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Bisphenol A triggers activation of ocular immune system and aggravates allergic airway inflammation

Tatsuo Ueda [a,b], Takumi Adachi [a], Tomoya Hayashi [c], Koubun Yasuda [a], Kazufumi Matsushita [a], Eiko Koike [d], Rie Yanagisawa [d], Takahiro Nagatake [e,f], Jun Kunisawa [f], Ken J. Ishii [c], Kenzo Tsuzuki [b], Etsushi Kuroda [a,*]

[a] Department of Immunology, Hyogo Medical University, School of Medicine, 1-1 Mukogawa-cho, Nishinomiya 663-8501, Japan
[b] Department of Otorhinolaryngology-Head and Neck Surgery, Hyogo Medical University School of Medicine, 1-1 Mukogawa-cho, Nishinomiya 663-8501, Japan
[c] Division of Vaccine Science, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo (IMSUT), Tokyo 108-8639, Japan
[d] Health and Environmental Risk Division, National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba 305-8506, Japan
[e] Laboratory of Functional Anatomy, Department of Life Sciences, School of Agriculture, Meiji University, 1-1-1 Higashi-Mita, Tama, Kawasaki 214-8571, Japan
[f] Laboratory of Vaccine Materials and Laboratory of Gut Environmental System, Microbial Research Center for Health and Medicine, National Institutes of Biomedical Innovation, Health and Nutrition, 7-6-8 Asagi-Saito, Ibaraki 567-0085, Japan

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ABSTRACT

Bisphenol A (BPA) is widely used in manufacturing plastic products, and it has been reported that exposure through the airway or orally aggravates allergic airway inflammation. Because BPA is detected in the atmosphere and indoor environments, the eyes can also be exposed to BPA. After ocular exposure to BPA and antigen via eye drops, we observed enhanced antigen uptake of antigen-presenting cells (APCs) in tear duct-associated lymphoid tissue (TALT). Additionally, we observed the formation of germinal center (GC) B cells in TALT and induction of allergic airway inflammation in mice sensitized with BPA and antigen via eye drops, followed by airway antigen exposure. We also found that DNAX-activating protein of 12 kDa (DAP12)-deficient mice displayed impaired activation of APCs enhanced by ocular exposure to BPA. These results indicate that ocular sensitization to BPA and allergen triggers allergic inflammation via TALT activation, and that DAP12 might be a key molecule for modulating the ocular immune system.

1. Introduction

Asthma is an allergic disease characterized by airway narrowing, bronchial hyperresponsiveness, and airway inflammation. In addition to genetic predisposition, environmental factors are thought to be associated with its development [1]. In particular, air pollution associated with industrialization is thought to be responsible for the rapid increase in allergic diseases in recent years [2,3].

Bisphenol A [4,4′-(propane-2,2-diyl) diphenol or BPA] is a pollutant of both outdoor and indoor air [4]. The plastic monomer and plasticizer BPA is one of the most abundantly produced chemicals worldwide. BPA is applied to make polycarbonate plastics and epoxy resins, which are used in many consumer products. Polycarbonate is employed as a material for reusable plastic bottles, baby bottles, plastic cups, microwave utensils, and storage containers, among others, while epoxy resins are used for internal coatings of food and beverage product containers. BPA is used not only in food and drink containers but also in a wide variety of other applications, including sunglasses, building materials, compact discs, medical equipment, dental materials, and thermal paper [5,6]. BPA is dispersed into the atmosphere through the thermal breakdown of these BPA-containing materials and emissions from facilities that handle BPA.

Environmental endocrine-disrupting chemicals such as BPA have immune system-modulating effects even at low concentrations [7]. BPA exhibits its endocrine-disrupting effects by binding to a variety of receptors, including estrogen receptors, androgen receptors, aryl hydrocarbon receptors, thyroid hormone receptors, and toll-like receptors [8,9]. It has been suggested that exposure to a low dose of BPA may exacerbate allergic airway inflammation by generating Th2-biased responses [10]. In fact, previous epidemiological studies have suggested that BPA is associated with the development of prenatal and postnatal asthma and allergies [11,12]. Various studies based on experimental

* Corresponding author at: Department of Immunology, School of Medicine, Hyogo Medical University, 1-1 Mukogawa-cho, Nishinomiya, Hyogo 663-8501, Japan.
E-mail address: kuroetu@hyo-med.ac.jp (E. Kuroda).

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animal models have also demonstrated that oral or airway exposure to BPA exacerbates allergic airway inflammation [10,13–18]. The primary route of exposure to BPA is through ingestion, as it leaches into foods and beverages. However, it has also been detected in ambient air and dust [4,6], meaning that BPA can enter the body through the eyes in addition to the mouth and nose. In environments contaminated with BPA, many people wear masks to protect their mouths and noses, but eye protection is often neglected, raising concerns about the effects of BPA on the eyes. In fact, there is growing evidence that air pollution, including BPA, can directly harm the eyes [19,20]. However, no experimental studies have demonstrated that the exposure to BPA via the ocular route exacerbates allergic airway inflammation.

Mucosa-associated lymphoid tissues (MALTs), including nasopharynx-associated lymphoid tissue (NALT) and Peyer's patches (PPs), are the portals of entry for the uptake of inhaled and orally ingested antigens and are considered the sites of induction of mucosal immune responses. Lymphoid tissue of the ocular mucosa is situated in the nasolacrimal duct, which can be found in humans and rodents and is called tear duct-associated lymphoid tissue (TALT) [21–24]. TALT has the cytomorphological and immunophenotypic characteristics of MALT and is thought to play an important role in the ocular immune system [25–28].

The ocular immune system plays an important role as site of entry of foreign allergens and serves as a primary sensitization site [29], and therefore, in this study, we investigated the role of TALT in ocular exposure to the chemical BPA. Exacerbated allergic airway inflammation was observed in mice that were sensitized with BPA and allergen via eye drops, followed by inhalation of the allergen. We also investigated the mechanism of action of BPA and found that it enhanced the antigen uptake and/or migration of antigen-presenting cells (APCs) and the formation of germinal center (GC) B cells in TALT. As a result, BPA provoked the production of antigen-specific antibody responses and type 2 inflammatory responses in the airways. Finally, we found that mice with the genetic ablation of DNAX-activating protein of 12 kDa (DAP12), an immune adaptor protein containing immunoreceptor tyrosine-based activation motif (ITAM), displayed impaired activation of APCs that was enhanced by ocular exposure to BPA. These results suggest that exposure to environmental chemicals through the eyes is an important sensitization route for airway inflammation and that DAP12 is a potential candidate molecule that senses and responds to environmental chemicals or endocrine disruptors.

2. Methods

2.1. Mice

C57BL/6 J mice were purchased from CLEA Japan (Tokyo, Japan). DAP12-deficient mice were provided by Dr. Toshiyuki Takai, Tohoku University, Sendai, Japan [30]. Il33-deficient mice were obtained as described previously [31]. Type 2 innate lymphoid cell (ILC2)-deficient mice were generated by crossing *Rora*$^{flox/flox}$ mice with Vav1-iCre mice. RORα is required for ILC2s development [32], but it is also involved in neuronal maturation, and mice lacking RORα in neuronal cells exhibit ataxia [33]. Since the Vav1-Cre transgene is used for hematopoietic linage-restricted [34], we utilized Vav1-iCre mice to induce hematopoietic cell-specific RORα deficiency. *Rora*$^{flox/flox}$ mice were provided by Dr. Shigeo Koyasu and Dr. Kazuyo Moro, RIKEN Center for Integrative Medical Science, Yokohama, Japan. Vav1-iCre mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were maintained under specific pathogen-free conditions at the animal facilities of Hyogo Medical University. All experiments were approved by the animal experimentation committee of Hyogo Medical University (Certification No. 23-043AG).

2.2. Reagents

Ovalbumin (OVA) were purchased from Kanto Chemical Co. Inc. (Osaka, Japan). OVA used for the in vivo experiments was prepared in endotoxin-free solution (<1 EU/ 1 mg OVA). Endotoxin levels were determined with Toxicolor® (Seikagaku Corp., Tokyo, Japan). Phosphate-buffered saline (PBS; pH 7.4) was purchased from Nacalai Tesque Inc. (Kyoto, Japan). BPA was purchased from Sigma-Aldrich (Saint Louis, MO, USA). Alhydrogel (Aluminum Hydroxide Gel Adjuvant) was obtained from InvivoGen (San Diego, CA, USA). Alexa Fluor 647-conjugated OVA was purchased from Invitrogen (Carlsbad, CA, USA). For ELISA, rat anti-mouse IgE-UNLB (clone: 23G3) was purchased from SouthernBiotech (Birmingham, AL, USA); mouse anti-ovalbumin IgE monoclonal antibody and mouse anti-ovalbumin IgG1 monoclonal antibody were purchased from Chondrex, inc. (Redmond, WA, USA). HRP-conjugated Ovalbumin was purchased from Bio-Rad (Hercules, CA, USA); goat anti-mouse IgG1 HRP conjugated was purchased BETHYL Laboratories, Inc. (Montgomery, TX, USA). The cells were cultured in RPMI-1640 medium (Nacalai Tesque Inc.). For flow cytometry, purified anti-mouse CD16/32 (clone: 93), 7-AAD Viability Staining Solution, Brilliant Violet 421 anti-mouse CD11c (clone: N418), and Brilliant Violet 421 anti-mouse CD45 (clone: 30-F11) were purchased from BioLegend (San Diego, CA, USA); fluorescein isothiocyanate (FITC) Rat anti-mouse CD45 (clone: 30-F11), FITC rat anti-mouse CD45R/B220 (clone: RA3-6B2), phycoerythrin (PE) rat anti-mouse Siglec-F (clone: E50–2440), PE hamster anti-mouse CD95, and PE anti-mouse I-A/I-E were purchased from BD Biosciences (San Jose, CA, USA); and anti-human/mouse GL7 Alexa Fluor 647 (clone: GL-7) was purchased from eBioscience (San Diego, CA, USA).

