

Triclosan-induced alteration of gut microbiome and aggravation of asthmatic airway response in aeroallergen-sensitized mice

To the Editor,

Triclosan (2,4,4'-trichloro-2'-hydroxydiphenyl ether) is a synthetic, broad-spectrum antimicrobial agent used extensively in various healthcare products and household consumer goods such as soap, detergents, toothpastes, and other products. Triclosan (TCS) was first introduced in the 1960s for the use in clinical applications. Reports have suggested that TCS is absorbed in human skin and oral mucosa; and it has been detected in various human tissues and fluids.^{1,2} In addition, toxicological studies have shown that TCS exposure induces physiological perturbations in animals and in humans.^{4,5} Recently, a risk assessment undertaken by the US Food and Drug Administration (FDA) revealed that TCS was present in the urine samples of 75% of the US general population, and, in 2016, the FDA banned its use in soap products.^{3,5} Over the last 6 years, a number of studies have identified a positive correlation between the urinary TCS level and sensitization to food, aeroallergens, and perturbations in asthmatic patients.^{1,6,7} In addition, an experimental study has shown that the topical application of TCS enhances allergic response in ovalbumin (OVA)-sensitized mice,⁸ suggesting that TCS might play an adjuvant role in the allergic response to environmental allergens. However, there has still been insufficient evidence to support a relationship between perturbation of the gut microbiome and allergic manifestations in relation to TCS exposure. The aim of this study was to evaluate the *in vivo* TCS-induced alteration of the gut microbiome and aggravation of asthmatic airway response in aeroallergen-sensitized mice.

We performed two independent experiments. In the first experiment, wild type BALB/cJ mice ($n = 36$) were randomly divided into 6 groups of six animals, including normal mice group (normal), *Dermatophagoides farinae* group (wild-type + Der f), Der f and diesel exhaust particle (DEP)-exposed group (asthma), vehicle-treated Der f-sensitized mice group (wild type + Der f), 5 mg/kg TCS-treated mice group (wild type + Der f + 5 mg/kg triclosan), 50 mg/kg TCS-treated mice group (wild type + Der f + 50 mg/kg triclosan), and 500 mg/kg TCS-treated mice group (wild type + Der f + 500 mg/kg triclosan). In the second experiment, knockout TLR-2 (–/–) mice ($n = 12$) were randomly divided into two groups of six animals that received 4 μ g of Der f intratracheally 2 days per

week for a total of 4 weeks. Both groups were treated either with vehicle (TLR-2 (–/–) mice + Der f group) or triclosan 500 mg/kg (TLR-2 (–/–) mice + Der f + triclosan group) through oral gavage 5 days per week for a total of 4 weeks. On the other hand, airway hyperresponsiveness (AHR) to intravenous acetylcholine (ACh) was performed as previously described,⁸ whereas bronchoconstriction was measured according to the overflow method using a bronchospasm transducer connected to the tracheal cannula. Changes in respiratory overflow volume were measured using an increasing dose of ACh. The area under the curve (AUC), calculated from the dose-response curves for ACh, was used to express the magnitude of AHR (ImageJ software, National Institutes of Health, Bethesda, Maryland, USA). On day 23, bronchoalveolar lavage (BAL) was performed and BAL fluid (BALF) was recovered for eosinophils enumeration and also IL-4 and IL-13 level measurements by ELISA. Blood samples and lung specimens were collected to measure the serum level of anti-Der f IgE and perform the histopathological examination, respectively. Furthermore, mouse fecal samples were collected to isolate microbial genomic DNA using a NucleoSpin® DNA Stool® Kit, and 16S rRNA gene sequencing was performed following procedures described by Shen et al.⁹ Data are presented as the mean $\pm$ SEM, and statistical comparisons among the treatment groups were performed with the use of one-way ANOVA test followed by the nonparametric Tukey test, whereas the abundance of bacterial genes was compared using Student's *t* test (SPSS software package). Detailed methodology is provided in Supporting informations.

