (2016), 64% of the 74 821 samples collected from 100 countries were contaminated with DON; DON demonstrated the highest median concentrations of all mycotoxins, reportedly 388 µg/kg. Therefore, the safety assessment of DON necessitates immediate attention.

DON is regarded as a vomitoxin, with many previous reports indicating its influence on the digestive system, resulting in vomiting and diarrhea (Amuzie et al., 2009; Kang et al., 2019; Luo et al., 2019; Meky et al., 2001; Metzler-Zebeli et al., 2020; Pierron et al., 2018; Wang et al., 2019). Additionally, several studies have demonstrated the immunotoxic effect of DON, including enhanced proinflammatory cytokine production in the immune system, resulting in vomiting, diarrhea, and immune disorders. These reports suggest that there exists a possible interaction between DON exposure and the development of immune disorders. Bol-Schoenmakers et al. (2016) have reported that DON facilitates allergic sensitization to food proteins and the development of sensitization can be induced by distinct molecular mechanisms and local immune responses. Recently, our group has investigated whether DON exposure is involved in the development of cutaneous allergy using a mouse model of allergic dermatitis (Aihara et al., 2020). Acute, as well as subacute, oral exposure to DON significantly exacerbates the proinflammatory responses, including the ear swelling response, histological observations, cellular infiltration into the auricular lymph nodes (LNs), and cytokine secretion in skin tissue; however, no influence was observed on pruritic responses. Furthermore, Aihara et al. (2020) have demonstrated that DON exposure significantly enhances cytokine release by dendritic cells (DCs) and keratinocytes, indicating the direct impact of DON on immunocytes and epithelium. Based on these findings, we hypothesized the probable involvement of DON exposure in the development of respiratory allergy. Previously, Schutze et al. (2010) have investigated the effect of mycotoxin patulin in a murine model of allergic asthma, demonstrating that intestinal exposure to patulin increases the allergic immune response in mice by modulating the Th1/Th2 balance via direct effects on interleukin (IL)-12 secretion in DCs, as well as by inducing oxidative stress. Hence, mycotoxins, which are secondary metabolites of molds, may be responsible for the exacerbation of respiratory allergy by interfering with immunocytes and the epithelium function, resulting in an increased Th2 differentiation.

Therefore, the aim of this study was to investigate the association between DON exposure and the development of respiratory allergy using a mite allergen (*Dermatophagoides farinae*, Derf)-induced mouse model of allergic asthma. In humans, as well as in companion animals, Derf has been recognized as a prevalent allergen responsible for atopic dermatitis and asthma (Sybilski et al., 2015). Additionally, an *in vitro* cytokine-release assay was used to elucidate the direct effect of DON exposure on the function of eosinophils and bronchial epithelial cells. Moreover, the goal of this study is to provide a proof of concept that oral exposure promotes allergic asthma rather than performing risk-assessment of DON. In line with the above

concept, we selected a rather high dose of DON particularly in acute-exposure protocol.

MATERIALS AND METHODS

Experimental animals. Six-week-old female BALB/c mice were kindly provided by Japan SLC, Inc. (Shizuoka, Japan). A maximum of 4 mice was housed per cage under controlled conditions (lighting, 12-h light-dark cycle; temperature, 22°C ± 3°C; humidity, 55% ± 15%). The mice received a certified pellet diet and tap water *ad libitum*. All aspects of this study were conducted in accordance with the Animal Care and Use Program of Azabu University (Approval No. 1910097).

Exposure to DON. The acute and subacute-exposure protocols were simultaneously performed in a mouse study (Figure 1). For the acute-exposure protocol, the mice were orally administered the vehicle-alone or DON at 0.03, 0.1, or 0.3 mg/kg body weight (b.w.; diluted in corn oil), at 48, 24, and 1 h before the final challenge with Derf to confirm the direct effect of DON exposure on the allergic reaction (Figure 1A). DON doses were selected based on a previous study (Aihara et al., 2020), as well as based on the fact that DON is occasionally detected at higher levels (5–20 mg/kg) in food intended for animal or human consumption (EFSA, 2017). DON (CAS No. 51481-10-8), procured from Sigma-Aldrich Co., LLC. (Tokyo, Japan), was used for the acute-exposure protocol (Supplementary Figure 1a) and *in vitro* experiments. In the subacute-exposure study, a diet contaminated with a low dose of DON (0.3 ppm) was continuously fed to mice during the experimental period to mimic real-world DON exposure (Figure 1B). A low dose of DON (0.3 ppm) was generated by mixing a standard rodent diet (CE-2, CLEA Japan, Inc., Tokyo, Japan) and wheat grain samples (Minaminokaori, a Japanese hard red spring wheat) to obtain a final DON concentration of 0.3 ppm based on a previously described method (Aihara et al., 2020). Detailed information including stability and representative HPLC chromatograms were also described in Aihara et al. (2020). The control diet also contained the same amount of normal wheat.

Mouse model of allergic asthma. A mouse model of allergic asthma was generated by repetitive intranasal sensitization and a house dust mite extract challenge (Derf extract for immunization, Derf, 1 mg/ml, ITEA Inc., Tokyo, Japan) based on the protocol described by Tashiro et al. (2016), with a slight modification. Briefly, mice were intranasally sensitized with Derf (25 µg in 25 µl phosphate-buffered saline [PBS]; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) under isoflurane anesthesia on days 1, 8, and 15. One week after the last sensitization, the mice were intranasally challenged with 5 µg Derf in 25 µl PBS under isoflurane anesthesia on consecutive 3 days (days 22–24). Twenty-four hours after the final Derf challenge, the mice were euthanized under isoflurane anesthesia. Subsequently, serum, bronchoalveolar lavage fluid (BALF), hilar LNs, and lung samples were isolated for further analysis. The reproducibility of results was confirmed by utilizing other settings with a limited number of animals (5 or 6 mice receiving 0.3 mg/kg DON and 0.3 ppm DON), and the other settings demonstrated the same tendencies as the main study.

Measurement of total and Derf-specific IgE in serum. Total serum immunoglobulin E was determined using an enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's protocol (Thermo Fisher Scientific, Inc., Kanagawa,

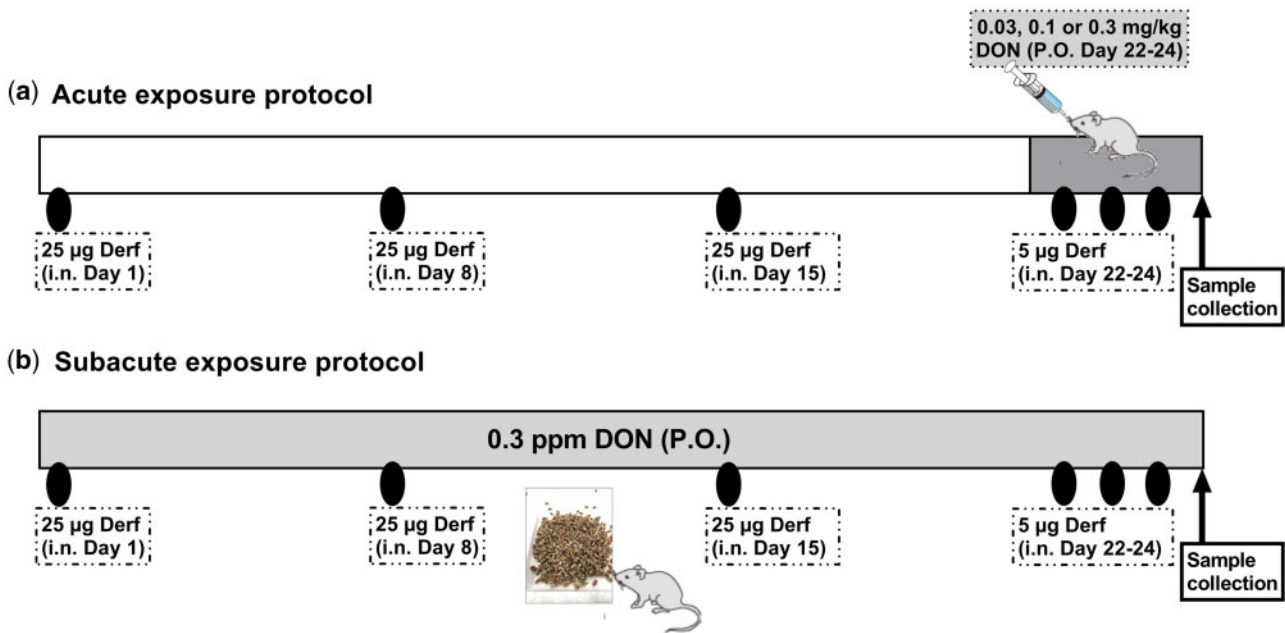


Figure 1. Experimental protocol used to sensitize and challenge BALB/c mice with Derf. Acute-exposure protocol (A); mice were orally administered vehicle-alone, 0.03, 0.1, or 0.3 mg/kg b.w. of DON (diluted in corn oil), at 48, 24, and 1 h before final Derf challenge. Subacute-exposure protocol (B); a diet containing DON-contaminated wheat (0.3 ppm DON as the final concentration) or a diet containing noncontaminated wheat was continuously fed to mice during the experimental period. Abbreviations: Derf, *Dermatophagoides farinae*; DON, deoxynivalenol; i.n., intranasal.

Japan). Derf-specific IgE were detected on plates coated with a 50 µg/ml solution of Derf. Mouse serum was diluted in PBS (1/2, 1/4, 1/8, 1/16, and 1/32). The diluted serum was added to each well and incubated for 2 h at room temperature. Biotin-conjugated rat anti-mouse IgE monoclonal antibody (BD Pharmingen, Tokyo, Japan) was added to each well and incubated for 1 h at room temperature. Optical density was read at a wavelength of 450 nm with a microplate reader (iMark micro plate reader, Bio-Rad Laboratories, Inc., Tokyo, Japan). Antibody titers were expressed as serum dilution factors to reach 0.5 of optical density.

