

# Childhood exposure to ambient polycyclic aromatic hydrocarbons is linked to epigenetic modifications and impaired systemic immunity in T cells

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## Clinical & Experimental Allergy

### Summary

**Background** Evidence suggests that exposure to polycyclic aromatic hydrocarbons (PAHs) increases atopy; it is unclear how PAH exposure is linked to increased severity of atopic diseases.

**Objective** We hypothesized that ambient PAH exposure is linked to impairment of immunity in atopic children (defined as children with asthma and/or allergic rhinitis) from Fresno, California, an area with elevated ambient PAHs.

**Methods** We recruited 256 subjects from Fresno, CA. Ambient PAH concentrations (ng/m<sup>3</sup>) were measured using a spatial-temporal regression model over multiple time periods. Asthma diagnosis was determined by current NHLBI criteria. Phenotyping and functional immune measurements were performed from isolated cells. For epigenetic measurements, DNA was isolated and pyrosequenced.

**Results** We show that higher average PAH exposure was significantly associated with impaired Treg function and increased methylation in the forkhead box protein 3 (*FOXP3*) locus ( $P < 0.05$ ), conditional on atopic status. These epigenetic modifications were significantly linked to differential protein expression of *FOXP3* ( $P < 0.001$ ). Methylation was associated with cellular functional changes, specifically Treg dysfunction, and an increase in total plasma IgE levels. Protein expression of IL-10 decreased and IFN- $\gamma$  increased as the extent of PAH exposure increased. The strength of the associations generally increased as the time window for average PAH exposure increased from 24 hr to 1 year, suggesting more of a chronic response. Significant associations with chronic PAH exposure and immune outcomes were also observed in subjects with allergic rhinitis.

**Conclusions and Clinical Relevance** Collectively, these results demonstrate that increased ambient PAH exposure is associated with impaired systemic immunity and epigenetic modifications in a key locus involved in atopy: *FOXP3*, with a higher impact on atopic children. The results suggest that increased atopic clinical symptoms in children could be linked to increased PAH exposure in air pollution.

**Keywords** epigenetics, *FOXP3*, IFN- $\gamma$ , polycyclic aromatic hydrocarbons, T regulatory cells, total IgE, Treg function

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### Introduction

Over 10 million children under the age of 21 years have atopy (i.e. asthma and/or allergic rhinitis and/or atopic dermatitis and/or food allergy, CDC Survey, 2009). The prevalence of asthma and respiratory allergy

and other allergies has also increased over the past decade [1, 2]. The reasons for this rise in atopic disease prevalence are unclear; one factor may be exposure to ambient air pollution (AAP). In the last decade, epidemiological studies have linked air pollution exposure to a variety of adverse health effects, including increased frequency and severity of atopic diseases such as allergic rhinitis and asthma [3–8].

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Polycyclic aromatic hydrocarbons (PAHs) are a highly toxic and carcinogenic class of compounds, characterized by fused aromatic rings. They form when organic matter, such as diesel fuel, coal, wood or tobacco, undergoes incomplete combustion [9–11]. More recently, PAHs have been associated with impaired immunological function [6, 12–15]; however, the precise molecular and cellular pathways leading to the immunological impairment remain largely unknown.

We focused our research on children (10–21 years) from Fresno, California, the second-most polluted city in the United States in terms of 24-hour particulate matter (PM) and the fifth-most polluted city for annual PM ('State of the Air: 2012', American Lung Association). Additionally, the prevalence of both paediatric asthma and allergic rhinitis is elevated in Fresno; 19.4% of children with asthma compared with 13.8% nationally and 43% prevalence of allergic rhinitis compared to 27% nationally [2, 16]. Therefore, the subjects from Fresno provided an optimal population to study the differential impacts of PAH exposure on the immune markers of both atopic and non-atopic children.

We hypothesized that ambient PAH exposure could worsen atopic diseases by inducing T cell changes at the epigenetic level leading to impairment of cellular and humoral immunity. Our prior research indicated impaired T cell function and FOXP3 downregulation in children exposed to high amounts of air pollution compared to children living in a low air pollution environment. Our research also showed that these effects were more pronounced in asthmatic children with high air pollution exposure compared to non-asthmatic children with similarly high levels of air pollution exposure. In this study, we hypothesized that atopic children would be at increased risk for the effects of ambient air PAH given the already-impaired T cell function associated with this disease [17, 18]. Finally, we hypothesized that PAH-associated epigenetic changes could be sustained overtime. To address these hypotheses, we developed daily estimates of ambient air PAH exposure [19] averaged over 24-hour (24 hr), 1-week, 1-month, 3-month, 6-month and 1-year time periods for each subject. We also quantified associations between PAH exposure and epigenetic modifications in a key genetic locus (i.e. *FOXP3* locus), IgE levels (total IgE) and atopic disease. Furthermore, in a subset of participants ( $n = 25$ ), we tested whether methylation in the same genetic loci was sustained after 8 months.

## Materials and methods

All methods and procedures were approved by the IRB of Stanford University and University of California, Berkeley (see Supplementary Methods for more details).

### *Measures of exposure to ambient polycyclic aromatic hydrocarbons*

For PAH measurements, based on our previous published methods [19], we developed a land use regression model for residential, outdoor, daily exposure to the sum of PAHs with 4, 5 or 6 rings (PAH456) with mixed effects regression modelling to incorporate both temporal and spatial covariates. The PAHs included are as follows: fluoranthene, benz[a]anthracene, chrysene, benzo[a]pyrene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[ghi]perylene, indeno[1,2,3-cd]pyrene and dibenz[a,h]anthracene (see Supplementary Methods for more details).

### *Collection and processing of blood specimens*

Blood samples were collected from all participants (see Supplementary Methods for more details).

### *Cytometry studies*

Phenotyping was performed with up to 13 colours of fluorescently conjugated antibodies (LSRII; BD Biosciences) (see Supplementary Methods for more details).