As results, simultaneous exposure of wild-type mice to TCS and Der f induced:

- 1). An increase in the production of anti-Der f IgE, IL-4, and IL-13 (Figure 1D-B-E), which resulted in the aggravation of airway hyperresponsiveness in aeroallergen-exposed wild type mice (Figure 1A), whereas these changes were not observed in non-sensitized mice;
- 2). Increased collagen deposition and infiltration of inflammatory cells (eosinophils) in the lungs of aeroallergen-exposed wild type mice as shown in Figure 1C;

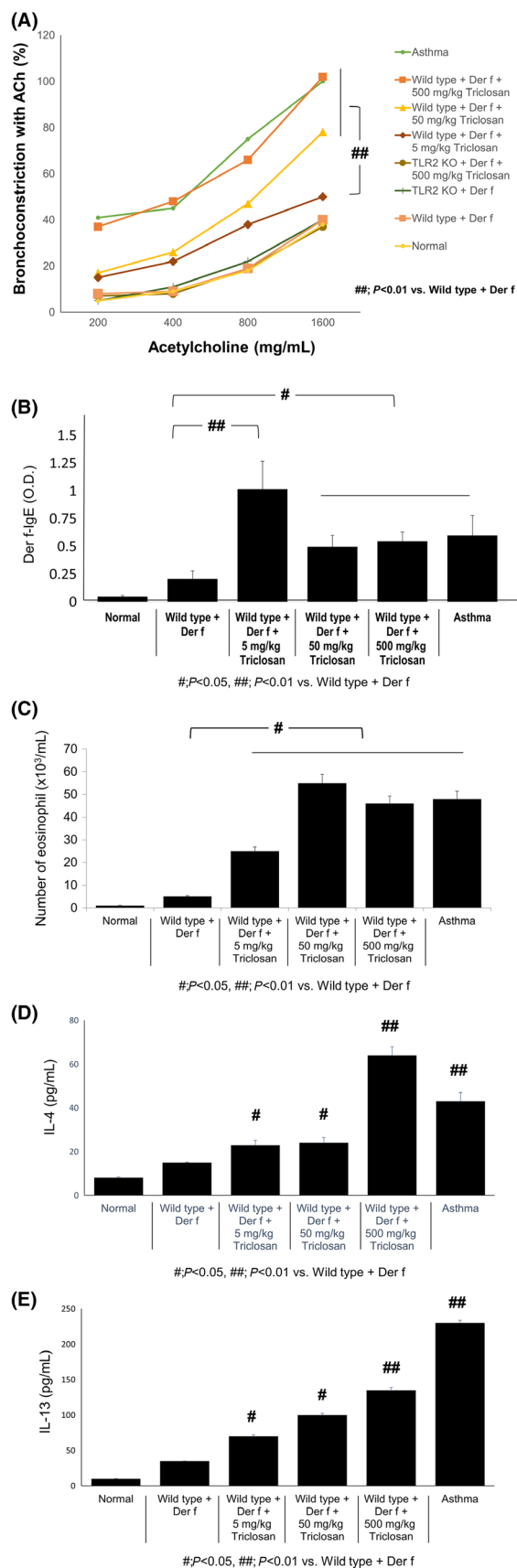


FIGURE 1 Induction of airway inflammation and hyperresponsiveness, eosinophil count, serum anti-Der f IgE level, and BAL fluid levels of IL-4 and IL-13 in wild-type BALB/c mice. Airway hyperresponsiveness was induced using acetylcholine (ACh). Normal mice group received PBS, whereas the allergen-exposed mice groups received either *Der f* only (*Der f* group), *Der f* + diesel-exhausted particles (asthma group), or *Der f* + triclosan (wild-type + *Der f* + 5 mg/kg triclosan group, wild-type + *Der f* + 50 mg/kg triclosan group, wild-type + *Der f* + 500 mg/kg triclosan group). In wild-type mice, AHR was significantly increased by simultaneous exposure to triclosan and *Der f* in comparison with wild-type + *Der f* mice ($P < 0.01$). The area under the curve (AUC) for the dose-response to ACh (range: 62.5–2000 mg/kg); $^{##}P < 0.01$ vs wild-type + *Der f* group (A). In addition, significantly higher serum anti-Der f IgE titers were observed in all triclosan-treated sensitized mice groups (5, 50, 500 mg/kg) as compared with wild-type + *Der f* group ($P < 0.01$, $P < 0.05$, and $P < 0.05$, respectively) (B). Furthermore, the number of eosinophils in BALF was significantly higher in of triclosan-treated mice groups (50 mg/kg triclosan and 500 mg/kg triclosan) than in wild-type + *Der f* mice group ($P < 0.05$) (C). The BALF levels of IL-4 (D) and IL-13 (D) (5, 50, 500 mg/kg) in triclosan-treated mice were significantly higher than those in wild-type + *Der f* mice ($P < 0.05$, $P < 0.05$, and $P < 0.01$, respectively). The results represent the mean $\pm$ SEM of data from each group; $^{#}P < 0.05$ vs wild-type + *Der f*. $^{##}P < 0.01$ vs wild-type + *Der f* [Colour figure can be viewed at wileyonlinelibrary.com]

