

Chlorinated Flame-Retardant Dechlorane 602 Potentiates Type 2 Innate Lymphoid Cells and Exacerbates Airway Inflammation

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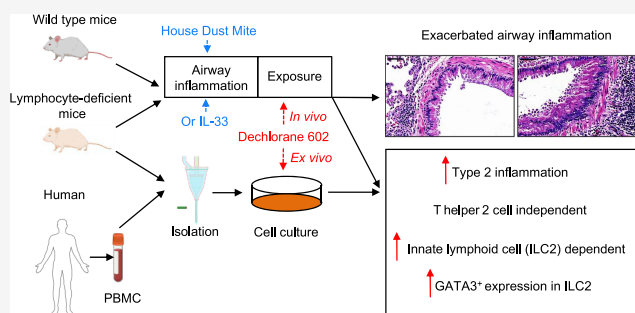


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Supporting Information

ABSTRACT: Chlorinated flame-retardant dechloranes are emerging substitutes for restricted flame retardants. Recent studies have demonstrated that they are accumulated in wildlife and detectable in humans; however, their effects on human health are poorly understood. Here, for the first time, we revealed that widely used chlorinated flame-retardant dechlorane 602 (Dec 602) exacerbated airway inflammation in two mouse models induced by house dust mite (HDM) or IL-33, respectively. Deteriorated airway inflammation by Dec 602 was associated with a higher production of type 2 cytokines including IL-4, IL-5, and IL-13, and IgE, accompanied by enhanced mRNA expression of proinflammatory cytokines such as TNF- α and IL-6. Mechanistically, we found that Dec 602 directly potentiated mouse and human group 2 innate lymphoid cells and, as such, promoted airway inflammation even in the absence of conventional T cells in *Rag*^{-/-} mice. These findings provide novel immunological insights necessary for further studies of the health impact of emerging flame-retardant dechloranes including Dec 602.



1. INTRODUCTION

Increased prevalence of asthma and other allergic disorders was shown to be associated with urbanization and industrialization, supported by evidence suggesting that air pollution can interact with airborne allergens and exacerbate atopic sensitization and allergic symptoms.¹ However, much less is known of the impacts of chemical pollution through other exposure pathways on human health, including their effects on the immune cells.²

Flame retardants are chemicals that are added to manufactured materials (e.g., electronics, textiles, and plastics) to inhibit combustion or delay the spread of fire. Due to the nature of being released to the environment such as through industrial manufacturing and recycling facilities, many of these substances are listed as persistent organic pollutants (POP),³ which can increase the risk of adverse health effects on wildlife and human.^{4–6} For instance, exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) enhances spontaneous IgE production from B cells, which may deteriorate allergic responses in patients.⁷ The logistic regression analysis showed that halogenated POPs *p,p'*-DDE was positively correlated with childhood asthma.⁸ Mice exposed to bisphenol A (BPA) also exhibited aggravated allergic airway inflammation.⁹ Classical flame retardants are banned or subjected to restrictions, resulting in the use of alternatives. The existing concentration of one of such alternatives in the dust, organophosphate flame retardants (PFRs), was shown to be

associated with allergic diseases such as allergic rhinitis, asthma, and atopic dermatitis in a population study in Japan.¹⁰ A follow-up study further demonstrated that the increased level of urine PFRs and their metabolites were associated with children's allergic symptoms.¹¹ Hexabromocyclododecane (HBCD), a brominated flame retardant (BFR), enhanced the production of inflammatory cytokine IL-4 or IL-17A in the house dust mite (HDM)-induced mouse asthma model.¹² Two other BFRs, DE71 and DE79, were reported to induce significant IgE production and exacerbated clinical scores of mice challenged with mite allergen.¹³ Another BFR, tetrabromobisphenol (TBBPA), induced cell death signaling in airway epithelial cells.¹⁴ These pieces of evidence collectively demonstrate that emerging POPs, including flame retardants, may profoundly affect airway inflammation. Therefore, investigation of the pathogenic effect of POPs and their impacts within an immune response is necessary, not only for better understandings of their health impacts but also for the development of assessing biomarkers.

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Airway inflammation is mainly driven by a type 2 immune response, which involves the production of cytokines such as IL-4, IL-5, IL-13, and IL-33 from immune cells including eosinophils, mast cells, macrophages, neutrophils, T helper 2 (T_H2) cells, and group 2 innate lymphoid cells (ILC2s).¹⁵ T_H2 cells are differentiated from naïve $CD4^+$ T cells upon antigen recognition and are pivotal for the adaptive immune response against extracellular pathogens or to induce an allergic response.¹⁶ Similar to T_H2 cells, ILC2s promote a type 2 immune response and can induce airway inflammation independent of T cells.^{17,18} Numerous studies have suggested that environmental factors could impact the properties and functions of immune cells, wherein particular bacterial metabolites, aryl hydrocarbon receptor (AhR) ligands, short-chain fatty acids, stearyl sulfate, and retinoic acid were known to regulate ILC2s.^{19–24}

Halogenated norbornenes, including dechlorane plus (DP) and dechlorane (Dec) 602, 603, and 604, represent a class of emerging flame retardants. Among them, Dec 602 ($C_{14}H_4Cl_{12}O$) is particularly concerning because it has a greater bioaccumulative potential than DP and other chlorinated flame retardants.²⁵ Dec 602 has been detected not only in the air, sediment, soil, and water in the North American Great Lakes area, Europe, and China²⁵ but also in milk, eggs, salmon, and other foods.²⁶ Importantly, Dec 602 was reported in over 60% of human milk and maternal serum in a Canadian population-based study,²⁷ raising concerns for human health risk.

