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Indoor pollutant Hexabromocyclododecane has a modest immunomodulatory effect in house dust mite induced allergic asthma in mice

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9 Keywords: Hexabromocyclododecane, house dust mite, dendritic cells, allergic asthma, IL-
10 17A.

11

12 ABSTRACT

13 Hexabromocyclododecane (HBCD) has been recognized as an indoor pollutant. HBCD is
14 added as flame retardant to many consumer products and leaches from the products into house
15 dust. HBCD might be potentially hazardous to the airways because of inhalation of house
16 dust. Sensitization to house dust mite (HDM) is a risk factor for the development of allergic
17 asthma. In this study, we examined whether HBCD can affect the immune response to HDM
18 allergens. Bone marrow derived dendritic cells (BMDCs) were exposed simultaneously to
19 HBCD and HDM *in vitro*. HBCD enhanced oxidative stress in HDM pulsed BMDCs which
20 was accompanied with a higher production of Interleukin (IL)-6 and -10. Adoptive transfer of
21 HDM/HBCD exposed BMDCs into naïve mice resulted in enhanced levels of IL-17A after
22 inhalational challenge with HDM. Direct mucosal exposure to HBCD during HDM inhalation
23 enhanced IL-4 or IL-17A production depending on the HDM extract used but did not
24 aggravate the eosinophilic airway inflammation or airway hyperreactivity. Our results indicate
25 that exposure to HBCD can have a mild immune-modulating effect by enhancing the
26 inflammatory cytokine production in response to inhaled HDM in mice.

27

28

29 INTRODUCTION

30 Allergic asthma is a chronic inflammatory airway disease of which prevalence and severity
31 has increased sharply since the 1970s.¹⁻³ During the last decades many chemicals have been
32 introduced into the indoor environment. Epidemiological studies point to a possible
33 correlation between exposure to chemical pollutants such as perfluorooctane sulfonate and
34 phthalates, present in indoor air and dust, and asthma in children.^{4,5}

35 During the last decades, flame retardants have become a major indoor pollutant which
36 accumulate in indoor dust and coexist with allergens.^{6, 7} Inhalation of dust exposes the
37 airways to allergens in the presence of these chemicals. The airways are covered with a
38 network of dendritic cells (DCs) sampling inhaled air continuously for incoming invaders in
39 order to initiate an appropriate immune response. DCs are capable of sensing endogenous
40 (produced by the organism) and exogenous danger signals (pathogens, allergens or toxic
41 chemicals)⁸ which will induce upregulation of co-stimulatory molecules and secretion of
42 cytokines in order to differentiate naïve T cells into a specific subset of effector T cells. In
43 allergic asthma, an aberrant T helper 2 (Th2) response is induced against a harmless antigen.
44 Th2 cells control the allergenic response by secreting cytokines IL (Interleukin)-4, IL-5 and
45 IL-13.^{9, 10} In severe forms of asthma, an aberrant production of Th17-derived cytokine IL-17A
46 has been associated with increased airway hyperreactivity and neutrophil levels. Th17 cells
47 are another subset of T helper cells.¹¹⁻¹³ The possible mechanisms involved in the
48 misinterpretation of the antigen by the immune system have to be elucidated.¹⁴ Simultaneous
49 exposure to the outdoor pollutant DEP (diesel exhaust particles) and house dust mite (HDM)
50 has been demonstrated to promote a mixed Th2/Th17 response in mice, which is associated
51 with increased airway hyperreactivity,¹⁵ indicating that pollutants can interfere with the
52 allergic immune response.

Hexabromocyclododecane (HBCD) is a cyclic aliphatic brominated flame retardant (BFR) widely used as an additive in thermal insulation of building materials, textiles and electronics to delay flammability. In 2006, HBCD was the second most frequently used BFR in Europe, following tetrabromobisphenol A (TBBPA)¹⁶. Flame retardants including HBCD leach from their product and several studies have shown its presence in house dust. Exposure of HBCD via house dust has been correlated with its concentrations in human serum, breast milk and adipose tissue.^{7, 17-19}

Based on its persistence, bioaccumulation and adverse health effects in laboratory animals, HBCD has been placed on the list of “substances of very high concern” by the European Chemicals Agency and under Annex A of the Stockholm Convention on Persistent Organic Pollutants. Animal studies have shown a diversity of possible health threats like endocrine-disrupting effects, developmental neurotoxicity in neuron cells and disruption of lipid metabolism.²⁰⁻²⁴ However, only limited animal studies have investigated immunological effects of HBCD and no study has investigated the potential effect of HBCD on the immune response to inhaled HDM.^{25, 26}

HBCD has been shown to affect cell proliferation in human hepatocytes via induction of oxidative stress.^{27, 28} Oxidative stress has been shown to mature DCs resulting in an enhanced proliferation and differentiation of allergen specific CD4⁺ expressing T helper cells.^{29, 30} In this study we aimed to investigate whether HBCD could induce oxidative stress in dendritic cells and its subsequent effect on the induced immune response to inhaled house dust mite allergens in mice.