3). Goblet cell hyperplasia in the lungs of sensitized wild type mice, whereas, in contrast, TLR-2 (–/–) mice did not show any allergic response after exposure to TCS (Figure 2A).

On the other hand, regarding the mouse microbial community analysis, the copy numbers of bacterial 16S rRNA gene of *c_Deltaproteobacteria*, *c_Clostridia*, and *c_Erysipelotrichi* in TCS-treated *Der f*-sensitized mice increased in a dose-dependent manner, whereas it markedly decreased in *c_Bacteroidia* (Figure 2B–C). Furthermore, for peripheral blood Treg cells (CD4+ CD25-high FOXP3+ T cells), no significant difference was observed among mice groups (Figure 2D).

This study showed that a simultaneous exposure to TCS and environmental aeroallergen induced an increased production of IL-4, IL-13, which are representatives Th-2 cytokines associated with allergic response, and anti-Der f IgE. Additionally, a perturbation of gut microbiome, an intense eosinophilic infiltration, and goblet cell hyperplasia, one of the prominent features of animal models of atopic asthma consecutive to repeated challenges with an allergen, were observed.

Our experiments were performed in duplicate, and the primary animal study showed high levels of the representative Th-2 cytokines, including IL-5, in the BALF of TCS and *Der f*-exposed animals; but the latter was not measured in the secondary experiments due to insufficient BALF samples. This constitutes a limitation for this work. Taken together, findings from this study indicate that exposure to TCS and aeroallergen exposure induces and/or aggravates AHR and inflammation and that there is a possible relationship between TCS-induced aggravation of AHR and the perturbation of gut microbiome in aeroallergen-sensitized mice. This suggests that TCS might play an adjuvant role in the induction or aggravation of allergic response in susceptible individuals.