### *DNA isolation, sodium bisulphite conversion and methylation studies*

Genomic DNA was isolated from Tregs using a DNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions and controlling for cell number. Genomic DNA was bisulphite-treated using published procedures [15, 20]. Direct quantification of methylated vs. unmethylated cytosine nucleotides for each analysed CpG site was determined by pyrosequencing with the PSQ HS 96 Pyrosequencing System and Pyro Gold CDT Reagents (Qiagen), as described previously [21, 22].

### *Functional assays*

<sup>3</sup>H-thymidine incorporation assays were performed according to previously published methods [15, 20], with fixed numbers of live cells per well for each cell type: CD4<sup>+</sup> T cells, Treg cells and antigen-presenting cells (APCs) (see Supplementary Methods for more details).

### *Statistical analysis*

Linear regression analysis was used to estimate associations with a series of ambient PAH exposure (ng/m<sup>3</sup>) variables averaged over different time periods prior to blood sample collection. To assess effect modification

of the associations between biomarkers and PAH exposure, we stratified by asthma (or rhinitis) status (Proc GLM in SAS). Non-linearity of the exposure response was examined with penalized splines in generalized additive models using R software (version 2.7.2, R Development core team, Vienna, Austria). A detailed description of the statistical methods used is provided in Supplementary Methods.

## Results

### *Changes in immune function with increasing averaging time for ambient polycyclic aromatic hydrocarbons exposure*

Demographic characteristics of subjects are summarized in Supplementary Material, Table S1. Our cohort for this analysis consisted of 256 subjects in Fresno ( $n = 171$  non-asthmatic and  $n = 85$  asthmatic). Of the subjects, 35 had allergic rhinitis (with concomitant asthma,  $n = 24$ , without concomitant asthma,  $n = 11$ ). Linear regression models were used to estimate the association between the six PAH exposures averaged over different time windows and measured immune outcomes (Supplementary Material, Tables S2 and S3). The immune outcomes included Treg transcription factor FOXP3 expression in CD4<sup>+</sup>CD25<sup>hi</sup> Tregs, Treg function, total IgE, IL-4 and IL-13 (Th2 cytokines) expression in CD4<sup>+</sup> T cells, IFN- $\gamma$  (Th1 cytokine) expression in gated CD4<sup>+</sup>CD25<sup>neg</sup> T cells (Teff) and IL-10 (immune regulatory cytokine) expression in gated CD4<sup>+</sup>CD25<sup>hi</sup> Tregs and chemokine receptor CCR8 expression in CD4<sup>+</sup> T cells according to published methods [15]. Table 1 summarizes the results of linear regression analysis between different immune outcomes and increasing PAH exposure time windows. Overall, we observed significant associations with both short-term and chronic PAH metrics and immune outcomes. The magnitude of the association (absolute value of the beta-coefficient) increased as the length of the time window of PAH exposure increased for both asthmatic and non-asthmatic subjects. Importantly, the magnitude of beta-coefficients for Treg function increased by almost threefold for asthmatic subjects and by more than 15-fold for non-asthmatic subjects as the averaging time increases from 24 hr to 1 year of ambient PAH exposure (Table 1 and Fig. 1).

Significant associations between higher PAH exposure and increased total IgE levels were observed for most all-time windows. As was observed with Treg function, the magnitude of the associations of ambient PAH exposure with total IgE levels increased substantially for all subjects with increasing averaging time (Table 1 and Fig. 2).

### *Epigenetic changes and increasing time for ambient polycyclic aromatic hydrocarbons exposure*

We next studied potential mechanisms through which PAH exposure could possibly modulate T cell responses. We investigated the association between average PAH exposure in different time windows and methylation of FOXP3 in PBMCs. Significant associations with average PAH exposure and increased FOXP3 methylation were not observed until 1 month for asthmatic subjects and 3 months for non-asthmatic subjects (Table 1 and Fig. 3). Furthermore, FOXP3 methylation was negatively correlated with FOXP3 protein expression in asthmatic subjects and FOXP3 transcript and protein expression in non-asthmatic subjects (Supplementary Material, Tables S4 A and B). In summary, epigenetic changes measured in FOXP3 associated with PAH exposure and the strength of association was highest with chronic PAH exposure in all subjects.

### *Cytokine expression in Teff cells and ambient polycyclic aromatic hydrocarbons exposure*

Our data also demonstrate that protein quantities of intracellular IFN- $\gamma$  in gated Teff cells increased as PAH exposure increased, with significant associations in asthmatic subjects at all-time windows but only chronic exposure windows in non-asthmatic subjects (Table 1). Increasing IL-4 and IL-13 protein expression was also significantly associated with increased PAH exposure more consistently with asthmatic subjects compared to non-asthmatic subjects (Table 1). In contrast, we observed decreased expression of IL-10 protein with increasing PAH exposure, but this was only significant in non-asthmatic subjects (Table 1).

In summary, among asthmatic subjects, all outcomes, with the exception of IL-10, were associated with annual PAH exposure. Among non-asthmatic subjects, annual PAH exposure was associated with most outcomes, with the exception of IL-10 and IFN- $\gamma$ . Our data suggest the strength of association increased with the length of the averaging time.