2.3. Sensitization and elicitation protocol

In this study, we established a mouse model of allergic airway inflammation by sensitization with BPA and allergen via eye drops. The experimental doses of BPA used in this study were equivalent to 10 times the estimated peak exposure dose from the general atmosphere in Japan of 0.3 ng/kg body weight/day reported by Ministry of the Environment of Japan (Fig. S1).

Eight-week-old female C57BL/6 J mice were randomly divided into three groups: control group, OVA alone group, and OVA with BPA group. We prepared three types of eye drop samples as follows: PBS for the control group, 10 μg of OVA in PBS for the OVA alone group, and 10 μg of OVA with 0.3 ng/kg body weight BPA for the OVA with BPA group. On Days 0, 3, 6, and 9, the mice were anesthetized by ketamine and xylazine and then sensitized with 10 μl of PBS, OVA, or OVA with BPA via eye drops in both eyes. On Days 16, 18, and 20, all groups of mice were exposed to OVA (10 mg/ml) via inhalation for 10 min using a nebulizer (Omron Healthcare, Kyoto, Japan). These mice were then used for the analysis of airway inflammation.

2.4. Detection of serum OVA-specific IgE and IgG_1 antibodies

Mice were sacrificed 24 h after the final antigen challenge, and blood was collected from each mouse by cardiac puncture. The collected blood was centrifuged at 10,000 rpm for 10 min twice to obtain serum. OVA-specific IgE and IgG_1 levels in the serum were measured using ELISA. For the detection of OVA-specific IgE, a 96-well flat-bottomed plate (Corning, Corning, NY, USA) was coated with 5 μg/ml rat anti-mouse IgE-UNLB in PBS as the capture reagent and incubated overnight at 4 °C. Nonspecific binding sites were blocked with blocking buffer (10 % FCS in RPMI) for 30 min at room temperature. After removing the blocking buffer, diluted serum, anti-OVA IgE or anti-OVA IgG_1 was added to each well and incubated overnight at 4 °C. HRP-conjugated Ovalbumin (1:200) as the detection reagent was added to each well and incubated for an additional hour at room temperature. After washing the plate, substrate solution was added to each well. The reaction was stopped by

adding 10 % H_2SO_4. Absorbance was read at 450 nm using a microplate reader (Bio Tek Instruments, Inc., Winooski, VT, USA) and antibody concentrations for each sample were interpolated from the standard curve. For the detection of OVA-specific IgG_1, 10 μg/ml OVA in PBS was used as the capture reagent and HRP-conjugated goat anti-mouse IgG1 (1:1000) was used as the detection reagent.

2.5. Collection and analysis of bronchoalveolar lavage fluid cells

A cannula was inserted into the trachea to collect bronchoalveolar lavage fluid (BALF). The lungs were washed with an injection of 1.0 ml of sterile saline using a 1 ml syringe (Terumo, Tokyo, Japan). The collected BALF was centrifuged at 2000 rpm for 3 min. The total cell count in the BALF was determined using a hemocytometer. The obtained cells were then stained with 7-AAD Viability Staining Solution, FITC rat anti-mouse CD45, Brilliant Violet 421 anti-mouse CD11c, and PE rat anti-mouse Siglec-F. $CD45^+$, $CD11c^+$, Siglec-F^+ cells were identified as eosinophils in the BALF using an LSRFortessaX-20 (BD Biosciences). The data were analyzed using FlowJo software (FlowJo LCC, Ashland, OR, USA).

2.6. Histological analysis

The lungs and the head were inflated and fixed in 10 % neutral phosphate-buffered formalin. To prepared paraffin sections, the tissues were gradually dehydrated and embedded in paraffin. The obtained blocks were sectioned at a thickness of 5 μm and stained with hematoxylin and eosin (H&E). Additionally, the lung sections were stained with periodic acid–Schiff (PAS) to evaluate the proliferation of goblet cells in the bronchial epithelium. Representative images were captured using a fluorescence microscope (BZ-X800; KEYENCE, Osaka, Japan).

2.7. Collection and analysis of cells in tear duct-associated lymphoid tissue

GC B cells in the TALT were evaluated using a flow cytometer. We initially identified the anatomical location of TALT in the tear duct by H&E staining of the mouse head (Fig. S2a and b). TALT was collected based on a previously reported method for isolating tear duct from the head of mouse [28]. Mouse was euthanized by cervical dislocation, and the head was isolated. After removing the epidermis and mandible from the head, the head was incised in the midline. The tear duct was exposed using forceps, and TALT was carefully dissected with the tear duct from the maxillary bone (Fig. S2c and d). Cells were prepared by passing through a 70 μm nylon cell strainer (Corning) in RPMI 1640. Isolated TALT cells were incubated with purified anti-mouse CD16/32 to block Fc receptors and then stained with 7-AAD Viability Staining Solution, Brilliant Violet 421 anti-mouse CD45, FITC rat anti-mouse CD45R/B220, PE hamster anti-mouse CD95, and anti-human/mouse GL7 Alexa Fluor 647. The GC B cells were identified as $CD45^+$, $B220^+$, $CD95^+$, and $GL7^+$ cells using an LSRFortessaX-20 (BD Biosciences). The data were analyzed using FlowJo software (FlowJo LCC).

2.8. Analysis of antigen uptake following ocular exposure

Eight-week-old female C57BL/6 J mice were randomly assigned to either a BPA-free group or a BPA-treated group. The BPA-free group received Alexa Fluor 647-conjugated OVA (40 μg in PBS), while the BPA-treated group received both Alexa Fluor 647-conjugated OVA and BPA (3.0 ng/kg body weight). TALT was collected from the head of the mouse 24 h after administering the eye drops; subsequently, the APCs in the TALT were analyzed using a flow cytometer. TALT cells were stained with 7-AAD Viability Staining Solution, FITC rat anti-mouse CD45, Brilliant Violet 421 anti-mouse CD11c, and PE anti-mouse I-A/I-E. Cells that had taken up labeled OVA were identified as $CD45^+$, $CD11c^+$, I-A/I-E^+, and Alexa Fluor 647^+ cells using an LSRFortessaX-20 (BD Biosciences). The data were analyzed using FlowJo software (FlowJo LCC).

2.9. Statistical analysis

The statistical significance of differences between two groups was determined using the non-parametric Mann–Whitney *U* test. We also performed one-way analysis of variance (ANOVA) followed by the non-parametric Kruskal–Wallis test. All statistical analyses were carried out using Prism 10 (GraphPad Software, La Jolla, CA, USA). *P* values of less than 0.05 were considered statistically significant. Data were combined from at least two independent experiments.

3. Results

3.1. Ocular exposure to BPA augments activation of antigen-presenting cells, enhancing soluble antigen uptake and/or cell migration to TALT

Some toxic chemicals can modulate immune responses by either activating or suppressing immune cells, which is often manifested as allergic inflammation [7]. For example, airborne pollutants such as acrylamide and ozone function as adjuvants to induce allergic airway inflammation [35,36]. Upon exposure to air pollution, the chemical primarily enters the body via the mouth, skin, and nose. However, the eye is also recognized as a significant site of exposure given that it features a mucosal surface [28]. It has been noted that TALT possesses an immunosurveillance capacity comparable to those of other well-known mucosal inductive tissues in the aerodigestive tract, such as NALT and PPs [25]. In this study, we focused on BPA as a semi-volatile environmental chemical and hypothesized that ocular exposure to it, along with an allergen, triggers immune responses as primary sensitization through the activation of TALT. Because previous studies showed that BPA modifies dendritic cell functions [10,37], we first investigated the function of APCs ($CD11c^+$, MHC II^+, $CD45^+$, $7AAD^-$ cells), such as their antigen uptake and migration, in TALT after exposure to BPA.

Mice were administered Alexa Fluor 647-labeled OVA with or without BPA via eye drops. Twenty-four hours later, the total numbers of APCs and labeled OVA-positive APCs in TALT were examined using flow cytometry. The experimental doses of BPA used in this study were equivalent to 10 times the estimated peak exposure dose from the general atmosphere in Japan of 0.3 ng/kg body weight/day. As shown in Fig. 1, the number of labeled OVA-positive APCs was significantly increased in TALT in the OVA+BPA group 24 h after instillation (Fig. 1a). On the other hand, there was no significant difference in the total number of APCs between OVA and OVA+BPA groups (Fig. 1b). TALT and NALT are connected via the nasolacrimal duct [27]; however, an increased number of OVA-positive APCs was observed in TALT after ocular exposure to OVA and BPA. Meanwhile, intranasal administration of OVA and BPA induced OVA-positive APCs in NALT, indicating that the activation of regional MALT depends on the site of sensitization, such as the eyes and nose (Fig. 1c, d). These results suggest that ocular exposure to BPA along with antigen induced the activation of APCs, leading to the enhancement of their antigen uptake and/or migration to TALT.