Previously, we examined mice exposed to Dec 602 by gavage and found negligible changes in total body weights and weights of multiple organs including brain, liver, thymus, spleen, and kidney.²⁸ In addition, similar to other hydrophobic chemicals such as TCDD, known to have significant *in vivo* effects,²⁹ we found that the uptake and accumulation of Dec 602 resulted in metabolic perturbations in the biosynthesis and metabolism of many essential chemicals including phenylalanine, tyrosine, tryptophan, pyrimidine, and purine in mice.³⁰ Dec 602 modestly enhanced the expression of genes associated with type 2 immune responses, such as IL-4, IL-13, and Gata3, and was associated with increased protein levels of IL-4 and IL-10 in the serum or spleen.²⁸ However, it remains unknown whether the immunomodulatory effect by Dec 602 exposure can potentially lead to a pathogenic consequence. This inspired us to evaluate Dec 602 exposure, for the first time, in airway inflammation mouse models induced by house dust mite (HDM) or IL-33, respectively, which are considered to be mainly driven by type 2 immune responses. In the present study, we revealed that Dec 602 exposure exacerbated airway inflammation in these two mouse models. We further found a new mechanism whereby Dec 602 directly potentiated ILC2s to enhance type 2 immune responses, which at least partially explained the deterioration of type 2 immune response-driven airway inflammation in mice exposed to Dec 602. The evidence urges the need for comprehensively assessing the risk of flame-retardant Dec 602 and suggests the function of ILC2 and airway inflammatory indicators as potential biomarkers for assessing Dec 602 in human health.

2. MATERIALS AND METHODS

2.1. Mice. All experimental C57BL/6 female mice were purchased from Vital River Laboratories (VRL). The RAG1-KO mice were purchased from Shanghai Model Animal Research Center or Australian Phenomics Facility. All

experimental mice were 6–8 weeks old and maintained at the Shandong Analysis and Test Center or The Australian National University (ANU), at a controlled temperature of 23 ± 2 °C, with humidity of $50 \pm 10\%$. All animals had *ad libitum* access to pellet feed and water and were randomly distributed into groups with a maximum of five adult mice per cage. All animal work was in accordance with protocols approved by the animal ethics committee of Shandong Analysis and Test Center or The Australian National University.

2.2. House Dust Mite-Induced Airway Inflammation Mouse Model.

For the house dust mite (HDM)-induced airway inflammation model, 6–8 weeks old female mice were sensitized with 50 μ g of PBS or HDM (Greer Labs) extract in 50 μ L of sterile saline through intranasal instillation on days 0, 1, 2, and challenged with 5 μ g of PBS or HDM extract in 50 μ L of sterile saline by intranasal instillation on days 19–22.³¹ Dechlorane 602 (from Toronto Research Chemicals Inc., 10 μ g/kg body weight) dissolved in DMSO was diluted in 100 μ L of PBS (DMSO < 0.1%) and administered in mice through gavage, as described before²⁸ on days 8, 12, 15, and 18 post HDM sensitization. After 24 h of final exposure, mice were euthanized by CO_2 asphyxiation, and bronchoalveolar lavage fluid (BALF), lung-draining mediastinal lymph nodes (MLN), lung, and spleen were harvested for further analysis.

2.3. IL-33-Induced Airway Inflammation Mouse Model.

For the IL-33-induced airway inflammation model, 6–8 weeks old female RAG1-KO mice were challenged with 500 ng of recombinant mouse IL-33 (BioLegend) in 50 μ L of sterile PBS through intraperitoneal injection on day 0 and intranasal injection on days 1, 2, and 5.^{32,33} Dechlorane 602 (from Toronto Research Chemicals Inc., 10 μ g/kg body weight) dissolved in DMSO was diluted in 100 μ L of PBS (DMSO < 0.1%) and administered in mice through gavage, as described before,²⁸ from day 1 to day 6 post initial IL-33 treatment. After 24 h of final exposure, mice were euthanized by CO_2 asphyxiation, and bronchoalveolar lavage fluid (BALF), lung, and spleen were harvested for further analysis.

2.4. Lung Histology. Whole right lungs were fully inflated with 4% paraformaldehyde, cut into 5 μ m sections, and stained with hematoxylin and eosin or periodic acid-Schiff (PAS) according to the manufacturer's protocol. Morphological evaluations of inflammatory infiltrates, structural alterations, and percentage of positive cells of total cells in the airway were determined at a magnification of 40 $\times$ or 10 $\times$. To score the lung histopathology, inflammatory cell counts were performed blind based on a five-point grading system for the following features: 1: normal, 2: few cells, 3: a ring of inflammatory cells one cell layer deep; 4: a ring of inflammatory cells two to five cell layers deep, 5: a ring of inflammatory cell layers of more than five cell layers deep. The thickness of the airway smooth muscle layer was evaluated by blinded scoring from 1 to 5, resulting in an average score for each section. Five randomized fields were counted for each slide and the mean score was calculated from each mouse within a group.³⁴ All histology results were measured by blind evaluation.

2.5. Bronchoalveolar Lavage Fluid. Upon exposure of the trachea, bronchoalveolar lavage fluid was obtained by injection of 1 mL of PBS into the lung, which was subsequently recovered again in the syringe. Total cell numbers were counted. Fluid (250 μ L) was centrifuged on glass slides at 600 rpm and May-Grunwald–Giemsa staining was applied for further analysis. Percentages of neutrophils, eosinophils, lymphocyte, and macrophages were determined by counting