### *Chronic ambient polycyclic aromatic hydrocarbons exposure and allergic rhinitis*

To determine the effect of 1-year average ambient PAH exposure in subjects with another atopic disease (i.e. allergic rhinitis), we evaluated immune function in non-asthmatic subjects that were diagnosed with allergic rhinitis ( $n = 11$ ). The results for associations between 1-year average PAH exposure and immune outcomes are summarized in Table 2 and Fig. 4. Briefly, most all immune outcomes were significantly associated

Table 1. Regression coefficients (95% CI) for each immune outcome with increasing averaging time for PAH

Biomarker	1 day	1 week	1 month	3 months	6 months	1 year
% Treg function						
Asthmatics	-2.95 (-5.11, -0.80)	-4.29 (-6.61, -1.98)	-3.80 (-6.29, -1.31)	-4.60 (-7.44, -1.76)	-7.86 (-11.44, -4.29)	-9.26 (-13.22, -5.30)
Non-asthmatics	-0.47 (-1.54, 0.60)	-0.52 (-1.96, 0.91)	-0.35 (-2.06, 1.36)	-0.10 (-2.09, 1.89)	-3.92 (-7.18, -0.66)	-7.31 (-10.82, -3.81)
Total IgE						
Asthmatics	12.83 (0.42, 25.24)	18.84 (5.14, 32.54)	17.91 (3.65, 32.17)	16.28 (-1.21, 33.76)	28.81 (6.48, 51.14)	35.62 (13.89, 57.36)
Non-asthmatics	5.24 (0.98, 9.51)	8.43 (2.82, 14.04)	10.49 (3.85, 17.12)	10.75 (3.08, 18.41)	28.65 (16.59, 40.70)	28.58 (15.35, 41.81)
FOXP3 methylation						
Asthmatics	1.34 (-0.22, 2.89)	1.71 (-0.01, 3.44)	2.45 (0.73, 4.17)	3.51 (1.56, 5.47)	4.98 (1.84, 8.13)	4.94 (1.22, 8.65)
Non-asthmatics	0.47 (-0.60, 1.54)	0.30 (-1.13, 1.73)	0.84 (-0.85, 2.54)	2.04 (0.12, 3.96)	3.30 (0.13, 6.46)	3.64 (0.21, 7.09)
FOXP3						
Asthmatics	-1.06 (-2.41, 0.30)	-1.30 (-2.88, 0.29)	-1.74 (-3.29, -0.19)	-2.39 (-4.16, -0.62)	-3.48 (-6.00, -0.97)	-3.78 (-6.48, -1.09)
Non-asthmatics	-0.48 (-1.52, 0.56)	-0.60 (-1.98, 0.78)	-1.22 (-2.85, 0.41)	-2.20 (-4.05, -0.35)	-4.33 (-7.36, -1.31)	-4.96 (-8.22, -1.71)
IFN- $\gamma$						
Asthmatics	1.62 (0.02, 3.22)	2.00 (0.20, 3.81)	2.50 (0.69, 4.30)	3.39 (1.27, 5.50)	4.69 (1.42, 7.96)	5.14 (1.45, 8.38)
Non-asthmatics	0.71 (-0.43, 1.86)	0.66 (-0.87, 2.19)	1.23 (-0.59, 3.04)	2.38 (0.33, 4.44)	4.04 (0.64, 7.44)	4.47 (0.77, 8.17)
IL-4						
Asthmatics	0.07 (-0.01, 0.14)	0.08 (-0.002, 0.17)	0.12 (0.04, 0.21)	0.18 (0.08, 0.27)	0.25 (0.09, 0.40)	0.24 (0.05, 0.43)
Non-asthmatics	0.02 (-0.03, 0.08)	0.02 (-0.06, 0.09)	0.04 (-0.04, 0.13)	0.10 (0.01, 0.20)	0.16 (0.01, 0.32)	0.18 (0.01, 0.35)
IL-13						
Asthmatics	0.03 (-0.01, 0.06)	0.04 (-0.001, 0.07)	0.05 (0.02, 0.09)	0.08 (0.03, 0.12)	0.11 (0.04, 0.18)	0.11 (0.03, 0.19)
Non-asthmatics	0.01 (-0.01, 0.03)	0.01 (-0.03, 0.04)	0.02 (-0.02, 0.05)	0.04 (-0.0002, 0.83)	0.07 (-0.002, 0.14)	0.07 (0.001, 1.15)
IL-10						
Asthmatics	-0.35 (-2.85, 2.15)	0.17 (-2.74, 3.08)	-1.49 (-4.50, 1.52)	-2.27 (-5.81, 1.27)	-4.04 (-8.79, 0.72)	-2.25 (-7.33, 2.83)
Non-asthmatics	-0.51 (-1.55, 0.54)	-0.84 (-2.25, 0.57)	-1.25 (-2.93, 0.43)	-2.07 (-4.01, -0.12)	-3.28 (-6.48, -0.07)	0.03 (-3.58, 3.64)
CCR8						
Asthmatics	1.08 (-0.17, 2.33)	1.40 (0.01, 2.78)	1.98 (0.60, 3.36)	2.84 (1.28, 4.41)	4.01 (1.49, 6.53)	3.95 (0.97, 6.92)
Non-asthmatics	0.42 (-0.45, 1.30)	0.32 (-0.85, 1.48)	0.78 (-0.60, 2.16)	1.83 (0.27, 3.39)	2.94 (0.37, 5.52)	3.19 (0.39, 5.99)

Data show regression coefficients and confidence intervals for each immune parameter units with increasing mean polycyclic aromatic hydrocarbons (PAH) concentrations at each time window stratified by asthma status. Model adjusted for age, sex, race, ethnicity, second-hand smoke and season. Estimates in bold are statistically significant,  $P < 0.05$ .

