Environ Sci Pollut Res (2017) 24:22579–22586
DOI 10.1007/s11356-017-9907-3

RESEARCH ARTICLE

A pilot study comparing T-regulatory cell function among healthy children in different areas of Gansu, China

Panhong Gou[1] · Xiaoru Chang[1] · Zhonghui Ye[1] · Yueli Yao[1] · Patton Khuu Nguyen[2] · Sally Katharine Hammond[2] · Junling Wang[1] · Sa Liu[2]

Received: 6 March 2017 / Accepted: 3 August 2017 / Published online: 14 August 2017

Abstract Immune system is critical to protecting human health from toxic substances. Our previously published research had found an important link between polycyclic aromatic hydrocarbons (PAHs) in ambient air and changes at the DNA level in immune cells that led to impaired function of regulatory T (Treg) cells in children living in California, USA. But molecular and cellular pathways of these changes remain unclear. The present study aims to explore whether exposure to PAHs leads to changes in Treg cells functions of children living in Gansu, China, where ambient air pollution levels are much higher than those in California, and to explore potential mechanisms of PAH-induced immunological dysfunctions. Air pollutions in Lanzhou and Lintao, Gansu Province, were measured from December 2015 to June 2016. Healthy children were recruited from both cities and enrolled in this pilot study. Demographic information was collected by questionnaires. Blood samples were collected. Peripheral blood Treg cells were analyzed for Treg cells percentage by flow cytometry. Gene expression of forkhead box transcription factor 3 (Foxp3), transforming growth factor-β (TGF-β), and interleukin 35 (IL35) were examined by reverse transcription-polymerase chain reaction (RT-PCR). The results indicated PAH concentration (as sum of 16 PAHs) in Lintao was over two times higher than that was in Lanzhou (707 vs. 326 ng/m^3), whereas $PM_{2.5}$ concentration was comparable in two cities (55.3 in Lintao vs. 65.7 μg/m^3 in Lanzhou). Notably, we observed lower gene expressions for Foxp3 ($P < 0.05$), IL35 ($P < 0.05$), and TGF-β, in children living in Lintao, suggesting an impairment of Treg cells function potentially associated with higher PAH exposure in Lintao. However, no significant difference was observed in Treg cells % among $CD4^+$ T cells between Lanzhou and Lintao groups.

Keywords Air pollution · Immune system · Treg cells · Immune system · Cytokine

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a class of organic compounds that consist of two or more fused benzene rings. They are ubiquitous in ambient air pollution and mainly generated by the incomplete combustion of organic matter such as coal, wood, tobacco, and fossil fuels (Kavouras et al. 2001; Pleil et al. 2004). Although the major concern for PAHs has been their carcinogenicity (IARC 1987), cumulative evidence indicates that PAHs in air pollution are associated with impaired immunological functions, which may lead to decreased pulmonary function, allergic disease, adverse birth outcomes (preterm birth and birth defects), and glucosedys regulation and obesity (Arnold et al. 2008; Lin et al. 2016). However, evidence on PAHs-induced immune-toxicity has been mainly from animal studies,

Panhong Gou and Xiaoru Chang contributed equally to receive first authorship.

Responsible editor: Philippe Garrigues

✉ Junling Wang
wangjl@lzu.edu.cn; 78097478@qq.com

✉ Sa Liu
saliu@berkeley.edu

[1] Department of Toxicology, School of Public Health, Lanzhou University, No 199 Donggang West Road, Lanzhou 730000, China

[2] Division of Environmental Health Sciences, School of Public Health, University of California, Berkeley, 50 University Hall, #7360, Berkeley, CA 94720-7360, USA

such as in mice or fish, and from in vitro human cell studies. Information is scarce on in vivo human health studies. Compared to adults, children are at a higher risk for adverse health consequences associated with ambient air pollution exposure. The critical period of growth and development in children increases their immune system's sensitivity and vulnerability to environmental exposures. The survey organized by American CDC showed that more than 10 million children below the age of 21 years old had atopy which including asthma, allergic rhinitis, and atopic dermatitis, and prevalence of these atopic disease had increased over the past years (Gupta et al. 2011; Moorman et al. 2012). Ambient air pollution is suspected to be one of the most important reasons for this problem (Perez et al. 2013).