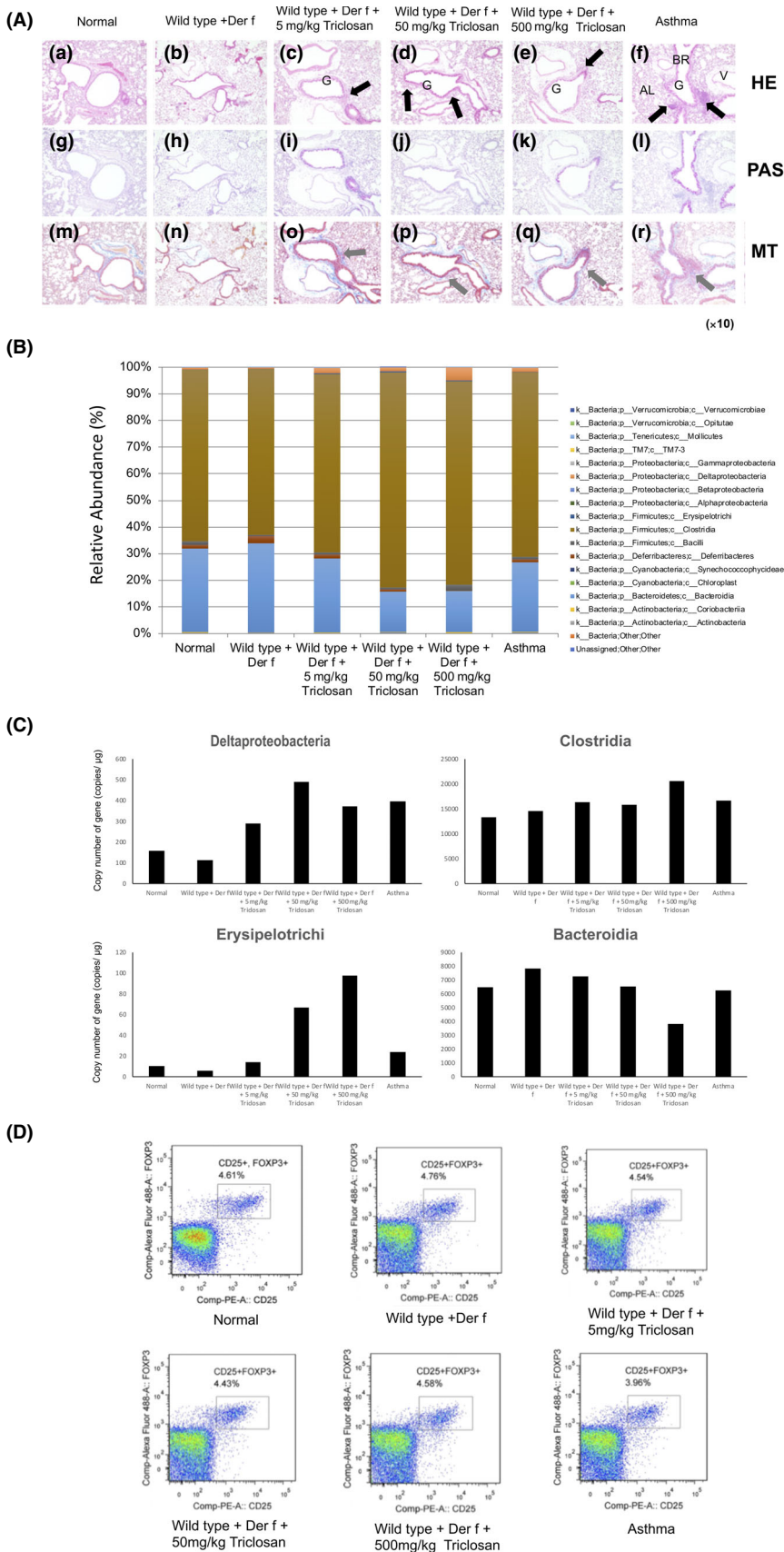


FIGURE 2 Histopathological images of lung specimens and taxonomic profiles of microbial communities in gut microbiota. The figure shows [1] representative photomicrographs at 10 times magnification (inset) of HE (histology) (A (a-f)), PAS (mucin) (A (g-l)), MT (fibrosis) (A (m-r)) staining of mouse lung specimens obtained 23 d after the intratracheal instillation of *Der f*. BALB/cJ mice were randomly divided into six groups ($n = 36$) and treated with saline (normal group) (A (a, g, m)) or 4 μg *Der f* (A (b, h, n)), or *Der f* + triclosan (5 mg/kg (A (c, i, o)), 50 mg/kg (A (d, j, p)), 500 mg/kg (A (e, k, q)) and *Der f* + DEP (asthma) (A (f, l, r)). The triclosan-treated mice (A (c-e, i-k, o-q)) showed a marked increase in goblet cell hyperplasia, eosinophil infiltration, and the numbers of PAS-positive cells and collagen fibers in the lung. Black arrows indicate representative eosinophils in the infiltrate (A (c, d, e, f)), yellow arrows indicate PAS-positive cells (A (i, j, k, l)), green arrows indicate subepithelial fibrosis (A (o, p, q, r)), magnification 10x. Abbreviations: AL, alveolus; BR, bronchiole; V, blood vessel; G, goblet cell hyperplasia; [2] the relative abundance of gut microbial species (B,C), and the proportions of Treg cells, defined as CD4+ CD25-high FOXP3+ T cells (D). The copy numbers of the bacterial 16S rRNA gene of *c_Deltaproteobacteria*, *c_Clostridia*, and *c_Erysipelotrichi* in triclosan-treated *Der f*-exposed mice increased in a dose-dependent manner. In contrast, the copy number of the bacterial 16S rRNA gene of *c_Bacteroidia* decreased (B,C). No significant difference was observed in terms of proportions of Treg cells, defined as CD4+ CD25-high FOXP3+ T cells (D)

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Omalizumab discontinuation in children with severe allergic asthma: An observational real-life study

To the Editor,

We previously showed the effectiveness and safety of omalizumab in children with severe allergic asthma.^{1,2} However, data on optimal treatment duration and discontinuation are limited.³ They mainly concern adults.⁴⁻⁷ This real-life study reports asthma outcomes comparing children maintained on omalizumab or discontinuing omalizumab after long-term treatment.