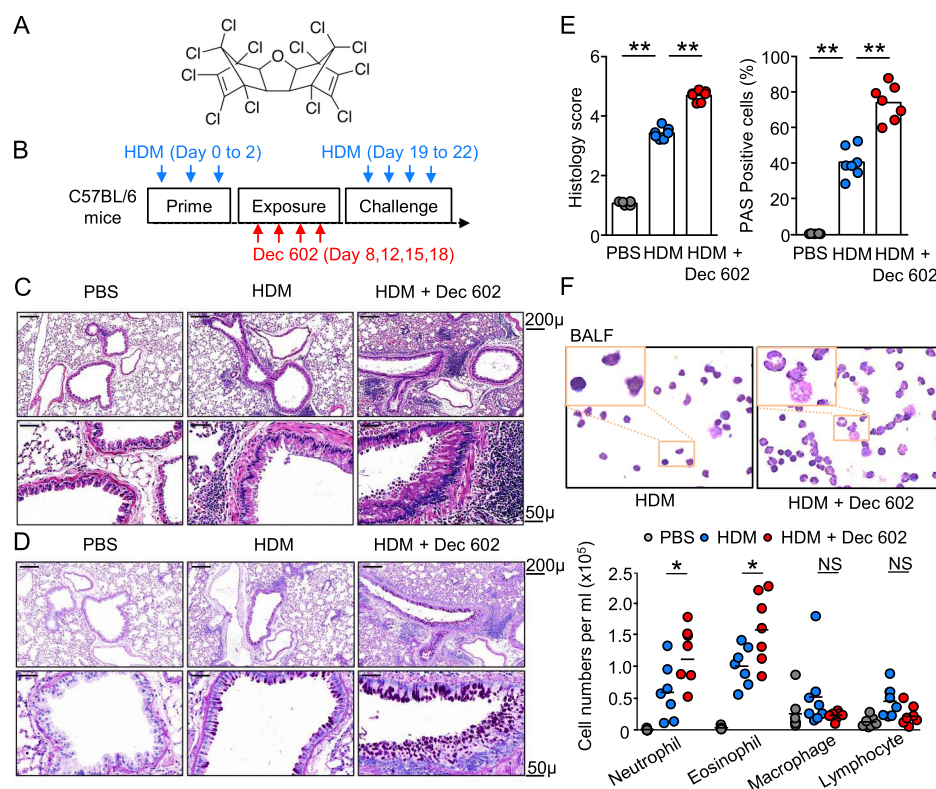


Figure 1. Dec 602 exacerbates airway inflammation induced by HDM in mice. (A) Chemical structural formula of Dec 602. (B) Schematic for (B–F). Mice were sensitized with HDM at days 0, 1, and 2 and then exposed to Dec 602 through gavage at days 8, 12, 15, and 18 before being challenged by HDM from day 19 to day 22. (C–E) Mice were euthanized 1 day after the final challenge for analysis. Lung histology was evaluated by hematoxylin and eosin (H&E) (C) or periodic acid-Schiff (PAS) staining (Scale bars, 50 or 200 μ m) (D). Pathology was blindly evaluated, and statistics are shown in (E). (F) Representative May-Grunwald–Giemsa staining (up) of cells infiltrated in bronchoalveolar lavage fluid (BALF), and statistics are shown (down). Each dot represents one mouse from three independent experiments and bars indicate mean values. Statistical significance was determined by one-way ANOVA, * p < 0.05, and ** p < 0.01.

their numbers in five randomly selected high-power fields (40 $\times$) in a blinded fashion. Cell numbers were divided by the total number of cells counted to calculate the percentage of each cell type. Total cell numbers of each cell type in BALF were then calculated by applying the percentage.³⁵

2.6. Enzyme-Linked Immunosorbent Assay (ELISA). ELISA for mouse IL-4 (BioLegend), IL-5 (BioLegend), IL-13 (Boster), and IgE (BioLegend) was performed according to the manufacturer's protocol. Briefly, assay plates were coated with capture antibodies, respectively, at 4 $^{\circ}$ C overnight. Plates were next washed with PBST (PBS + 0.05% Tween-20) and blocked with bovine serum albumin (BSA) at room temperature for 90 min. Samples from BALF and cell culture supernatant were added into the plate with recombinant mouse IL-4, IL-5, IL-13, and IgE used as standards. Next, biotinylated detection antibodies (BioLegend or Boster) were added and incubated at room temperature for 60 min. Avidin-HRP (BioLegend, B221242 or Boster) conjugates were added and incubated at room temperature for 45 min. Lastly, TMB (Invitrogen) was used for the development and 0.5 M H₂SO₄ was used to stop the reaction. Absorbance was detected at the wavelength of 450 nm using a PerkinElmer EnSpire.

2.7. Tissue Digestion. The whole left lungs were collected from mice for isolation of mononuclear cells. The lung tissue was minced and enzymatically digested using 1 mg/mL collagenase IV (Roche) and 1 μ g/mL DNase (Solarbio) in an RPMI-1640 medium (Hyclone) at 37 $^{\circ}$ C for 30 min. Cells were filtered through a cell strainer (70 μ m) and centrifuged at

500g for 5 min at 4 $^{\circ}$ C. After using RBC lysis buffer (BD), cells were further harvested with the RPMI-1640 medium and enriched by 40% Percoll (GE) gradient centrifugation at 2000 rpm for 10 min at room temperature. Lung mononuclear cells were collected, counted, and pooled for further analysis.

2.8. In Vitro Polarization of Naïve CD4 T Cells. Naïve CD4 T cells were purified from C57BL/6 mice and polarized under T_H0 (anti-CD3 5 μ g/mL, anti-CD28 5 μ g/mL), T_H1 (anti-CD3 5 μ g/mL, anti-CD28 5 μ g/mL, anti-IL-4 10 μ g/mL, IL-12 20 ng/mL), and T_H2 (anti-CD3 5 μ g/mL, anti-CD28 5 μ g/mL, anti-IFN γ 1 μ g/mL, IL-4 10 ng/mL) conditions for 3–5 days.

2.9. Isolation and Expansion of Mouse Lung ILC2s In Vitro. The lung tissue was collected and digested for isolation of mononuclear cells. Cells were then stained with antibodies to lineage markers, as well as antibodies to CD25, CD45, CD90, CD127, Sca-1, and ST2 (Supporting Information Table 1). ILC2s were sorted as Lin[−]CD45⁺CD127⁺Sca-1⁺ST2⁺ cells. Sorted cells were expanded in a complete medium (RPMI-1640 medium containing 10% FBS, L-glutamine, penicillin–streptomycin, HEPES, nonessential amino acids, sodium pyruvate, and 2-mercaptoethanol), 50 ng/mL IL-2, 50 ng/mL IL-7, 50 ng/mL IL-25, and 50 ng/mL IL-33.³⁶ After 3 days of expansion, cells were cultured in the presence or absence of Dec 602 for 2 days and analyzed by flow cytometry.

