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Dibutyl phthalate exposure alters T-cell subsets in blood from allergen-sensitized volunteers

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Abstract

Phthalates are ubiquitous environmental contaminants associated with allergic disease in epidemiological and animal studies. This investigation aims to support these associations by interrogating systemic immune effects in allergen-sensitized volunteers after controlled indoor air exposure to a known concentration of dibutyl phthalate (DBP). The phthalate-allergen immune response (PAIR) study enrolled 16 allergen-sensitized participants to a double-blinded, randomized, crossover exposure to two conditions (DBP or control air for 3 hr), each followed immediately by inhaled allergen challenge. Peripheral blood immune cell composition and activation along with inflammatory mediators were measured before and after exposure. DBP exposure prior to the inhaled allergen challenge increased the percentage of $CD4^+$ T helper cells and decreased the percentage of regulatory T cells (3 hr and 20 hr post-exposure), while only modest overall effects were observed for inflammatory mediators. The cells and mediators affected by the phthalate exposure were generally not overlapping with the endpoints affected by allergen inhalation alone. Thus, in distinction to our previously published effects on lung function, DBP appears to alter endpoints in peripheral blood that are not necessarily enhanced by allergen alone. Further studies are needed to clarify the role of phthalate-induced systemic effects in disease pathogenesis.

KEYWORDS
allergen, blood immunology, crossover study, human controlled exposure, phthalates, T cells

1 | INTRODUCTION

Phthalates are synthetic diesters of phthalic acid, present in a variety of consumer products to enhance properties such as flexibility, transparency, durability, and longevity.[1,2] Industrial applications of phthalates include building materials, household furnishings, clothing, cosmetics, personal care products, pharmaceuticals, medical devices, children's toys, food packaging, and cleaning materials amongst others.[3] Due to their weak, non-covalent binding, phthalates leak out of their carrier plastics and become ubiquitous environmental contaminants.[2]

The presence of polyvinyl chloride (PVC) materials in homes and the phthalate levels in house dust have been associated with allergic diseases such as asthma and rhinitis in a range of epidemiological

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studies.[4,5] Experimental animal studies suggest that phthalates may exert adjuvant effects on basic mechanisms of allergic sensitization.[5] For example, adjuvant effects have been reported in the airways both in terms of increased recruitment of inflammatory cells and airway hyper-responsiveness.[6,7] However, systemic effects are not frequently described in animal models, except for adjuvant effects on production of total or allergen-specific serum IgE.[4,5]

The inflammatory response, usually triggered by infection or tissue injury, is driven by a complex network of cells, mediators, and signaling pathways. Dysregulation of cytokines and chemokines is linked to the onset and exacerbation of pathologies such as asthma and other allergic diseases,[8] while allergic diseases such as rhinitis and asthma alter the levels and phenotypes of circulatory cells, and inflammatory mediators in serum.[9,10] Controlled allergen inhalation is a research tool that induces several clinical and pathophysiologic features of asthma in sensitized individuals.[11] Systemic effects have been reported due to allergen inhalation both in terms of altered levels of circulating cells and inflammatory mediators.[12,13]

Although systemic effects have been given limited attention, exposures to environmentally relevant levels of phthalates are reported to affect both innate and adaptive immune cells and mediators in animal and cell culture models.[4] Phthalates either enhanced or attenuated the production and release of inflammatory mediators from macrophages and monocytes, while phagocytosis and antigen-presenting capacity were reduced.[4,14-17] Moreover, phthalates were reported to modulate differentiation of dendritic cells and their interaction with T cells, and also increase the release of Th2 mediators from lymphocytes in vitro.[18-23] Thus, phthalates may affect both innate and adaptive immune cells and mediators.

Human exposure to phthalates occurs through ingestion, inhalation, and dermal absorption, with ingestion being the dominant route for most phthalates.[24] Inhaled exposure is highest for low molecular weight phthalates, such as dibutyl phthalate (DBP), and these are also found in higher concentrations in indoor air than the high molecular weight phthalates.[25] Inhalation exposure may contribute to more than 20% of the daily internal dose for DBP, and this phthalate has also been shown to exhibit high inflammatory potential in vitro.[2,26] The role of inhalation and dermal uptake from indoor air is challenging to address in epidemiological studies.[4] However, Beko et al. (2015)[27] identified an important role for low molecular weight phthalates through indoor exposure routes in allergic sensitization by utilizing a modeling approach. Since there is a paucity of direct evidence of the effects of phthalates in humans, studies using an inhalation exposure to phthalates through controlled chambers have been recommended.[28]

The current study reports on systemic effects of exposure to DBP in indoor air prior to allergen inhalation in allergic individuals. The phthalate-allergen immune response (PAIR) study is the first randomized, controlled, crossover study utilizing exposure to a known air concentration of phthalate. In the airways, DBP exposure augmented allergen-induced lung function decline and exhibited immuno-modulatory effects.[29] Here, we tested the hypothesis that exposure to DBP in indoor air, prior to allergen inhalation, enhances allergen-induced systemic inflammation and alters immune cell composition and activation in peripheral blood.

Practical implications

- This study demonstrates that indoor exposure to dibutyl phthalate can alter circulating lymphocytes in sensitized individuals. Specifically, the results suggest that inhalation and dermal phthalate exposure can affect fundamental aspects of the systemic immune response.
- The results provide support for the associations between phthalate exposure and allergic diseases reported in epidemiological studies, highlighting the need for further studies addressing the role of the indoor exposure route.

2 | METHODS

2.1 | Study design and participant characteristics

The study design is previously reported,[29] but relevant elements are repeated here. Written informed consent was signed by study participants who enrolled in a double-blind, randomized, crossover study that was counter-balanced to order between two exposure conditions (DBP or control air, CA, Figure 1). Sixteen allergen-sensitized, non-asthmatic, or mildly asthmatic participants between the ages of 19 and 49 years were recruited (Table 1). Allergen sensitization was determined by 3 mm or greater wheal after skin prick to birch (Cat# LH1169ED; Omega Laboratories, Montreal QC, Hollister Stier), house dust mite (Cat# LH6692UP) or grass (Cat# LH0831TS). During screening, participants were classified based on their airway hyper-responsiveness (AHR) status as either hyper-responsive (methacholine PC_{20} ≤ 16 mg/mL) or normally responsive (methacholine PC_{20} > 16 mg/mL).[29]

Participants with asthma using oral or inhaled corticosteroids were excluded from the study. Throughout the study period, all study participants stopped bronchodilator, antihistamine, and/or nonsteroidal anti-inflammatory use 7 days prior to each exposure and during exposure days (with the exception of Ventolin, 2 inhalations of 100 mg each by metered dose inhaler, which was given routinely after methacholine challenge [by protocol, 20 hr after exposure]).