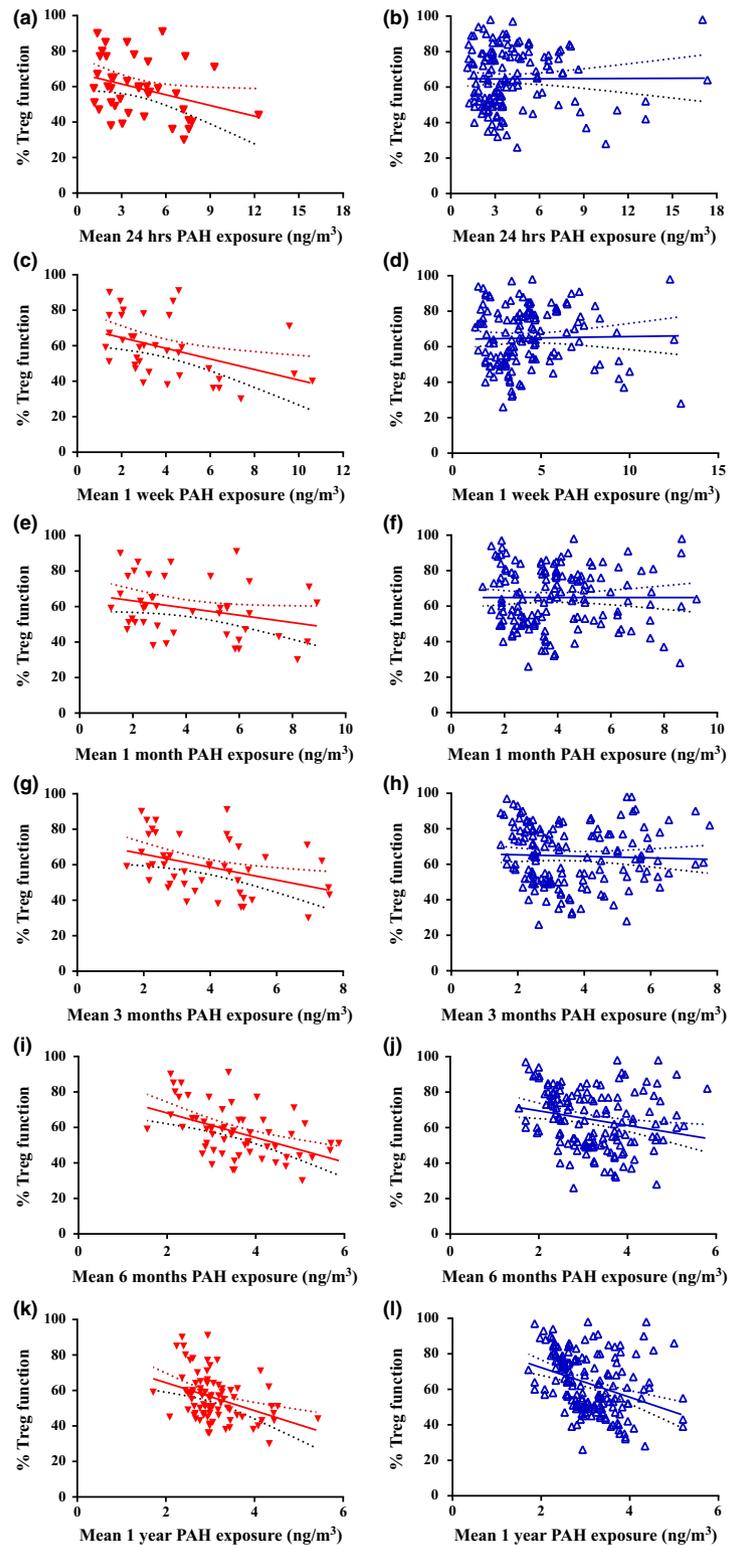


Fig. 1. Ambient polycyclic aromatic hydrocarbons (PAH) exposure is associated with changes in Treg function. Linear regression analysis of ambient average PAH exposure at different time periods (in  $\text{ng}/\text{m}^3$ ) and Treg function. Treg function reported as % of suppressive function. (a, b) 24-hr mean PAH exposure ( $n = 41$  asthmatic,  $n = 146$  non-asthmatic), (c, d) 1-week mean PAH exposure ( $n = 38$  asthmatic,  $n = 149$  non-asthmatic), (e, f) 1-month mean PAH exposure ( $n = 44$  asthmatic,  $n = 150$  non-asthmatic), (g, h) 3-month mean PAH exposure ( $n = 47$  asthmatic,  $n = 153$  non-asthmatic), (i, j) 6-month mean PAH exposure ( $n = 61$  asthmatic,  $n = 156$  non-asthmatic) and (k, l) 1-year mean PAH exposure ( $n = 85$  asthmatic,  $n = 171$  non-asthmatic). Data points represent individuals. Red = asthmatic subjects, Blue = non-asthmatic subjects. Dotted lines represent 95% confidence intervals.