2.10. Isolation of Human Peripheral Blood Mononuclear Cells (PBMCs). The buffy coat from healthy donors was collected and diluted with PBS (1:1). The diluted buffy

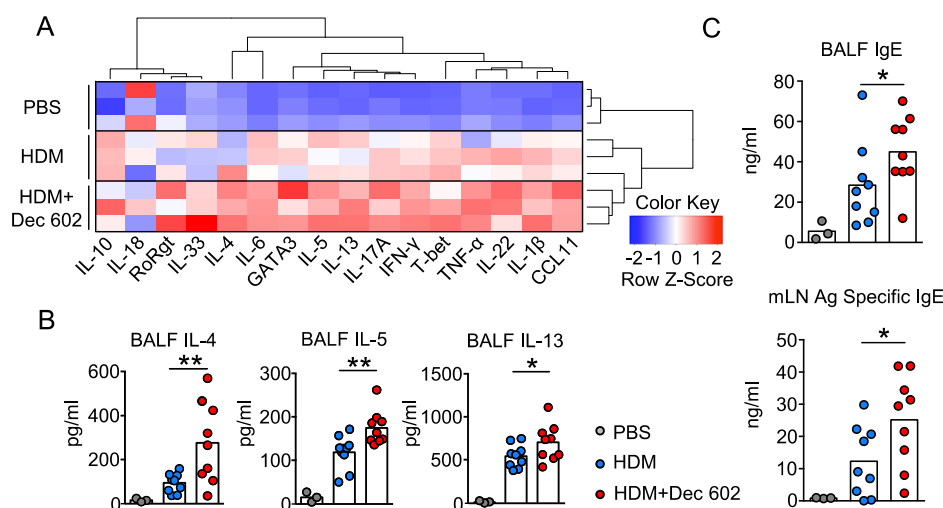


Figure 2. Dec 602 promotes type 2 immune response induced by HDM in mice. Mice were sensitized with HDM at days 0, 1, and 2 and then exposed to Dec 602 through gavage at days 8, 12, 15, and 18 before being challenged by HDM from day 19 to day 22. (A) Gene expressions in lung homogenates from three individual mice in each group were measured by qPCR and are shown in a heatmap. Blue represents low expression and red represents high expression of each gene compared among the three groups, with row Z-Score (−2 to 2). β -actin was used as the reference gene. (B) Type 2 cytokines in BALF were measured by ELISA. (C) IgE antibody production from BALF (up) and from ex vivo HDM-stimulated mLN cells (down) was measured by the ELISA assay. Each dot represents one mouse from three independent experiments and bars indicate mean values. Statistical significance was determined by one-way ANOVA, * p < 0.05, and ** p < 0.01.

coat was then gently loaded onto the Ficoll-Paque Plus (GE) layer at the ratio of 1:1 followed by density gradient centrifugation with brakes off (760g, 20 °C, 20 min). Mononuclear cells were collected and processed in FAC sorting buffer for further analysis. All protocols were approved by Nanjing University Drum Tower Hospital's human ethics committee.

2.11. In Vitro Expansion of Human Innate Lymphoid Cells (ILCs). Human PBMC CD45⁺Lin[−]CD127⁺ cells were sorted by flow cytometry and expanded in vitro in the presence of IL-2 (10 ng/mL), IL-7 (10 ng/mL), and IL-33 (10 ng/mL) in a complete medium (RPMI-1640 medium containing 10% FBS, L-glutamine, penicillin–streptomycin, HEPES, nonessential amino acids, sodium pyruvate, and 2-mercaptoethanol) for 5 days.³⁷ Expanded cells were rested for 2 days in the complete medium and then treated with Dec 602 (800 nM) or DMSO for 2 days.

2.12. Flow Cytometry. For surface staining, cells were washed once with PBS and stained in an appropriately diluted antibody solution for 45 min on ice. 7-Amino-actinomycin D (7-AAD) (Biolegend) staining was used to exclude dead cells. For intracellular/intranuclear staining, cells were washed once after surface staining and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) for 30 min on ice. The intracellular/intranuclear-specific antibodies were diluted in permeabilization buffer and incubated for 60 min on ice. Data were collected on a BD Aria III flow cytometer (BD) and analyzed using FlowJo software. Antibody information is presented in the Supporting Information Table 1.

2.13. RNA Extraction and Quantitative Real-Time PCR. Total RNA was purified from the lung tissue using the TRIzol reagent (Invitrogen), and cDNA was synthesized from 5 μ g of the total RNA using the RevertAid first-strand cDNA synthesis Kit (Thermo Scientific) and amplified using quantitative real-time PCR according to the manufacturer's instructions. The sequences of primers used are presented in the Supplementary Table 2.

2.14. Statistical Analysis. The algorithm from “Heatmap.2” (gplots package version 3.0.1.1) was used to generate a heatmap of mRNA expression of genes via R version 3.6.1. Experimental data were analyzed by one-way ANOVA or Student's *t*-test using GraphPad Prism 7.0a software and all values were expressed as the mean. Bar graphs indicate the mean values. Differences were considered to be statistically different at * p < 0.05 and ** p < 0.01.