The 3-hr indoor air exposure took place at the Air Pollution Exposure Laboratory (APEL). The room's air exchange rate, temperature, volatile organic compounds, and humidity were measured and controlled throughout the duration of exposure. The chosen target level of 150 (mean 147.5, standard deviation 63.4) μg/m^3 of DBP was 10 times the maximum reported (15 μg/m^3) indoor level, to ensure a controlled exposure level significantly higher than the environmental background exposure.[25,29] The time-weighted

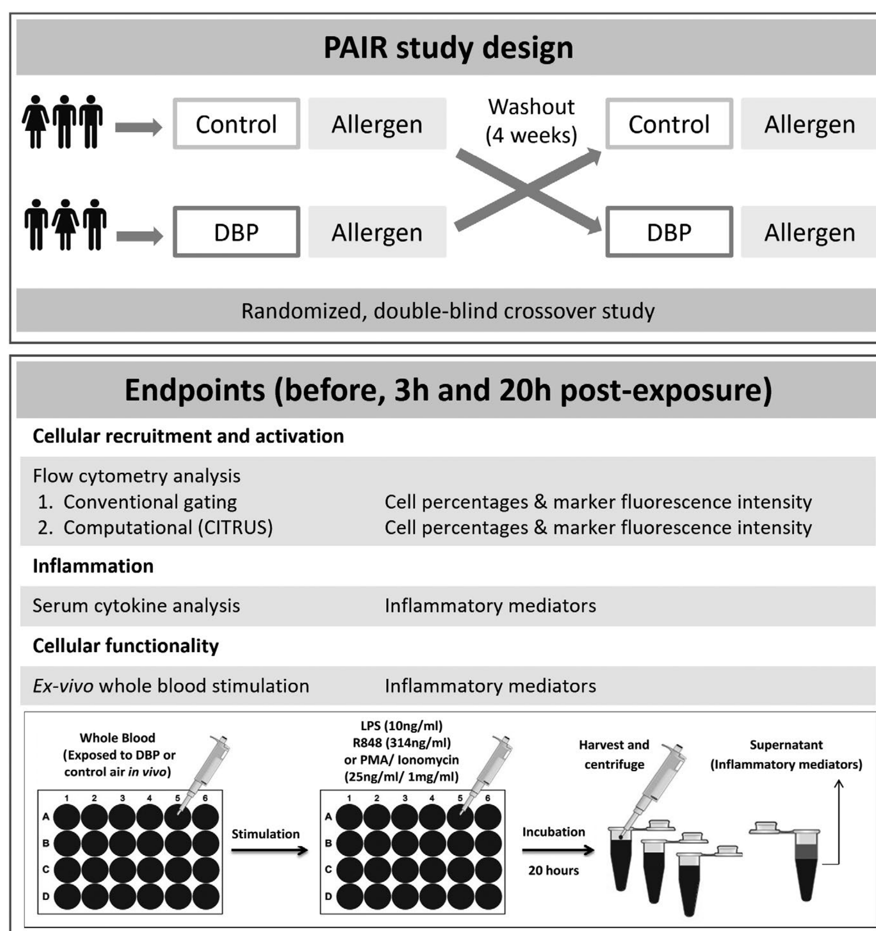

FIGURE 1 Overview of study design and endpoints. Consenting participants were screened and randomly assigned to two groups for a double-blinded crossover exposure to DBP and control. Cellular and inflammatory endpoints were assessed before, 3 and 20 hr post-exposure

average concentration for DBP exposure was well below the 8 hr time-weighted exposure limit of 5000 μg/m^3 recommended by the National Institute for Occupational Safety and Health.

A well-characterized doped latex paint exposure method[30] used previously in a human DBP exposure study[31] was followed, adapted to the APEL room specifications using paint doped with 10% DBP (Sigma Aldrich #18281) by weight. For the control exposures, latex paint without DBP was applied. The previous human exposure study assessed dermal and inhalation exposure and, to mimic their exposure conditions, our participants wore a short hospital gown and shorts during exposures, resulting in both inhalation and dermal exposure to DBP.

To assess the early allergen response, an inhaled allergen challenge to the specific allergen determined at screening was conducted immediately after each exposure (DBP or control air). The specific allergen inhalation dose was estimated based on minimal concentration to provoke a 3 mm wheal size in the skin prick test and methacholine PC_{20} percent drop and concentration, as previously described.[32] Then, an allergen challenge was conducted, starting with a saline dilution 4 times lower than the estimated allergen PC_{20} dose, until a 20% drop in FEV_1 was achieved. During the exposure visits, a participant-specific 2-min inhaled allergen challenge was performed immediately after exposure. Each exposure, for a given individual, was separated by at least 4 weeks to avoid carryover effects. Controlled inhalation challenges to grass allergen were only performed outside the pollen season.

The primary outcomes for the previously published PAIR study, as reported to ClinicalTrials.gov, were all related to airway responses,[29] while the systemic responses reported here were listed as secondary endpoints.

2.2 | Sample analysis

Peripheral blood was collected into sodium heparin and serum tubes (Vacutainer), before (−4 hr), 3 and 20 hr post-DBP/CA exposure and processed immediately, where 0 hr reflects the allergen inhalation time point. Whole blood from sodium heparin tubes was used to analyze the cellular fraction (see below), while serum samples were prepared from serum tubes by a 10-minute centrifugation at 500*g*, and stored at −80°C until inflammatory mediator analysis.