Histopathological evaluation of lung tissue. A portion of the right upper lobe of the lung was fixed in a 10% formalin solution, embedded in paraffin wax, sectioned to 5 µm thickness, and stained with hematoxylin and eosin. A semiquantitative histopathological evaluation was performed for alveolar inflammation, alveolar multinucleated giant cell, alveolar fibrosis, and perivascular inflammation, in a blinded fashion using the following grading system: 0, within normal limits; 1, mild; 2, moderate; and 3, severe. The total score of lesions was statistically evaluated.

BALF analysis. BALF was collected by cannulating the trachea and lavaging the lungs 3 times with 1 ml PBS containing 0.1% bovine serum albumin (Thermo Fisher Scientific, Inc.; Tajiki-Nishino et al., 2018; Watanabe et al., 2019). The supernatant from the first fraction of BALF was used for measuring chemokine levels. Eotaxin and RANTES levels were measured using ELISA, according to the manufacturer's protocol (R&D Systems, Minneapolis, Minnesota). Furthermore, the cell pellets of all 3 fractions were used for measuring the total number of live cells, as well as the differentiation of eosinophils, neutrophils, and macrophages. The total number of live cells was measured using the Tali Image-Based Cytometer (Thermo Fisher Scientific, Inc.). For flow

cytometric analysis, 5×10^5 cells were first incubated with 1 µg of mouse Fc Block (Miltenyi Biotec K.K., Tokyo, Japan) prior to incubation with monoclonal antibodies (antimouse CCR3, antimouse CD11c, antimouse CD11b, and antimouse Gr-1 for neutrophils, Miltenyi Biotec K.K.). The cells were washed and analyzed using an EC800 flow cytometer (Sony Imaging Products & Solutions Inc., Tokyo, Japan); 10 000 events were collected from the disaggregated BALF to analyze cell surface marker expression.

Analysis of hilar LNs. Single-cell suspensions were prepared from LNs obtained from each mouse and were used for flow cytometric and cytokine analysis (Tajiki-Nishino et al., 2018; Watanabe et al., 2019). The total number of live cells was measured using Tali Image-Based Cytometer. Flow cytometric analysis was conducted as described in the BALF analysis, using monoclonal antibodies (antimouse CD3, antimouse CD4, antimouse IgE, antimouse CD19, antimouse CD11c, and antimouse CD40, Miltenyi Biotec K.K.). For cytokine evaluation, single-cell suspensions of LNs (5×10^5 cells/well) were incubated with Dynabeads mouse T-Activator CD3/CD28 (Thermo Fisher Scientific, Inc.) for 24 or 96 h. IL-4, IL-5, IL-9, IL-13, and IL-17 concentrations in the supernatant were evaluated using ELISA according to the manufacturer protocols (R&D Systems).

Cytokine levels in the lung tissue. Frozen lung tissue was homogenized with an electric homogenizer in RPMI-1640 medium (FUJIFILM Wako Pure Chemical Corporation) containing Halt Protease and Phosphatase Inhibitor Cocktail (Bio-Rad Laboratories, Inc.), as described by Watanabe et al. (2019). The protein content in the isolated supernatant was determined using a DC protein assay kit (Bio-Rad Laboratories, Inc.) according to the manufacturer's protocol. The levels of IL-1β, IL-4, IL-9, IL-13, IL-17, IL-33, and thymic stromal lymphopoietin (TSLP) were measured using ELISA, following the manufacturer's instructions (R&D Systems).

Table 1. Histological Evaluation of the Lung Following Acute or Subacute Oral Administration of DON in a Derf-Induced Mouse Model of Allergic Asthma

Group	Derf Vehicle	Acute DON 0.03 mg/kg	Acute DON 0.1 mg/kg	Acute DON 0.3 mg/kg	Subacute DON 0.3 ppm
Alveolar inflammation	1.20 ± 0.20	1.40 ± 0.40	1.83 ± 0.17	1.60 ± 0.24	1.83 ± 0.17*
Alveolar multinucleated giant cell	0.00 ± 0.00	0.20 ± 0.20	0.00 ± 0.00	0.20 ± 0.20	0.17 ± 0.17
Alveolar fibrosis	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.40 ± 0.24	0.33 ± 0.21
Perivascular inflammation	1.40 ± 0.40	1.60 ± 0.24	1.33 ± 0.21	1.40 ± 0.24	1.17 ± 0.17
Total score	2.60 ± 0.51	3.20 ± 0.49	3.17 ± 0.31	3.60 ± 0.24	3.50 ± 0.56

A histological score (0, within normal limits; 1, mild; 2, moderate; and 3, severe) was given to each observation. Results are expressed as the mean ± SEM (n = 6 per group).

Abbreviations: Derf, *Dermatophagoides farinae*; DON, deoxynivalenol.

*p < .05 (Dunnett's multiple comparison test) versus the Derf + vehicle control group.

Cytokine-release assay in the EOL-1 cell line (human eosinophilic leukemia). EOL-1 cells were obtained from the European Collection of Cell Cultures (ECACC) and cultured in RPMI 1640 medium (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 10% fetal calf serum (Sigma-Aldrich Co. LLC.) and penicillin-streptomycin (FUJIFILM Wako Pure Chemical Corporation). EOL-1 cells (5×10^4 cells) were seeded in 200 μ l complete medium at 70% confluency in 96-well culture plates and were treated with 0.01, 0.1, or 1 μ mol/l DON for 24 h. Next, the cells were stimulated with toll-like receptor (TLR) ligand lipopolysaccharide (LPS, 1 μ g/ml, Sigma-Aldrich Co., LLC) or Derf (20 μ g/ml) for an additional 24 h. The concentrations of IL-1 β , IL-8, and tumor necrosis factor (TNF)- α in the supernatant were quantified using ELISA (R&D Systems). The DON concentrations used in this experiment were determined based on a cytotoxicity test conducted before the experiments (data not shown). Cytotoxicity was not observed even at the highest concentrations. To verify experimental reproducibility, a minimum of 3 independent experiments were performed using different passages of EOL-1 cells.

Cytokine-release assay in BEAS-2B (human bronchial epithelium). The BEAS-2B cell line, obtained from the ECACC, was cultured in the bronchial epithelial cell growth media (BEGM; Lonza Japan Ltd., Tokyo, Japan), according to a previously reported protocol (Tajiki-Nishino et al., 2018; Watanabe et al., 2019). Cells (1×10^4 cells/200 μ l) at a 70% confluence were seeded in a 96-well culture plate and were exposed to DON at 0.4, 2, or 10 μ mol/l for 24 h, followed by stimulation with LPS (1 μ g/ml) or Derf (20 μ g/ml) for another 24 h. The concentrations of IL-1 β , IL-6, and IL-8 in the supernatant were quantified using ELISA (R&D Systems). The DON concentrations used in this experiment were determined based on a cytotoxicity test conducted before experimentation (data not shown). Cytotoxicity was not observed even at the highest concentrations. To verify experimental reproducibility, a minimum of 3 independent experiments were performed using different passages of BEAS-2B cells.

Statistical analyses. Data are expressed as the mean ± standard error of the mean (SEM). Concerning the Derf-specific IgE titers in serum, a probit method was used to draw a linear line then calculate the dilution factor to reach 0.5 of optical density. Fisher's exact test (1-sided analysis) was used for the evaluation of the incidence of pathological findings. Other *in vivo* and *in vitro* findings were analyzed using analysis of variance, followed by Dunnett's multiple comparison test. Statistical significance was estimated at 5% and 1% probability levels. The data

were analyzed using GraphPad Prism version 8 (GraphPad Software, San Diego, California).

RESULTS

Effect of Acute or Subacute DON Exposure on Inflammatory Responses and Total IgE Levels in a Derf-Induced Mouse Model of Allergic Asthma

Initially, we investigated the influence of DON exposure on inflammatory responses in the lung and total serum IgE levels. Table 1, Supplementary Table 1, Figure 2A, and Supplementary Figure 1 present the histological evaluation of lung after acute or subacute oral DON exposure in a Derf-induced mouse model of allergic asthma. In the acute DON exposure groups, a slightly higher extent of alveolar inflammation, alveolar multinucleated giant cells, alveolar fibrosis, and perivascular inflammation were observed when compared with the Derf + vehicle control group; however, no significant changes were observed. In the subacute DON exposure group, a significant exacerbation was observed only in alveolar inflammation when compared with the Derf + vehicle control group. Additionally, total serum IgE was greater in both acute and subacute DON exposure groups; however, no significant difference was observed when compared with the Derf + vehicle control group (Figure 2B). On the other hand, Derf-specific IgE in serum was significantly enhanced by acute (0.3 mg/kg) and subacute (0.3 ppm) DON exposure compared with the Derf + vehicle control group (Figure 2C). In the second setting, both total IgE and Derf-specific IgE levels were significantly upregulated by the acute and subacute DON exposure (Supplementary Figure 2).

Acute and Subacute Oral Exposure to DON Promotes Eosinophil, Neutrophil, and Macrophage Infiltration, but Not Chemokine

Secretion in BALF of a Derf-Induced Mouse Model of Allergic Asthma

To confirm the proinflammatory response following DON exposure, we analyzed eosinophil, neutrophil, and macrophage infiltration, as well as chemokine (eotaxin and RANTES) secretion, in BALF of the Derf-induced mouse model of allergic asthma. In BALF, acute oral DON exposure significantly induced eosinophil (CD11c^{int}CCR3⁺), neutrophil (Gr-1⁺), and macrophage (CD11b^{int}CD11c^{int}) infiltration in a dose-dependent manner (Figs. 3A–C). Furthermore, subacute oral DON exposure significantly induced eosinophil and macrophage infiltration (Figs. 3A and 3C). Conversely, the chemokine levels in BALF were not impacted following DON exposure (Figs. 3D and 3E).