3. RESULTS

3.1. Dec 602 Exacerbates Airway Inflammation and Promotes a Type 2 Immune Response. Based on the concentrations of Dec 602 in sediments and fish, the environmentally relevant doses range from 1 to 10 μ g/kg body weight.^{38,28,39} Previous mouse exposure studies chose 10 μ g/kg body weight, which showed an immunomodulatory effect,²⁸ therefore this mouse exposure dose was adopted in this study. Mice were exposed to Dec 602 by gavage following sensitization and prior to HDM challenge (Figure 1A,B). We found exacerbated airway remodeling and increased infiltration of immune cells in lung tissues from mice exposed to Dec 602 compared with non-Dec 602-exposed littermates (Figure 1C,E). Exposed mice also showed enhanced secretion of mucus, indicated by higher numbers of goblet cells stained positively by PAS (Figure 1D,E). In line with the aggravated inflammation in the lung, Dec 602 exposure also caused significant increases of leukocyte cellularity in the bronchoalveolar lavage fluid (BALF) and mediastinal lymph nodes (mLNs) but not in spleens (Figures 1F and S1). In BALF, the increase in leukocytes was attributed to the major cell types, eosinophils and neutrophils, with a ~2-fold increase evident in exposed mice relative to nonexposed mice (Figure 1F).

3.2. Dec 602 Promotes a Type 2 Immune Response Induced by HDM in Mice. By examining the expression of a range of cytokines and chemokines involved in lung inflammation, we recorded increased mRNAs for proinflammatory cytokines TNF- α , IL-6, and profibrogenic eotaxin/

CCL11 in lung tissues from exposed mice (Figure 2A). The airway inflammation within the HDM-induced model is driven by a type 2 response.⁴⁰ Indeed, severe pulmonary pathology in Dec 602-exposed mice was associated with an increase in type 2 immune response-associated gene expression in lung tissues including IL-5, IL-13, and GATA3 (Figure 2A). We observed only mild changes in gene expression related to T_H1 or T_H17 cells. Correspondingly, higher amounts (approximately 30%) of IL-5 and IL-13 proteins were detected in BALF and mLN of exposed mice than those from unexposed mice (Figures 2B and S2). Furthermore, we found a ~ 1.5 -fold increase of IgE in BALF from exposed mice compared to unexposed mice (Figure 2C). We also measured the production of antigen-specific IgE by stimulating cells from mLNs with HDM. Compared to unexposed mice, the production of HDM-stimulated IgE by cells from exposed mice was ~ 2 -fold higher. (Figure 2C). These pieces of evidence collectively suggest that Dec 602 promotes type 2 immune responses.

3.3. Dec 602 Increases T Helper 2 (T_H2) Cells Induced by HDM in a Cell-Extrinsic Manner. To further dissect the mechanisms underlying a Dec 602-promoted type 2 immune response, we evaluated T_H2 cells in vivo and in vitro that are essential for a type 2 immune response against infection and allergen. During HDM-induced airway inflammation, Dec 602-treated mice showed a 2–3-fold increase of IL-4⁺ T_H2 cells in the lung and mLN compared to PBS-treated mice (Figure 3A), as measured by the frequencies in total CD4⁺ T cells or the absolute cell numbers using flow cytometry (Figure 3B). To test whether Dec 602 can directly enhance the generation of T_H2 cells, Dec 602 was added to cultured naïve CD4⁺ T cells under T_H2 conditions. It was found, however, that Dec 602 was unable to enhance T_H2 differentiation (Figure 3C). These pieces of evidence suggest that while Dec 602 can promote T_H2 cells during airway inflammation, this regulation may occur through a cell-extrinsic manner.

3.4. Dec 602 Directly Potentiates Mouse ILC2s. Next, we evaluated ILC2s, which are known to regulate a type 2 immune response in the context of airway diseases.⁴¹ We found that HDM sensitization and challenge in mice drastically induced the generation of Lin[−]CD127⁺GATA3⁺ST2⁺ ILC2s, which also expressed the ILC2 markers KLRG1, CD90, and CD25 (Figure 4A,B). Importantly, Dec 602 exposure showed a 2-fold increase in the frequency and number of ILC2s (Figure 4C,D). In contrast to the negligible effect of Dec 602 on T_H2 differentiation, Dec 602 directly promoted the generation of ILC2s in cell culture (Figure 4E), suggesting that Dec 602 might enhance type 2 immune responses by potentiating ILC2s.

3.5. Dec 602 Promotes the Generation of Human ILC2s. To further validate whether Dec 602 can promote the generation of human ILC2s, we collected blood from healthy donors and sorted human ILCs from peripheral blood mononuclear cells (PBMC) using FACS (Figure 5A,B). After in vitro expansion and resting, Dec 602 was added to the cultured human ILCs for 48 h. It was found that Dec 602 directly increased the expression of GATA3, consistent with our data and previous studies in mouse²⁸ (Figure 5C). Upregulated GATA3 expression gave rise to approximately a 50% increase in the generation of human c-Kit⁺GATA3⁺ ILC2s in cell culture (Figure 5C).

3.6. Dec 602 Potentiates ILC2s and Exacerbates Airway Inflammation in RAG-KO Mice. To test whether Dec 602 can enhance a type 2 immune response in a T_H2 -