For immune cell phenotyping and assessment of activation of cell-surface markers, blood cells were stained with fluorochrome-conjugated antibodies against surface and intracellular proteins (Table S1), following supplier's instructions (BD Biosciences), and single cell data acquired on a BD FACSCanto II flow cytometer (BD Biosciences). Conventional flow cytometry analysis was performed using FCS Express version 6.04.0034 (De Novo Software), following

a manual gating strategy to identify the frequency of major cell subpopulations (Figure S1).

For analysis of inflammatory mediators in serum, samples were thawed immediately before analysis with a human cytokine/chemokine 65-plex panel (Eve Technologies, AB). The 65 mediators included chemokines, Th2-related cytokines, regulators of normal immune cell function and maturation, and growth factors (Table S2).

Cellular functionality was assessed by *ex vivo* stimulation of whole blood as previously described.[15] Briefly, whole blood diluted 1:1 vol/vol with RPMI-1640 Medium (Millipore-Sigma) plus penicillin-streptomycin (Thermo Fisher Scientific) was stimulated with LPS (10 ng/ml, from E. coli 026:B6, Millipore-Sigma), R848 (314 ng/ml, Enzo Life Sciences) or PMA/Ionomycin (25/1000 ng/ml/Abcam), as shown in Figure 1. After a 20-hr incubation at 37°C, 5% CO_2, the samples were centrifuged at 450*g* for 10 min and supernatants were collected, stored at −80°C and examined for the presence of 10 inflammatory mediators (Custom Human Cytokine 10-Plex Panel, Eve Technologies), as noted in Table S2.

TABLE 1 Overview of participant characteristics

Participant	Methacholine PC_{20} (mg/mL)	AHR status	Allergen	Sex	Age
1	>128	No	Grass	M	26
2	>128	No	Birch	F	29
3	6.9	Yes	HDM	F	45
4	2	Yes	Grass	M	29
5	>128	No	Grass	F	26
6	>128	No	HDM	M	31
7	2.9	Yes	Grass	F	46
8	9.1	Yes	HDM	F	45
9	64	No	Birch	F	34
10	>128	No	Grass	M	26
11	1.7	Yes	HDM	F	33
12	121.1	No	HDM	F	21
13	47.9	No	Birch	F	21
14	0.3	Yes	Birch	F	23
15	16	Yes	Birch	M	27
16	14.5	Yes	HDM	M	36

Abbreviations: AHR, airway hyper-responsiveness; F, female; HDM, house dust mite; M, male;PC20, provocative concentration (that causes a 20% drop).

2.3 | Statistical analysis

Statistical analysis to assess effects of DBP and effect modifiers was performed in RStudio (R version 3.4.1). To evaluate the overall effect of DBP exposure, a linear mixed effects (LME) model was applied, with participant ID as a random effect, and exposure condition (DBP or CA) as a fixed effect. All LME analyses were performed for delta values, calculated for 3 and 20 hr timepoints relative to the baseline (−4 hr), for each individual and each exposure condition. To explore the impact of effect modification, three potential effect modifiers, AHR-status (yes or no), sex (male or female), and the type of allergen inhaled (grass, house dust mite or birch), were included in separate models where exposure-by-modifier interaction was considered as an additional fixed effect. For all analyses, specific unadjusted $p < 0.05$ are reported.

In this novel study, we purposefully applied these statistical models to a wide range of endpoints, spanning from immune cellular phenotypes and activation markers, to inflammatory mediators and cellular functionality. We included such a range as it was not the primary objective of our study to assess effects of DBP on a particular systemic marker for definitive clinical or policy implementation, that is why we explicitly considered these as secondary at study conception and so designate them here as exploratory endpoints. Therefore, no adjustment for multiple comparisons was performed in the LME analysis.

In addition to the conventional analysis of the flow cytometry data, unsupervised computational analysis was performed with Cytobank (http://www.cytobank.org) to assess the effect of phthalate exposure. The CITRUS (cluster identification, characterization, and regression) algorithm was applied, designed for the fully automated discovery of statistically significant biological signatures within single cell datasets.

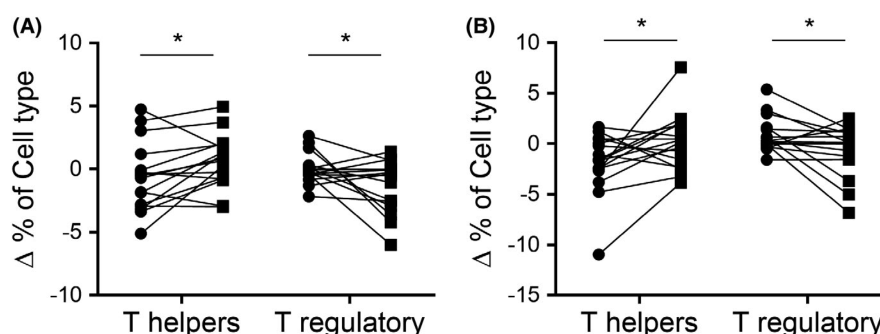

FIGURE 2 Effect of DBP exposure on blood cells. The figures show the overall DBP effect (squares) compared with control air (circles). Data reflect delta values relative to baseline for (A) 3 hr and (B) 20 hr post-exposure for all the participants, n = 16. Note that the 0 hr timepoint reflects the time of the allergen inhalation immediately after the end of the 3 hr controlled indoor air exposure. (*) $p < 0.05$ for effect of DBP over control for unadjusted *p*-values

To evaluate whether the effects of DBP were associated with the allergen inhalation challenge, analysis of systemic cellular and humoral endpoints effects of the allergen inhalation per se was performed. Data from the control air exposure condition were analyzed by repeated measures one-way ANOVA with a Dunnet post hoc test, in GraphPad (v.6.01), to assess significant changes relative to the −4 hr time-point. In doing so, we recognize that differences between 3 and −4 hr could be due to either allergen-induced or diurnal effects, as these paired samples were collected at different times of the day.