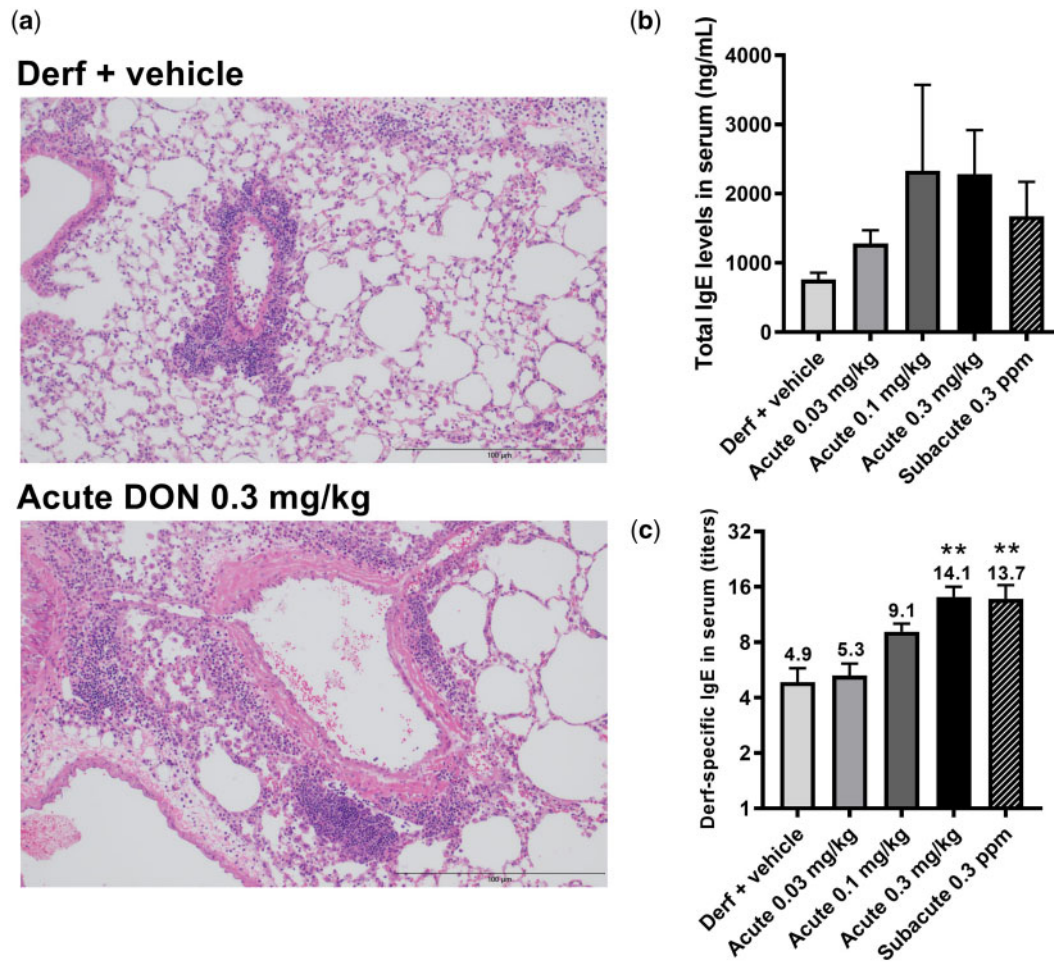


Figure 2. Histological evaluation and serum IgE levels in the Derf-induced mouse model of allergic asthma. Representative microscopic features of the lung of the Derf + vehicle control group and the 0.3 mg/kg DON group (A). A slight upwards trend in total serum IgE levels (ng/mL) (B) and significant upregulation in Derf-specific IgE levels (titers) are observed after acute and subacute DON administration; however, no significant changes are observed. Bar = 100 μ m. Each result is presented as the mean $\pm$ SEM ($n = 6$ per group). Abbreviations: Derf, *Dermatophagoides farinae*; DON, deoxynivalenol.

Influence of Oral DON Exposure on Immunocyte Infiltration and Cytokine Production in LN After Derf Challenge. To examine the influence of DON exposure on the local immune reaction, the number of DCs (CD11c⁺CD40⁺), helper T cells (CD3⁺CD4⁺), and IgE-positive B cells (CD19⁺IgE⁺) in the hilar LN were measured using flow cytometry. Acute oral DON exposure dose-dependently upregulated the number of DCs, helper T cells, and IgE-positive B cells (Figs. 4A–D). Subacute oral DON exposure also significantly induced DCs, helper T cells, and IgE-positive B cells when compared with the Derf + vehicle control group (Figs. 4A–C). To further elucidate whether DON affects T cell function, we analyzed IL-4, IL-5, IL-9, IL-13, and IL-17 production by T cells stimulated with the CD3/CD28 antibody. IL-4, IL-5, IL-9, and IL-13 were increased following acute DON exposure in a dose-dependent manner, and significant changes were observed in DON 0.1 and 0.3 mg/kg exposure groups when compared with the Derf + vehicle control group (Figs. 5A–D). Subacute oral DON exposure significantly enhanced all cytokines when compared with the Derf + vehicle control group (Figs. 5A–E).

Impact of Oral DON Exposure on Cytokine Levels in the Lung Tissue of a Derf-Induced Mouse Model of Allergic Asthma

In the acute-exposure protocol, mice treated with 0.3 mg/kg DON exhibited significantly higher levels of IL-1 β and TSLP in

the lung homogenate 24 h after the final Derf challenge when compared with those treated with Derf + vehicle (Table 2). Subacute oral DON exposure significantly upregulated the levels of IL-33 and TSLP (Table 2).

Effect of DON Pre-Exposure on LPS- or Derf-Stimulated Eosinophils In Vitro

To assess whether exposure to DON directly affects eosinophil function in vitro, we evaluated LPS- or Derf-induced IL-1 β , IL-8, and TNF- α production in EOL-1 cells. In DON pretreated EOL-1 cells, the production of IL-1 β , IL-8, and TNF- α increased in a dose-dependent manner; these values differed significantly from those observed in the vehicle-alone control group (Figs. 6A–C). To confirm the upregulatory effect of DON using another stimulus, we additionally measured Derf-induced IL-8 production. In DON-exposure groups, Derf-induced IL-8 production was significantly enhanced in a dose-dependent manner (Figure 6D).

Cytokine Secretions in LPS- or Derf-Stimulated Human Bronchial Epithelium (BEAS-2B) Are Significantly Increased by DON Pre-Exposure In Vitro

Comparable with EOL-1 cells, IL-1 β , IL-6, and IL-8 secretions were significantly increased in LPS- or Derf-stimulated BEAS-2B cells following 24 h of DON pre-exposure (Figs. 7A–D).

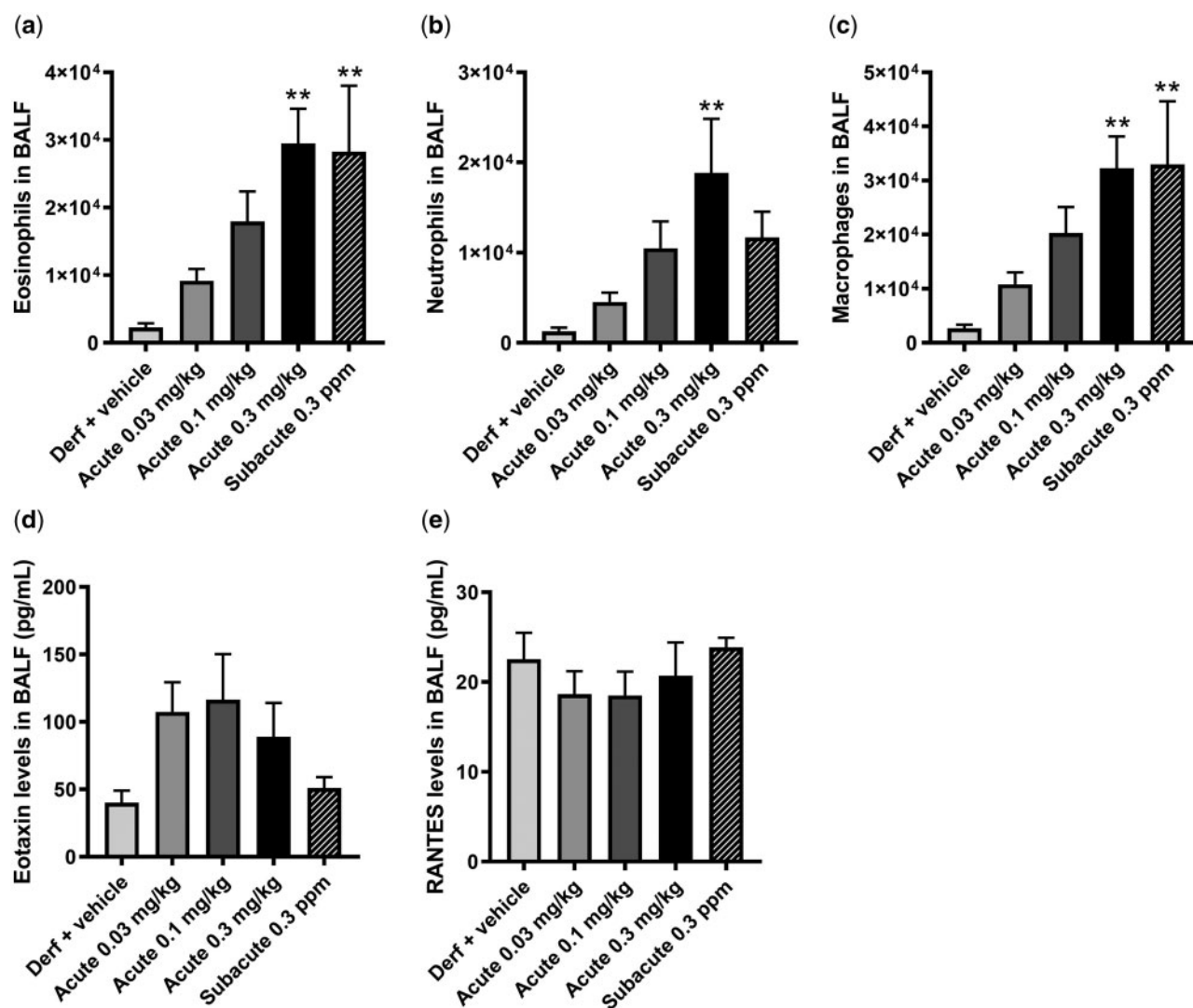


Figure 3. Acute and subacute oral DON administration induces cellular infiltration but not cytokine secretion in BALF of the Derf-induced mouse model of allergic asthma. Eosinophil, neutrophil, and macrophage infiltration in BALF following DON administration (A–C). Eotaxin (D, pg/ml) and RANTES (E, pg/ml) levels in BALF are not affected by DON exposure. Each result is presented as the mean $\pm$ SEM ($n=6$ per group). ** $p < .01$ (Dunnett's multiple comparison test) versus the Derf + vehicle control group. Abbreviations: Derf, *Dermatophagoides farinae*; DON, deoxynivalenol; BALF, bronchoalveolar lavage fluid.