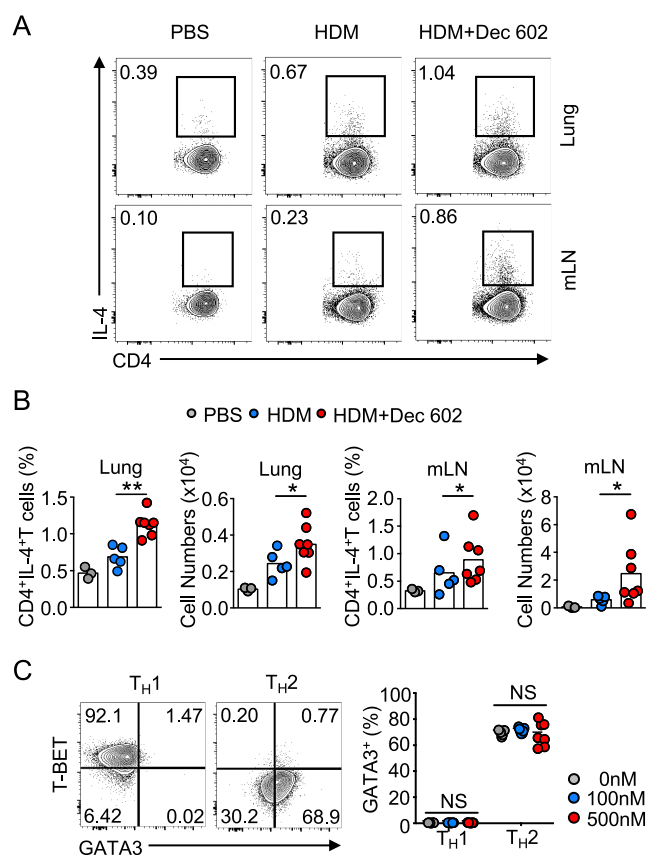


Figure 3. Dec 602 increases T helper 2 (T_H2) cells induced by HDM in a cell-extrinsic manner. Mice were sensitized with HDM at days 0, 1, and 2 and then exposed to Dec 602 through gavage at days 8, 12, 15, and 18 before being challenged by HDM from day 19 to day 22. (A) IL-4-secreting CD4⁺ T cells in the lung and mLN were evaluated by FACS. Differences in the frequency and total cell number of IL-4-secreting CD4⁺ T cells between groups were measured, and statistics are shown in (B). (C) Naïve CD4⁺ T cells were polarized in T_H1 or T_H2 condition for 3 days with Dec 602 at indicated concentrations. Each dot represents an individual mouse. Results are representative of three independent experiments and bars indicate mean values. Statistical significance was determined by one-way ANOVA, * p < 0.05, and ** p < 0.01.

independent manner, we induced type 2 inflammation by the administration of IL-33 in RAG1-deficient mice, which lack both T cells and B cells (Figure S3). One group of IL-33-treated RAG1-deficient mice were exposed to Dec 602 (Figure 6A). Lin[−]CD127⁺GATA3⁺ST2⁺ ILC2s were induced by IL-33 and further increased following Dec 602 exposure by approximately 2-fold (Figure 6B). Compared to unexposed mice, Dec 602-exposed mice displayed exacerbated airway remodeling, increased infiltration of immune cells, and enhanced mucus secretion (Figure 6C–E). The aggravated inflammation in the lung of exposed mice was accompanied by increased numbers of eosinophils and macrophages in BALF (Figure 6F,G). Together, these results support the conclusion that Dec 602 can potentiate type 2 ILCs in vivo and exacerbate airway inflammation independent of T_H2 cells.

4. DISCUSSION

Despite the increasing evidence showing that flame-retardant dechlorane 602 is detectable in milk, eggs, salmon, and other food,²⁶ there are limited studies that have evaluated its

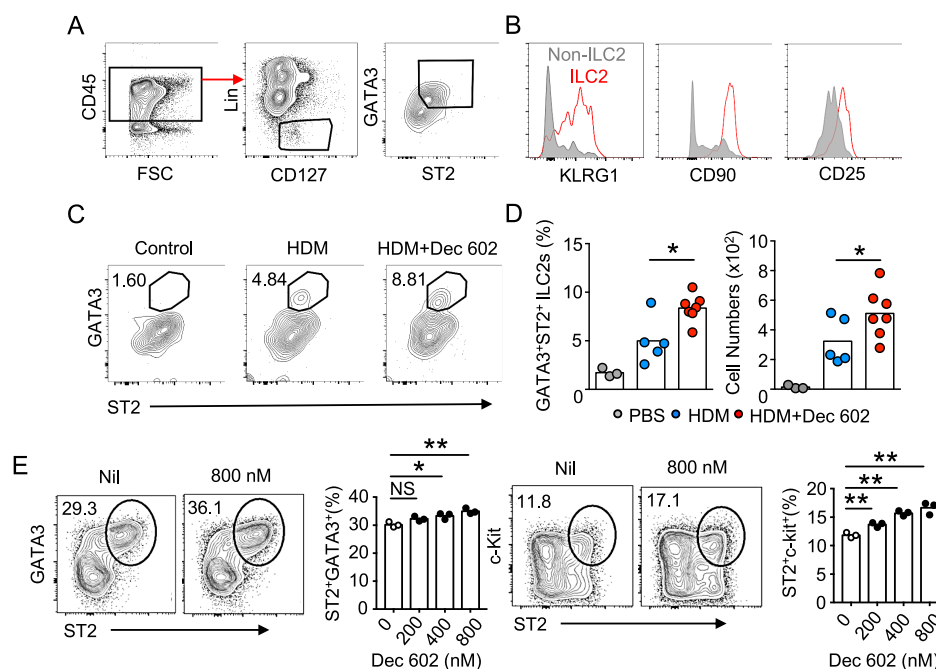


Figure 4. Dec 602 directly potentiates mouse ILC2s. Mice were exposed to Dec 602 through gavage between sensitization and being challenged by HDM. (A) Gating strategy for the ILC2s. (B) Histogram plots indicated the phenotypes of the ILC2s and the non-ILC2s. (C) Representative contour plots of GATA3⁺ST2⁺ ILC2s in lungs from different groups. (D) Frequency and cell numbers of GATA3⁺ST2⁺ ILC2s. (E) Lin⁻CD127⁺ST2⁺Sca-1⁺ ILC2s were sorted using FACS from lung tissues of RAG1-deficient mice treated with IL-33, and then cultured in vitro to expand with IL-2, IL-7, IL-25, and IL-33 for 2 days. Cultured ILC2s were then maintained in the presence or absence of Dec 602 with indicated concentrations for 2 days. FACS plots and statistics showing the percentages of GATA3⁺ST2⁺ ILC2s (left) or c-Kit⁺ST2⁺ ILC2s (right) (E). Each dot represents an individual mouse. Results are representative of three independent experiments and bars indicate mean values. Statistical significance was determined by one-way ANOVA, **p* < 0.05, and ***p* < 0.01.