3 | RESULTS

3.1 | Acute responses in blood after DBP exposure

Compared with air, DBP exposure followed by inhaled allergen challenge increased the percentage of $CD4^+$ T helper cells ($p = 0.02$) and decreased the percentage of regulatory T cells ($p = 0.03$) in blood 3 hr post-exposure, and these effects persisted at 20 hr post-exposure ($p = 0.03$ for both) (Figure 2). DBP also decreased the percentage of non-classical monocytes at 20 hr post-exposure ($p = 0.05$), while activation markers and other leukocyte populations were not significantly altered by DBP exposure (Table S3). The data-driven unsupervised CITRUS analysis supported these findings from conventional gating, showing an increase in the percentage of four clusters characterized as $CD4^+$ T helper cells. In addition, the CITRUS analysis showed an increase in the percentage of a cluster characterized as $CD8^+$ T cytotoxic cells and the activation state (increased MFI for CD69) for two of the $CD4^+$ T helper subpopulations, at 3 hr post-exposure (Figure S2).

Few of the 65 serum inflammatory markers were statistically significantly affected by exposure to DBP compared with air, except for an increase in the chemoattractant SDF-1α+β ($p = 0.03$) and a reduction in Th2-mediator TSLP ($p = 0.04$). Similarly, the effect of DBP inhalation on *ex vivo* mediator release after stimulation of whole blood with LPS, R848, or PMA/Ionomycin was limited except reduced IL-1β ($p = 0.02$) release after stimulation with R848 in blood collected at 20 hr (note that *ex vivo* mediator analysis was not included in further analysis).

3.2 | Interaction effects of sex and allergen inhaled on cellular endpoints

In the interaction model for sex, the effect of DBP in the decreased percentage of regulatory T cells was stronger in male subjects at both timepoints ($p = 0.04$ at 3 hr; $p = 0.03$ at 20 hr), although the interaction effect was not significant (Figure 3). In Th17 cells, there was a significant interaction effect at 3 hr for the activation marker CD196 ($p = 0.003$), while the effect of DBP was significant in males at 3 hr ($p = 0.01$) and in females at 20 hr ($p = 0.04$) (Figure 3). Some

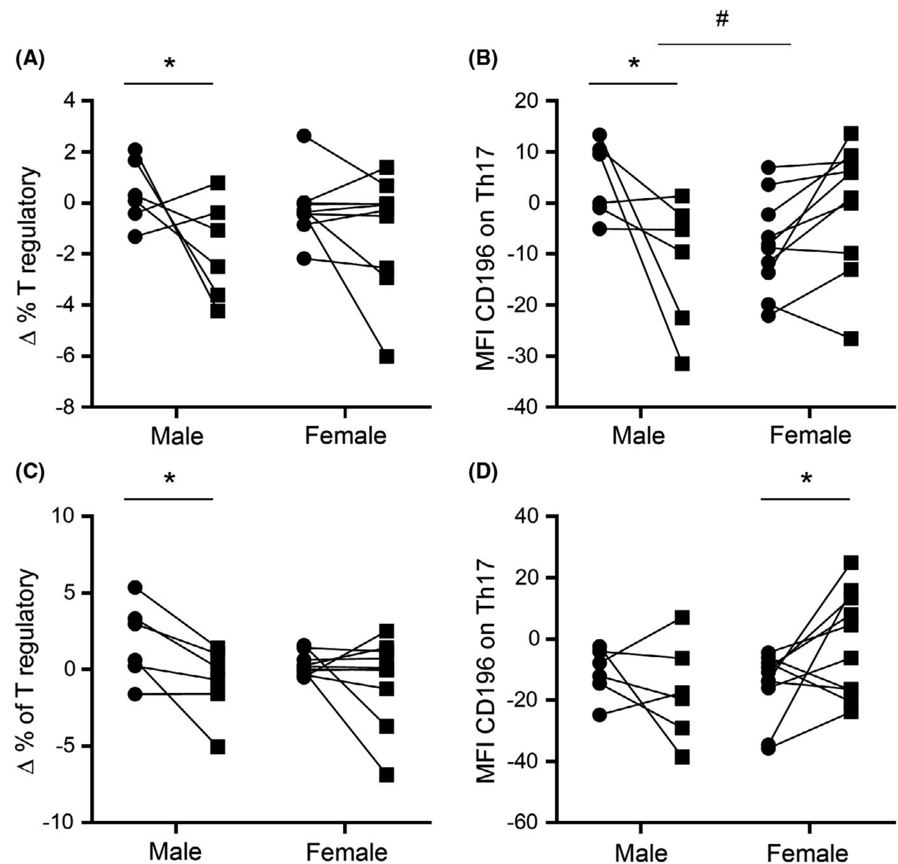

FIGURE 3 Sex modification of DBP effect on blood cells. The figures show the effect of DBP effect (squares) compared with control air (circles) stratified for males and females for the (A-B) 3 hr and (C-D) 20 hr timepoints. Data indicate delta values relative to baseline for 3- and 20 hr post-exposure for all the participants, n = 16. Note that the 0 hr timepoint reflects the time of the allergen inhalation immediately after the end of the 3 hr controlled indoor air exposure. The figure shows results from the interaction LME model where exposure-by-sex interaction was considered as a fixed effect in addition to the exposure; (*) $p < 0.05$ for effect of DBP compared with control air in males or females, respectively, and (#) $p < 0.05$ for interaction effect of sex, for unadjusted p-values

activation markers such as CD24 expression on eosinophils and neutrophils, and CD196 in Th17 cells, were significantly reduced in males only at the 3 hr time-point ($p = 0.03$ for all) (Table S4).

The type of allergen inhaled also modified the effect of DBP on cellular endpoints primarily at the 20 hr timepoint, and the effect of DBP was stronger in subjects exposed to grass (Table 2). There was a significant interaction effect at 20 hr for percentages of monocytes, non-classical monocytes, eosinophils, neutrophils and B cells, and also the neutrophil granularity (Table 2). The percentage of non-classical monocytes was reduced in subjects exposed to grass at 3 hr ($p = 0.02$) and 20 hr ($p = 0.002$), while percentages of monocytes and eosinophils were only reduced at 20 hr ($p = 0.02$ and $p = 0.01$) (Table 2). There were no significant interaction effects for AHR-status for the cellular data (data not shown).