78 MATERIALS AND METHODS

79 **Mice.** 6-8 week old female Balb/c mice (Harlan, Horst, The Netherlands) were housed
80 under specific pathogen-free conditions at the animal care facility of the AMC. All
81 experiments were approved by the AMC Animal Ethics Committee, The Netherlands.

82 ***In vitro* exposure to HBCD of bone marrow derived DCs.** BMDCs were obtained as
83 described earlier.³¹ Briefly, BM cells were isolated from Balb/c mice. After red blood cell
84 lysis, BM cells were cultured for ten days with 20 ng/ml granulocyte-macrophage colony
85 stimulating factor (GM-CSF, a gift from Prof. K. Thielemans, VUB, Brussels, Belgium). On
86 day 9 of culture, cells were exposed to 100 µg HDM/ml (equivalent to 1 µg Der p 1
87 (peptidase variant 1 of mite *Dermatophagoides pteronyssinus*)/ml, one of the major allergens
88 in HDM) (*Dermatophagoides pteronyssinus*, XPB82D3A2.5, Greer Laboratories; Lenoir, NC,
89 USA) with or without 10 µM HBCD (Technical mixture (TM) from Dead Sea Bromine
90 Group, Beer-Sheva, Israel) overnight (Figure 1A). Alternatively, cells were incubated with or
91 without 1 mM N-acetyl-L-cysteine (NAC) (Sigma Aldrich Corp. St. Louis, MO, USA) for 30
92 min before the exposure to HDM +/- HBCD at 37°C in 5%CO₂. The concentration of HBCD
93 (10 µM) was determined based on a dose response assay (0 µM, 0.1 µM, 1 µM or 10 µM
94 HBCD with 100 µg/ml HDM or 10 µM HBCD) on DC activation and survival (Figure S1).
95 Since HBCD was dissolved in DMSO, controls received 0.4% DMSO to match the final
96 concentration achieved in the culture medium. This concentration did not affect cell survival
97 and/or activation of BMDCs compared with untreated BMDCs (Figure S1).

98 **BMDC phenotyping and cytokine production.** At day 10 of BMDC culture, cells were
99 stained with antibodies against MHCII(FITC), CD11c(APC), in combination with PE labeled
100 antibodies against co-stimulatory molecules (cell surface proteins) CD80, CD86, CD40,
101 ICAM-1, ICOS-L or isotypes Rat IgG2a and, Armenian Hamster IgG (eBioscience Inc, San
102 Diego, CA, USA). To prevent non-specific antibody binding, anti-FcγII/III antibody (clone:

2.4G2, provided by Louis Boon, Bioceros B.V., Utrecht, The Netherlands) was added to the monoclonal antibody mixture. Propidium iodide (Sigma Aldrich Corp. St. Louis, MO, USA) was used to distinguish between living and dead cells. Expression of co-stimulatory molecules were analyzed with FlowJo software (Tree Star Inc., Ashland, OR). IL-6, IL-10, IL-1 β and TGF- β production by BMDC was determined in culture supernatant by ELISA according to the manufacturer's instructions (Ready-SET-Go ELISA kit, eBioscience, San Diego, CA).

Real Time PCR on BMDCs. To determine oxidative stress induced by HBCD, the level of mRNA coding for heme-oxygenase-1 (HO-1), a protective anti-oxidant enzyme used as a marker for oxidative stress,³² was determined by RT-PCR. Cultured BMDCs were exposed 7 hours to HDM/HBCD, HDM/DMSO or DMSO alone before BMDCs were macerated in Trizol (Invitrogen Life Technologies, Carlsbad, CA, USA) for total RNA extraction according to the manufacturer's recommendations. After synthesis of the complementary DNA (cDNA) using First strand cDNA Synthesis Kit (Thermo Fisher Scientific, Vilnius, Lithuania), real-time PCR was performed in a 10 μ l reaction volume that included 5 μ l of SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) and 1 μ M of FW and RV primers (Table 1). All reactions were performed in optical 96-well reaction plates using StepOne Plus RealTime PCR System (Applied Biosystems, Warrington, UK). HO-1 mRNA concentrations from each sample were calculated based on the standard curve as previously described and were normalized to the concentration of the housekeeping gene HPRT.³³