3.2. Ocular exposure to BPA enhances GC formation in TALT

In general, MALT is located at mucosal surfaces and contributes to antigen uptake and the induction of local immune responses. Meanwhile, TALT forms adjacent to the nasolacrimal duct and is considered a lymphoid tissue that senses ocularly exposed antigens similar to NALT, which senses antigens in the nasal and oral cavities [25].

To clarify the importance of TALT as a regional MALT following ocular exposure to BPA + OVA, we developed an allergic airway inflammation model. In this model, mice were sensitized to OVA with or without BPA via the ocular route, followed by OVA exposure by

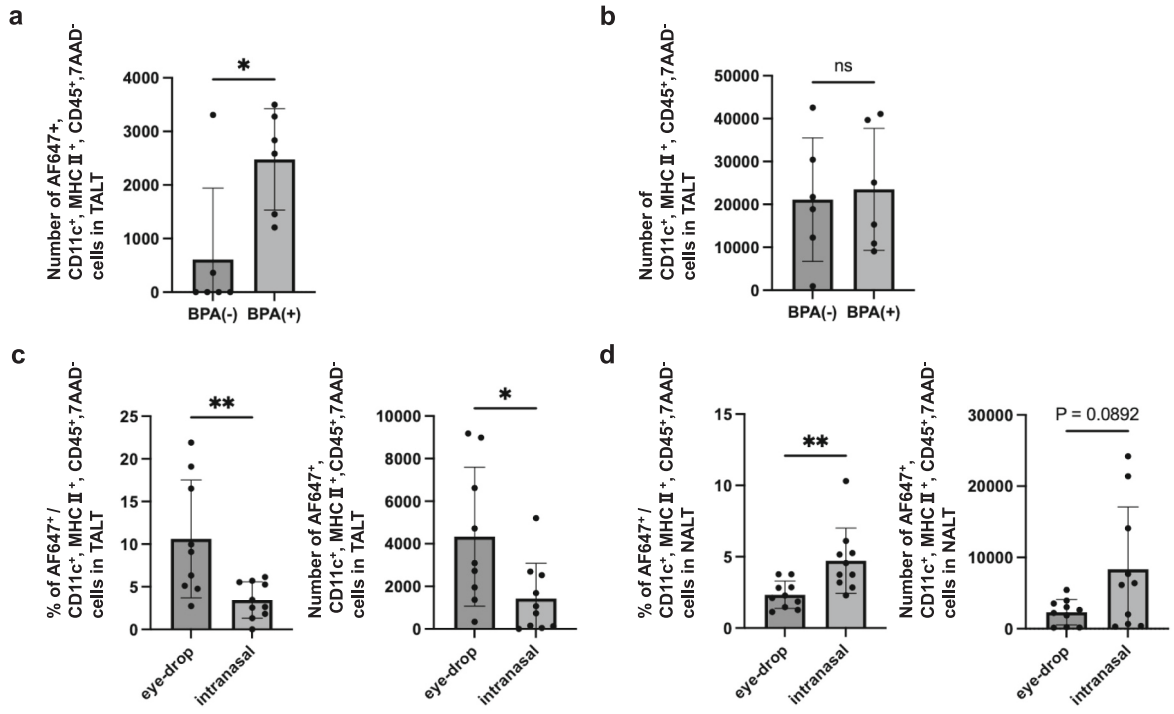

Fig. 1. Effects of ocular exposure to BPA on activation of antigen-presenting cells in TALT.
C57BL6/J wild type mice were administered AF647 OVA (40 μg) with or without BPA (3.0 ng/kg body weight) via eye drops and analyzed 24 h later. The total numbers of AF647 OVA^+ APCs ($CD11c^+$, MHC II^+, $CD45^+$, $7AAD^-$ cells) (a) and overall APCs (b) in TALT after the ocular administration of OVA with BPA were analyzed by flow cytometry ($n = 6$ mice per group). The percentages and numbers of AF647 OVA^+ APCs in TALT (c) or NALT (d) were analyzed by flow cytometry after intranasal or ocular administration of OVA with BPA ($n = 10$ mice per group). Gating strategies for the flow cytometry analyses are shown in Supplementary Fig. 3a and b. Bars represent mean ± standard deviation. $^*P < 0.05$, $^{**}P < 0.01$ (Mann–Whitney *U* test).
BPA = bisphenol A; TALT = tear duct-associated lymphoid tissue; NALT = nasopharynx-associated lymphoid tissue; AF647 = Alexa Fluor 647; OVA = ovalbumin; APC = antigen-presenting cell; ns = not significant.

inhalation (Fig. 2a). Twenty-four hours after the last inhalation, we analyzed TALT and NALT for the formation of GC B cells. As shown in Fig. 2b, the percentage of GC B-cells populations in TALT was significantly increased following ocular sensitization with BPA + OVA, compared with the findings in both the PBS group and the OVA alone group (Fig. 2b). In contrast, ocular sensitization to BPA + OVA did not lead to an increase in GC B cells in NALT (Fig. 2c).

These results clearly demonstrated that ocular exposure to BPA, along with antigen, induces the formation and proliferation of GC B cells in TALT, indicating that TALT is the primary site of entry of ocularly exposed antigens and an inducer of immune responses. This suggests that TALT is anatomically and functionally distinct from NALT.

3.3. Ocular exposure to BPA during the sensitization phase induces allergic airway inflammation

Next, we investigated the immunomodulatory effects of ocular exposure to BPA on acquired immunity, with a particular focus on the allergic lung inflammatory response, using the mouse model shown in Fig. 2a. The mice were sacrificed 24 h after the final OVA inhalation, and the levels of serum OVA-specific antibodies and BALF cells were analyzed. As shown in Fig. 3a and b, the mice sensitized ocularly to OVA and BPA displayed significantly increased levels of serum OVA-specific IgE and IgG_1 compared with those sensitized to PBS or OVA alone.

Twenty-four hours after the final OVA inhalation, we also examined the numbers of total cells and eosinophils in the BALF using a flow cytometer. The results revealed that the numbers of $CD45^+$ cells and eosinophils in the BALF were significantly increased in mice sensitized to both OVA and BPA compared with those sensitized to PBS or OVA alone (Fig. 3c and d).

As shown in Fig. 3e and f, H&E staining indicated that eosinophil and lymphocyte infiltration increased in the lungs from mice sensitized to both OVA and BPA. Additionally, PAS staining revealed that goblet cell hyperplasia were also observed in the airway of these mice.

Next, we investigated the dose-responses of ocular sensitization to BPA for inducing allergic inflammation after OVA inhalation. Mice were administered varying amounts of BPA (low: 0.3 ng/kg body weight/day, medium 3.0 ng/kg body weight/day, high: 30 ng/kg body weight/day) via eye drops. We then examined the level of serum OVA-specific antibodies 24 h after the final OVA inhalation. As shown in Fig. 3g, although an increase in OVA-specific IgE antibody production was observed with BPA eye drops, no significant difference was observed. Meanwhile, as shown in Fig. 3h, a significant increase in OVA-specific IgG_1 was observed at both high and medium doses of BPA. Similarly, we examined the numbers of total cells and eosinophils in the BALF 24 h after the final OVA inhalation. An increased number of eosinophils in the BALF were observed in mice sensitized to both high and medium doses of BPA, and a higher number of total BALF cells was observed in those sensitized to a high dose of BPA.

These results demonstrated that ocular exposure to BPA with OVA during the sensitization phase substantially induced allergic airway inflammation in the mice in a BPA-dose-dependent manner.

3.4. IL-33 and ILC2 are insufficient for the induction of allergic airway inflammation via ocular exposure to BPA

IL-33 stimulates ILC2 and aggravates eosinophilic asthma [38]. IL-33 serves as a growth factor for ILC2s, promoting their differentiation, maturation, and expression of type 2 cytokines [39]. We investigated whether IL-33 and ILC2s are involved in the exacerbation of allergic airway inflammation mediated by the ocular immune system.