3.3 | Interaction effects of sex and AHR-status on inflammatory mediators

The effect modification by sex was significant for several chemoattractants at 3 hr with significantly increased levels in males relative to control air (Eotaxin-1, $p = 0.01$; MCP-1, $p = 0.02$; MCP-2, $p = 0.01$) and for pro-inflammatory mediators at 20 hr with decreased levels in males relative to control air (IL-6, $p = 0.07$, IL-8, $p = 0.03$; IL-17A, $p = 0.07$) (Table 3). Moreover, effect modification by sex was significant for the Th2-cytokine IL-13 for both timepoints and for TSLP at 20 hr (Table 3). While DBP exposure reduced the IL-13 and TSLP levels significantly in males at both timepoints, the decrease seemed to be driven by two-three subjects and should be interpreted with caution.

The effect modification by AHR-status was significant for the regulatory cytokine TGFβ1 for both timepoints, and DBP increased the TGFβ1 in non-AHR participants with 163% ($p = 0.03$) and 132% ($p = 0.03$) relative to control air (Table 4). At the 20 hr timepoint, effect modification by AHR-status was also significant for several Th2 cytokines that were all reduced in non-AHR participants by DBP exposure relative to control (IL-13, $p = 0.04$; IL-33, $p = 0.02$; TSLP, $p = 0.004$). The IL-13 levels were also significantly reduced in non-AHR participants at 3 hr ($p = 0.048$).

The effect modification by type of allergen inhaled was less consistent than for sex and AHR-status, and there were no clear patterns across the two timepoints (data not shown).

3.4 | Effects due to the allergen inhalation alone

To evaluate whether the effects of DBP reflect an enhancement of the allergen-induced effects, the significant findings reported above were compared to the endpoints affected by allergen inhalation per se. There was no overlap between the endpoints affected by DBP exposure in the main LME model and the cellular and mediator responses affected by allergen inhalation alone in the one-way ANOVA (Table 5).

4 | DISCUSSION

Indoor air exposure to DBP, followed by an allergen inhalation challenge, increased the percentage of CD^+ T helper cells in peripheral blood and reduced the proportion of regulatory T cells at both 3 and 20 hr post-exposure. Assessment of inflammatory mediator levels and cellular functionality revealed a limited number of additional DBP-induced effects. Assessment of effect modification revealed that effects of DBP on cellular endpoints were generally stronger in males and grass-sensitized individuals, while the effects on inflammatory mediators were stronger in males and non-AHR participants.

Epidemiological studies linking phthalate exposure to allergic diseases have been complemented by experimental animal studies, in which allergic adjuvant effects are presented as a possible cellular mechanism, that is, that repeated phthalate exposure prior to sensitization results in promoted or aggravated allergic responses toward the allergen.[4,5] In the present study, the acute systemic effects on T helper subsets, inflammatory mediators and cellular functionality associated with exposure to both DBP and allergen showed a pattern distinct from that in response to allergen inhalation alone. Therefore, these systemic DBP effects up to 20 hr after exposure did not appear to simply reflect an enhancement of allergen-induced effects. Although most studies report limited or no effects of phthalates in the absence of a model allergen, a recent study in mice reported effects of diethylhexyl phthalate (DEHP) alone. Specifically, DEHP nasal instillation increased the dendritic and Th17 cells numbers, and IL-17A, IL-6, and MCP-1 inflammatory mediator levels.[33] The current observation of a significant effect modification by the type of allergen inhaled on DBP's effect on cell percentages suggests that the phthalate effect is somehow influenced by the allergen challenge or, alternatively, simply a function of the subject's inherent sensitization status. Further studies are required to determine definitively whether the effects observed in the PAIR study are a result of DBP and allergen co-exposure, or a response toward the chemical itself in sensitized individuals.

The overall increase in the proportion of $CD4^+$ T helper cells, at 3 and 20 hr post-DBP exposure, could be the result of an expansion of already present and previously primed memory T cells. T helper cell functions are complex and heterogeneous, and the original characterization of the Th1 and Th2 pathways has now been expanded to include additional subsets, such as Th9 (not measured), Th17, and regulatory T cells, each with their own cytokine repertoire and transcription factors.[34] In addition, memory T cells are a long-lived subtype that provide an enhanced, faster and stronger immune response upon re-exposure to a pathogen or antigen.[35] Since our study participants are sensitized and previously exposed to the allergen, it is plausible that they have this population ready for expansion. Based on the current results, further controlled exposure studies including specific memory T cell markers are recommended.

Our finding of reduced numbers of regulatory T cells and non-classical monocytes in peripheral blood at 3 hr and/or 20 hr could reflect a migration of these cells to the affected tissue, specifically the lungs. In support of this hypothesis, our group previously reported

