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Immunotoxicity of organophosphate flame retardants TPHP and TDCIPP on murine dendritic cells *in vitro*

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HIGHLIGHTS

- TPHP and TDCIPP can be immunotoxic for dendritic cells.
- TPHP has an immunomodulatory effect dependent on the maturation status of the dendritic cell.
- TDCIPP suppressed the house dust mite allergen induced maturation of dendritic cells.
- TPHP and TDCIPP induced oxidative stress in dendritic cells.

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ABSTRACT

Organophosphate flame retardants (PFRs) are commonly used as alternatives for the banned polybrominated diphenyl ethers (PBDEs) and are ubiquitously detected in indoor dust. PFRs can be potentially hazardous to respiratory health via the inhalation of house dust. Dendritic cells (DCs) are crucial in the immunological defense against pathogens in the airways. In respiratory allergy however, an aberrant immune response is induced against innocuous proteins, like house dust mite allergens. In this study, we examined whether exposure to PFRs Triphenylphosphate (TPHP) and Tris(1,3-dichloroisopropyl) phosphate (TDCIPP) affected activation/maturation of DCs at steady state and during exposure to house dust mite allergens (HDM). Bone marrow-derived dendritic cells (BMDCs) were exposed to a concentration range of each PFR (0.1–100 μM) with or without HDM *in vitro* to analyze the effect on the expression of major histocompatibility complex class II (MHCII), co-stimulatory molecules and cytokine production. Concentrations of TPHP and TDCIPP of ≥50 μM were cytotoxic to BMDCs. At these cytotoxic concentrations, TPHP exposure induced an activated phenotype in steady state DCs, while HDM exposed DCs acquired a tolerogenic phenotype. In contrast, TDCIPP exposure had no effect at steady state DCs but suppressed the expression of MHCII, costimulatory molecules, and the IL-6 production in HDM exposed DCs. The cytotoxic concentrations induced the anti-oxidant enzyme hemeoxigenase-1, which is a marker for oxidative stress. These results demonstrate that PFRs can be immunotoxic for DCs and suggest the necessity to evaluate the effects on the immune system on a cellular level during the risk assessment of these alternative flame retardants.

1. Introduction

Flame retardants have become a major indoor pollutant during the last decades. Until 2004, polybrominated diphenyl ethers (PBDEs) were the primary flame retardants, but they have been phased out in Europe and USA because of their properties of persistence and bio-accumulation as well as their potential to cause adverse health effects. The ban of PBDEs has resulted in an increase in the global use of organophosphate flame retardants (PFRs) which are often proposed as alternatives for the banned PBDEs (Van den Eede et al., 2011; van der Veen and de Boer, 2012). Environmental levels of PFRs have been well investigated and concentrations of PFRs such as tris(1,3-dichloroisopropyl)-phosphate (TDCIPP) and triphenylphosphate (TPHP) were found to exceed that of PBDEs (van der Veen and de Boer, 2012). It has been postulated that this

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was representing the increased usage. However, besides the use of TDCIPP and TPHP as additive flame retardants in polymers, resins, latexes and foams, they are also used in plasticizers, lacquer, paint, lubricant and TPHP in hydraulic fluids (van der Veen and de Boer, 2012). We have recently reported that in mattress dust collected more than two decades ago, PFRs exceeded the levels of PBDEs around sixty fold. TDCIPP and TPHP were present in 100% and 91% of the analyzed dust samples, respectively (Canbaz et al., 2016b). Thus, even before the ban, these "alternative" flame retardants were present in greater concentrations than the brominated flame retardants. The concentration of TDCIPP in house dust has been correlated with concentrations of its metabolite in human urine (Meeker et al., 2013).

Inhalation of house dust is one of the human exposure routes besides dermal contact and unintended ingestion of dust (Abdallah et al., 2008). Recently, total intake of chlorinated PFRs such as TDCIPP via inhalation has been estimated to exceed intake via dust ingestion (Schreder et al., 2016). The respiratory mucosal immune system is exposed continuously to inhaled proteins which has to result in the appropriate immune response, being either tolerogenic or immunogenic. The airways are covered with a network of dendritic cells (DCs) that monitor inhaled air continuously for microorganisms and other substances. DCs are able to recognize pathogens via different pattern recognition receptors. They process a large variety of antigens for presentation to naïve T cells. In this process, they display co-stimulatory molecules and secrete pro-inflammatory cytokines to interact and differentiate naïve T cells into a specific subset of effector T cells (von Garnier and Nicod, 2009; Willart and Hammad, 2010). In house dust mite allergy, a T helper 2 (Th2) type cell response is induced against innocuous proteins derived from the house dust mite feces (Lambrecht and Hammad, 2003, 2009). Environmental co-exposures have been implicated to play an important role in skewing the immune system towards (or away from) an inflammatory immune response to harmless allergens. Efforts are being made to elucidate how these co-factors contribute to Th2-skewing (Maes et al., 2010). Respiratory allergic disorders have become very common diseases in Western countries with an increasing prevalence since the 1970s (Eder et al., 2006). Although house dust mite allergy has been associated with the development of respiratory allergic diseases, the underlying mechanism for these immune system disorders are complex and the risk factors are multiple (Eder et al., 2006; Beasley et al., 2015). Ubiquitous detection of diverse chemical pollutants in indoor dust and increasing exposure levels have resulted in many studies aiming to elucidate their possible health effects. Indoor air pollutants have been recognized as risk factors for allergic airway diseases such as asthma (Nielsen et al., 2007; Hulin et al., 2012; Patelarou et al., 2015; Jiang et al., 2016).