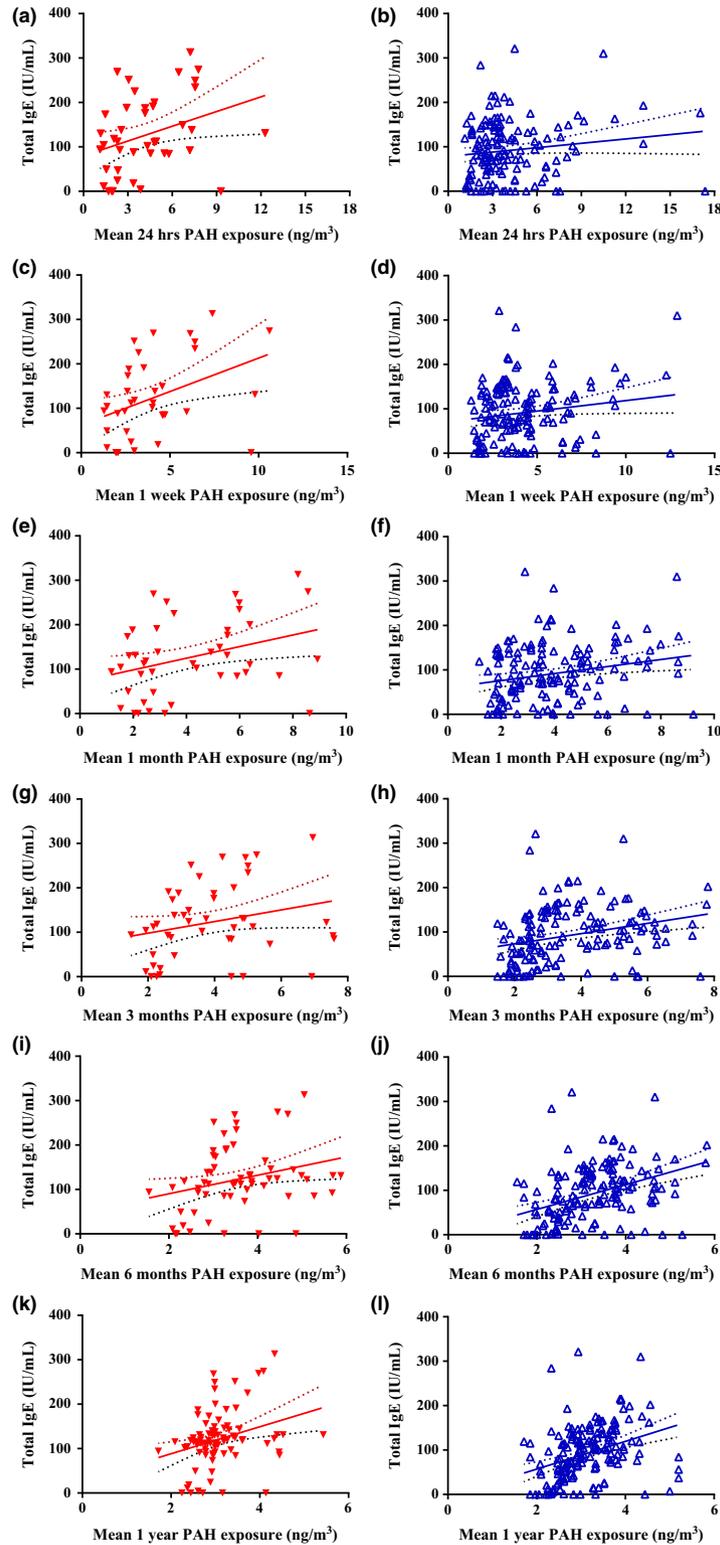


Fig. 2. Ambient polycyclic aromatic hydrocarbons (PAH) exposure is associated with changes in total IgE. Linear regression analysis of ambient average PAH exposure at different time periods (in  $\text{ng}/\text{m}^3$ ) and plasma total IgE levels. IgE levels reported as IU/mL. (a, b) 24-hr mean PAH exposure ( $n = 41$  asthmatic,  $n = 146$  non-asthmatic), (c, d) 1-week mean PAH exposure ( $n = 38$  asthmatic,  $n = 149$  non-asthmatic), (e, f) 1-month mean PAH exposure ( $n = 44$  asthmatic,  $n = 150$  non-asthmatic), (G, H) 3-month mean PAH exposure ( $n = 47$  asthmatic,  $n = 153$  non-asthmatic), (i, j) 6-month mean PAH exposure ( $n = 61$  asthmatic,  $n = 156$  non-asthmatic) and (k, l) 1-year mean PAH exposure ( $n = 85$  asthmatic,  $n = 171$  non-asthmatic). Data points represent individuals. Red = asthmatic subjects, Blue = non-asthmatic subjects. Dotted lines represent 95% confidence intervals.