Due to the rapid economic development, China is experiencing serious air pollution. Unlike in developed countries, where traffic pollution is the major contributor of ambient air pollution in most cities, in China, biomass and coal burning contributes greatly to the emissions of fine particulate matter ($PM_{2.5}$, particles with aerodynamic diameter equal or less than 2.5 μm) and PAHs. In fact, residential biomass combustion was estimated to account for 47% of the total PAH emission in China (Li et al. 2014). So far, majority of air pollution studies in China has been focused on large cities. Major control measures have also been taken to fight air pollution in urban areas. Air quality in small cities and rural areas has been largely overlooked where exposure to PAHs could be worse, because biomass and coal burning are not regulated as they are in major cities. This leaves children in less developed area at a great risk of increased PAH exposure, but meanwhile provides a unique opportunity to examine potential immunological effects of PAH exposure over a wider concentration range, as well as with different composition, than possible in developed countries.

Regulatory T (Treg) cells are suppressors of immune responses and play an essential role in maintaining immunological homeostasis. Treg cells establish early in life, which is critical for immunological self-tolerance in children (Verhasselt et al. 2008). Ambient air pollution has been found to potentially lead to hypermethylation of forkhead box transcription factor 3 (Foxp3) gene, a key transcription factor of Treg cells activity, causing impaired Treg cells suppression (Nadeau et al. 2010). These researchers also reported an association between number of methylated CpG islands in Treg cells Foxp3 gene and PAH exposure, an important link between PAHs in ambient air pollution and epigenetic changes in immune cells that led to impaired Treg cells function in children living in California, USA (Nadeau et al. 2010). It was the first published paper reporting these findings. However, these results were inconclusive due to unmeasured confounders such as ethnicity and social economic status. Meanwhile, the molecular and cellular pathways of PAH-induced Treg cells dysfunction remained unclear.

This pilot study aimed to explore whether exposure to air pollution, specifically PAHs, lead to impaired immune function among children living in Lanzhou, Gansu Province, a typical large city in northwest China, and Lintao, a small city near Lanzhou, where (in both cities) ambient air pollution levels are much higher than those in California, USA, and to explore potential mechanisms of PAH-induced immunological impairments. Lanzhou was located in a narrow valley and was once among ten most polluted cities in China (Wang et al. 2012). Since 2012, many air pollution control measures have been implemented to reduce industrial emission, coal combustion, and automobile exhaust to curb severe air pollution in the city. In contrast, Lintao is a small city 80 km southwest of Lanzhou and has only 5% of Lanzhou's population (0.13 vs. 2.5 million in the urban area). It is encompassed by flatlands compared mountains surrounding Lanzhou. There is little industrial emission of air pollutants in Lintao. However, coal and biomass burning are not regulated in Lintao. Immunological outcomes examined included Treg cells count, as % of $CD4^+$ T cells and gene expression of Foxp3, transforming growth factor-β (TGF-β) and interleukin 35 (IL35). Foxp3 and TGF-β are key transcription factors that are implicated in Treg cells function (Marson et al. 2007; Sakaguchi et al. 2009; Cohen et al. 2006). IL35 is a cytokine produced by Treg cells and suppresses inflammatory responses of immune cells. Although the results from a pilot study cannot be generalized for larger populations, this exploratory evaluation provides important information on the immune responses to high level PAH exposure in pollution cities of northern China.

Materials and methods

All procedures and methods of this study were approved by the Committee for the Protection of Human Subjects at the University of California, Berkeley (Project identification code: 00006252), and Lanzhou University (Project identification code: IRB150626-1).

Subjects

Children (10–13 years old) from Chengguan District, a major residential and commercial area of the Lanzhou City ($n = 21$) and Lintao City ($n = 21$), were enrolled in this pilot study. All participants were healthy children recruited during their regular checkup at local health examination centers. Children from Lanzhou all lived within 5 km of Lanzhou EPA compliance monitoring site in Chengguan District. Whereas, all participants from Lintao had resided in the immediate vicinity of the air monitoring site set by the present study. No government

air-monitoring site was established in Lintao. Subjects had lived at their residences for at least 1 year when recruited. There was no major highway near either study site. Two groups were frequency-matched by age and gender. A questionnaire was administrated by trained researchers to collect basic information on demographic and general health history. Subjects were excluded if they had been taking oral immunosuppressives within 5 days of the blood sample collection, had a chronic disease, and/or had acute infection.