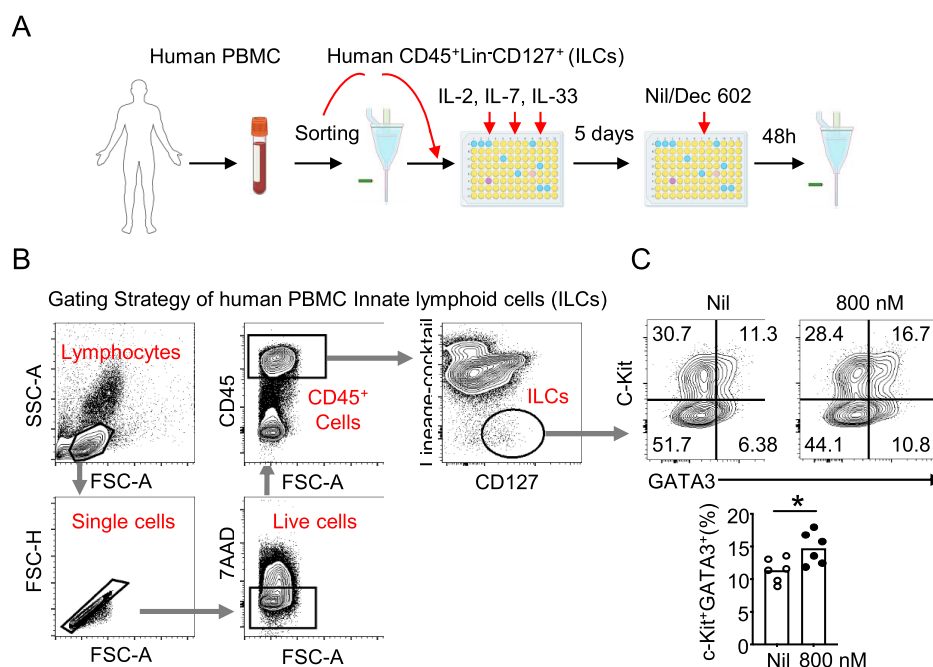


Figure 5. Dec 602 promotes the generation of human ILC2s. Human PBMC CD45⁺Lin⁻CD127⁺ cells were sorted by flow cytometry and in vitro expanded in the presence of IL-2, IL-7, and IL-33, followed by the treatment of Dec 602 for 48 h, shown in schematic (A). (B) Gating strategy of human innate lymphoid cells (ILCs) utilized in both cell sorting and analysis. (C) FACS plots and statistics of in vitro-expanded human ILCs that were treated with or without Dec 602. Results are representative of three independent experiments and bars indicate mean values. Statistical significance was determined by Student's *t*-test, **p* < 0.05, and ***p* < 0.01.

potential impact on health and disease. Previously, we reported that Dec 602 favored a “T_H2” phenotype when characterizing

the production of type 2 cytokines and the expression of transcription factors at the mRNA level in mice exposed to Dec