First, we examined the role of IL-33 in allergic airway inflammation

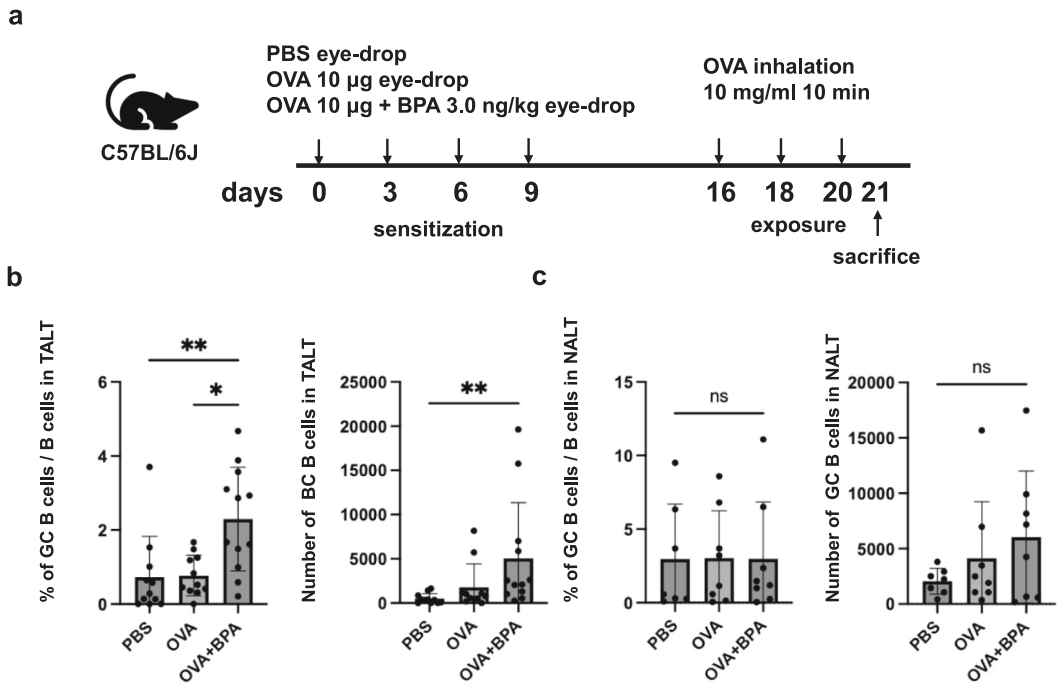

Fig. 2. Effects of ocular exposure to BPA on GC formation in TALT.
In this model, C57BL6/J wild type mice were ocularly sensitized to PBS, OVA (10 μg), or OVA (10 μg) + BPA (3.0 ng/ml) on Days 0, 3, 6, and 9, followed by exposure to aerosolized OVA on Days 16, 18, and 20 (a). The percentage of GC B cells ($CD95^+$ $GL7^+$ cells) among $CD45^+$ $B220^+$ $7AAD^-$ lymphocytes and the numbers of GC B cells in TALT and NALT were analyzed using a flow cytometer 1 day after the last exposure (b, c). Gating strategies for the flow cytometry analyses are shown in Supplementary Fig. 4. Bars represent mean ± standard deviation. $^*P < 0.05$, $^{**}P < 0.01$ (one-way ANOVA by Kruskal–Wallis test, $n = 7$–12 mice per group). Data were combined from at least three experiments.
PBS = phosphate-buffered saline; OVA = ovalbumin; BPA = bisphenol A; GC = germinal center; TALT = tear duct-associated lymphoid tissue; NALT = nasopharynx-associated lymphoid tissue; ANOVA = analysis of variance.

triggered by ocular exposure to OVA and BPA, using IL-33-deficient mice. Both wild type (WT) mice and IL-33-deficient mice were sensitized to OVA and BPA via eye drops, followed by exposure to OVA aerosol.

The OVA-specific antibody responses and the number of eosinophils in the BALF were comparable between WT and IL-33-deficient mice (Fig. 4a–d). We performed similar experiments using ILC2-deficient mice. ILC2-deficient mice were generated by crossing *Rora*$^{flox/flox}$ mice with Vav1-iCre mice. RORα is required for ILC2s development [32], but it is also involved in neuronal maturation, and mice lacking RORα in neuronal cells exhibit ataxia [33]. Since the Vav1-Cre transgene is used for hematopoietic linage-restricted [34], we utilized Vav1-iCre mice to induce hematopoietic cell-specific RORα deficiency. The results showed that, similarly to IL-33-deficient mice, the antibody responses and the number of eosinophils in the BALF were also comparable between controls (*Rora*$^{flox/flox}$ mice) and ILC2-deficient mice (Fig. 4e–h). These results suggest that the type 2 inflammation mediated by the IL-33–ILC2 axis was not a main contributor to the allergic airway inflammation triggered by ocular exposure to OVA and BPA.

3.5. *DAP12 is required for antigen uptake and migration in antigen-presenting cells and is specific to the immune response in TALT*

Fig. 1 demonstrates that BPA augments the activation of APCs, enhancing antigen uptake and/or cell migration to TALT. Subsequently, we investigated the factors that mediate the activation of APC function by BPA and serendipitously discovered that DAP12 plays a role in the activation of dendritic cells (DCs) by BPA.

DAP12 is an ITAM-containing adaptor molecule. It is a transmembrane adaptor known for its role in transducing activation signals for a wide range of receptors in NK cells, granulocytes, monocytes, macrophages, and DCs [40,41].

Fluorescence-labeled OVA with BPA was administered via eye drops to WT or DAP12-deficient mice. Twenty-four hours later, TALT was collected and used for the analysis of APC function by flow cytometry. The number of labeled OVA-positive APCs in TALT was decreased in DAP12-deficient mice compared with that in WT (Fig. 5a). The numbers of APCs in TALT were comparable between WT and DAP12-deficient mice (Fig. 5b).

Next, we investigated whether the impaired function of ocular APCs in DAP12-deficient mice contributes to the activation of acquired immunity, followed by the induction of allergic inflammation. We examined the formation of GC B cells in TALT using flow cytometry. TALT was collected from both WT and DAP12-deficient mice 24 h after the final OVA inhalation. In WT mice, we observed the formation of GC B cells in TALT, whereas in DAP12-deficient mice, this formation was significantly suppressed (Fig. 5c). Additionally, we measured the levels of OVA-specific antibodies. The results showed that OVA-specific IgE and IgG_1 production was significantly decreased in DAP12-deficient mice compared with that in WT mice (Fig. 5d and e). We also investigated whether DAP12-deficient mice exhibited impaired immune responses to antigen sensitization that was not mediated via TALT. WT or DAP12-deficient mice were sensitized by the intraperitoneal injection of OVA alone or OVA mixed with the Th2 adjuvant aluminum salts (Alum), followed by intranasal OVA challenge. Twenty-four hours after the final OVA inhalation, serum was collected and the levels of serum OVA-specific IgE and IgG_1 were analyzed (Fig. S6a). We found no significant difference in antigen-specific IgE and IgG_1 production between WT and DAP12-deficient mice (Fig. S6b and c). These results indicate that a reduced immune response in DAP12-deficient mice is not a universal phenomenon across all immune responses.

We also examined the number of eosinophils in the BALF 24 h after the final OVA inhalation. In DAP12-deficient mice, the number of eosinophils in the BALF was significantly lower than in WT mice, as was