This study was conducted in 10 French pediatric pulmonology tertiary centers (Hauts de France, Normandie, Paris) and approved by the Research Ethics Committee of the French Society of Pediatrics and by the French Data Protection Authority (CNIL). Children, aged 6-18 years with severe allergic asthma and a minimum 24-month course of omalizumab, were included.⁸ Omalizumab indication was based on the recommendations of the French Health Drug

Supplementary File 1.

Triclosan-induced Alteration of Gut Microbiome and Aggravation of Asthmatic Airway Response in Aeroallergen-sensitized mice

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Methods

1. Animals and drug administration

All of the experimental procedures were performed in accordance with the guidelines of the Laboratory Animal Research Kochi Medical School and were approved by the Institutional Animal Care and Use Committee of Kochi Medical School (J-0058, 28-0039). Thirty-six 5-week-old male BALB/cJ mice (wild-type) were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). Twelve 5-week-old male Toll-like Receptor (TLR)-2 knockout BALB/c (TLR-2 (-/-) mice) were purchased from Oriental Bio-service (Kyoto, Japan). Animals, weighing 20-25 g, were housed under conventional conditions at the animal facility of Kochi Medical School: in filter-topped macrolon cages with wood chips bedding, at a temperature of 23° C with 50-60% relative humidity, a 12-h light/dark cycle, and ad libitum access to standard chow. In the first experiment, wild type BALB/cJ mice (n=36) were randomly divided into six groups (n = 6). They received either saline (Normal group), 4 µg of *Dermatophagoides farinae* (*Der f*) (4 allergy induction groups), or 4 µg of *Der f* and diesel exhaust particles as an adjuvant (Asthma group) intratracheally for two days a week for a total of four weeks. The four allergy-induction groups were treated simultaneously with vehicle (Wild type + *Der f* group) or Triclosan (5 mg/kg, 50 mg/kg, 500 mg/kg Triclosan groups) by oral gavage, five days a week for a total of four weeks. In the second experiment, knockout BALB/c (TLR-2 (-/-)) mice (n=12) were randomly divided into 2 groups (n = 6) and received 4 µg of *Der f* intratracheally for two days a week for four weeks in total. The 2 groups were treated simultaneously either with vehicle (TLR-2 (-/-) mice + *Der f* group) or Triclosan 500 mg/kg (TLR-2 (-/-) mice + *Der f* + Triclosan group) by oral gavage, five days a week for four weeks.