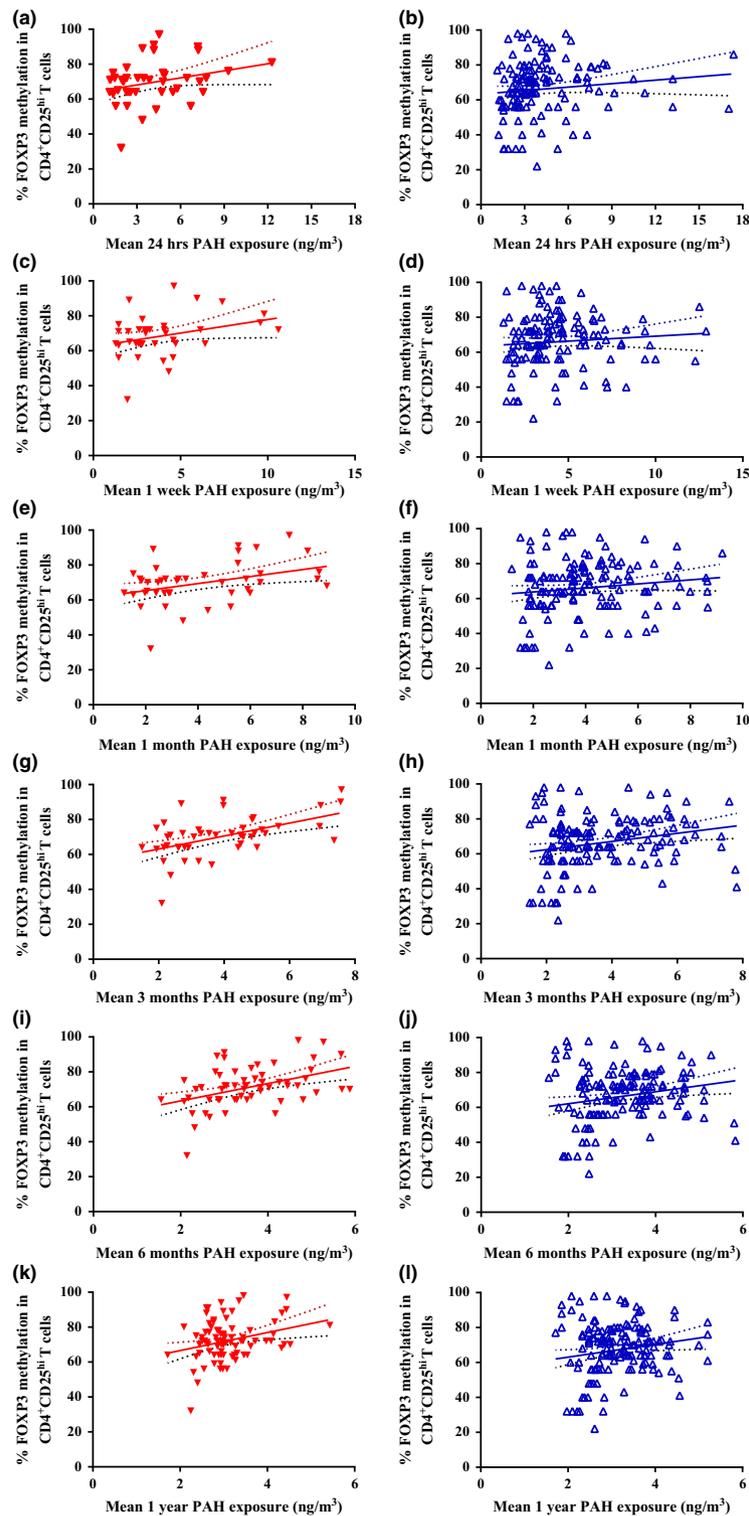


Fig. 3. Ambient polycyclic aromatic hydrocarbons (PAH) exposure is associated with changes in *FOXP3* methylation. Linear regression analysis of ambient average PAH exposure at different time periods ( $\text{ng}/\text{m}^3$ ) and *FOXP3* methylation in  $\text{CD4}^+\text{CD25}^{\text{hi}}\text{CD127}^-$  T cells. Methylation is reported as percent methylated CpG sites of 13 total examined CpG sites within the *FOXP3* locus; the cut-off for positive methylation at each site was set at 70% of all sequencing reactions. (a, b) 24-hr mean PAH exposure ( $n = 41$  asthmatic,  $n = 146$  non-asthmatic), (c, d) 1-week mean PAH exposure ( $n = 38$  asthmatic,  $n = 149$  non-asthmatic), (e, f) 1-month mean PAH exposure ( $n = 44$  asthmatic,  $n = 150$  non-asthmatic), (g, h) 3-month mean PAH exposure ( $n = 47$  asthmatic,  $n = 153$  non-asthmatic), (i, j) 6-month mean PAH exposure ( $n = 61$  asthmatic,  $n = 156$  non-asthmatic) and (k, l) 1-year mean PAH exposure ( $n = 85$  asthmatic,  $n = 171$  non-asthmatic). Data points represent individuals. Red = asthmatic subjects, Blue = non-asthmatic subjects. Dotted lines represent 95% confidence intervals.

Table 2. Effect of mean 1 year PAH exposure on immune outcomes stratified by allergic rhinitis status

Biomarker	Non-atopics ( <i>n</i> = 160)			Rhinitis in non-asthmatics ( <i>n</i> = 11)			Asthmatics without rhinitis ( <i>n</i> = 61)			Asthmatics with rhinitis ( <i>n</i> = 24)		
	Estimate	LCI	UCI	Estimate	LCI	UCI	Estimate	LCI	UCI	Estimate	LCI	UCI
Treg Function	-8.15	-11.57	-4.73	<b>-16.65</b>	-40.63	7.32	<b>-8.41</b>	-13.37	-3.45	-6.27	-16.56	4.03
Total IgE	<b>31.04</b>	17.99	44.09	36.61	-5.11	78.33	<b>33.20</b>	8.58	57.82	32.71	-15.37	80.78
<i>FOXP3</i> Methylation	3.03	-0.24	6.30	<b>29.04</b>	12.00	46.07	<b>5.79</b>	1.66	9.92	1.82	-9.27	12.92
<i>FOXP3</i>	<b>-4.73</b>	-7.79	-1.68	<b>-30.39</b>	-48.07	-12.7	<b>-4.49</b>	-7.74	-1.24	-1.01	-7.31	5.28
<i>IFN-γ</i>	<b>3.79</b>	0.29	7.30	<b>32.49</b>	12.34	52.63	<b>6.14</b>	1.92	10.36	0.71	-9.72	11.14
IL-4	0.15	-0.01	0.32	<b>1.43</b>	0.58	2.28	<b>0.28</b>	0.07	0.49	0.1	-0.47	0.67
IL-13	0.06	-0.01	0.13	<b>0.63</b>	0.27	1.00	<b>0.13</b>	0.04	0.22	0.04	-0.2	0.28
IL-10	-4.15	-8.29	-0.02	<b>-6.09</b>	-24.47	12.29	<b>-0.63</b>	-7.34	6.07	<b>-15.04</b>	-28.95	-1.14
CCR8	2.59	-0.07	5.24	<b>23.21</b>	9.61	36.81	<b>4.66</b>	1.35	7.97	1.34	-7.45	10.14

Data shows regression coefficients and confidence intervals for each immune parameter units with increasing mean 1 year polycyclic aromatic hydrocarbons (PAH) concentration. Estimates in bold are statistically significant,  $P < 0.05$ .

with annual average PAH exposure. Interestingly, subjects with concomitant asthma and rhinitis ( $n = 24$ ) did not show statistically significant associations between changes in immune outcomes and annual average PAH exposure except for decreased IL-10 cytokine expression, while asthmatic subjects without allergic rhinitis did show statistically significant associations ( $n = 62$ , Table 2 and Figure 4C).

In conclusion, our allergic rhinitis groups were relatively small, but these initial differences between a single atopic condition (i.e. asthma or allergic rhinitis) vs. multiple atopic conditions (i.e. asthma with allergic rhinitis) might elucidate our understanding of immunological differences in atopy.

#### Sustained changes in immune parameters

In a random subset of 25 subjects, which included both atopic ( $n = 6$ ) and non-atopic ( $n = 19$ ), we examined longitudinal effects of PAH exposure on immune function and methylation of *FOXP3* through comparison of each subject's initial visit data with those obtained at a follow-up visit approximately 8 months later (average interval:  $252.1 \pm 13.4$  days). No significant changes were seen in levels of total IgE or *FOXP3* methylation (Fig. 5) between the two visits. Together, these data suggest that the molecular and cellular changes measured over time in our assays were sustained in the subjects tested.