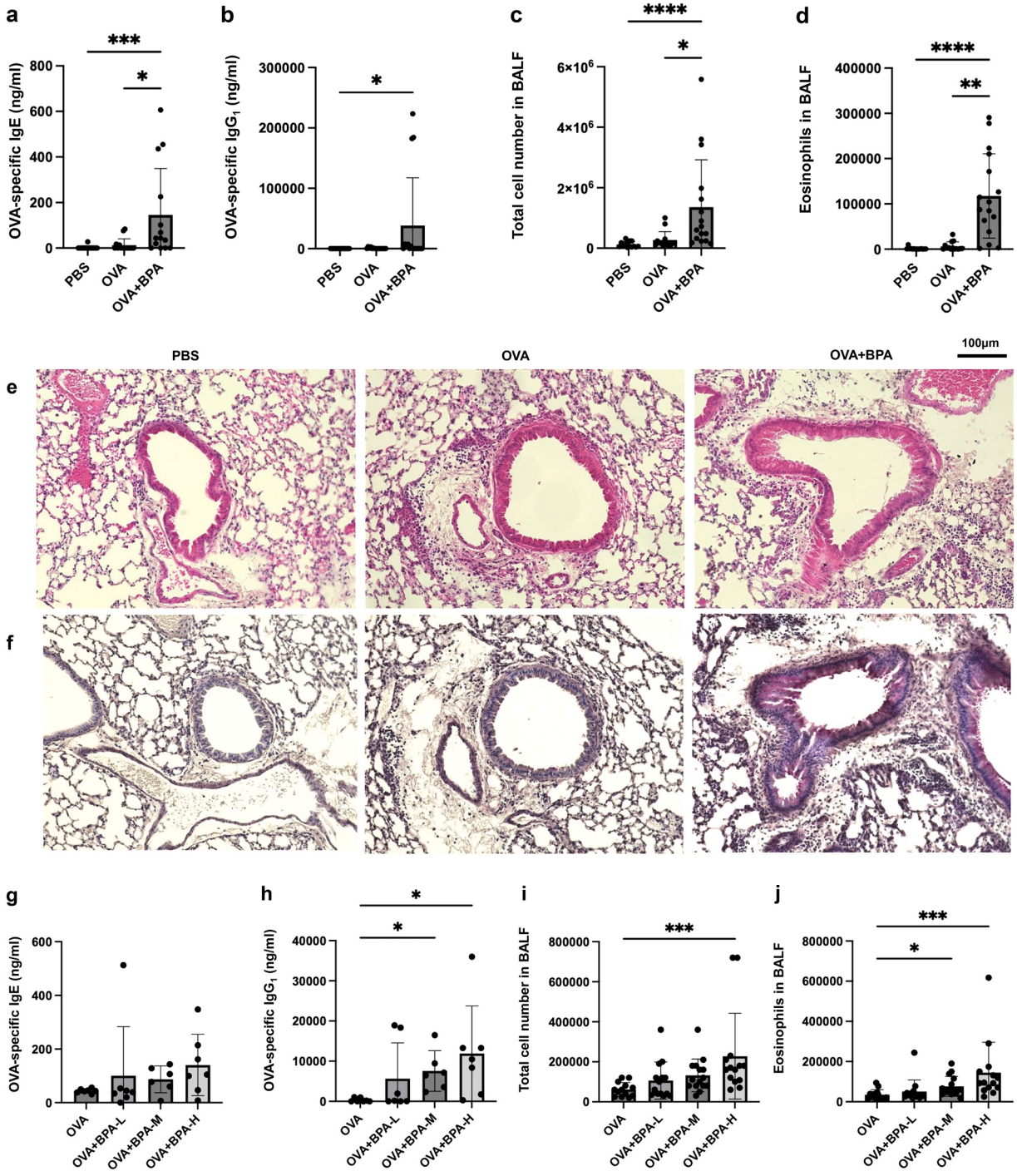

Fig. 3. The effects of ocular exposure to BPA during the sensitization phase on the induction of allergic airway inflammation.
C57BL6/J wild type mice were ocularly sensitized to PBS, OVA (10 μg), or OVA (10 μg) + BPA (3.0 ng/ml) on Days 0, 3, 6, and 9, followed by exposure to aerosolized OVA on Days 16, 18, and 20. Serum and BALF were collected 24 h after the last exposure. The serum levels of OVA-specific IgE (a) and OVA-specific IgG_1 (b) were measured by ELISA. Data were combined from four experiments (n = 14–15 mice per group). Flow cytometric analysis of total immune cells and eosinophils in the BALF (c, d). Gating strategies for the flow cytometry analyses are shown in Supplementary Fig. 5. Data were combined from four experiments (n = 14–16 mice per group). Bars represent mean ± standard deviation. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (one-way ANOVA by Kruskal–Wallis test). Histological changes in the lungs were investigated 24 h after the last exposure. Representative photomicrographs of lung sections of H&E staining (e) and PAS staining (f). C57BL6/J wild type mice were ocularly sensitized to OVA (10 μg) or OVA (10 μg) + BPA (0.3, 3.0, or 30 ng/kg body weight) on Days 0, 3, 6, and 9, followed by exposure to aerosolized OVA on Days 16, 18, and 20. Serum and BALF were collected 24 h after the last exposure. The serum levels of OVA-specific IgE (g) and OVA-specific IgG_1 (h) were measured by ELISA. Data were combined from two experiments (n = 6–7 mice per group). Flow cytometric analysis of total immune cells and eosinophils in the BALF (i, j). Data were combined from four experiments (n = 13–15 mice per group). Bars represent mean ± standard deviation. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (one-way ANOVA by Kruskal–Wallis test).
PBS = phosphate-buffered saline; OVA = ovalbumin; BPA = bisphenol A; ELISA = enzyme-linked immunosorbent assay; H&E = hematoxylin and eosin; PAS = periodic acid–Schiff; BALF = bronchoalveolar lavage fluid; BPA-L = low-dose bisphenol A; BPA-M = medium-dose bisphenol A; BPA-H = high-dose bisphenol A; ANOVA = analysis of variance.

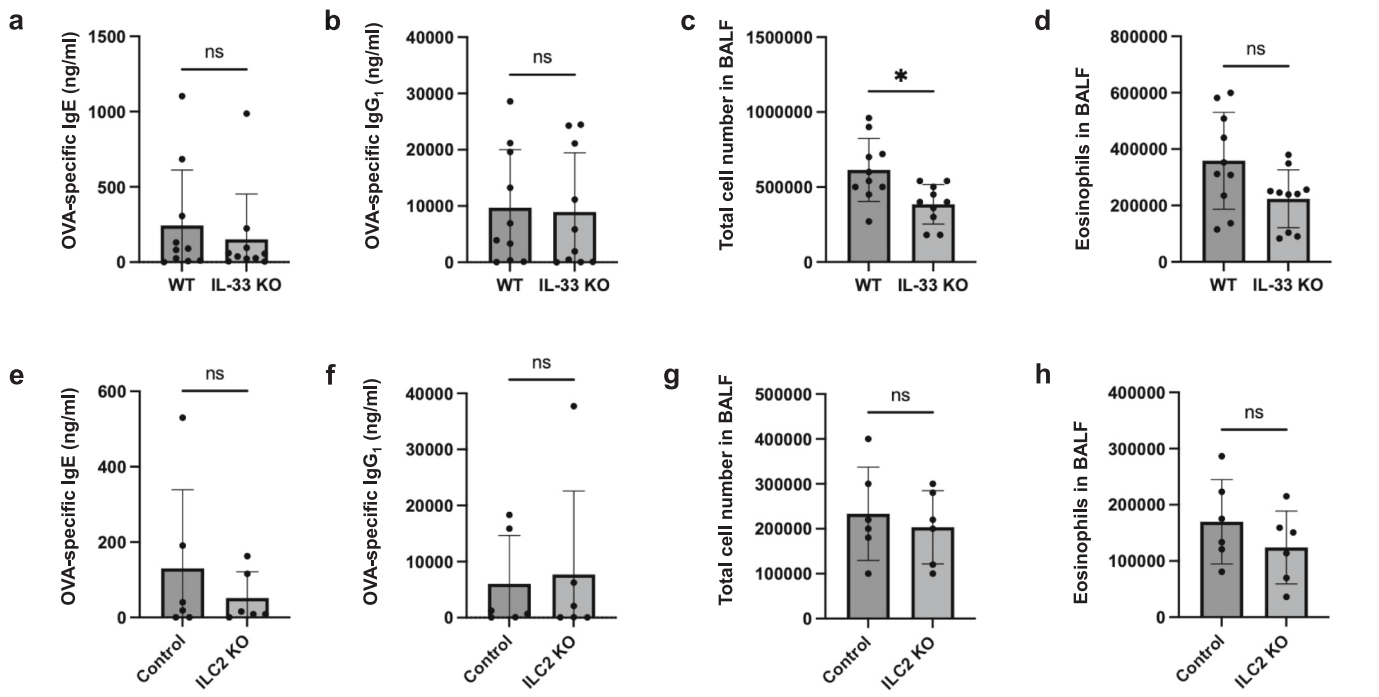

Fig. 4. Involvement of IL-33 and ILC2 in the induction of allergic airway inflammation induced via ocular exposure to BPA.
IL-33-deficient mice (C57BL6/J background) and C57BL6/J wild type mice were ocularly sensitized to OVA (10 μg) + BPA (3.0 ng/ml) on Days 0, 3, 6, and 9, followed by exposure to aerosolized OVA on Days 16, 18, and 20 (n = 10 mice per group). Serum and BALF were collected 24 h after the last exposure. The serum levels of OVA-specific IgE (a) and OVA-specific IgG_1 (b) were measured by ELISA. Flow cytometric analysis of total immune cells and eosinophils in the BALF (c, d). Bars represent mean ± standard deviation. *$P < 0.05$ (Mann–Whitney U test). Data were combined from two experiments. ILC2-deficient mice (C57BL6/J background) and controls ($Rora^{flox/flox}$ mice) were ocularly sensitized to OVA (10 μg) + BPA (3.0 ng/ml) on Days 0, 3, 6, and 9, followed by exposure to aerosolized OVA on Days 16, 18, and 20 (n = 6 mice per group). Serum and BALF were collected 24 h after the last exposure. The serum levels of OVA-specific IgE (e) and OVA-specific IgG_1 (f) were measured by ELISA. Flow cytometric analysis of total immune cells and eosinophils in the BALF (g, h). Bars represent mean ± standard deviation. *P < 0.05 (Mann–Whitney U test). Data were combined from two experiments.
IL-33 = interleukin-33; ILC2 = group 2 innate lymphoid cell; KO = knockout; WT = wild type; OVA = ovalbumin; BPA = bisphenol A; BALF = bronchoalveolar lavage fluid; ELISA = enzyme-linked immunosorbent assay; ns = not significant.

the total number of BALF cells (Fig. 5f and g).

These results suggest that the impaired function of APCs of TALT in DAP12-deficient mice affects acquired immunity, including the formation of GC B cells, antigen-specific antibody responses, and pulmonary eosinophilia following OVA inhalation. Taken together, these findings suggest that DAP12 is a unique adaptor molecule required for the type 2 inflammatory allergic response triggered by sensitization (exposure) of the ocular immune system via TALT to BPA.

4. Discussion

In recent years, various experimental studies have addressed the issue of whether bisphenols influence the development and outcome of allergic airway diseases, but a comprehensive understanding of this has yet to be obtained. One study showed that oral BPA exposure in combination with OVA challenge increased the severity of lung eosinophilia in adult mice by promoting a Th2-biased immune response [14]. Another study indicated that low-dose BPA airway exposure may enhance Th2 responses and exacerbate allergic airway inflammation by disrupting the immune system [10]. Furthermore, many epidemiological studies on humans have reported the relationship between BPA exposure and asthma in humans [11,12,42].

BPA is widely detected in both the atmosphere and indoor air [4], and therefore we hypothesized that exposure to it can occur not only through oral and respiratory routes but also ocularly. To the best of our knowledge, this is the first study to demonstrate that ocular exposure to BPA with allergen during sensitization promotes allergic airway inflammation through activation of the ocular immune system.