2. Induction of airway hyperresponsiveness

Measurement of airway hyperresponsiveness (AHR) to intravenous acetylcholine (ACh) was performed as previously described (1). Briefly, to measure AHR, the mice were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and the jugular vein was cannulated for the intravenous injection of ACh. They were injected with pancuronium bromide (0.1 mg/kg, i.v.) to stop spontaneous respiration and then ventilated with a rodent ventilator (New England Medical Instruments, Inc., Medway, MA, USA). Bronchoconstriction was measured according to the overflow method, using a bronchospasm transducer (Ugo Basil 7020, Milan, Italy) connected to the tracheal cannula. Changes in respiratory overflow volume were measured using an increasing dose of ACh. The increase in respiratory overflow volume induced by ACh was represented as a percentage of the maximal overflow volume (100%) obtained by clamping the tracheal cannula. We performed two independent experiments. Since similar results were obtained, we used the data from the second experiment in this report.

The area under the curve (AUC), calculated from the dose-response curves for ACh, was used to express the magnitude of AHR. Briefly, the AHR chart was saved as a bmp file, then the AUC was selected and calculated using the ImageJ software program (1.44p; National Institutes of Health, USA) with the value of each dose converted logarithmically and represented as arbitrary units.

3. Collection of blood samples and measurement of serum anti-*Der f*IgE

Blood samples were drawn from animals on day 23 and kept at -80° C for subsequent analyses of the serum anti-*Der f*IgE levels. The serum levels of anti-*Der f*IgE were determined as follows: serum IgE was bound with coated *Der f* antigen, and was then detected with horse radish peroxidase conjugated antibodies. The data were expressed as optical density (O.D.) values.

4. Bronchoalveolar lavage, cell count and enzyme-linked Immunosorbent assay (ELISA)

On day 23, the animals were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). Bronchoalveolar lavage (BAL) was performed using 1.5 ml of 0.9% saline solution and 80% (1.2 ml) of BAL fluid (BALF) was recovered. The numbers of eosinophils in the BALF were enumerated. The levels of inflammatory cytokines (IL-4, IL13) in BALF were measured using a commercially available enzyme immunoassay (EIA) kit (DuoSet, R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

5. Histopathological analysis of lung specimens

On day 23, after completion of BALF and blood sample collection, the animals were sacrificed and lung specimens from representative mice were collected. The tissue samples were fixed in 10% formalin, embedded in paraffin, sectioned at 10 µm, and stained with haematoxylin and eosin (HE), periodic acid-Schiff (PAS) and Masson tri-chrome (MT). The histological examination was performed using a light microscope (Olympus BX51, Olympus, Japan) at 10x magnification.

6. 16S rRNA gene sequencing

16S rRNA gene sequencing was performed as described in Shen et al. (2). On Day 23, we collected fecal samples from each mouse and isolated DNA using a NucleoSpin® DNA Stool® kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) according to the manufacturer's instruction. We amplified DNA using 515 F and 806R primers¹¹ targeting the V4 regions of the 16S rRNA of bacteria, followed by normalization, a barcoding procedure, and finally pooling for the construction of the sequencing library. The resultant DNA was quantified using a Qubit 2.0 Fluorometer, and then sequenced using an Illumina MiSeq (500 cycles v2 kit) in the Hokkaido System Science Co., Ltd. Paired-reads were assembled using the Geneious software program (Biomatters, Auckland, New Zealand), and taxonomic unit (OTU) picking and a diversity analysis were conducted using the Quantitative Insights into Microbial Ecology (QIIME) software program.