***In vivo* exposure to HBCD in different asthma models.** Mice were instilled intratracheally (i.t.) with 1×10^6 *in vitro* HDM/HBCD exposed BMDCs. As a control, mice received HDM/DMSO pulsed BMDCs or DMSO pulsed BMDCs at day 0. At day 10, mice were challenged intranasally (i.n.) with 100 μ g HDM. At day 12 airway hyperreactivity (AHR) to the inhaled bronchoconstrictor metacholine (0, 3.125, 6.25, 12.5, 25 and, 50 mg/ml for 3 min) was measured by whole body plethysmography (EMKA Technologies) as

described previously.³⁴ The enhanced pause (PenH) is an index of airway hyperreactivity that is derived from the equation:

$$\text{PenH} = (\text{peak expiratory flow} / \text{peak inspiratory flow}) \times [(\text{Expiratory time} - \text{time for expiring 64\% volume}) / \text{time for expiring 64\% volume}]$$

One day later, mice were sacrificed (Figure 1A). To investigate the direct effect of HBCD, we exposed mice with two different HDM extracts containing an equal amount of one of the major HDM allergens Der p 1 (1 μg) together with HBCD. Mice were exposed to 100 μg HDM (*Dermatophagoides pteronyssinus*, XPB82D3A2.5, Greer Laboratories; Lenoir, NC, USA) or 8.2 μg HDM with low endotoxin levels (*Dermatophagoides pteronyssinus*, LoToxTM LTN-DPE-4, INDOOR biotechnologies, Cardiff, UK referred to as LT-HDM) together with 5.2 μg HBCD on day 0 i.t. and challenged with either HDM/HBCD or LT-HDM/HBCD i.n. on day 7. As control groups, mice received HDM/DMSO or LT-HDM/DMSO or DMSO at day 0 and 7. AHR was measured on day 8 (Figure 1B).

Collection and analysis of cells and tissues. *Bronchoalveolar lavage fluid:* Bronchoalveolar lavage fluid inflammatory cells were determined by airway lumen lavage with 3x1 ml PBS supplemented with 0.1 mM EDTA. Cell differentiation was made by flow cytometric analysis as described earlier.³⁵ *Ex vivo re-stimulation of lung-draining lymph node cells:* Lung draining lymph node cell suspensions were plated in 96 well round bottom plates at a density of 2×10^5 cells per well and were re-stimulated for 4 days with 100 $\mu\text{g}/\text{ml}$ HDM or 8.2 $\mu\text{g}/\text{ml}$ LT-HDM (both concentrations are equivalent to 1 μg Der p 1/ml). Supernatants were analyzed for IL-4, IL-5, IL-13, IFN γ and, IL-17A production by ELISA (Ready-SET-Go ELISA KIT, eBioscience, San Diego, CA) according to manufacturer's instructions. To detect intracellular cytokine production, cells were cultured with 100 $\mu\text{g}/\text{ml}$ HDM and 1 $\mu\text{l}/\text{ml}$ brefeldin A for 12 h. Then, cells were first stained for T (helper) cell marker CD4-FITC and activation marker CD25-APC (eBioscience, San Diego, CA) and then for the intracellular

153 cytokine production with anti-mouse IL-17A-PE (eBioscience, San Diego, CA) using BD
154 Cytotfix/ CytopermTM Plus Fixation/ Permeabilization Kit (with BD GolgiPlugTM protein
155 transport inhibitor containing brefeldin A) (BD Biosciences, San Jose, CA, USA) according to
156 manufacturer's instructions. The stained cells were analyzed with a flowcytometer
157 FACSCalibur (BD Biosciences, San Jose, CA, USA). *Serum immunoglobulins:* Serum was
158 analyzed for the level of total IgE and HDM specific IgG1 by ELISA (Opteia; BD, San
159 Diego, CA, USA). A standard curve of murine IgE or IgG1 was used as a quantitative
160 reference. *Statistical analysis:* For statistical analysis, the Mann-Whitney U-test was
161 performed using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA).
162 *In vitro* experiments were performed with 1-6 simultaneous cultures and repeated 2-6 times.
163 All experiments were performed 2-3 times with 4–6 animals per group. Data from repeated
164 experiments were pooled for statistical analysis. Differences were considered to be significant
165 at a P value of <0.05.