Exposure to air pollutants

Air pollution samples were collected weekly in both Lanzhou and Lintao from December 2015 to June 2016. While few samples were lost during the sampling, there were a total of 81 PAH and 61 $PM_{2.5}$ samples. Details of sample collection and analysis methodologies are described elsewhere (Liu et al. 2017). In brief, $PM_{2.5}$ was collected on glass fiber filters (Whatman, Maidstone, UK) by intellectual medium volume total suspended particle (TSP) sampling-meter (Model: WT10-TH-150AII, Wuhan, China) at a flowrate of 100 l/min for 24 h and followed by gravimetric analysis. PAHs were collected on 47 mm XAD-4 coated quartz filters at a flowrate of 5 l/min for 24 h with $PM_{2.5}$ impactor installed. Sixteen PAHs were analyzed in filter samples using GC/MS (Noth et al. 2016).

Separation of peripheral blood mononuclear cells and detection of gene expressions

Flow cytometer analysis

Two microliters (μl) of antibody (Becton Dickinson, America), including CD4-, CD25-APC, and CD127-PE, was added into 50 μl whole blood sample in a 1.5-ml tube and incubated at 4 °C in the dark for 15 min. Then, 450 μl red cell lysing reagent (Beyotime, China) was added into the tube and pyrolysed in the dark again for 15 min at 4 °C. Next, the solution was centrifuged with 400*g* for 10 min, and the supernatant was removed to obtain peripheral blood mononuclear cells (PBMCs). Finally, the cells were gently resuspended with 500 μl PBS and ready for detection by flow cytometer. Data were analyzed by FlowJo analysis software (Tree Star, Inc. Ashland, OR). The percentage of Treg cells was calculated as the number of $CD4^+CD25^+CD127^{low}$/the number $CD4^+$. The above operations were performed on ice and repeated three times for each blood sample.

Separation of PBMCs

Anticoagulant EDTA-K_2 was added into 2 ml whole blood for each blood sample. The mixture was centrifuged at 4 °C, 400*g* for 5 min to remove plasma. Then, the sample was diluted by approximately 2.5 ml RPMI 1640 (HyClone, America) to 4 ml, and the mixture was added into a new glass tube containing 4 ml Ficoll-Paque (GE Healthcare, England); then, the sample mentioned above was centrifuged at 18 °C, 400*g* for 40 min to isolate PBMCs. Supernatant was removed, and the PBMCs were collected into a 10-ml glass tube. Then, 6 ml cold PBS was added into collected PBMCs to wash. After a gentle mixing, the cell suspension solution was centrifuged at 18 °C, 100*g*, for 10 min. Supernatant was removed, and cell dumps were suspended with 1 ml RPMI 1640. With a gentle mixing, PBMCs were ready to be used to detect gene expression.

The mRNA expression detection by reverse transcription-polymerase chain reaction

The mRNA expressions of IL-35, TGF-β, and Foxp3 were examined using reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from PBMCs with TRIzol Reagent (Ambion, America), avant in and chloroform, diethyl pyrocarbonate DEPC, and 75% ethanol. Reverse transcription reactions were performed with PrimeScript TMRT Master Mix kit (TaKaRa, Japan) according to the manufacturer's protocol. Briefly, 1 μg of the total RNA was incubated with 5 × PrimeScript RT Master Mix and had RNAase Free ddH_2O added up to 10 μl. The reverse transcription reaction was performed at 37 °C for 15 min then inactivated by heating the samples at 85 °C for 5 s. The complementary DNA (cDNA) was stored at − 80 °C for PCR. All PCRs were performed using SYBR Premix Ex Taq kit (TaKaRa, Japan) on iQ5 (Bio-Rad, America). The total volume of 25 μl RT-PCR reaction mixture included 12.5 μl of SYBR Premix Ex Taq, 1 μl (10 μmol/L) of forward primers, 1 μl (10 μmol/L) of reverse primers, 2 μl of cDNA, and 8.5 μl of ddH_2O. The cDNA of β-actin, used for internal control, was also amplified. The amplification conditions were used for all genes and consisted of a predegeneration cycle of 30 s at 95 °C, 40 cycles of PCR consisting of 5 s at 95 °C for denaturation, and 60 s for annealing/extension. The melt curve analysis program consisted of 95 °C for 10 s, 72 °C for 5 min, and a step cycle starting at 65 °C with a 0.2 °C/s transition rate to 95 °C. Negative controls and internal control were also run in each plate. The primers of genes are as follows (Table 1):