References

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Supplementary file 2. Experimental protocol

Wild type BALB/cJ mouse
TLR2 (-/-) BALB/c mouse
Five-week-old male

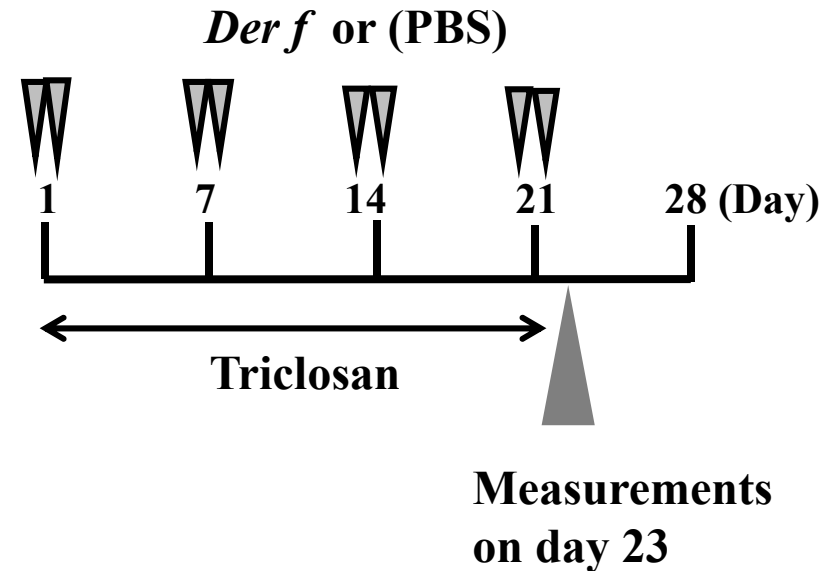
Experient 1: Wild type + *Der f* + Triclosan

Triclosan 0, 5, 50, 500 mg/kg/d
(oral gavage)

Experient 2: TLR2 (-/-) + *Der f* + Triclosan

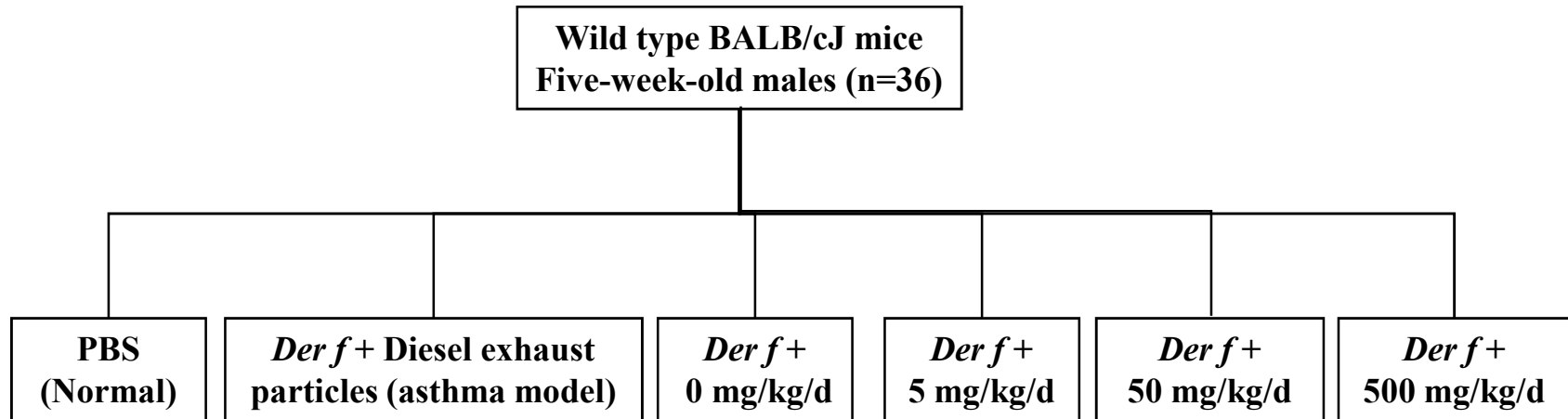
Triclosan 500 mg/kg/d
(oral gavage)

- Allergen (*Der f*) or PBS
- Positive: *Der f* + Diesel exhaust particles (adjuvant)



Supplementary file 3. Mice grouping

(Experiment 1) Wild type + *Der f* + Triclosan



(Experiment 2) TLR2 KO + *Der f* + Triclosan