TABLE 2 Effect modification by type of allergen inhaled

Cell population/activation marker	Control intercept	DBP effect (Δ ± SE)	DBP effect (% change)	*p*-value (DBP)	*p*-value (interaction)
3 hr					
% Monocytes	−0.03	−1.01 ± 0.84	−3366.67	0.25	0.08
% Classical Mo	−2.18	3.69 ± 6.77	169.27	0.60	0.45
% Non-classical Mo	1.18	−7.32 ± 2.62	−620.34	0.02*	0.05
MFI CD16	255.66	−524.27 ± 186.95	−205.07	0.02*	0.01*
% Eosinophils	−2.74	−0.55 ± 1.47	−20.07	0.71	0.19
MFI CD24	166.96	−281.39 ± 124.74	−168.54	0.04*	0.15
Granularity	−14.20	22.20 ± 16.57	156.34	0.20	0.15
% Neutrophils	3.53	−0.28 ± 1.51	−7.93	0.86	0.38
MFI CD24	44.91	−103.74 ± 49.87	−231.00	0.06	0.21
Granularity	−5.80	15.40 ± 16.69	265.52	0.37	0.16
% T helpers	0.37	1.38 ± 0.91	372.97	0.15	0.49
% Th1	0.90	−1.12 ± 2.53	−124.44	0.67	0.79
% Th2	−0.98	−1.08 ± 3.20	−110.20	0.74	0.60
%Th17	1.66	−2.01 ± 1.35	−121.08	0.16	0.20
MFI CD196	−1.27	−11.29 ± 7.57	−888.98	0.16	0.25
% regulatory T cells	−0.35	−0.21 ± 0.98	−60.00	0.83	0.91
% NK cells	0.08	−0.20 ± 0.28	−250.00	0.51	0.55
% B cells	−2.13	0.34 ± 1.09	15.96	0.76	0.39
20 hr					
% Monocytes	0.90	−2.28 ± 0.88	−253.33	0.02*	0.04*
% Classical Mo	−3.54	1.72 ± 8.63	48.6	0.85	0.58
% Non-classical Mo	5.33	−12.67 ± 3.34	−237.7	0.002*	0.01*
MFI CD16	260.80	−554.49 ± 255.58	−212.6	0.049*	0.16
% Eosinophils	2.51	−1.89 ± 0.61	−75.3	0.01*	0.001*
MFI CD24	178.98	−224.69 ± 119.86	−125.5	0.08	0.17
Granularity	−9.90	38.30 ± 24.46	386.87	0.14	0.14
% Neutrophils	−1.59	0.94 ± 0.65	59.1	0.17	0.01*
MFI CD24	63.39	−76.77 ± 61.89	−121.1	0.24	0.38
Granularity	−24.40	51.40 ± 20.28	210.66	0.03*	0.047*
% T helpers	−1.04	2.33 ± 1.66	224.0	0.19	0.54
% Th1	−0.64	3.70 ± 3.09	578.1	0.25	0.07
% Th2	−1.13	4.27 ± 3.61	377.9	0.26	0.26
%Th17	−2.26	2.48 ± 1.38	109.7	0.10	0.12
MFI CD196	−13.51	−3.27 ± 9.29	−24.2	0.73	0.34
% regulatory T cells	1.03	−1.40 ± 1.29	−135.9	0.30	0.92
% NK cells	0.26	−0.33 ± 0.34	−126.9	0.34	0.22
% B cells	1.90	−1.16 ± 0.66	−61.05	0.10	0.04*

Note: The table shows results from the interaction LME model for cellular endpoints in blood at 3 and 20 hr, where exposure-by-allergen interaction was considered as a fixed effect in addition to the exposure. The effects of DBP in grass sensitized individuals are listed for the main classes of immune cells, as well as activation markers. The table lists the control intercept, the DBP effect in grass sensitized individuals (increase or decrease in numerical mean relative to control air (intercept)) ± standard error, percent change (relative to control air), unadjusted *p*-values for the effect of DBP, and the interaction effect. The listed results reflect LME analysis for delta values, calculated for 3 and 20 hr timepoints relative to the baseline (−4 hr) with n = 16.

Abbreviations: CD, cluster of differentiation; DBP, dibutyl phthalate; LME, linear mixed effect; MFI, mean fluorescence intensity; Mo, monocytes; NK, natural killer; SE, standard error; Th1, T helper type 1; Th17, T helper type 17; Th2, T helper type 2.

*Reflects significant effects, that is, $p < 0.05$.

TABLE 3 Effect modification by type of sex

Mediator	Control intercept	DBP effect (Δ ± SE)	DBP effect (% change)	*p*-value (DBP)	*p*-value (interaction)
3 hr					
Eotaxin-1	−16.73	24.11 ± 8.37	147.28	0.01*	0.045*
MCP-1	−78.13	108.67 ± 42.03	139.09	0.02*	0.04*
MCP-2	−1.57	6.10 ± 1.90	388.54	0.01*	0.01*
RANTES	−86.44	91.11 ± 52.18	105.40	0.10	0.15
IL-4	6.43	−4.62 ± 2.09	−71.85	0.04*	0.07
IL-13	1.51	−3.27 ± 1.27	−216.56	0.02*	0.03*
IL-33	−1.29	1.17 ± 4.45	90.70	0.80	0.90
TSLP	9.82	−16.29 ± 6.58	−165.89	0.03*	0.08
IL-1b	1.59	0.60 ± 0.38	37.74	0.14	0.28
IL-6	−0.56	0.31 ± 0.83	55.36	0.72	0.81
IL-8	1.22	−1.20 ± 1.12	−98.36	0.30	0.42
IL-17A	3.57	−3.90 ± 1.95	−109.24	0.07	0.07
TNFα	3.98	−2.19 ± 1.04	−55.03	0.05	0.02*
TGFβ1	189.57	415.09 ± 2740.11	218.96	0.88	0.60
IFNα2	5.44	−5.78 ± 5.93	−106.25	0.35	0.31
PDGF-BB	−140.19	−51.66 ± 1035.91	−36.85	0.96	0.65
20 hr					
Eotaxin-1	19.43	−10.74 ± 14.51	−55.53	0.47	0.86
MCP-1	67.62	−60.59 ± 45.16	−89.60	0.20	0.38
MCP-2	1.33	4.42 ± 2.03	332.33	0.047*	0.14
RANTES	−67.19	38.37 ± 41.03	57.11	0.37	0.13
IL-4	4.93	0.28 ± 2.23	5.68	0.90	0.38
IL-13	6.55	−4.98 ± 1.65	−76.03	0.01*	0.01*
IL-33	0.92	−1.99 ± 7.11	−216.30	0.78	0.51
TSLP	13.19	−21.45 ± 6.75	−162.62	0.01*	0.04*
IL-1b	1.91	−2.78 ± 1.35	−145.55	0.06	0.17
IL-6	1.00	−1.11 ± 0.56	−111.00	0.07	0.02*
IL-8	3.24	−3.68 ± 1.47	−113.58	0.03*	0.02*
IL-17A	4.80	−4.41 ± 2.28	−91.88	0.07	0.04*
TNFα	4.48	−3.89 ± 2.54	−86.83	0.15	0.08
TGFβ1	15.51	−509.36 ± 2401.95	−3284.07	0.84	0.34
IFNα2	1.47	−9.02 ± 3.88	−613.61	0.04*	0.19
PDGF-BB	373.82	1025.12 ± 1069.91	274.23	0.35	0.05

Note: The table shows results from the interaction LME model for cellular endpoints in blood at 3 and 20 hr, where exposure-by-sex interaction was considered as a fixed effect in addition to the exposure. The effects of DBP in males are listed in pg/ml for a selection of mediators (see online supplemental material for 65-plex panel analyzed). The table lists the control intercept, the DBP effect in males (increase or decrease in numerical mean relative to control air (intercept)) ± standard error, percent change (relative to control air), unadjusted *p*-values for the effect of DBP and the interaction effect. The listed results reflect LME analysis for delta values, calculated for 3 and 20 hr timepoints relative to the baseline (−4 hr). n = 16.