DISCUSSION

The aim of this study was to identify the possible association between exposure to the mycotoxin DON and the development of allergic asthma. We elucidated the possible interaction between acute or subacute DON exposure and allergic asthma development. Our findings identified several crucial aspects: (1) in a mouse model of Derf-induced allergic asthma, acute oral treatment with DON significantly increased inflammatory responses, including cellular infiltrations in BALF, with increased numbers of DCs, helper T cells, and IgE-positive B cells in LN, as well as enhanced cytokine production; however, chemokine levels in BALF and total serum IgE levels were not significantly upregulated; (2) subacute oral exposure to a low dose of DON significantly exacerbated the inflammatory responses in the mouse model of allergic asthma; (3) these *in vivo* findings were confirmed by the enhanced cytokine secretion demonstrated by EOL-1 and BEAS-2B cells pretreated with DON.

Previously, the World Health Organization has reported that approximately 235 million individuals globally suffer from

asthma (Chuchalin, 1994), and currently 1–18% of populations across various countries still experience asthma (D'Amato et al., 2016). Asthma, characterized by hyper-responsiveness of the respiratory tract, is emerging as one of the most serious medical conditions worldwide (Backman et al., 2017; Gern 2015; Jackson et al., 2016; Lundback et al., 2016; Rehman et al., 2018). Nevertheless, pathogenesis and triggers of asthma remain elusive as a variety of factors can trigger asthma, including viral infections, air pollution, exercise, as well as physical and emotional stress. Therefore, there is a growing need to discover and explore biomarkers providing further insight into factors that may predispose individuals to asthma. From this perspective, our research group has recently investigated the allergic properties of environmental factors, including endocrine disruptors, certain pesticides, and ligands of the aryl hydrocarbon receptor, which could be immediate threats in humans, as well as in companion animals (Tajiki-Nishino et al., 2018; Tajima et al., 2019; Watanabe et al., 2019).

Among these environmental factors, we currently focused on the interaction between mycotoxin exposure and immune

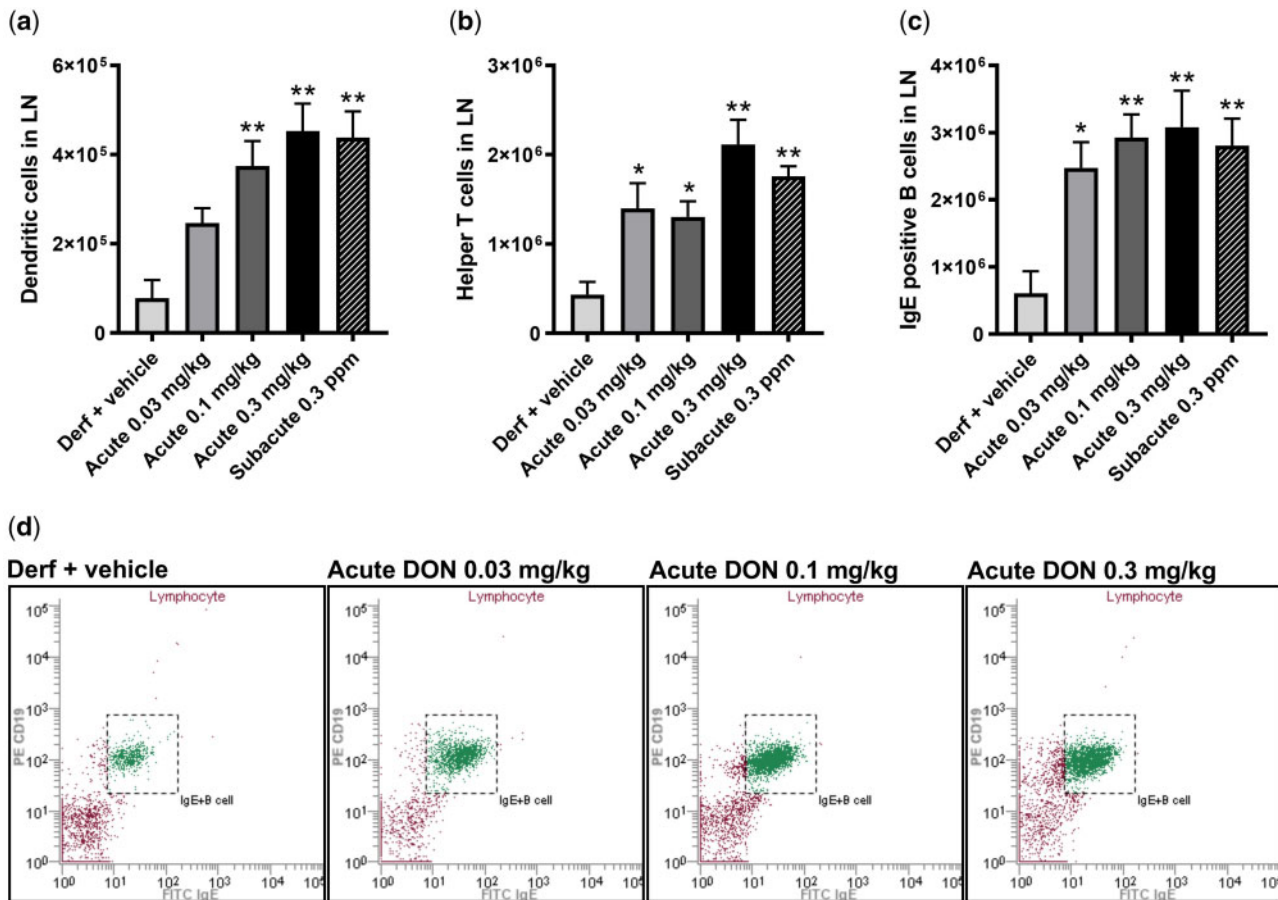


Figure 4. Impact of acute and subacute oral DON administration on immunocytes infiltration in LN of a Derf-induced mouse model of allergic asthma. A significant increase in dendritic cells (A), helper T cells (B), and IgE-positive B cells (C) is observed in the DON-exposure group. Representative dot plot images of IgE-positive B cells in the Derf + vehicle control group and the acute DON exposure group (D). Each result is presented as the mean $\pm$ SEM ($n=6$ per group). * $p < .05$, ** $p < .01$ (Dunnett's multiple comparison test) versus the Derf + vehicle control group. Abbreviations: Derf, *Dermatophagoides farinae*; DON, deoxynivalenol; LN, lymph node.

disfunction. Exposure to indoor allergens, such as from house dust mites, animals, and molds, is considered an important risk factor for asthma (Platts-Mills et al., 1997). Epidemiological evidence suggests that molds found in indoor environments are associated with an increased prevalence and exacerbation of respiratory symptoms and asthma (Langley et al., 2003), with several previous studies demonstrating the association between exposure to mycotoxins, which are secondary metabolites of molds, and observed health effects (Bunger et al., 2004; Jarvis and Miller 2005). However, the mechanisms underlying the allergy-promoting effects of mycotoxins remain unclear, particularly, when mycotoxins enter the body via the ingestion of contaminated food. Recently, we have documented the direct proinflammatory effects of oral mycotoxin DON exposure on cutaneous allergy development in a mouse model of Th2-driven allergic dermatitis (Aihara et al., 2020), and our findings indicate that DON exacerbates similar Th2-dependent allergic diseases, such as allergic asthma. The aim of this study was to confirm the link between DON exposure and the development of allergic asthma.

In this study, we investigated the direct and chronic effects of DON using acute and subacute oral exposure protocols in a highly reliable mouse model of Derf-induced allergic asthma, widely used for evaluating toxicological and pharmacological effects of agents, including a β_2 adrenergic agonist and saturated fatty acid (Kato et al., 2014; Tashiro et al., 2016, 2017). The

proinflammatory effects of acute and subacute DON exposure were demonstrated by IgE levels in serum, an enhanced infiltration of eosinophils, neutrophils, and macrophages in BALF, confirmed by the significantly increased number of DCs, helper T cells, and IgE-positive B cells in hilar LNs. In stimulated T cells and lung tissues, the enhanced cytokine production (IL-4, IL-5, IL-13, IL-9, IL-17, IL-33, and TSLP) was supported by the proinflammatory responses observed following acute DON exposure. Several reports have revealed the crucial role of eosinophils, neutrophils, and macrophages in patients with asthma and antieosinophilic drugs have recently been investigated as a novel therapeutic option for asthma therapy (Bel and Ten Brinke 2017; Busse et al., 2019; Maslan and Mims 2014; Patel et al., 2018). Our findings revealed that acute and subacute DON exposure directly influences the infiltration of these cells into lung tissues. Conversely, eotaxin and RANTES, which are major chemotactic factors for eosinophils, neutrophils, and macrophages, were not influenced by DON exposure in this study. One possible reason is the time course of necropsy. Chemokines are secreted before the immigration of these cells, and thus modifying the protocol might allow better detection of chemokines affected by DON exposure. Additionally, this observation could be attributed to other factors infiltrating these cells. In this study, we monitored IL-33 and TSLP which are mainly secreted by the bronchial epithelial cells and play a pivotal role in the pathogenesis of allergic airway diseases (Penberthy et al.,