The results of this study showed that ocular sensitization of mice to BPA and OVA, followed by OVA inhalation, led to increased levels of OVA-specific antibodies, along with marked infiltration of eosinophils into the lungs. These results suggest that BPA may act as an immunogenic adjuvant to induce type 2 immunity via ocular exposure to the antigen during the sensitization phase. Additionally, an adjuvant effect of BPA was observed even at low concentrations. Ministry of the Environment of Japan reports a total amount of BPA exposure through various routes (Fig. S1). However, there are currently no reports on BPA exposure specific to the ocular surface, so we cannot accurately estimate the ocular exposure, which is a limitation of this study. In the preliminary study, we observed dose-dependent allergic responses in this model, with a BPA concentration of 1.0×10^4 pg/m^3 (3.0 ng/kg body weight/day), inducing a marked eosinophilic infiltration and IgE production, so we decided to use this dose for the experiments. Although the dose of BPA used in this study is much higher than the estimated peak exposure dose from the general atmosphere in Japan (1000 pg/m^3: 0.3 ng/kg body weight/day), Vasiljevic et al. reported that the BPA concentrations in the indoor air in North Carolina, US and outdoor ambient air in Xi'an, China were 1.0×10^5 pg/ m^3 and 1.1×10^4 pg/ m^3 respectively [4], therefore we think that the dose we have used in this study is somewhat acceptable. Furthermore, this study revealed that eye drop sensitization with antigens and environmental chemicals promoted the formation of GC B cells in TALT. It is speculated that this activation of TALT is the starting point for the onset of allergic airway inflammation.

Various studies have focused on the effects of bisphenols on DCs. One study showed that DCs exposed to BPA generate a Th2-dominant immune response in allergic reactions [43]. Another study showed that BPA increases MHC II and CD86 expression on bone marrow-derived

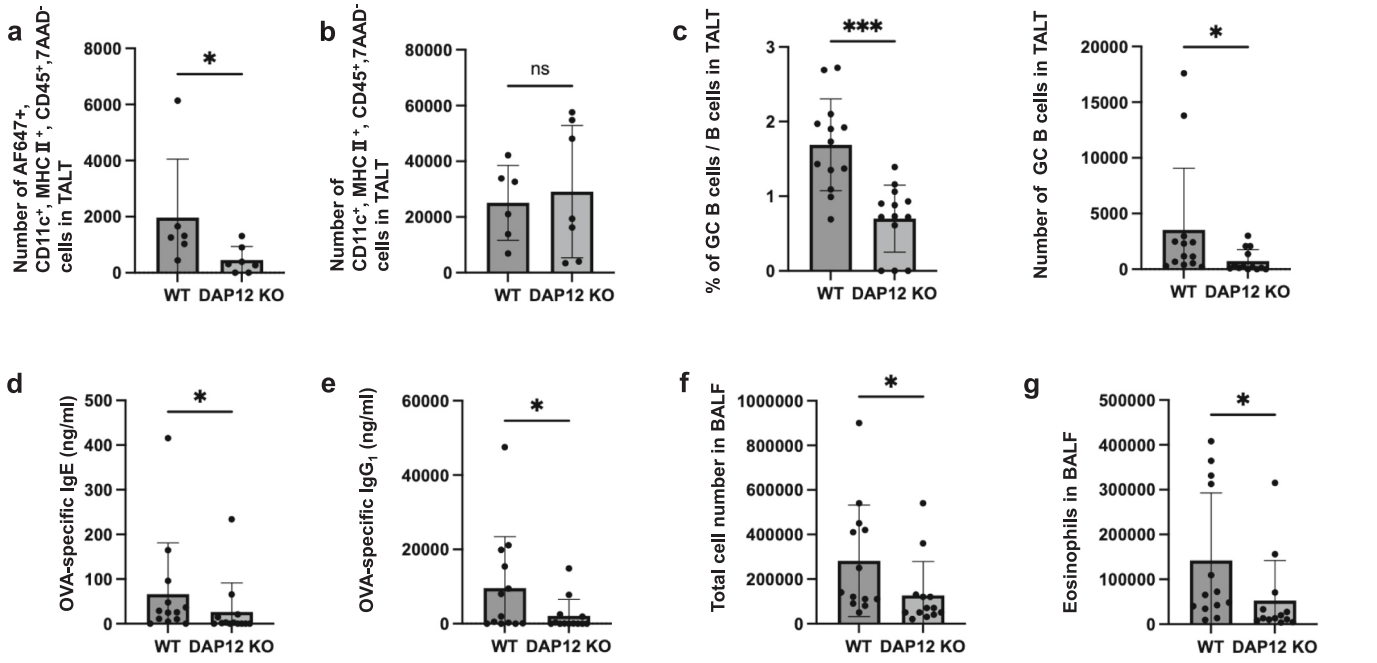

Fig. 5. Involvement of DAP12 in antigen-presenting cells in TALT and its role in activating the ocular immune system.
Ocular sensitization induced DAP12-dependent antigen uptake by mucosa-associated APCs, subsequent type 2 inflammation, and IgE production. (a, b) DAP12-deficient mice (C57BL6/J background) and C57BL6/J wild type mice were administered AF647 OVA (40 μg) with BPA (3.0 ng/ml) via the eye drops and TALT was analyzed 24 h later. The total numbers of AF647 OVA$^+$ APCs (CD11c$^+$, MHC II$^+$, CD45$^+$, 7AAD$^-$ cells) (a) and overall APCs (b) in TALT after the ocular administration of OVA with BPA were analyzed by flow cytometry (n = 6–7 mice per group). Data were combined from two experiments. Bars represent mean ± standard deviation. *P < 0.05 (Mann–Whitney U test). (c–g) DAP12-deficient mice (C57BL6/J background) and C57BL6/J wild type mice were ocularly sensitized to OVA (10 μg) + BPA (3.0 ng/ml) on Days 0, 3, 6, and 9, followed by exposure to aerosolized OVA on Days 16, 18, and 20 (n = 13 mice per group). Serum, BALF, and TALT were collected 24 h after the last exposure. The percentage of GC B cells (CD95$^+$ GL7$^+$ cells) among CD45$^+$ B220$^+$ 7AAD$^-$ lymphocytes and the number of GC B cells in TALT were analyzed using a flow cytometer (c). The serum levels of OVA-specific IgE (d) and OVA-specific IgG_1 (e) were measured by ELISA. Flow cytometric analysis of total immune cells and eosinophils in the BALF (f, g). Data were combined from three experiments. Bars represent mean ± standard deviation. *P < 0.05, ***P < 0.001 (Mann–Whitney U test).
DAP12 = DNAX-activating protein of 12 kDa; WT = wild type; APC = antigen-presenting cell; OVA = ovalbumin; BPA = bisphenol A; TALT = tear duct-associated lymphoid tissue; AF647 = Alexa Fluor 647; GC = germinal center; ELISA = enzyme-linked immunosorbent assay; BALF = bronchoalveolar lavage fluid.

dendritic cells (BMDCs) [37]. Our results showed an increased number of DCs as MHC II-high and CD11c-positive cells that took up antigens and migrated to individual mucosa-associated tissues (TALT and NALT) after the ocular or intranasal administration of OVA with BPA, respectively. We thus conclude that TALT can function as a primary site for recognizing environmental antigens and immunogens via ocular exposure. We further examined the effect of BPA on APC function, specifically using mouse BMDCs in vitro. However, no significant responses were identified, which differs completely from the findings in in vivo experiments. This suggests that BPA affects immune responses through a mechanism that cannot be fully replicated in vitro.

IL-33 activates ILC2s and aggravates airway inflammation through adaptive Th2 immune responses [44]. In addition, IL-33 is released by dying cells as damage-associated molecular patterns (DAMPs). Given that BPA is a toxic substance and DAMPs serve as endogenous adjuvants [45], we investigated the impact of IL-33 and ILC2s on allergic airway inflammation mediated by the ocular immune system. Unexpectedly, IL-33 and ILC2s were not responsible for allergic inflammation caused by ocular exposure to BPA. On the other hand, we observed that BPA somewhat influenced the activation of DCs, as mentioned earlier. Similar to previous findings, our results suggest that BPA may directly or indirectly influence DC function to skew the Th2 response.

In this study, we serendipitously discovered that DAP12 plays a role in the activation of DCs by BPA. DAP12 is a signaling adaptor containing an immunoreceptor ITAM found on innate immune cells [41], and it is reported to be expressed on APCs such as macrophages [46] and DCs [40]. However, DAP12 has been reported to activate or inhibit immune responses, and its immunoregulatory function may be determined by the ligand, the receptor, and the cell type, although no consensus has yet been reached on this [47]. Using DAP12-deficient mice, we observed impaired function of DCs, leading to decreased allergic inflammation such as GC formation in TALT, antigen-specific IgE and IgG_1 responses, and the infiltration of eosinophils into the lung. These results suggest that DAP12 is involved in the development of allergic airway inflammation mediated by the ocular immune system, mainly by regulating the ability of APCs in TALT to take up antigens. Furthermore, DAP12-deficient mice did not show a decrease in antigen-specific IgE and IgG_1 antibodies via intraperitoneal antigen sensitization, suggesting that reduced immune responses in DAP12-deficient mice are not a universal phenomenon. Additionally, recent studies have shown that triggering receptor expressed on myeloid cells 2 (TREM-2) requires association with DAP12 in microglia within the central nervous system, and this receptor complex is involved in the activation and phagocytosis of microglia [48]. The specific receptors, including TREM-2, involved in activation of the ocular immune system are currently under investigation.

In conclusion, we revealed that BPA affects the ocular immune system by enhancing antigen uptake by APCs and GC B-cell formation, which subsequently induces the allergic response in the respiratory tract after antigen inhalation. Additionally, we observed that DAP12 is required for antigen uptake and presentation by APCs in TALT, suggesting that DAP12 is the molecule responsible for recognizing foreign antigens and initiating allergic immune responses. Based on these results, we propose that DAP12 is a key molecule for controlling the ocular immune system and subsequently developing allergic inflammation.