RESULTS

HBCD enhanced oxidative stress and cytokine production of BMDCs. An initial dose response experiment showed that BMDCs which were exposed to 0, 0.1, 1, 10 μ M HBCD together with HDM, had an enhanced IL-6 and IL-10 production at the highest dose of 10 μ M HBCD while survival was not affected compared with HDM/DMSO exposure. Exposure to 10 μ M HBCD in the absence of HDM showed a slight decrease in viability and a small increase in IL-6 compared to DMSO or medium control while the phenotype was not affected (Figure S1). To investigate whether 10 μ M HBCD induced oxidative stress in HDM presenting BMDCs, cells were pulsed *in vitro* with HDM and simultaneously exposed to 10 μ M of HBCD or the solvent DMSO (Figure 1A). DMSO exposed BMDCs were used as a control. Expression of the protective anti-oxidant enzyme HO-1 was used as a marker for oxidative stress.³² Exposure of BMDCs to HDM induced HO-1 mRNA, but this was not significant. Simultaneous exposure to HBCD and HDM increased the level of HO-1 significantly (Figure 2A).

Next, we investigated whether this enhanced oxidative stress in the HDM pulsed BMDCs boosted expression of co-stimulatory molecules and cytokine production involved in T cell differentiation. As expected, exposure to HDM (HDM/DMSO) increased strongly the expression of the co-stimulatory molecules CD80, CD86, CD40 and, ICAM-1 and not of ICOS-L compared with unpulsed BMDCs (DMSO).³⁶ However, simultaneous addition of HBCD and HDM to the BMDC culture did not enhance the expression of the co-stimulatory molecules of BMDCs or the percentage of the cells expressing these co-stimulatory molecules compared to HDM/DMSO BMDCs (Figure 2B and S2A). Although the co-stimulatory molecule expression was not affected, HBCD increased significantly the production of the cytokines IL-6 and IL-10 by HDM exposed BMDCs (Figure 2C, left panel). Pre-treatment of BMDCs with the anti-oxidant N-acetyl-L-cysteine (NAC), a potent ROS scavenger,

significantly decreased the production of both cytokines by HDM/HBCD exposed BMDCs (Figure 2C, right panel). Importantly, exposure to HBCD did not lower viability of BMDCs (Figure S2B).

HBCD enhanced IL-17A cytokine production in DC driven asthma model. To explore whether the induced oxidative stress and increased pro-inflammatory cytokine production by HBCD would have an effect on the differentiation of naïve T cells *in vivo*, we adoptively transferred HDM/HBCD exposed BMDCs by intratracheal instillation in the airways of naïve Balb/c. As a control, mice received HDM/DMSO pulsed BMDCs or DMSO pulsed BMDCs. All mice were challenged with HDM via a single intranasal exposure. As expected, HDM/DMSO BMDCs induced all characteristics of allergic eosinophilic airway inflammation as seen in allergic asthma.³⁷ The mice developed airway hyperreactivity and recruited more eosinophils to the bronchoalveolar compartment compared with mice instilled with unpulsed (DMSO) BMDCs (Figure S3A-B). In concordance, IL-4, IL-5, IL-13, IFN γ and, IL-17A production by lung draining lymph node cells was increased (Figure 3A-B) and the scores of mucus production and peribronchial inflammatory infiltrates were increased (Figure S3C). Although total IgE levels were increased, HDM specific IgG1 (HDM IgG1) was not enhanced by HDM/DMSO pulsed BMDCs compared with unpulsed BMDCs (DMSO) (Figure S3D). Mice transferred with HDM/HBCD BMDCs, showed a similar eosinophilic inflammation, Th2 cytokine production and serum level of total IgE and HDM IgG1 as mice transferred with HDM/DMSO exposed BMDCs (Figures S3B-D and 3A). Remarkably, production of IL-17A was increased. The percentage of activated Th17 cells (identified as CD4⁺ CD25⁺ IL-17A⁺) were increased in the lung draining lymph nodes of HDM/HBCD BMDC transferred mice compared with HDM/DMSO BMDC transferred mice (Figure 3B). However, the increase of IL-17A production by HBCD did not lead to an enhancement of airway hyperreactivity in these mice (Figure S3A).