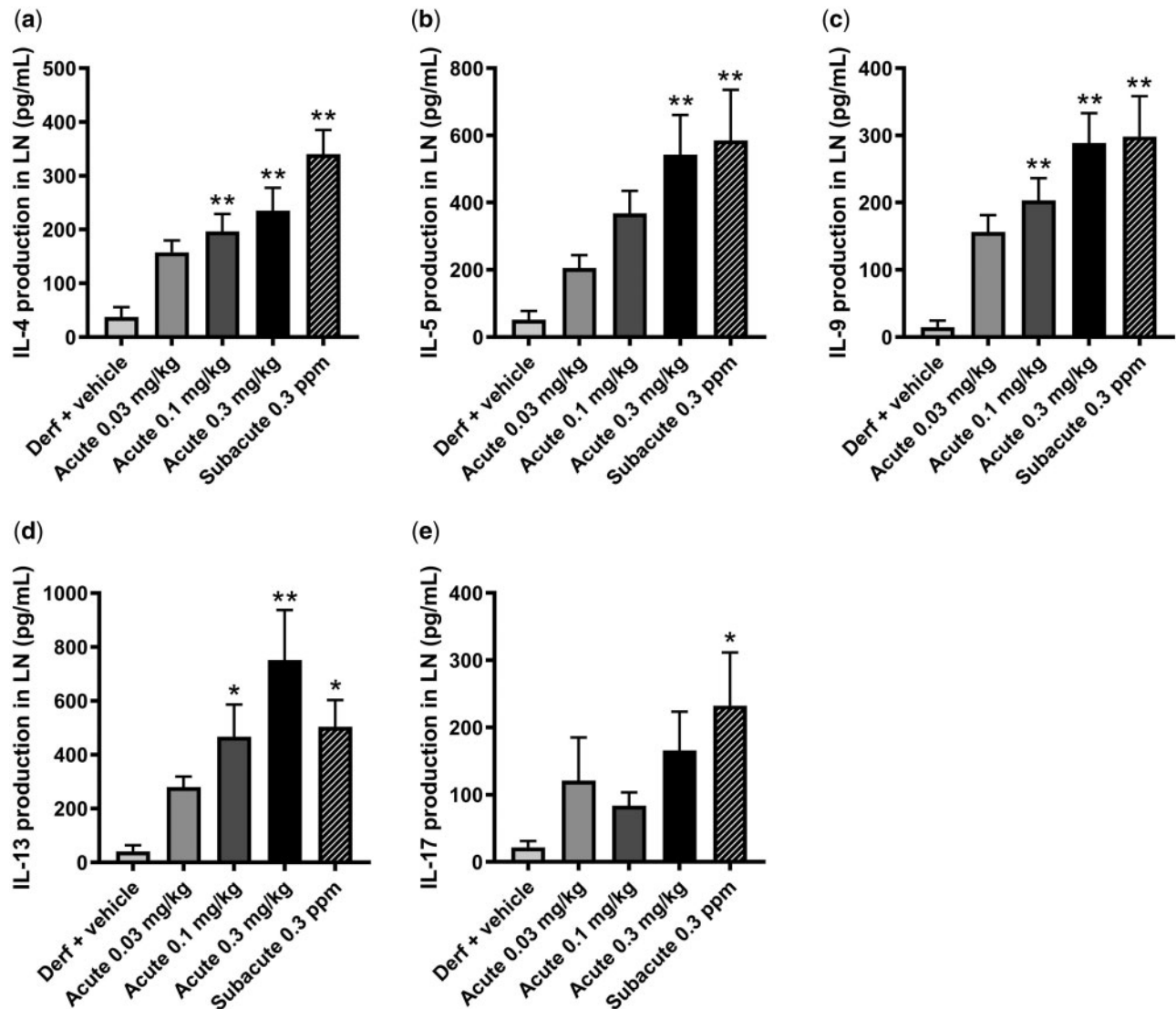


Figure 5. Cytokine production induced by activated T cells in the auricular LN after acute or subacute oral DON administration in a Derf-induced mouse model of allergic asthma. A significant increase in interleukin (IL)-4 (A), IL-5 (B), IL-9 (C), IL-13 (D), and IL-17 (E) is observed in the DON-exposure group. Each result is presented as the mean (pg/ml) $\pm$ SEM ($n=6$ per group). * $p < .05$, ** $p < .01$ (Dunnett's multiple comparison test) versus the Derf + vehicle control group. Abbreviations: Derf, *Dermatophagoides farinae*; DON, deoxynivalenol; LN, lymph node.

Table 2. Responses in the Lung Tissue to Acute or Subacute Oral Administration of DON in a Derf-Induced Mouse Model of Asthma

Group	Derf Vehicle	Acute DON 0.03 mg/kg	Acute DON 0.1 mg/kg	Acute DON 0.3 mg/kg	Subacute DON 0.3 ppm
IL-1 β (pg/mg protein)	330 $\pm$ 44	336 $\pm$ 28	440 $\pm$ 67	548 $\pm$ 65*	415 $\pm$ 29
IL-4 (pg/mg protein)	941 $\pm$ 144	922 $\pm$ 88	1026 $\pm$ 203	1485 $\pm$ 196	1026 $\pm$ 201
IL-9 (pg/mg protein)	392 $\pm$ 27	575 $\pm$ 49	615 $\pm$ 66	661 $\pm$ 119	599 $\pm$ 122
IL-13 (pg/mg protein)	307 $\pm$ 91	286 $\pm$ 13	328 $\pm$ 40	384 $\pm$ 86	558 $\pm$ 185
IL-17 (pg/mg protein)	595 $\pm$ 14	796 $\pm$ 60	996 $\pm$ 104	1188 $\pm$ 373	806 $\pm$ 231
IL-33 (pg/mg protein)	868 $\pm$ 45	2136 $\pm$ 405*	1865 $\pm$ 280	1865 $\pm$ 269	2114 $\pm$ 467*
TSLP (pg/mg protein)	33 $\pm$ 3	41 $\pm$ 9	48 $\pm$ 6	77 $\pm$ 18*	77 $\pm$ 11*

Lung samples were collected 24 h after the Derf challenge. Results are expressed as the means $\pm$ SEM ($n=6$ per group).

Abbreviations: Derf, *Dermatophagoides farinae*; DON, deoxynivalenol; IL, interleukin; TSLP, thymic stromal lymphopoietin.

* $p < .05$ (Dunnett's multiple comparison test) versus the Derf + vehicle control group.

2014; Willart et al., 2012; Yao et al., 2018). The major targets of IL-33 in vivo are tissue-resident immune cells, including mast cells, group 2 innate lymphoid cells, and regulatory T cells. Other

cellular targets include Th2 cells, eosinophils, basophils, DCs, Th1 cells, CD8⁺ T cells, NK cells, iNKT cells, B cells, neutrophils, and macrophages. Thus, IL-33 is emerging as a crucial immune

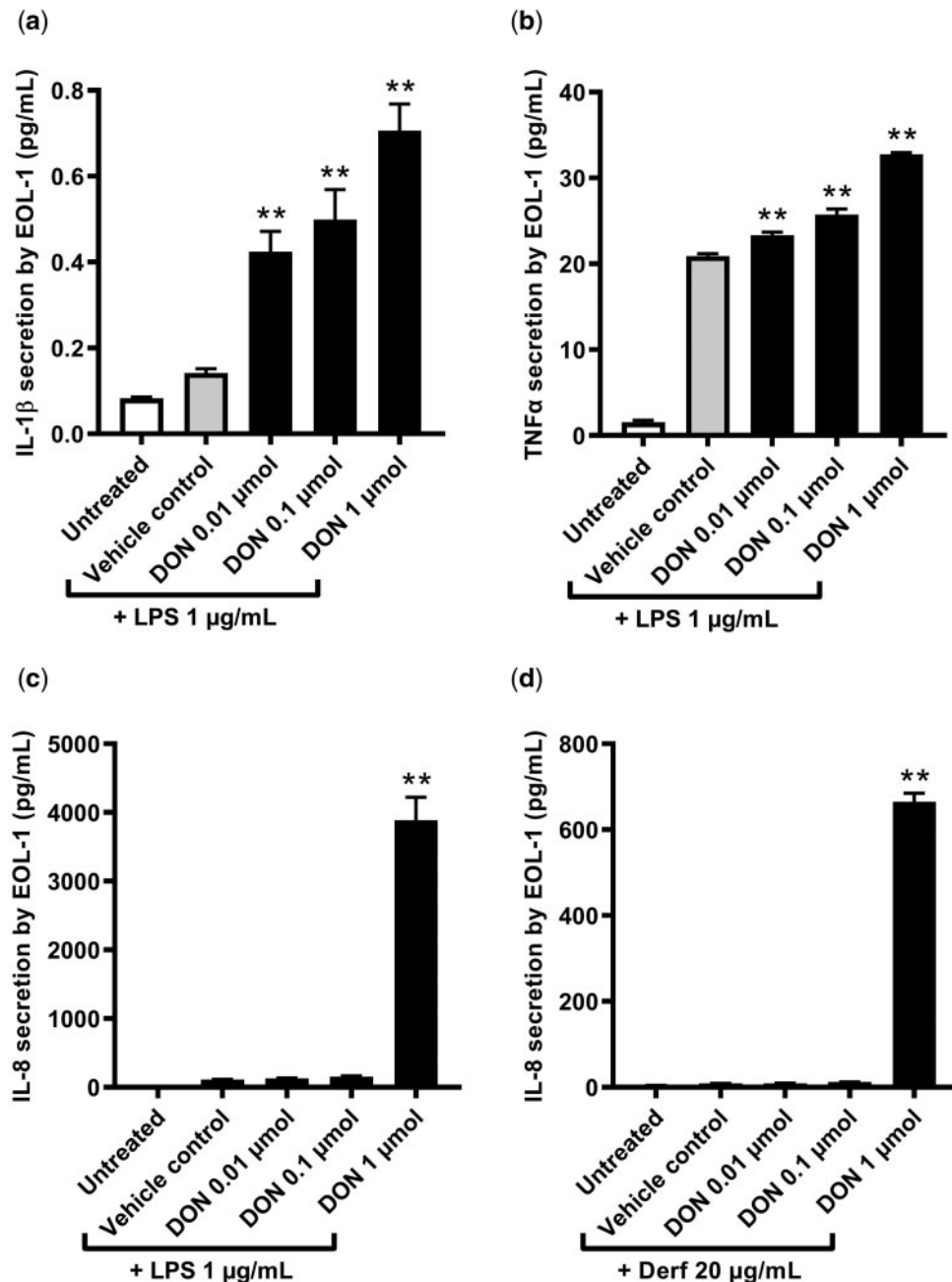


Figure 6. Direct effects of DON exposure on human eosinophilic leukemia (EOL-1) functions in vitro. Levels of IL-1 β (A, pg/ml), tumor necrosis factor- α (B, pg/ml), and interleukin (IL)-8 (C, pg/ml) in LPS-induced EOL-1 are significantly enhanced by pre-exposure to DON in a dose-dependent manner. DON pre-exposure significantly induces IL-8 secretion by Derf-stimulated EOL-1 (D, pg/ml). Each result is presented as the mean $\pm$ SEM ($n=7$ per group). ** $p < .01$ (Dunnett's multiple comparison test) versus the vehicle-alone control group. Abbreviations: Derf, *Dermatophagoides farinae*; DON, deoxynivalenol; LPS, lipopolysaccharide.