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CRediT authorship contribution statement

Tatsuo Ueda: Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Takumi Adachi:** Methodology, Formal analysis, Investigation, Data curation, Writing – review & editing, Visualization. **Tomoya Hayashi:** Methodology, Investigation, Writing – review & editing. **Koubun Yasuda:** Methodology, Formal analysis, Investigation, Writing – review & editing. **Kazufumi Matsushita:** Methodology, Formal analysis, Investigation, Writing – review & editing. **Eiko Koike:** Methodology, Writing – review & editing. **Rie Yanagisawa:** Methodology, Writing – review & editing. **Takahiro Nagatake:** Methodology, Writing – review & editing. **Jun Kunisawa:** Writing – review & editing, Funding acquisition. **Ken J. Ishii:** Writing – review & editing, Funding acquisition. **Kenzo Tsuzuki:** Writing – review & editing, Supervision. **Etsushi Kuroda:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors have no competing interests to declare.

Data availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2024.110370.

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Supplementary information

Supplemental method

Intraperitoneal sensitization and intranasal challenge of mice

C57BL6/J wild-type and DAP12-deficient mice were intraperitoneally sensitized to OVA (10 μg) or OVA (10 μg) + Alum (100 μg) on Day 0, followed by intranasal exposure to OVA (10 μg) on Days 16, 18, and 20. The serum was collected the day after the last exposure. Mice were sacrificed 24 h after the final antigen challenge and blood was collected from each mouse. OVA-specific IgE and IgG_1 levels in the serum were measured using ELISA.

Ministry of the Environment of Japan (2004)

Profiles of the Initial Environmental Risk Assessment of Chemicals, vol.3 [15] Bisphenol A. (in Japanese), https://www.env.go.jp/chemi/report/h16-01/pdf/chap01/02_2_15.pdf

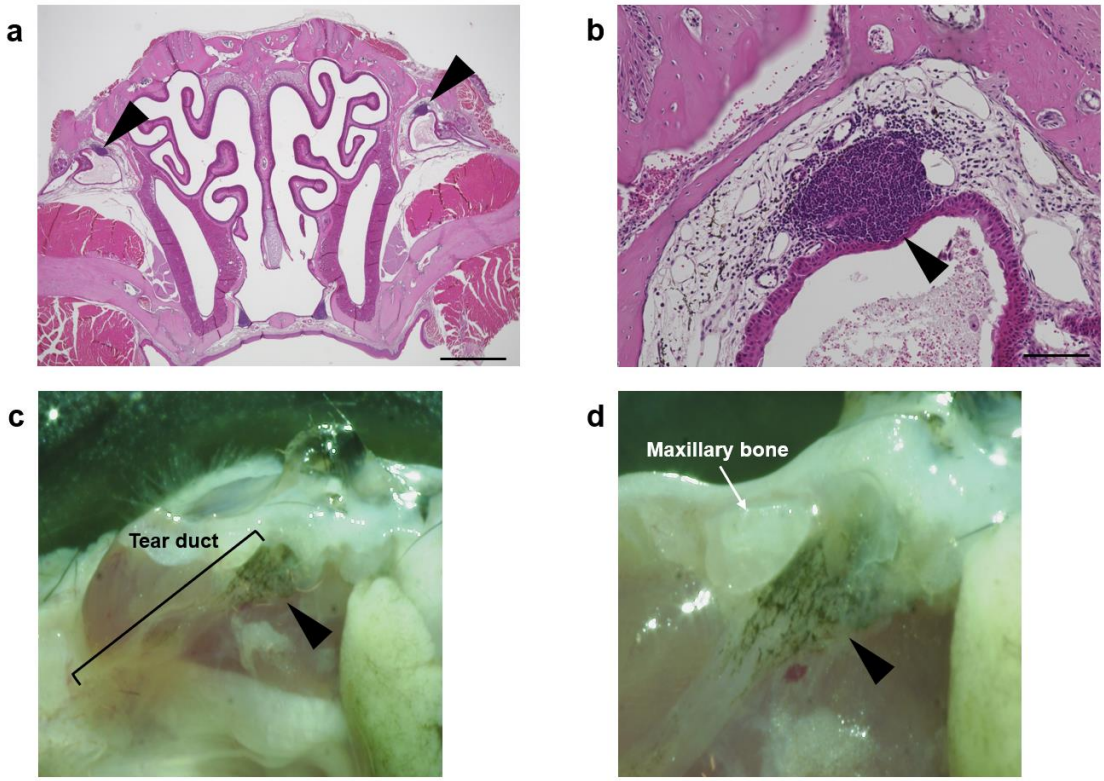
	Concentration (average)	**Daily exposure dose (average)**	**Concentration (maximum)**	**Daily exposure dose (maximum)**
General ambient air	**0.0005 μg/m^3**	**0.00015 μg/kg/day**	**0.001 μg/m^3**	**0.0003 μg/kg/day**

Body weight: 50 kg, respiratory rate: 15 m^3/person/day

Supplementary Figure 1. Maximum daily exposure to BPA

Ministry of the Environment of Japan reports that maximum daily exposure to BPA is calculated based on the maximum concentration of BPA in general ambient air (0.001 mg/m^3), along with human body weight and respiratory rate (estimated at 50 kg, 15m^3/person/day).

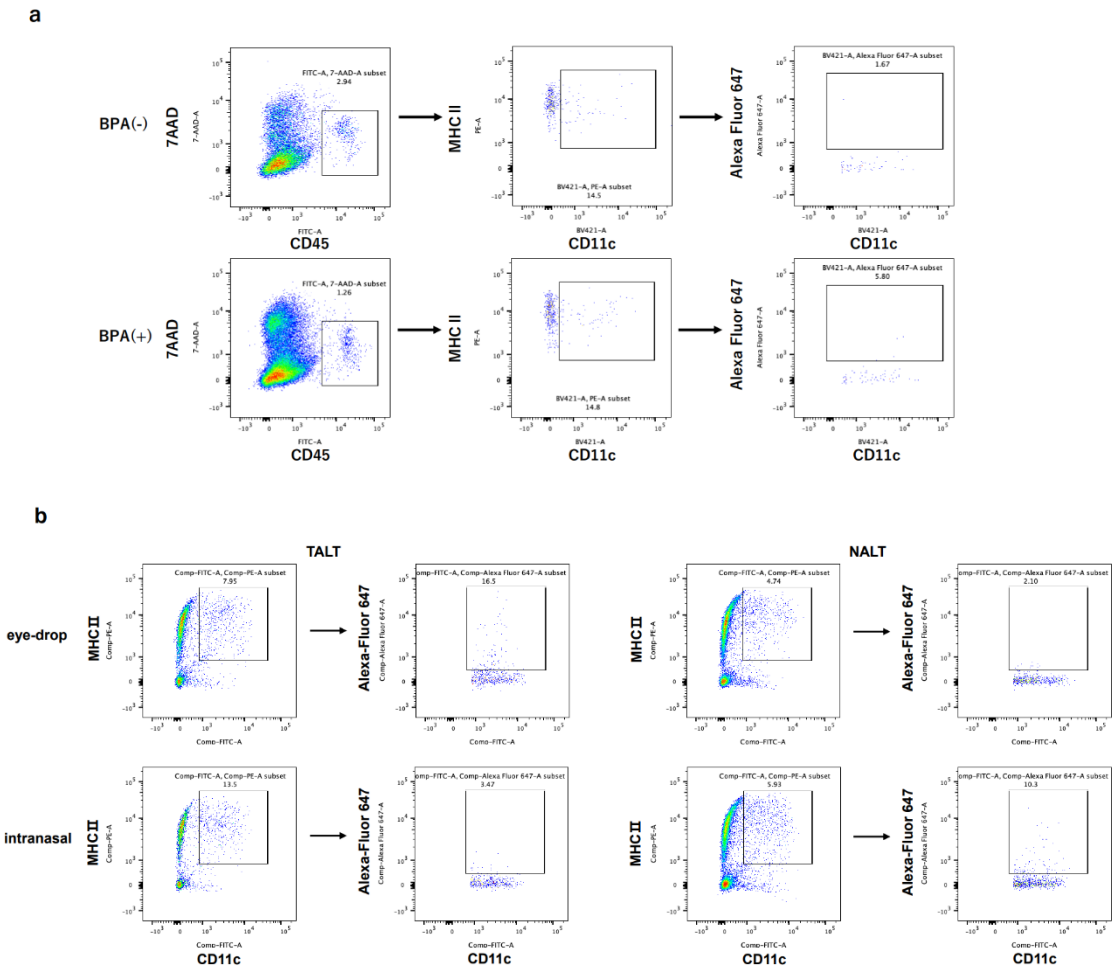
BPA = bisphenol A.



Supplementary Figure 2. Identification of TALT from mouse head

H&E-stained sections of mice head (a, b). H&E staining identified TALT in the tear duct (a). Panel (b) shows highly magnified image of the tear duct. Microscopic images of the tear duct (c, d). The tear duct was exposed from the mouse head using forceps (c). Panel (d) shows highly magnified image of the tear duct. TALT was carefully dissected with the tear duct from the maxillary bone (d). An arrowhead indicates TALT. Bars: 1000 μm (a), 100 μm (b).

TALT = tear duct-associated lymphoid tissue; H&E = hematoxylin and eosin.