Mucosal exposure to HBCD and HDM increased IL-4 or IL-17A depending on the strength of the HDM stimulus. The above described experiments showed that HBCD exposure of HDM pulsed BMDCs boosted the Th17 response but did not show that direct exposure to HBCD via the airways would have an immune-modulatory effect. In a new experiment, mice were challenged simultaneously with HDM and HBCD into the airways at day 0 and day 7 (Figure 1B; upper experiment). Inhalation of HDM/HBCD resulted in a significant increase in Th2 cytokine IL-4 but not in IL-5, IL-13, IFN γ or IL-17A production by lung draining lymph nodes compared with HDM/DMSO exposed mice (Figure 4A). This increase was accompanied with a trend for an increased level of HDM specific, Th2 associated IgG1 but not of total IgE in serum (Figure 4B). HBCD did not aggravate HDM induced AHR or airway inflammation (Figure S4A-C).

We hypothesized that the strong immune response induced by the HDM allergy model, might overpower a possible effect of HBCD. HDM extract is a complex mixture of HDM allergens but also of endotoxins, which are derived from the microbial flora of the HDM. Especially the lipopolysaccharide (LPS) present in the outer membrane of gram-negative bacteria is a strong stimulus for the immune system. Therefore, we used a HDM extract containing trace amounts of endotoxin (LT-HDM), which induces a much milder immune response compared with regular HDM extract.³⁷ This allowed us to investigate whether HBCD can provide an additional ‘danger signal’ to enhance the HDM induced immune response.

Similar to the previous experimental protocol, we sensitized and challenged mice with LT-HDM in the presence or absence of HBCD on day 0 and 7, referred to as LT-HDM/HBCD or LT-HDM/DMSO respectively. As a control, mice were exposed with HBCD alone or PBS/DMSO. As described before,³⁷ exposure to LT-HDM/DMSO was not sufficient to induce AHR, eosinophil recruitment, peribronchial inflammatory infiltrates or an

immunoglobulin response (data not shown). However, LT-HDM/DMSO did induce an IL-4, -5, -13 and IL-17A cytokine production by lung draining lymph nodes after *ex vivo* re-stimulation with LT-HDM compared with PBS/DMSO control mice. In concordance with the DC transfer model, an additional exposure to HBCD induced an increased IL-17A production while IL-4, -5 or -13 were not affected, although HBCD alone induced a small but significant increase in both IL-4 and IL-17A (Figure 5). The increase in IL-17A was not accompanied by an aggravation of any other inflammatory parameter (data not shown).

DISCUSSION

The effect of HBCD on the allergic immune response has been investigated very scarcely. HBCD and HDM allergens are encountered together by the immune system when dust is inhaled. Here, we show that HBCD can increase HDM induced IL-6 and IL-10 production by BMDCs, by enhancing oxidative stress. Transfer of HDM/HBCD exposed BMDCs in the airways of naïve mice increased IL-17A production, but not of Th2 cytokines, after inhalation of HDM. The increase in IL-17A production was also observed after direct administration of HDM and HBCD to the airways but only when a HDM extract was used with a low level of endotoxins. HBCD together with a HDM extract with endotoxins showed an increase in IL-4 production. Recently, IL-17A has been implicated in more severe asthma, but in our study this increase was not sufficient to enhance airway hyperreactivity.

HBCD has been reported to induce reactive oxygen species formation in the human hepatocyte L02 cells.²⁷ Oxidative stress can enhance inflammation by increasing expression of co-stimulatory molecules and pro-inflammatory cytokine IL-6 production in BMDCs leading to enhanced proliferation of allergen specific T helper cells.³⁰ Koike et al. demonstrated that HBCD accelerates BMDC differentiation when added for 6 days during culture but the role of HBCD in relation to allergen induced maturation was not elucidated.²⁵ In this study, the increase in IL-6 production after exposure to HBCD alone, was negligible compared with the level induced after HDM/HBCD, suggesting a strong synergistic effect of HBCD on HDM induced IL-6 (Figure S1A). IL-6 is a pro-inflammatory cytokine which has been associated with an increase in Th17 differentiation in T cells.^{13, 38, 39} Other Th17 related cytokines like TGF- β and IL-1 β were produced by BMDCs, but the production was not enhanced by HBCD exposure (Figure S5). Zhou et. al. demonstrated that IL-6 in the presence of TGF- β programs Th17 differentiation.⁴⁰ Sensitization to HDM via adoptive transfer of HDM/HBCD exposed BMDCs in airways of naïve mice, resulted in a higher IL-17A

production and an increased number of Th17 cells in the lung draining lymph nodes after subsequent inhalational challenge with HDM. In concordance, direct exposure of HBCD and HDM allergens to the airways boosted the IL-17A production as well, but only when a HDM extract was used which was low in endotoxins, like LPS (Figure 5). When mice were exposed to a commonly used HDM extract which contains endotoxins, simultaneous exposure to HBCD did not increase IL-17A but instead increased the Th2 cytokine IL-4 which was accompanied by a higher trend of HDM IgG1 levels in serum. It is important to note that the endotoxin LPS, which is present in the commonly used HDM extract, is a strong danger signal that can convey a possible effect of HBCD. A driving role for LPS has been described both for IL-17A and Th2 cytokine production in the immune response to allergens.^{39, 41} Further research is needed to elucidate the differential effect of HBCD on Th2 or Th17 mediated immune responses to inhaled HDM allergens but this might depend on the presence of endotoxins in the HDM extract used.