modulator with pleiotropic activities in type-2, -1, and regulatory immune responses; furthermore, it may play important roles in allergic and chronic inflammatory diseases (Cayrol and Girard 2014; Liew et al., 2016; Molofsky et al., 2015). Additionally, TSLP is an epithelial cell-derived cytokine, synthesized in response to various stimuli, including protease allergens and microorganisms such as viruses and bacteria. TSLP polarizes DCs to induce type 2 inflammation and directly expands and/or activates Th2 cells, ILC2s, basophils, and other immune cells. Thus, TSLP is considered a master regulator of type 2 immune responses (Bell et al., 2013; Pandey et al., 2000). In this study, our findings indicated that the significant enhancement of IL-33

and TSLP secretion, via acute and subacute DON exposure, might directly activate eosinophils, neutrophils, macrophages, and other immune cells, resulting in the exacerbation of proinflammatory responses in respiratory allergy. Our findings also show the absence of a dose response detection of IL-33 while TSLP and other cytokines in the lung tissue were elevated in a dose-dependent manner. IL-33 is distinguished from other cytokines by the extensive posttranslational modifications that profoundly modulate its ability to activate ST2-expressing cells, thus the time course of secretion possibly different from other cytokines. Different time courses may cause the peak out of the IL-33 levels in higher concentrations of DON.

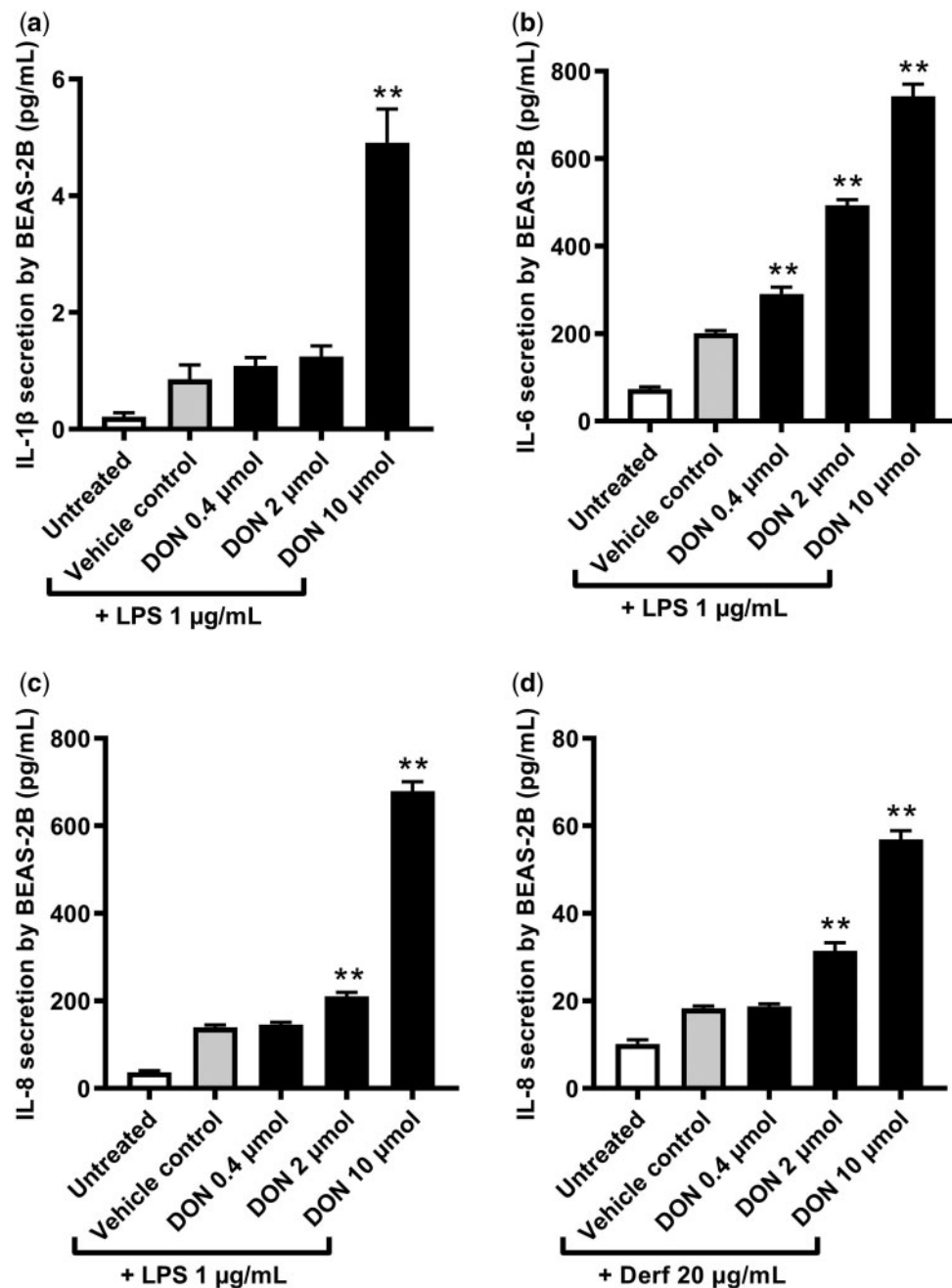


Figure 7. Proinflammatory cytokine secretion in human bronchial epithelium cell line (BEAS-2B) following DON exposure. Levels of interleukin (IL)-1 β (A, pg/ml), IL-6 (B, pg/ml), and IL-8 (C, pg/ml) in LPS-induced BEAS-2B are significantly enhanced by pre-exposure to DON in a dose-dependent manner. DON pre-exposure significantly induces IL-8 secretion by Derf-stimulated BEAS-2B (D, pg/ml). Each result is presented as the mean $\pm$ SEM ($n=7$ per group). * $p < .01$ (Dunnett's multiple comparison test) versus the vehicle-alone control group. Abbreviations: Derf, *Dermatophagoides farinae*; DON, deoxynivalenol; LPS, lipopolysaccharide.

The mouse model used in this study mainly represents the Th2 immune responses in allergic asthma. Hence, the significant enhancement of Th2 cytokines, including IL-4, IL-5, and IL-13, reflect the direct link between DON exposure and Th2 immune responses. Conversely, IL-9 is reportedly released from Th9 cells in allergic asthma models. Additionally, IL-9 plays an important role in mast-cell accumulation and activation (Sehra et al., 2015). Th17 cells, a helper T-cell subset that secretes IL-17, play an important role in the development of allergic asthma (Ahn et al., 2020; McGeachy and Cua 2008). Although the mouse model used in this study was a Th2-dominated allergy model,

Th9 and Th17 immunoreactions were also influenced following acute and subacute DON exposure.

We demonstrated that *in vivo* proinflammatory responses were associated with eosinophil infiltration and IL-33/TSLP expression in bronchial epithelial cells. Therefore, we performed *in vitro* experiments using EOL-1 and BEAS-2B cell lines to confirm the results observed in the *in vivo* experiments. We previously used TLR-activated human eosinophils and bronchial epithelium to demonstrate the proinflammatory effect of Bisphenol A and estrogen-receptor agonist (Tajiki-Nishino et al., 2018; Watanabe et al., 2019). In the assay examining TLR- or

Derf-stimulated cytokine production, DON exposure significantly influenced the production of IL-1 β , IL-6, IL-8, and/or TNF- α while there was no influence on cytokine production only by DON without any stimulus (data not shown). Our *in vitro* findings indicated that several pathways are involved in the DON-mediated development of allergic airway inflammation. Notably, overexpression of IL-33 and TSLP, induced by regulating the airway epithelium function, indicates a direct association with eosinophil functions (Figure 7).

Collectively, the findings of this study indicated that acute and subacute oral DON administration significantly exacerbates allergic asthma responses by enhancing the migration of eosinophils, neutrophils, and macrophage into the lung, activating Th2-, Th9-, and Th17-immune responses, and elevating IL-33 and TSLP secretion in a mouse model of allergic asthma. Furthermore, *in vitro* evidence demonstrated that DON exposure significantly increases cytokine release in activated eosinophils and bronchial epithelial cells. Further studies using IL-33/TSLP-deficient mice should be performed to additionally characterize the underlying mechanism by which DON induces proinflammatory responses.

SUPPLEMENTARY DATA

Supplementary data are available at Toxicological Sciences online.

ACKNOWLEDGMENT

We would like to thank Editage (www.editage.jp) for English language editing.

FUNDING

A part of this study was conducted under the research project on “Toxic Compounds and Foodborne Pathogens for Food Safety Risk Management” funded by the Ministry of Agriculture, Forestry, and Fisheries of Japan. Additionally, a part of this study was funded by the grant-in-aid for scientific research of The Tojuro Iijima Foundation for Food Science and Technology.