The specificity products of RT-PCR were confirmed by melting curve analysis. Threshold cycle (CT) value measured the fluorescence and corresponded to the fixed threshold on the amplification curve. The CT value of β-actin acts as the inner control for the target genes. △CT is the CT value of β-actin minus the CT value of the target gene. Fold changes in gene expression for each sample were calculated with the 2^(− △CT) method relative to control after normalization of gene-specific CT values to β-actin CT value. All samples

Table 1 The primers of the genes

Genes	Primer sequences
β-actin	Forward: 5′-GACATCCGCAAAGACCTG-3′
	Reverse: 5′-GGAAGGTGGACAGCGAG-3′
Foxp3	Forward: 5′-GGTGCTGGAGAAGGAGAA-3′
	Reverse: 5′-GATGATGCCACAGATGAAG-3′
TGF-β	Forward: 5′-AGCAATCTGTGGGTTGTGACT-3′
	Reverse: 5′-GGTAGAGCGATTACGACTCTGTT-3′
IL-35	Forward: 5′-CCTTGCACTTCTGAAGAGATTGA-3′
	Reverse: 5′-ACAAGGCCATCATAAAAGAGGT-3′

were run at least three times, and the relative expression of mRNA was normalized to the mRNA of β-actin.

Statistical analysis

All data were indicated as the mean ± standard deviation (SD). Statistical analysis was performed with GraphPad Prism software 5.0. Comparisons between two groups and was made by using Wilcoxon Test. $P < 0.05$ were considered to be statistically significant.

Results

Subjects' characteristics

Ambient air pollution data were shown in Table 2, which confirmed contrasts in pollution concentrations between Lanzhou and Lintao. The study included 42 children, of which half were from Lanzhou (including 8 boys, 13 girls), and half were from Lintao (including 7 boys, 14 girls). The subjects were mainly Han Chinese [21 subjects (100%) from Lanzhou and 18 subjects (85.7%) from Lintao], while three (14.3%) subjects from Lintao were Tibetan. The age of subjects was 11.67 ± 0.86 in Lanzhou and 11.38 ± 0.59 in Lintao, with no significant difference between groups. Several variables were significantly different between two groups, including educational level, family income, parental smoking rate, and household fuel use (Table 3). For example, the educational background for parents of subjects from Lanzhou were higher than those from Lintao ($P < 0.01$), with 17 (81%) parents graduated from universities in the Lanzhou group, whereas only 2 (9.6%) parents graduated from universities in the Lintao group. All the rest parents were high school graduates. These differences may have influences on exposure and/or outcomes measured.

Table 2 The concentration of PAHs and $PM_{2.5}$ in Lanzhou and Lintao (December 2015–June 2016)

	PAHs (sum of 16), ng/m^3	$PM_{2.5}$, μg/m^3
Lanzhou	388.5 ± 336.9	65.7 ± 26.5
Lintao	707.3 ± 743.4	55.3 ± 26.7

Percentage of Treg cells

Treg cells play an important role in immune regulation. Flow cytometry analysis demonstrated that the number of Treg cells in the box of the flow cytometry chart was similar between the Lintao and Lanzhou groups (Fig. 1a). The percentage of Treg cells was represented by the number of $CD4^+CD25^+CD127^{low}$ T cells divided by the number of $CD4^+$ T cells. Data analysis revealed that the mean percentage of Treg cells was 7.623 in the Lintao group and 7.437 in Lanzhou group, which were not statistically different (Fig. 1b).

The expression of cytokines

Foxp3 (Bennett et al. 2001; Hori et al. 2003), TGF-β (Yamagiwa et al. 2001), and IL-35 (Collison et al. 2007) all produce human Treg cells. They also regulate Treg cells' function. RT-PCR was performed to compare the relative transcript level of Foxp3, TGF-β, and IL-35 gene in different groups. The data showed that expression of Foxp3 mRNA was 0.00008 and 0.00015 in Lintao and Lanzhou groups, respectively. The statistical analysis indicated that the relative expression of Foxp3 mRNA was significantly decreased in the Lintao group ($P < 0.05$) (Fig. 2a). The IL-35 mRNA expression was also significantly lower in the Lintao group (0.00035) compared to that of the Lanzhou (0.00172) group ($P < 0.01$) (Fig. 2c). However, the difference in the expression of TGF-β mRNA was not significant, although it was lower in the Lintao group (0.00219) compared to the Lanzhou group (0.00328) (Fig. 2b).