#### Discussion

Our study aimed to determine whether increased exposure to ambient PAH over different time periods was associated with cellular (i.e. T cell) and humoral (i.e. IgE) changes, specifically in relation to atopic disease. We found significant associations between ambient PAH levels and immune dysregulation, favouring an increase in proinflammatory, Th2-associated immune parameters

and a concurrent decrease in some regulatory immune parameters (IL-10), but an increase in Th1-associated immune parameters (IFN- $\gamma$ ). These associations were more pronounced in subjects with atopic diseases, specifically asthma and allergic rhinitis, compared to subjects without atopic disease.

One major finding of our study is the significant increases in methylation and decreased expression of *FOXP3* in Tregs, and also the differences in IFN- $\gamma$  protein expression in Teff between asthmatic and non-asthmatic subjects at multiple time windows of exposure and the sustainability of this effect. This study showed the prominent effects of PAH exposure on atopy (i.e. asthma and allergic rhinitis). Recent studies show evidence of epigenetic modifications, particularly methylation changes, associated with air pollution exposure [23–25]. Our data provide a plausible molecular mechanism that explains the pathology linked to air pollution, that is, PAH exposure may alter methylation patterns of genes involved in immune regulation, which leads to adverse immune effects linked to atopy.

There have been inconsistent data regarding the association between total IgE levels and air pollution exposure [26–29]. Our study shows a significant association between increased PAH exposure at multiple time windows and elevated total IgE, with a more pronounced effect in asthmatic subjects compared to non-asthmatic subjects. IL-4 and IL-13, along with IL-5, are the major proinflammatory cytokines released by Th2 cells and are heavily implicated in atopic disease due to their role in induction of IgE production by B cells [30–32]. Our finding of increased IL-4 and IL-13 expression combined with decreased Treg function after PAH exposure could be directly or indirectly related to the induction of isotype switching to IgE resulting in increased total IgE in the plasma. This study is the first, to our knowledge, to show an increase in Th2-associated markers, specifically expression of CCR8, IL-4 and IL-13 in CD4<sup>+</sup> T cells in both asthmatic and non-asthmatic subjects

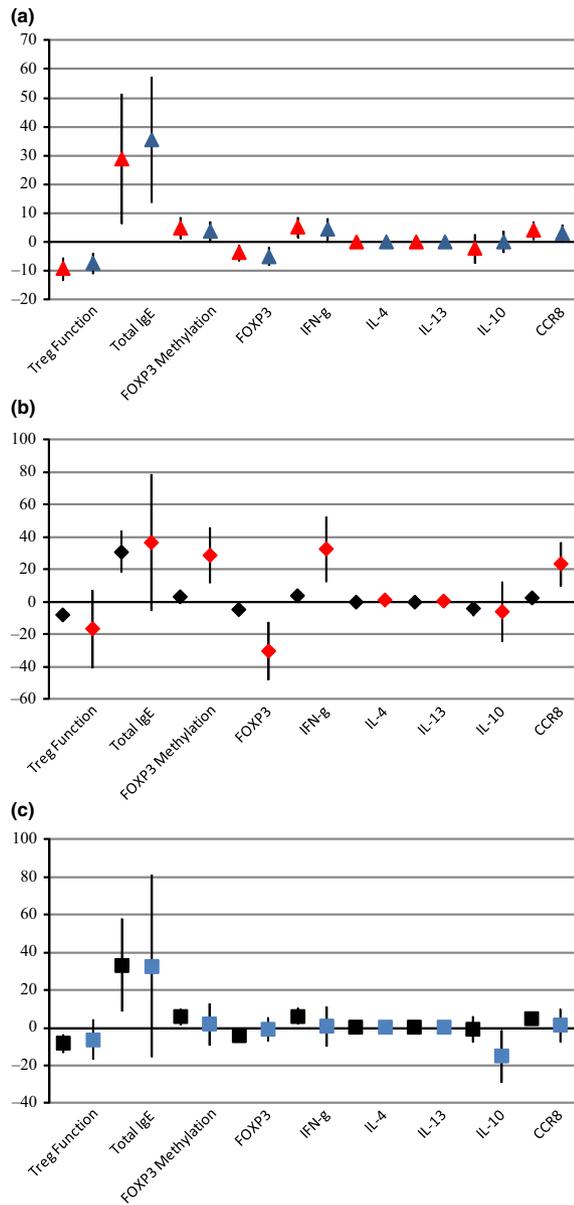


Fig. 4. Effect of  $1 \text{ ng/m}^3$  mean annual polycyclic aromatic hydrocarbons (PAH) exposure on changes in immune function in subjects with asthma and/or allergic rhinitis. Stock plots showing estimates and confidence intervals for changes in immune parameter units with a  $1 \text{ ng/m}^3$  increase in mean annual PAH concentration. (a) Stratified by asthma. Red triangles = asthmatic subjects ( $n = 85$ ); Blue triangles = non-asthmatic subjects ( $n = 171$ ). (b) Stratified by allergic rhinitis. Black diamonds = non-asthmatic subjects without rhinitis ( $n = 160$ ); Red diamonds = non-asthmatic subjects with allergic rhinitis ( $n = 11$ ). (c) Stratified by asthma and allergic rhinitis. Black squares = asthmatic subjects without rhinitis ( $n = 61$ ); Blue squares = asthmatic subjects with allergic rhinitis ( $n = 24$ ).

with exposure to AAP. As CCR8 is a chemokine receptor important for migration of T cells into the lung parenchyma, its increase could be linked to more activated T cells in the lungs of atopic individuals exposed to AAP.

In addition to Th2 parameter changes, we found changes in Th1 marker IFN- $\gamma$ . Our data on IFN- $\gamma$  protein expression show increased IFN- $\gamma$  with increasing PAH time windows that is significant in asthmatic subjects, but not in non-asthmatic subjects. This could indicate increased inflammation in asthmatic subjects compared to non-asthmatic subjects. In another air pollutant exposure study, Hernandez *et al.* [33] found increased inflammatory cytokine expression, IL-6 and IL-1b in the sputum of atopic asthmatics compared to healthy volunteers and non-atopic asthmatics postzone exposure. Further studies are needed to understand this differential regulation of Th1 inflammatory immune responses between asthmatics and non-asthmatics.