DECLARATION OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

- Ahn, K., Kim, B. E., Kim, J., and Leung, D. Y. (2020). Recent advances in atopic dermatitis. *Curr. Opin. Immunol.* **66**, 14–21.
- Aihara, R., Ookawara, T., Morimoto, A., Iwashita, N., Takagi, Y., Miyasaka, A., Kushiro, M., Miyake, S., and Fukuyama, T. (2020). Acute and subacute oral administration of mycotoxin deoxynivalenol exacerbates the pro-inflammatory and pruritic responses in a mouse model of allergic dermatitis. *Arch. Toxicol.* **94**, 4197–4207.
- Amuzie, C. J., Shinozuka, J., and Pestka, J. J. (2009). Induction of suppressors of cytokine signaling by the trichothecene deoxynivalenol in the mouse. *Toxicol. Sci.* **111**, 277–287.
- Backman, H., Raisanen, P., Hedman, L., Stridsman, C., Andersson, M., Lindberg, A., Lundback, B., and Ronmark, E. (2017). Increased prevalence of allergic asthma from 1996 to 2006 and further to 2016—results from three population surveys. *Clin. Exp. Allergy* **47**, 1426–1435.
- Becker-Algeri, T. A., Castagnaro, D., de Bortoli, K., de Souza, C., Drunkler, D. A., and Badiale-Furlong, E. (2016). Mycotoxins in bovine milk and dairy products: A review. *J. Food Sci.* **81**, R544–552.
- Bel, E. H., and Ten Brinke, A. (2017). New anti-eosinophil drugs for asthma and copd: Targeting the trait! *Chest* **152**, 1276–1282.
- Bell, B. D., Kitajima, M., Larson, R. P., Stoklasek, T. A., Dang, K., Sakamoto, K., Wagner, K. U., Kaplan, D. H., Reizis, B., Hennighausen, L., et al. (2013). The transcription factor stat5 is critical in dendritic cells for the development of th2 but not th1 responses. *Nat. Immunol.* **14**, 364–371.
- Bol-Schoenmakers, M., Braber, S., Akbari, P., de Graaff, P., van Roest, M., Kruijssen, L., Smit, J. J., van Esch, B. C., Jeurink, P. V., Garssen, J., et al. (2016). The mycotoxin deoxynivalenol facilitates allergic sensitization to whey in mice. *Mucosal Immunol.* **9**, 1477–1486.
- Bunger, J., Westphal, G., Monnich, A., Hinnendahl, B., Hallier, E., and Muller, M. (2004). Cytotoxicity of occupationally and environmentally relevant mycotoxins. *Toxicology* **202**, 199–211.
- Busse, W., Chupp, G., Nagase, H., Albers, F. C., Doyle, S., Shen, Q., Bratton, D. J., and Gunsoy, N. B. (2019). Anti-il-5 treatments in patients with severe asthma by blood eosinophil thresholds: Indirect treatment comparison. *J. Allergy Clin. Immunol.* **143**, 190–200 e120.
- Cayrol, C., and Girard, J. P. (2014). IL-33: An alarmin cytokine with crucial roles in innate immunity, inflammation and allergy. *Curr. Opin. Immunol.* **31**, 31–37.
- Chuchalin, A. G. (1994). Bronchial asthma: A global strategy. *Ter Arkh* **66**, 3–8.
- D’Amato, G., Vitale, C., Molino, A., Stanziola, A., Sanduzzi, A., Vatrella, A., Mormile, M., Lanza, M., Calabrese, G., Antonicelli, L., et al. (2016). Asthma-related deaths. *Multidiscip. Respir. Med.* **11**, 37.
- EFSA. (2017). Risks to human and animal health related to the presence of deoxynivalenol and its acetylated and modified forms in food and feed. *EFSA J.* **15**, 4718.
- Gern, J. E. (2015). Virus/allergen interaction in asthma exacerbation. *Ann. Am. Thorac. Soc.* **12**, S137–S143.
- Gruber-Dorninger, C., Jenkins, T., and Schatzmayr, G. (2019). Global mycotoxin occurrence in feed: A ten-year survey. *Toxins (Basel)* **11**, 375.
- Jackson, D. J., Gern, J. E., and Lemanske, R. F. Jr. (2016). The contributions of allergic sensitization and respiratory pathogens to asthma inception. *J. Allergy Clin. Immunol.* **137**, 659–665. quiz 666.
- Jarvis, B. B., and Miller, J. D. (2005). Mycotoxins as harmful indoor air contaminants. *Appl. Microbiol. Biotechnol.* **66**, 367–372.
- Kang, R., Li, R., Dai, P., Li, Z., Li, Y., and Li, C. (2019). Deoxynivalenol induced apoptosis and inflammation of IPEC-J2 cells by promoting ROS production. *Environ. Pollut.* **251**, 689–698.
- Kato, G., Takahashi, K., Tashiro, H., Kurata, K., Shirai, H., Kimura, S., and Hayashi, S. (2014). Beta2 adrenergic agonist attenuates house dust mite-induced allergic airway inflammation through dendritic cells. *BMC Immunol.* **15**, 39.
- Langley, S. J., Goldthorpe, S., Craven, M., Morris, J., Woodcock, A., and Custovic, A. (2003). Exposure and sensitization to indoor allergens: Association with lung function, bronchial reactivity, and exhaled nitric oxide measures in asthma. *J. Allergy Clin. Immunol.* **112**, 362–368.

- Liew, F. Y., Girard, J. P., and Turnquist, H. R. (2016). Interleukin-33 in health and disease. *Nat. Rev. Immunol.* **16**, 676–689.
- Lundback, B., Backman, H., Lotvall, J., and Ronmark, E. (2016). Is asthma prevalence still increasing? *Expert Rev. Respir. Med.* **10**, 39–51.
- Luo, S., Terziolo, C., Bracarense, A., Payros, D., Pinton, P., and Oswald, I. P. (2019). In vitro and in vivo effects of a mycotoxin, deoxynivalenol, and a trace metal, cadmium, alone or in a mixture on the intestinal barrier. *Environ. Int.* **132**, 105082.
- Maslan, J., and Mims, J. W. (2014). What is asthma? Pathophysiology, demographics, and health care costs. *Otolaryngol. Clin. North Am.* **47**, 13–22.
- McGeachy, M. J., and Cua, D. J. (2008). Th17 cell differentiation: The long and winding road. *Immunity* **28**, 445–453.
- Meky, F. A., Hardie, L. J., Evans, S. W., and Wild, C. P. (2001). Deoxynivalenol-induced immunomodulation of human lymphocyte proliferation and cytokine production. *Food Chem. Toxicol.* **39**, 827–836.
- Metzler-Zebeli, B. U., Lucke, A., Doupovec, B., Zebeli, Q., and Bohm, J. (2020). A multicomponent mycotoxin deactivator modifies the response of the jejunal mucosal and cecal bacterial community to deoxynivalenol contaminated feed and oral lipopolysaccharide challenge in chickens. *J. Anim. Sci.* **98**, skz377.
- Molofsky, A. B., Savage, A. K., and Locksley, R. M. (2015). Interleukin-33 in tissue homeostasis, injury, and inflammation. *Immunity* **42**, 1005–1019.
- Pandey, A., Ozaki, K., Baumann, H., Levin, S. D., Puel, A., Farr, A. G., Ziegler, S. F., Leonard, W. J., and Lodish, H. F. (2000). Cloning of a receptor subunit required for signaling by thymic stromal lymphopoietin. *Nat. Immunol.* **1**, 59–64.
- Patel, S. S., Casale, T. B., and Cardet, J. C. (2018). Biological therapies for eosinophilic asthma. *Expert Opin. Biol. Ther.* **18**, 747–754.
- Penberthy, K. K., Juncadella, I. J., and Ravichandran, K. S. (2014). Apoptosis and Engulfment by Bronchial Epithelial Cells. Implications for Allergic Airway Inflammation. *Ann. Am. Thorac. Soc.* **11** (Suppl. 5), S259–S262.
- Pierron, A., Bracarense, A., Cossalter, A. M., Laffitte, J., Schwartz-Zimmermann, H. E., Schatzmayr, G., Pinton, P., Moll, W. D., and Oswald, I. P. (2018). Deepoxy-deoxynivalenol retains some immune-modulatory properties of the parent molecule deoxynivalenol in piglets. *Arch. Toxicol.* **92**, 3381–3389.
- Platts-Mills, T. A., Vervloet, D., Thomas, W. R., Aalberse, R. C., and Chapman, M. D. (1997). Indoor allergens and asthma: Report of the third international workshop. *J. Allergy Clin. Immunol.* **100**, S2–S24.
- Rehman, A., Amin, F., and Sadeeqa, S. (2018). Prevalence of asthma and its management: A review. *J. Pak. Med. Assoc.* **68**, 1823–1827.
- Schutze, N., Lehmann, I., Bonisch, U., Simon, J. C., and Polte, T. (2010). Exposure to mycotoxins increases the allergic immune response in a murine asthma model. *Am. J. Respir. Crit. Care Med.* **181**, 1188–1199.
- Sehra, S., Yao, W., Nguyen, E. T., Glosson-Byers, N. L., Akhtar, N., Zhou, B., and Kaplan, M. H. (2015). Th9 cells are required for tissue mast cell accumulation during allergic inflammation. *J. Allergy Clin. Immunol.* **136**, 433–440 e431.
- Sybilski, A. J., Zalewska, M., Furmańczyk, K., Lipiec, A., Krzych-Falta, E., and Samoliński, B. (2015). The prevalence of sensitization to inhalant allergens in children with atopic dermatitis. *Allergy Asthma Proc.* **36**, e81–e85.
- Tajiki-Nishino, R., Makino, E., Watanabe, Y., Tajima, H., Ishimota, M., and Fukuyama, T. (2018). Oral administration of bisphenol A directly exacerbates allergic airway inflammation but not allergic skin inflammation in mice. *Toxicol. Sci.* **165**, 314–321.
- Tajima, H., Tajiki-Nishino, R., Watanabe, Y., and Fukuyama, T. (2019). Direct activation of aryl hydrocarbon receptor by benzo[a]pyrene elicits T-helper 2-driven proinflammatory responses in a mouse model of allergic dermatitis. *J. Appl. Toxicol.* **39**, 936–944.
- Tashiro, H., Takahashi, K., Hayashi, S., Kato, G., Kurata, K., Kimura, S., and Sueoka-Aragane, N. (2016). Interleukin-33 from monocytes recruited to the lung contributes to house dust mite-induced airway inflammation in a mouse model. *PLoS One* **11**, e0157571.
- Tashiro, H., Takahashi, K., Sadamatsu, H., Kato, G., Kurata, K., Kimura, S., and Sueoka-Aragane, N. (2017). Saturated fatty acid increases lung macrophages and augments house dust mite-induced airway inflammation in mice fed with high-fat diet. *Inflammation* **40**, 1072–1086.
- Wang, H., Zong, Q., Wang, S., Zhao, C., Wu, S., and Bao, W. (2019). Genome-wide DNA methylome and transcriptome analysis of porcine intestinal epithelial cells upon deoxynivalenol exposure. *J. Agric. Food Chem.* **67**, 6423–6431.
- Watanabe, Y., Tajiki-Nishino, R., Tajima, H., and Fukuyama, T. (2019). Role of estrogen receptors alpha and beta in the development of allergic airway inflammation in mice: A possible involvement of interleukin 33 and eosinophils. *Toxicology* **411**, 93–100.
- Willart, M. A. M., Deswarte, K., Pouliot, P., Braun, H., Beyaert, R., Lambrecht, B. N., and Hammad, H. (2012). Interleukin-1 α controls allergic sensitization to inhaled house dust mite via the epithelial release of GM-CSF and IL-33. *J. Exp. Med.* **209**, 1505–1517.
- Yao, X. J., Liu, X. F., and Wang, X. D. (2018). Potential role of interleukin-25/interleukin-33/thymic stromallymphopoietin-fibrocyte axis in the pathogenesis of allergic airway diseases. *Chin. Med. J. (Engl.)* **131**, 1983–1989.