Discussion

Treg cells, formerly known as suppressor T cells, are very important for keeping the homeostasis of the immune system. It is also involved in many immune diseases under abnormal conditions. There are varieties of studies indicating that the number of Treg cells is reduced in subjects of systemic lupus erythematosus (Shimon et al. 2008), multiple sclerosis, and immune dysregulation (Huter et al. 2008). PAHs-induced immune-toxicity has been studied for many years, with evidence being mainly from animal studies, such as in mice or fish, or from in vitro human cell studies. There is little evidence from human epidemiological studies of environmental exposure to PAHs in ambient air pollution. Molecular and cellular mechanisms largely remain unclear. Epigenetic modification plays an important role in regulating the expression of genes (Miller

Table 3 Characteristics of subject groups

Characteristics	Category	Lanzhou $n = 21$(%)	Lintao $n = 21$(%)
Sex	Male	8 (38.1%)	7 (33.3%)
	Female	13 (61.9%)	14 (66.7%)
Ethnicity	Han nationality	21 (100%)	18 (85.7%)
	Tibetan	0 (0)	3 (14.3%)
Age	Mean ± SD	11.67 ± 0.86	11.38 ± 0.59
Education	University	17 (81%)	2 (9.6%)*
	High school	4 (19%)	19 (90.4%)*
Family income	≧ 30,000 RMB	21 (100%)	3 (14.3%)*
	< 30,000 RMB	0 (0)	18 (85.7%)
Cooking and heating fuel	Coal, firewood	0 (0)	8 (38.1%)*
	Natural gas, electricity	21 (100%)	13 (61.9%)
Parental smoking	Yes	11 (52.4%)	18 (85.7%)*
	No	10 (47.6%)	3 (14.3%)

*$P < 0.01$

and Ho 2008; Baccarelli et al. 2009). Nadeau et al. (2010) reported ambient air pollution exposure led to hypermethylation of the Foxp3 locus and impaired Treg cells function, as well as an association between PAH exposure and total number of methylated CpG islands in Treg cell Foxp3 gene among children living in California, USA, which was the first published paper reporting these findings. However, strong arguments have been made on unmeasured confounding from non-homogenous ethnicity and unbalanced social economic status between groups in Nadeau et al. (2010). In the present study, we observed lower mRNA expression for Foxp3, IL35, and TGF-β in children living in Lintao than those of in Lanzhou. Our air monitoring data indicated that ambient PAH concentration was two times higher in Lintao than that was in Lanzhou. These results are consistent with observations reported by Nadeau et al. (2010) in Fresno, CA. The strengths of the present study are, first, Lintao is only 80 km from Lanzhou. Lifestyle and dietary habits are very similar among people living in these two cities. Secondly, the ethnicity was highly similar in two groups studied. The present study also provides more discrimination between $PM_{2.5}$ and PAH exposures. In air pollution studies, pollutants are often correlated. In this study, while PAH concentration was two times higher in Lintao, $PM_{2.5}$ concentrations were actually comparable, indicating PAHs might have played a more important role in air pollution-induced immunological impairments. It is interesting to find that immunological changes were in the same direction and at similar magnitudes when particulate matter and PAHs were three times and 100 times higher, respectively, in these two Chinese cities than those were in Fresno, California (annual average were 21.2 μg/m^3 and 4.4 ng/m^3 for$PM_{2.5}$ and PAHs, respectively) (Nadeau et al. 2010). Mechanisms of how immune system copes with such high pollution exposures warrant further investigation.