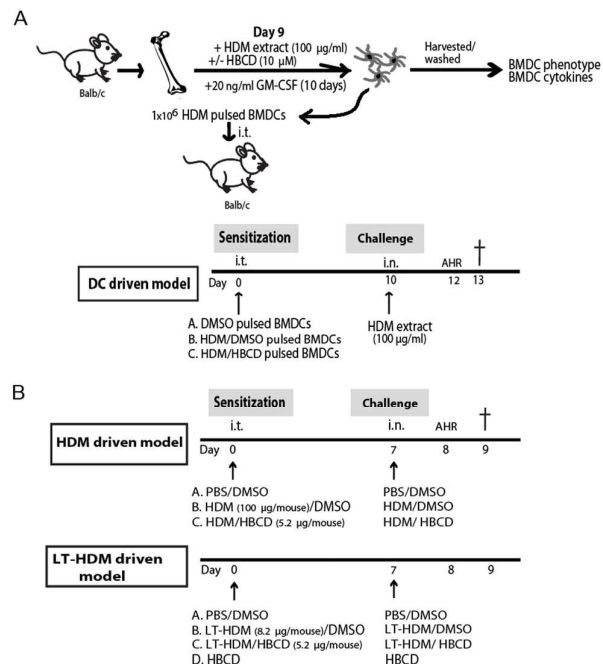
Increased levels of IL-17A have been associated with the more severe phenotypes of asthma which are characterized by increased neutrophil infiltration and airway hyperreactivity.^{11, 42} However, in our study, the increase in IL-17A levels by HBCD was not sufficient to enhance airway hyperreactivity (AHR) or to induce neutrophilia (Figure 3, S3 and 5 and data not shown).

A potential weakness of our study is the experimental high dose of HBCD (5.2 μg) used in our model, considering that humans are continuously exposed to a low dose via inhaled air (5 ng day^{-1} in UK homes). The total exposure to HBCD via unintended dust ingestion, diet and air was estimated to be 549 ng day^{-1} (average) up to 2022 ng day^{-1} (for high exposure).⁴³ However, as we wanted to investigate the immune response to inhaled HDM, we chose to expose exclusively via the airways. Preliminary data indicated that a high dose was needed to show an effect. A preliminary experiment with a 52 fold lower concentration of HBCD (100

ng) did not affect the outcome of the measured immunological and clinical parameters (Figure S6). Also *in vitro* effects were only observed when HDM pulsed DCs were exposed to the highest concentration of 10 μ M HBCD. Together this indicates that an experimental high exposure was needed to demonstrate the immuno-modulatory effect of HBCD in this model. These experimental data might support further investigation of the respiratory health effects of HBCD in human.

In conclusion, the present study demonstrates that simultaneous exposure to HBCD during inhalation of HDM allergens can have a mild immune-modulating effect on the subsequent immune response. In different murine models for HDM allergy, exposure to HBCD increased either IL-17A production or IL-4 production. However, HBCD exposure did not increase airway hyperreactivity or recruitment of inflammatory cells. These data suggest that although the overall effect is very modest, HBCD can act as an unwanted danger signal which might contribute to the aberrant immune response which is induced in HDM allergy.

324 FIGURES



325

326 **Figure 1.** Study designs to investigate the effects of HBCD exposure to HDM induced
 327 immune response after (A) Adoptive DC transfer model: administration of HDM+/- HBCD
 328 pulsed BMDCs or unpulsed (DMSO pulsed) BMDCs in the airways at day 0. Mice were
 329 challenged with HDM extract at day 10. (B) Inhalation of HDM or LT-HDM (with only trace
 330 levels of endotoxin) +/- HBCD at day 0 and 7, followed by AHR measurement. All control
 331 groups received the same volume of DMSO (solvent of HBCD) instead of HBCD.