The phosphate backbone is an important characteristic of PFRs, which they share with organophosphate-based pesticides that have been associated with exacerbations in allergic asthma (Proskocil et al., 2008; Hoppin et al., 2009; Hernandez et al., 2011). In concordance, a recent epidemiological study in Japan has revealed an association between increased concentrations of PFRs in house dust with allergic disorders such as allergic asthma, allergic rhinitis and atopic dermatitis (Araki et al., 2014). Although toxicological studies have revealed health threats of PFRs such as neurotoxic effects of TPHP, adverse effects of TPHP and TDCIPP on reproduction, and carcinogenicity of TDCIPP (van der Veen and de Boer, 2012; Bruchajzer et al., 2015), no study has investigated their potential immunomodulating effects.

Here we investigated whether exposure of DCs to PFRs affects the expression of MHCII, costimulatory molecules and the production of cytokines, at steady state and during exposure to house dust mite (HDM) allergens.

2. Materials and methods

2.1. Mice

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

2.2. Chemicals

TPHP (CAS no. 115-86-6; 99% purity) and TDCIPP (CAS no. 13674-87-8; 95.7% purity) were purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany). Stock solutions were prepared in dimethyl sulfoxide (DMSO).

2.3. Generation of bone marrow-derived DCs

Bone marrow-derived DCs (BMDCs) were obtained as described earlier (van Rijt et al., 2002). Briefly, BM cells were isolated from the tibiae and femurs of Balb/c mice. After red blood cell lysis, BM cells were cultured for ten days in Tissue Culture Medium (RPMI 1640, 5% FCS, 50 μg/ml gentamicin (Invitrogen), 0.05 mM 2-ME) with 20 ng/ml recombinant mouse granulocyte-macrophage colony stimulating factor (GM-CSF, Thermo Scientific, Rockford, IL).

2.4. In vitro exposure of BMDCs with PFRs

On day 9, BMDCs were exposed for 24 h to 0, 0.1, 1, 10, 50 or 100 μM TPHP or TDCIPP with or without 100 μg HDM/ml (Canbaz et al., 2016a), containing 1 μg/ml of Der p 1, a cysteine protease and one of the major allergens of *Dermatophagoides pteronyssinus* HDM (XPB82D3A2.5, Greer Laboratories; Lenoir, NC). Control exposures contained 0.1% DMSO to match the final concentration achieved in the culture medium in the experimental exposures.

2.5. BMDC phenotyping and cytokine production

At day 10, BMDCs were harvested, washed in FACS buffer (PBS containing 0.25% BSA, 0.05% sodium azide, 0.5 mM EDTA) and stained with antibodies against MHCII(FITC labeled), CD11c(APC labeled), in combination with PE labeled antibodies against costimulatory molecules (cell surface proteins) CD80, CD86 and CD40, or with isotype controls for rat IgG2a and, armenian hamster IgG (eBioscience Inc, San Diego, CA). To prevent non-specific antibody binding, anti-FcRγII/III antibody (clone: 2.4G2, provided by Louis Boon, Bioceros, Utrecht, The Netherlands) was added to the monoclonal antibody mixture. 10 μg/ml propidium iodide (PI, Sigma Aldrich Corp. St. Louis, MO) was used to distinguish between living (PI^-) and dead cells (PI^+). Flow cytometry was performed using a FACS Calibur (BD Biosciences, San Jose, CA). In all cases, 50000 events/sample was acquired. Expression of costimulatory molecules was analyzed on alive dendritic cells ($MHCII^+CD11c^+PI^-$) with FlowJo software (Tree Star Inc., Ashland, OR). IL-6 and IL-10 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).

2.6. Real time PCR on BMDCs

To determine oxidative stress induced by PFRs, the level of mRNA coding for heme-oxigenase-1 (HO-1), a protective anti-oxidant enzyme, was used as a marker for oxidative stress (Ryter and Tyrrell, 2000). Cultured BMDCs were exposed 7 h to HDM/