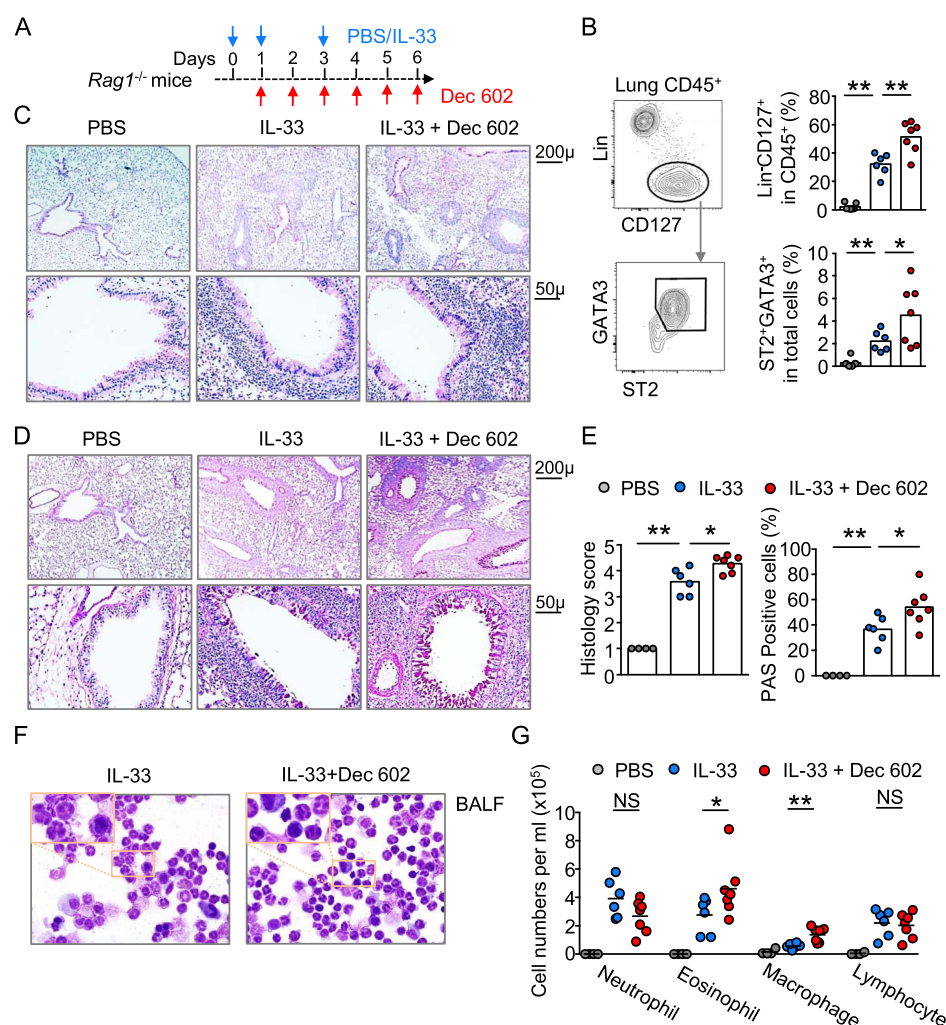


Figure 6. Dec 602 enhances ILC2s and exacerbates airway inflammation in RAG-KO mice. (A) Schematic for (A–G). RAG1-KO mice were treated with IL-33 and were exposed to Dec 602 from day 1 to day 6. (B) Mice were euthanized 1 day after the final challenge for analysis. Gating strategy and statistics for Lin[−]CD127⁺ ILCs and GATA3⁺ST2⁺ ILC2s in the lung. (C–E) Lung histology was evaluated by hematoxylin and eosin (H&E) (C) or periodic acid-Schiff (PAS) staining (Scale bars, 50 or 200 μ m) (D). Pathology was blindly evaluated, and statistics are shown in (E). (F) Representative May-Grunwald–Giemsa staining of cells infiltrated in bronchoalveolar lavage fluid (BALF), and statistics are shown in (G). Each dot represents one mouse from three independent experiments and bars indicate mean values. Statistical significance was determined by one-way ANOVA, * p < 0.05, and ** p < 0.01.

602 for 7 days.²⁸ One limitation of this Dec 602 exposure study was the lack of investigation on the source of type 2 cytokines, which can be produced by T_H2 cells or other immune cell types such as ILC2s. Moreover, Dec 602-promoted T_H2 responses might increase the risk for type 2 inflammation in diseases, such as asthma, chronic sinusitis, as well as atopic dermatitis,⁴² which requires further investigation. In this study, we demonstrated that exposure of Dec 602 exacerbated type 2 inflammation in the airway in two mouse models, and this was at least partially mediated by the direct potentiation of ILC2s by Dec 602.

Type 2 inflammation in the airway is driven by both innate and adaptive immune cells.^{43,44} Innate immune cells such as eosinophils and neutrophils play forefront roles in regulating inflammation in the tissue through interaction with epithelial cells as well as the production of cytokines and chemokines that can coordinate with adaptive immune cells.^{45,46} We found that Dec 602 markedly promoted the accumulation of neutrophils and eosinophils in the airway associated with enhanced airway remodeling. It remains unknown whether this

increased accumulation is through direct regulation by Dec 602 or cellular cross talk.

Elevated protein levels of IL-4, IL-5, and IL-13 in BALF were noticed in mice challenged with HDM and exposed to Dec 602. Among them, IL-4 is known to induce B cell class-switching to IgE, which is a hallmark of airway inflammation in both mice and humans.^{47–49} Interestingly, increased production of overall and antigen-specific IgE antibodies suggests that Dec 602 can alter the outcome of antibody development into IgE production, which is primarily regulated by germinal center (GC) B cells and follicular helper T (T_{FH}) cells⁵⁰ in airway diseases.^{51–54} It is important to know in future studies whether Dec 602 can directly regulate a GC response, especially in the airway and gastrointestinal tract where environmental stimuli constantly interact with host cells, and whether elevated IgE occurs in humans exposed to Dec 602.

ILC2s are a recently identified innate lymphoid subset that responds to environmental stimuli.^{55,56} ILC2s produce canonical type 2 cytokines and synergize with T_H2 cells to orchestrate type 2 responses including airway inflammation.⁴⁰

Unlike T_H2 cells, Dec 602 can directly promote the generation of both human and mouse ILC2s in vitro. Moreover, Dec 602 can promote type 2 immune responses independent of T_H2 cells in an IL-33-induced airway inflammation model in RAG-KO mice and exacerbate airway inflammation with clearly increased ILC2s in the lung tissue. In our previous study, the exposure of Dec 602 in mice changed the T_H1/T_H2 balance.²⁸ The discovery of the direct effect of Dec 602 on ILC2s instead of T_H2 cells suggested that the change in the T_H1/T_H2 balance by Dec 602 exposure could be indirect and mediated by ILC2s. While the molecular mechanisms that underlie the promotion of ILC2s by Dec 602 require more investigation, it is clear that Dec 602 can enhance the generation of these cells in vivo. Immune cells can directly sense environmental compounds such as fatty acids, bile acids, and dioxin by membrane or intracellular molecules.^{57–59} It will be interesting to reveal the responding cellular receptors to Dec 602 in ILC2s in future studies and intervene such pathway to control potential risk exposure.

In summary, our findings indicate that Dec 602 can potentiate the generation of both human and mouse ILC2s in vitro. Furthermore, in two mouse models, we demonstrated that the exposure of Dec 602 exacerbated type 2 inflammation in the airway, and ILC2s, which can be directly potentiated by Dec 602, were critical for this process. In both mouse models, the ILC2-potentiating function of Dec 602 was associated with increased BALF eosinophils. This observation aligns with the report that ILC2s promote the persistence of airway eosinophilia in patients with asthma.⁶⁰ Further studies are required to dissect the molecular mechanism underlying the potentiation of ILC2s by Dec 602 and to examine the immunomodulatory effects of other chlorinated flame retardants including DP, Dec 603, and 604. To our best knowledge, the study investigating the exposure of these emerging chlorinated flame retardants and the risk on the airway or cardiovascular disease has not been conducted. The global demand for flame retardants including Dec 602 is increasing dramatically, and the annual use is measured to be around two million tons.³ Therefore, the risks they pose to human health needs to be evaluated urgently and carefully.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.est.0c03758>.

Antibodies used for FACS staining of mouse and human cells (Table S1); real-time PCR primers (Table S2); scoring rubric for lung histology (Table S3); Dec 602 treatment enhances the infiltration of leukocytes in the BALF and mLNs (Figure S1); Dec 602 treatment promotes type 2 cytokine secretion (Figure S2); and the deficiency of T and B cells in RAG-KO mice (Figure S3) (PDF)

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Notes

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