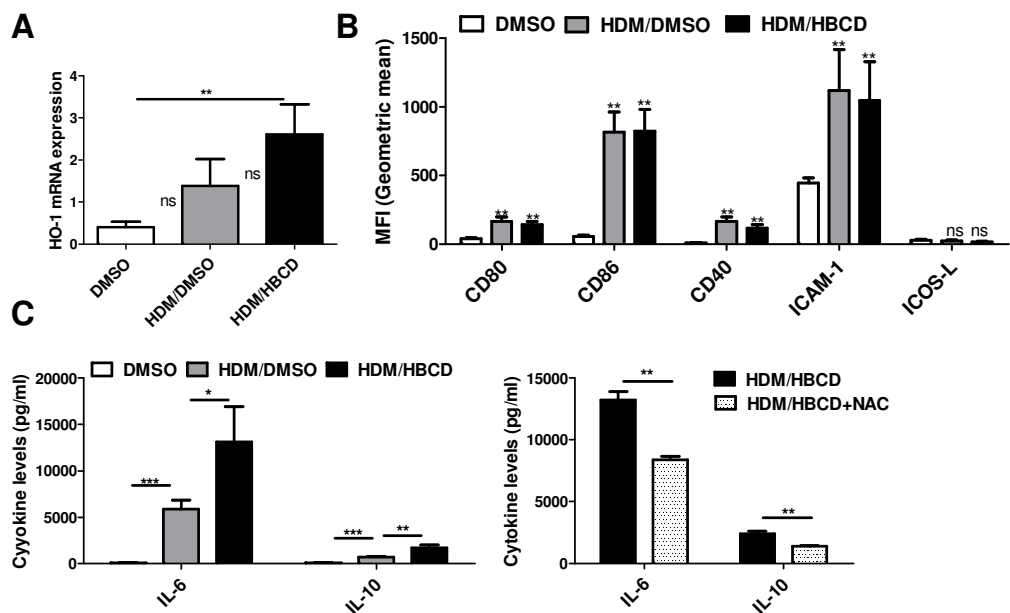


Figure 2. Activation of BMDCs following o/n exposure to HDM/DMSO, HDM/HBCD or as a control DMSO. A) mRNA expression of anti-oxidant enzyme Heme Oxygenase-1 (HO-1) in BMDCs. B) Expression of co-stimulatory molecules CD80, CD86, CD40, ICAM-1 and ICOS-L of BMDCs. C) Production of pro-inflammatory cytokines IL-6 and IL-10 by BMDCs in the presence or absence of ROS scavenger NAC. Data are presented as means $\pm$ SEM. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ vs DMSO exposed BMDCs. ns =not significant.

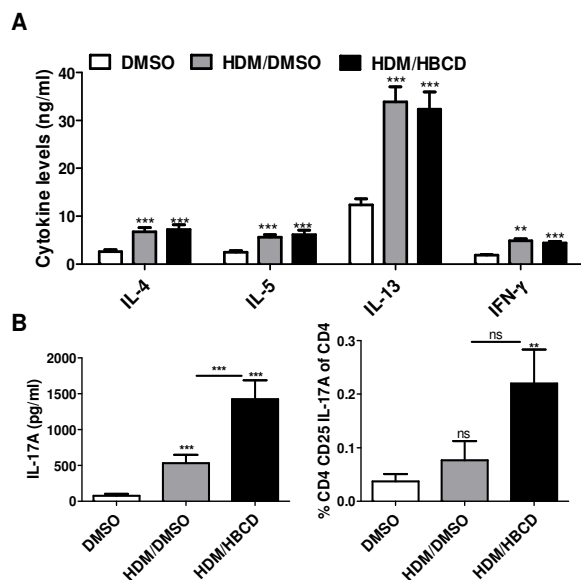


Figure 3. Mice were intratracheally instilled with HDM/DMSO, HDM/HBCD or DMSO exposed BMDCs. To investigate their potential to induce an immune response to inhaled HDM, mice were intranasally rechallenged with HDM at day 10. A) Production of cytokines IL-4, IL-5, IL-13 and IFN- γ in supernatants of *ex vivo* restimulated lung-draining lymph node cells with HDM B) Production of IL-17A (left panel) and the percentage of CD4⁺ CD25⁺ IL-17A of CD4 cells in lung-draining lymph node cells (right panel). Data are presented as means $\pm$ SEM. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ vs DMSO. ns =not significant.