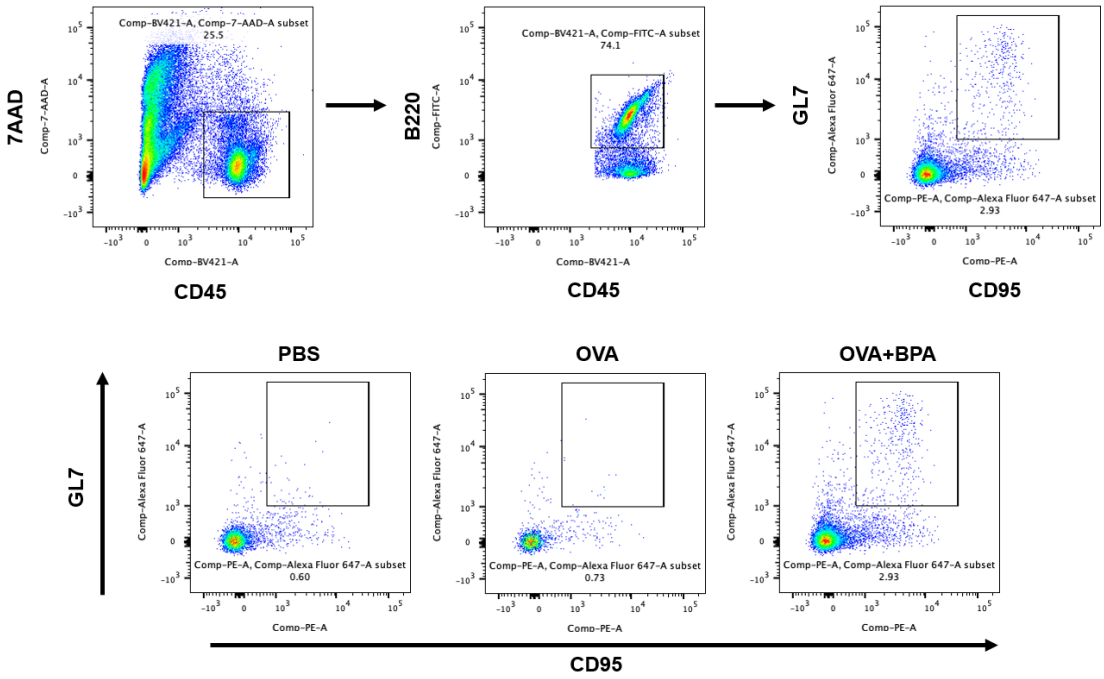


Supplementary Figure 3. FACS gating strategies for antigen-presenting cells in TALT and NALT

FACS gating strategies for AF647^{+} cells / CD11c^{+}, MHCII^{+}, CD45^{+}, 7AAD^{-} cells in TALT (a).

FACS gating strategies for AF647^{+}, CD11c^{+}, MHCII^{+}, CD45^{+}, 7AAD^{-} cells in TALT and NALT (b).

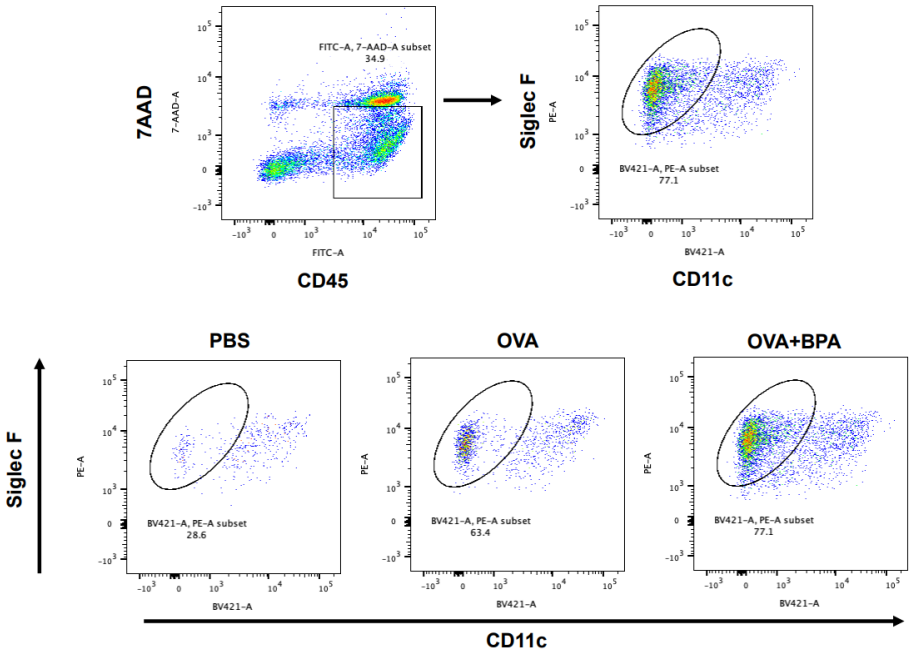
FACS = fluorescence-activated cell sorting; BPA = bisphenol A; TALT = tear duct-associated lymphoid tissue; NALT = nasopharynx-associated lymphoid tissue; AF647 = Alexa Fluor 647.



Supplementary Figure 4. FACS gating strategies for GC B cells in TALT

FACS gating strategies for GC B cells in TALT. GC B cells were identified as $GL7^+$, $CD95^+$, $B220^+$, $CD45^+$, $7AAD^-$ cells.

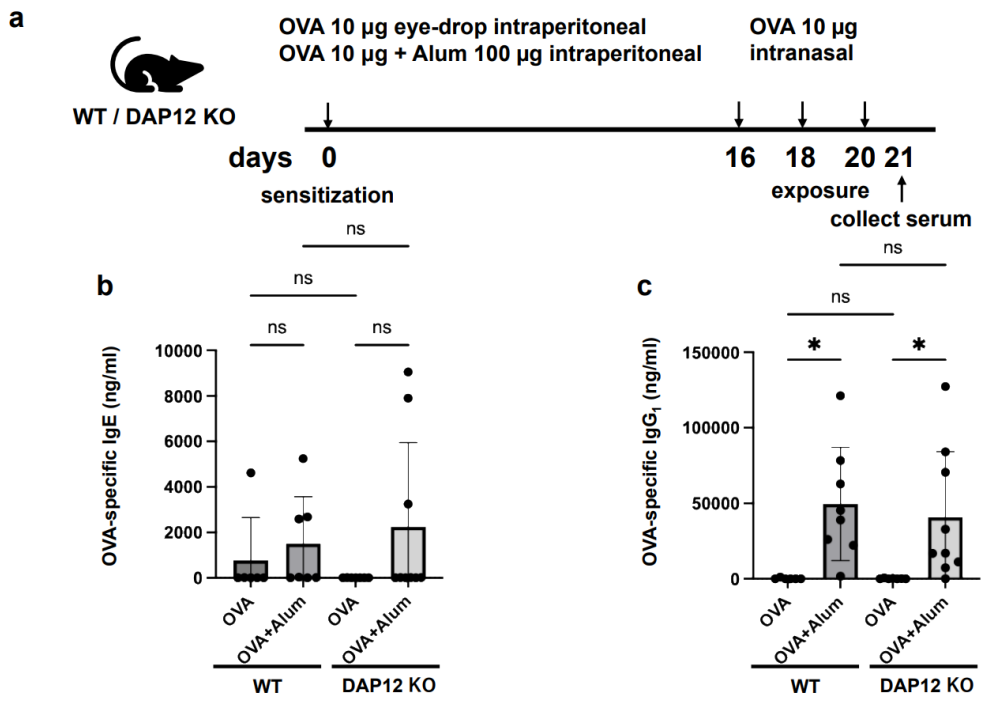
FACS = fluorescence-activated cell sorting; PBS = phosphate-buffered saline; OVA = ovalbumin; BPA = bisphenol A; GC = germinal center; TALT = tear duct-associated lymphoid tissue.



Supplementary Figure 5. FACS gating strategies for eosinophils in bronchoalveolar lavage fluid

FACS gating strategies for eosinophils in bronchoalveolar lavage fluid (BALF). Eosinophils were identified as Siglec F^+, $CD11c^+$, $CD45^+$, $7AAD^-$ cells.

FACS = fluorescence-activated cell sorting; PBS = phosphate-buffered saline; OVA = ovalbumin; BPA = bisphenol A; BALF = bronchoalveolar lavage fluid.



Supplementary Figure 6. Effects of intraperitoneal administration of antigen on the serum levels of antigen-specific antibody in DAP12-deficient mice

Schematic diagram of the mouse model of exposure (a).

In this model, C57BL6/J wild type and DAP12-deficient mice were intraperitoneally sensitized to OVA (10 μg) or OVA (10 μg) + Alum (100 μg) on Day 0, followed by intranasal exposure to OVA on Days 16, 18, and 20 (a). Serum was collected 24 h after the last exposure. The serum levels of OVA-specific IgE (b) and OVA-specific IgG_1 (c) were measured by ELISA (n = 7–9 mice per group). Data were combined from two experiments. Bars represent the mean ± standard deviation. *P < 0.05 (one-way ANOVA by Kruskal–Wallis test).

WT = wild type; DAP12 = DNAX-activating protein of 12 kDa; KO = knockout; OVA = ovalbumin; Alum = aluminum salts; ELISA = enzyme-linked immunosorbent assay; ns = not significant; ANOVA = analysis of variance.