We found some evidence for a significant decrease in IL-10 expression in  $\text{CD4}^+\text{CD25}^{\text{hi}}$  Treg only for the 3- and 6-month PAH in non-asthmatic, but not asthmatic subjects (Table 1). Although decreases in IL-10 have been associated with asthma [33, 34], our findings do not support a clear association between increased PAH exposure and decreased IL-10 expression in asthmatic subjects. We did see a significant association between increased PAH exposure and decreased Treg function in both asthmatic and non-asthmatic groups and speculate that this may be through IL-10 independent mechanisms.

There are limitations to our study that should be acknowledged. We focused on understanding the effects of acute and chronic ambient PAH exposure on atopy in children from Fresno. But atopy in our study was confined to allergic rhinitis and asthma; other atopic conditions such as food allergy and atopic dermatitis are currently being studied. We also excluded any children with second-hand or primary tobacco smoke exposure. Other exposures such as  $\text{PM}_{2.5}$ ,  $\text{PM}_{10}$ , ozone, endotoxin, and their potential interaction with PAH, are also of interest and will be studied in the future, as these exposures could be associated with immune cell dysregulation, epigenetic modifications and atopic disease. We recognize that we tested some, not all, immune markers and not all genes. Environmental exposures have been found to affect many aspects of the immune system. Future studies addressing the important role of other immune cells will further our understanding of the effects of AAP exposure on the immune system. Our findings suggest that long-term ambient PAH exposure affects immune responses differentially depending on atopic disease status. More studies are needed to understand the aetiology of these differences. We note that our allergic rhinitis sub-cohort was small ( $n = 26$ ) such that we had limited statistical power to study this group. That we observed any statistically significant differences in such a small population speaks to the potential impact of PAH exposure on the immune system.

In summary, ambient PAH exposure was found to be associated with increased methylation of CpG sites in

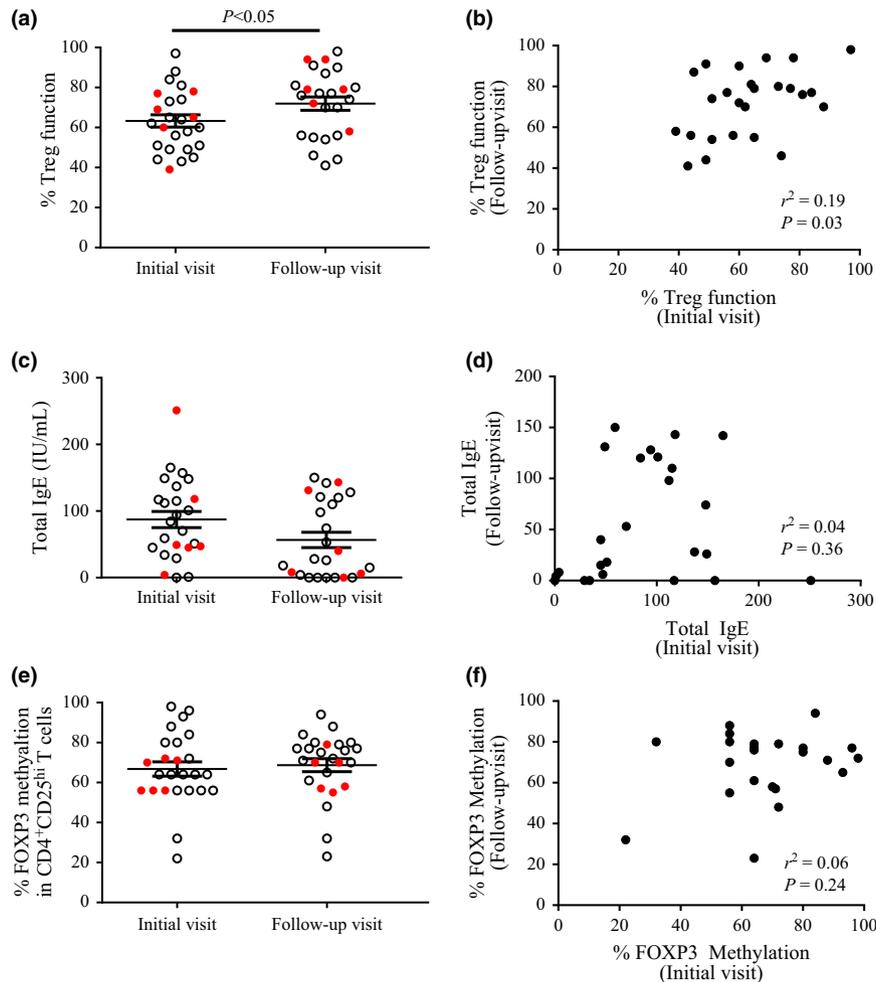


Fig. 5. Changes in immune parameters in a follow-up subset. Comparison (a–c) and correlation (d–f) of (a, b) % Treg function, (c, d) levels of total IgE and (e, f) FOXP3 methylation between initial and follow-up visit. The average time between visits was 252.1 + 13.4 days. (a–c) Data are presented as mean + standard error of mean (SEM). Black circles = non-asthmatic subjects ( $n = 19$ ), Red circles = asthmatic subjects ( $n = 6$ ). Paired  $t$ -test \*,  $P < 0.05$ ; (d–f) data are presented as correlation between initial and follow-up visit. Pearson's correlation test,  $r^2$ , \* $P < 0.05$ .

key loci in T cells and this was sustained in a random subset of subjects. Associations with ambient PAH exposure and immune parameters were more pronounced in subjects with atopic vs. non-atopic diseases (i.e. asthma and allergic rhinitis).

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### Conflict of interest

The authors declare that they have no competing interests.

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