Abbreviations: AHR, airway hyper-responsiveness; DBP, dibutyl phthalate; IFN, interferon; IL, interleukin; MCP, monocyte chemoattractant protein; PDGF-BB, platelet-derived growth factor subunits BB; RANTES, regulated on activation, normal T cell expressed and secreted; SE, standard error; TGF, transforming growth factor; TNF, tumor necrosis factor; TSLP, thymic stromal lymphopoietin.

*Reflects significant effects, that is, $p < 0.05$. Dotted lines separate mediators into 4 main functionality groups; chemoattractants, Th2-related, pro-inflammatory, and regulatory (acknowledging that some have a wide range of biological activity and cross-function).

increased levels of MIP-1α and fractalkine in lung lavage samples 20 hr post-DBP exposure in the same subjects,[29] both implicated in the migration of leukocytes to the lung.[36,37] Moreover, the percentage of macrophages was increased in bronchoalveolar lavage (BAL) accompanied by a skewing toward M2-type macrophages, consistent with the migration of non-classical monocytes out of the circulation and to the lung. The observed reduction in regulatory T cells may further support a pro-allergic effect of DBP, since reduced

TABLE 4 Effect modification by AHR-status

Mediator	Control intercept	DBP effect (Δ ± SE)	DBP effect (% change)	*p*-value (DBP)	*p*-value (interaction)
3 hr					
Eotaxin-1	−11.96	10.95 ± 8.39	91.56	0.21	0.82
MCP-1	−11.90	24.58 ± 42.69	206.55	0.57	0.81
MCP-2	−0.52	0.45 ± 2.00	86.54	0.82	0.35
RANTES	12.06	−48.55 ± 45.04	−402.57	0.30	0.03*
IL-4	2.86	−0.69 ± 2.04	−24.13	0.74	0.67
IL-13	0.89	−2.46 ± 1.13	−276.40	0.048*	0.05
IL-33	−5.58	1.61 ± 3.70	28.85	0.67	0.74
TSLP	6.87	−11.59 ± 5.91	−168.70	0.07	0.23
IL-1b	1.02	0.57 ± 0.32	55.88	0.10	0.20
IL-6	−0.44	−0.05 ± 0.72	−11.36	0.94	0.70
IL-8	0.91	−1.27 ± 0.94	−139.56	0.20	0.25
IL-17A	1.50	−1.19 ± 1.91	−79.33	0.54	0.79
TNFα	1.91	−0.96 ± 1.04	−50.26	0.37	0.27
TGFβ1	−3150.15	5134.58 ± 2171.69	162.99	0.03*	0.04*
IFNα2	2.84	−2.23 ± 5.31	−78.52	0.68	0.72
PDGF-BB	215.98	354.77 ± 853.23	164.26	0.68	0.21
20 hr					
Eotaxin-1	1.93	5.90 ± 11.57	305.70	0.62	0.04*
MCP-1	35.97	−25.44 ± 40.25	−70.73	0.54	0.93
MCP-2	−0.84	0.86 ± 1.87	102.38	0.65	0.46
RANTES	8.03	−39.91 ± 36.56	−497.01	0.29	0.33
IL-4	5.52	−1.39 ± 1.98	−25.18	0.49	0.97
IL-13	4.28	−3.57 ± 1.57	−83.41	0.04*	0.046*
IL-33	5.48	−14.85 ± 5.66	−270.99	0.02*	0.04*
TSLP	11.63	−19.67 ± 5.75	−169.13	0.004*	0.03*
IL-1b	1.39	−2.37 ± 1.18	−170.50	0.07	0.19
IL-6	0.81	0.05 ± 0.59	6.17	0.94	0.95
IL-8	0.10	−0.64 ± 1.56	−640.00	0.69	0.96
IL-17A	1.31	−1.38 ± 2.26	−105.34	0.55	0.56
TNFα	2.21	−1.47 ± 2.41	−66.52	0.55	0.45
TGFβ1	−3439.02	4553.72 ± 1935.78	132.41	0.03*	0.04*
IFNα2	2.84	−8.88 ± 3.37	−312.68	0.02*	0.11
PDGF-BB	438.58	−653.07 ± 1062.78	−148.91	0.55	0.89

Note: The table shows results from the interaction LME model for cellular endpoints in blood at 3 and 20 hr, where exposure-by-AHR-status interaction was considered as a fixed effect in addition to the exposure. The effects of DBP in non-AHR participants are listed in pg/ml for a selection of mediators (see online supplemental material for 65-plex panel analyzed). The table lists the control intercept, the DBP effect in non-AHR participants (increase or decrease in numerical mean relative to control air (intercept)) ± standard error, percent change (relative to control air), unadjusted *p*-values for the effect of DBP and the interaction effect. The listed results reflect LME analysis for delta values, calculated for 3 and 20 hr timepoints relative to the baseline (−4 hr). n = 16.

Abbreviations: AHR, airway hyper-responsiveness; DBP, dibutyl phthalate; IFN, interferon; IL, interleukin; MCP, monocyte chemoattractant protein; normal T cell expressed and secreted; PDGF-BB, platelet-derived growth factor subunits BB; RANTES, regulated on activation; SE, standard error; TGF, transforming growth factor; TNF, tumor necrosis factor; TSLP, thymic stromal lymphopoietin.