Supplemental Material

Acute and subacute oral toxicity of deoxynivalenol exposure in a *Dermatophagoides farinae* induced murine asthma model.

Toa Ookawara¹, Ryota Aihara¹, Ai Morimoto¹, Naoki Iwashita^{1,2}, Keigo Kurata³, Yoshiichi Takagi^{1,4}, Atsushi Miyasaka⁵, Masayo Kushiro⁶, Shiro Miyake⁷, and Tomoki Fukuyama^{1*}

¹Laboratory of Veterinary Pharmacology, School of Veterinary Medicine, Azabu University, 1-17-71 Fuchinobe, Chuo-ku, Sagamihara-shi, Kanagawa 252-5201 Japan.

²Bioalchemis, 3-28-61 Honshuku-cho, Fuchu-shi, Tokyo 183-0032 Japan.

³Institute of Tokyo Environmental Allergy, ITEA Inc., 1-2-5, Yushima, Bunkyo-ku, Tokyo, Japan

⁴Japan SLC, Inc., 85 Ohara-cho, Kita-ku, Hamamatsu-shi, Shizuoka 433-8102 Japan.

⁵Kyushu Okinawa Agricultural Research Center, National Agriculture and Food Research Organization (NARO), Suyu 2421, Koshi, Kumamoto 861-1192, Japan.

⁶Food Research Institute, National Agriculture and Food Research Organization (NARO), 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan.

⁷Laboratory of Food and Hygiene, School of Food and Life Science, Azabu University, 1-17-71 Fuchinobe, Chuo-ku, Sagamihara-shi, Kanagawa 252-5201 Japan.

*Corresponding author

Tomoki Fukuyama

Laboratory of Veterinary Pharmacology, School of Veterinary Medicine, Azabu University, 1-17-71 Fuchinobe, Chuo-ku, Sagamihara-shi, Kanagawa 252-5201 Japan.

Fax: 81-427-52-3415. E-mail: t-fukuyama@azabu-u.ac.jp.

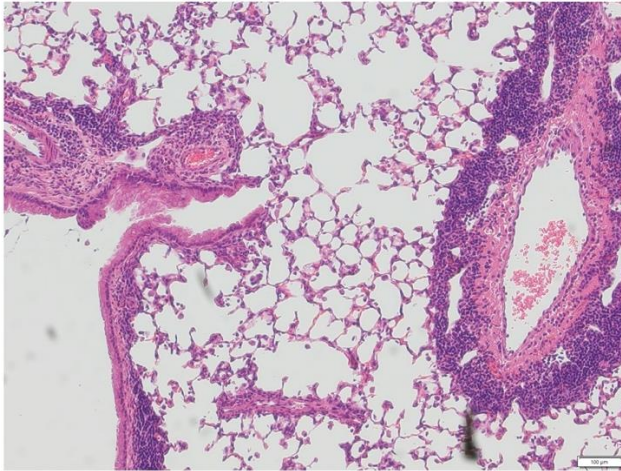
Supplemental TABLE 1

Histological evaluation of the lung following acute or subacute oral administration of DON in a Derf-induced mouse model of allergic asthma (2nd experiment)

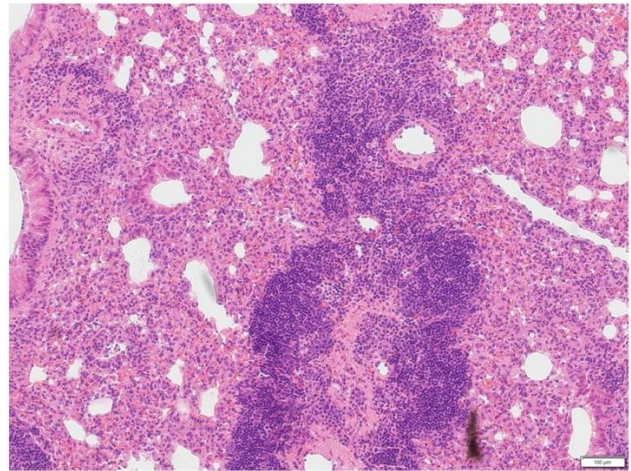
A histological score (0, within normal limits; 1, mild; 2, moderate; and 3, severe) was given to each observation. Results are expressed as the mean $\pm$ SEM. n = 6 per group. * p < 0.05 (Dunnett's multiple comparison test) vs. the Derf + vehicle control group.

Group	Derf vehicle	Acute DON 0.3 mg/kg	Subacute DON 0.3 ppm
Alveolar inflammation	0.75 $\pm$ 0.25	1.25 $\pm$ 0.25	2.00 $\pm$ 0.58
Bronchial excessive mucoid	0.75 $\pm$ 0.25	1.25 $\pm$ 0.25	1.75 $\pm$ 0.75
Perivascular inflammation	1.50 $\pm$ 0.29	2.25 $\pm$ 0.25	2.25 $\pm$ 0.48
Total score	3.00 $\pm$ 0.58	4.75 $\pm$ 0.75	6.00 $\pm$ 1.78

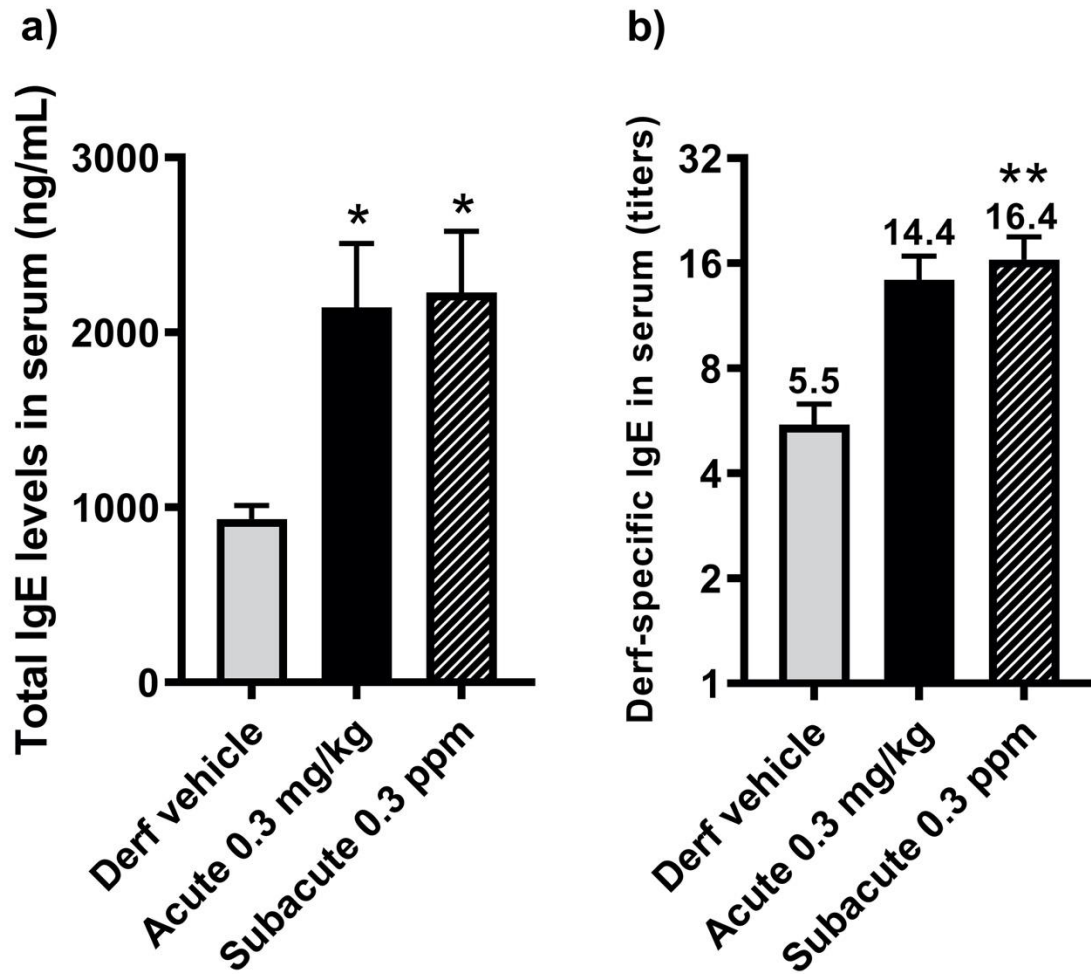
Derf + vehicle



Subacute DON 0.3 ppm



Supplemental Figure. 1 Representative microscopic features of the lung of the Derf + vehicle control group and the subacute 0.3 ppm DON group in the additional setting.



Supplemental Figure. 2 Total (a) and Derf-specific (b) IgE levels in the Derf-induced mouse model of allergic asthma of the second setting. Significant increase in total and Derf-specific serum IgE levels (ng/mL) are observed after acute and subacute DON administration. Each result is presented as the mean $\pm$ SEM. $n = 6$ per group. Derf, *Dermatophagoides farinae*; DON, deoxynivalenol.