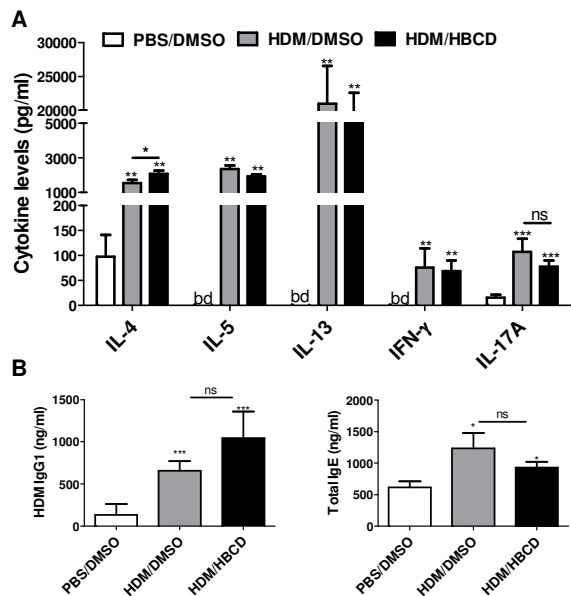


Figure 4. Mice were exposed via the airways with PBS/DMSO, HDM/DMSO or HDM/HBCD at day 0 and 7. A) Production of Th2 cytokines IL-4, IL-5, IL-13, Th1 cytokine IFN- γ and Th17 cytokine IL-17A in supernatants of *ex vivo* restimulated lung-draining lymph node cells with HDM. B) Concentration HDM specific IgG1 and total IgE in serum.

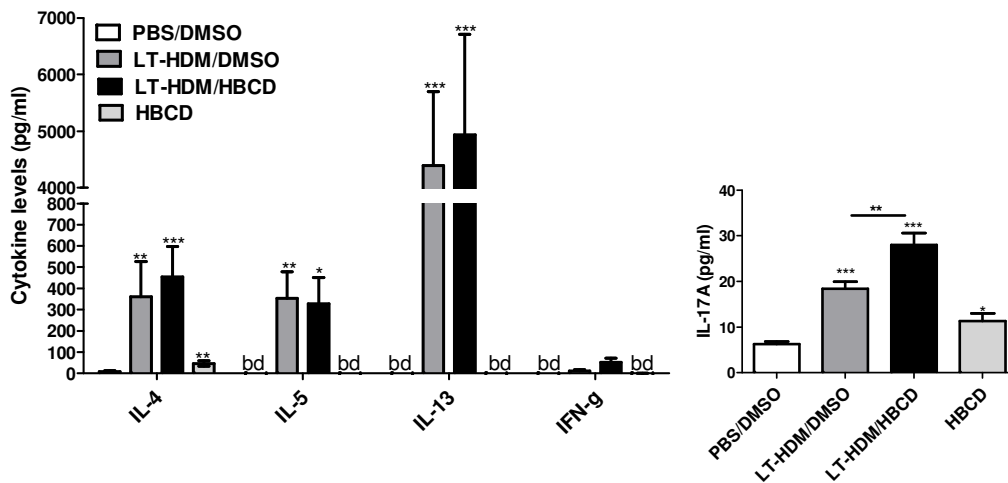


Figure 5. Direct effect of HBCD in the immune response to HDM containing trace amounts of endotoxin (LT-HDM). Mice were exposed via airways with PBS/DMSO, LT-HDM/DMSO, LT-HDM/HBCD or HBCD alone on day 0 and 7. Production of Th2 cytokines

IL-4, IL-5, IL-13, Th1 cytokine IFN- γ and, Th17 cytokine IL-17A was determined in supernatants of *ex vivo* re-stimulated lung-draining lymph node cells with HDM. Data are presented as means $\pm$ SEM. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ vs PBS/DMSO).

TABLES

Table 1. Primer sequences used for RT-PCR

Name	Sequence (5' $\rightarrow$ 3')
HPRT Fw:	GTC CTG TGG CCA TCT GCC TAG TA
HPRT Rv:	AAG TCT GGG GAC GCA GCA ACT
HO-1 Fw:	AGC AGA ACC CAG TCT ATG CCC CA
HO-1 Rv:	TGC CAG TGA GGC CCA TAC CAG AA

ASSOCIATED CONTENT

Supporting Information. Determination of HBCD concentration based on a dose response assay (Figure S1), the percentage of BMDCs expressing co-stimulatory molecules (Figure S2), effects of HDM/HBCD exposed BMDCs on induction of allergic immune response to inhaled HDM (Figure S3), effect of HBCD exposure on airway hyperreactivity and eosinophilic inflammation (Figure S4), analysis of IL-1 β and TGF- β production by BMDCs (Figure S5), and experimental design and analysis of allergenic markers for the effect of 100 ng HBCD exposure on the HDM induced allergic airway inflammation (Figure S6). This information is available free of charge via the Internet at <http://pubs.acs.org/>.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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