*Reflects significant effects, that is, $p < 0.05$. Dotted lines separate mediators into 4 main functionality groups (acknowledging that some have a wide range of biological activity and cross-function); chemoattractants, Th2-related, pro-inflammatory, and regulatory (acknowledging that some have a wide range of biological activity and cross-function).

blood levels of this T-cell subtype has been associated with allergic phenotypes in some studies.[38-40]

DBP exposure only affected a few of the inflammatory mediators in serum, but the increased levels of leukocyte chemoattractant SDF-1α+β support the interpretation of a possible leukocyte migration to the affected tissue.[41] The epithelial derived mediator TSLP, is associated with Th2 inflammation and airway hyper-responsiveness, and plays a major role in asthma pathogenesis.[42] However, the

TABLE 5 Comparison of significant endpoints for DBP exposure vs. allergen inhalation alone

Endpoint	Timepoint, hr	Significant DBP effect	Significant allergen effect
Acute cellular effects			
% T helpers	3	X	
% regulatory T cells	3	X	
% Eosinophils	3		X
% Neutrophils	3		X
% Th17	3		X
MFI CD169 (on Th17)	3		X
% B cells	3		X
% T helpers	20	X	
% regulatory T cells	20	X	
Inflammatory mediators			
BCA	3		X
MIP-1d	3		X
SDF-1α+β	20	X	
TSLP	20	X	
IL-5	20		X
IL-7	20		X
IL-10	20		X
TGF-α	20		X
I-309	20		X
sCD40L	20		X
EGF	20		X

Note: The table shows the endpoints where the overall effect of DBP was significant in the LME analysis for 3 and 20 hr, as well as the endpoints where the one-way ANOVA showed a significant effect of allergen inhalation alone. Note that the significant effects at 3 hr could be due to either allergen-induced or diurnal effects, while effects at 20 hr reflect allergen-induced effects since these samples were collected at the same time of day. See supplemental material for results from one-way ANOVA.

Abbreviations: BCA, B cell-attracting chemokine; CD, cluster of differentiation; DBP, dibutyl phthalate; EGF, epidermal growth factor; IFN, interferon; IL, interleukin; MFI, mean fluorescence intensity; MIP, macrophage inflammatory protein; SDF-1α+β, stromal cell-derived factor-1α and β; SE, standard error; T helper type 17; TGF, transforming growth factor; Th17.

clinical relevance of systemic TSLP is still unclear, as research has focused on airway levels of TSLP.[42] In our study, exposure to DBP reduced the serum TSLP levels significantly, and decreased serum TSLP, IL-13, and IL-33 in non-AHR participants in the interaction model. In contrast, TSLP has been suggested to be a critical mediator in phthalate-induced effects on the Th2 inflammation and airway hyper-responsiveness induced by the model allergen ovalbumin (OVA) in mice.[43-45] It should be noted that our participants were healthy or mildly asthmatic, and although they were sensitized and were subjected to an allergen inhalation challenge, the serum Th2 mediators were generally not affected by the controlled allergen inhalation in our analysis. Thus, while the animal models assess the adjuvant effects due to repeated oral exposure to phthalates, our findings reflect acute effects that may result from a response toward the chemical itself.

Interestingly, effects on Th17 cells had a different pattern for males and females, suggesting sex differences in the effects of DBP therein. Moreover, the effect of DBP exposure on cell percentages, activation markers, and a range of mediators was consistently stronger in males although effect modification by sex was not always significant. Phthalates are endocrine disruptors that interfere with hormone pathways[46] and androgen-receptor interactions have been suggested,[47] providing a possible mechanism for the stronger effects observed in males. Several studies have reported sex differences regarding phthalate and allergen effects on airway diseases. For instance, a cross-sectional epidemiological study found that urinary levels of MBP were associated with reduced FEV_1, and decreased pulmonary function in males but not females.[48] Epidemiological studies in children reported that pre-natal exposure to phthalates were associated with increased asthma occurrence, and development of eczema, in boys but not in girls.[49-51] Our results for DBP provide support for possible sex-specific mechanisms underlying the observed epidemiological associations.

Cellular endpoints, such as percentages of cell subtypes in peripheral blood and expression of activation markers, showed consistently stronger DBP effects in grass-sensitized individuals. Recently, mice studies have highlighted the importance of the applied model allergen, suggesting that cellular mechanisms involved in experimental models may be allergen-dependent.[33,52] For example, in a cockroach-allergen extract model, DEHP induced a skewing from eosinophilic to neutrophilic inflammation, promoting a Th2 and Th17 immune response[33] whereas DEHP induced an eosinophilic Th2 response in the ovalbumin model.[6] It should be noted though, that these animal models assess effects of repeated exposure to phthalates prior to and during sensitization, while our human exposure model represents an acute exposure in sensitized individuals.

The main sources for human DBP exposure are diet (ingestion), personal care products (inhalation and dermal absorption), and indoor environments (ingestion of dust, inhalation and dermal).[2] Uptake, metabolism, and biological effects of phthalates are likely to differ between exposure pathways, but the implications of these differences are generally not considered when assessing human health effects associated with phthalate exposure.[4] For instance, epidemiological studies commonly use total exposure measured through urinary phthalate metabolites, and there are no experimental studies directly comparing the effects of multiple exposure routes. The results from the PAIR study, presented here and in Maestre-Batlle et al 2020[29], suggest that inhalation and dermal exposure to DBP can affect immune cells in both airways and blood, and also augment allergen-induced lung function decline. A better understanding of the relative importance of the exposure routes in human health effects, is essential for the implementation of more targeted abatement strategies for the reduction of phthalate exposure. Thus, the current findings underline the importance of considering the effects of the indoor exposure route in future studies.

In conclusion, the PAIR study is the first human phthalate exposure study to demonstrate that a controlled indoor air exposure to a specific phthalate induces systemic changes in immune cell profiles and inflammation, specifically in T-cell subsets. Moreover, DBP effects were influenced by sex and type of allergen inhaled and support epidemiological associations by providing possible underlying sex-specific mechanisms. Since the effects of DBP and allergen exposure differed from the effects of allergen alone, our data suggest a direct effect of DBP rather than enhancement of allergen-induced effects. However, further studies are required to clarify the biological effects of DBP alone versus its effects in the context of allergen exposure.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

DMB contributed substantially to the acquisition, analysis, and interpretation of data, and drafted the original version of the manuscript. UCN, RDF, NEA, SJT, and ST contributed to the interpretation of data. AKB and CC contributed substantially to the conception and design of the study, interpretation of data, and development of the final version of the manuscript. All authors revised the manuscript critically and are accountable for all aspects of the work.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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