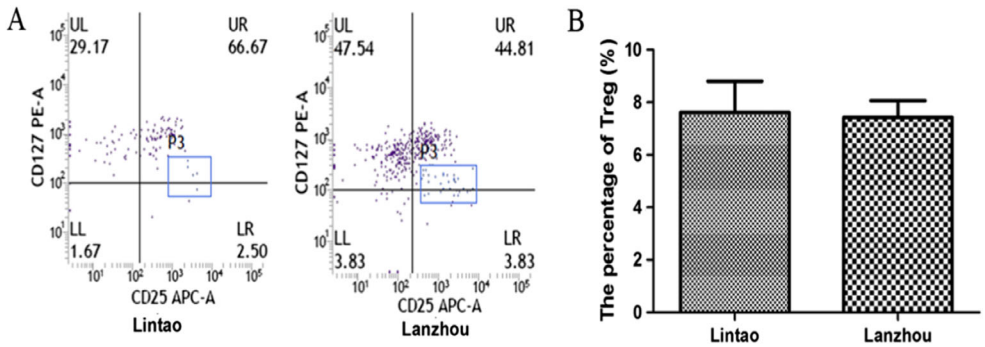

Fig. 1 The number of Treg cells in different groups. PBMCs were obtained from peripheral blood samples, and Treg cells were sorted by flow cytometry. Treg cells were marked as $CD4^+CD25^+CD127^{low}$ (Lintao, $n = 21$; Lanzhou, $n = 21$). The percentage of Treg cells equals the number of $CD4^+CD25^+CD127^{low}$ T cell divided by the number of $CD4^+$ T cell. **a** The result of flow cytometry analysis. **b** Average of % of Treg cells among $CD4^+$ T cells. Vertical bars represent SD from the mean

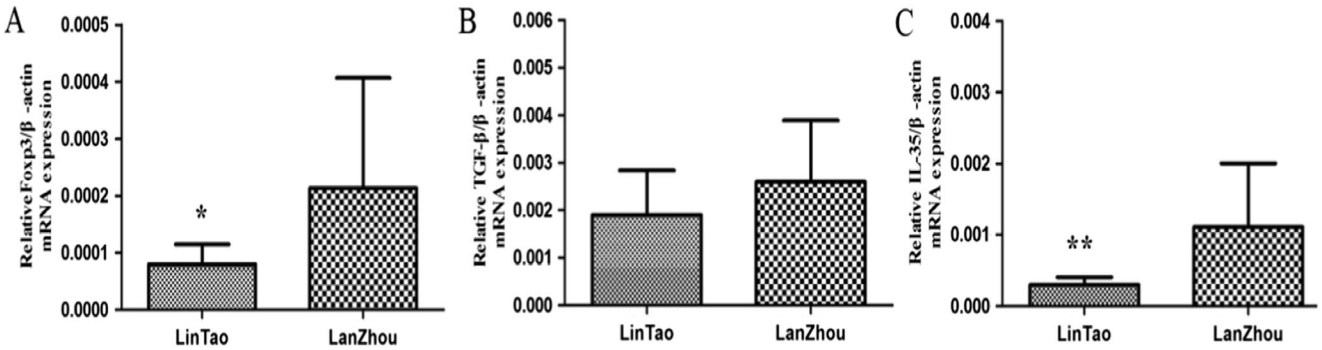

Fig. 2 Relative expression of cytokines mRNA. The serum was obtained from peripheral blood samples. The cytokines expression was detected by RT-PCR (Lintao, $n = 21$; Lanzhou, $n = 21$). **a** Expression of Foxp3 mRNA in Lintao and Lanzhou groups. **b** Expression of TGF-β mRNA in the Lintao and Lanzhou groups. **c** Expression of IL-35 mRNA in the Lintao and the Lanzhou groups. $^{*}P < 0.05$ and $^{**}P < 0.01$ based on Wilcoxon test. Vertical bars represent SD from the mean

Functions of cytokines in immune system

Foxp3 is a transcription factor that is related to Treg cells function and development (Fragale et al. 2008). Meanwhile, it is also a specific intracellular marker of Treg cells (Takeuchi and Nishikawa 2016). Treg cells manage the function of immune inhibition through expression of Foxp3 (Hill et al. 2007). Studies indicated air pollution could initiate transformation of Th1 to Th2 cells, which would lead to the secretion of Th2 cytokines such as IL-4, IL-13, and IL-35 (Truyen et al. 2006; Miller and Ho 2008; Baccarelli et al. 2009). Research has reported exposure to PAHs increases DNA methylation of CpG islands of several genes in asthmatic children (Perera et al. 2009). Human cellular toxicity studies further indicated that increased DNA methylation of Foxp3 led to a lower mRNA expression of Foxp3, which was associated with dysfunction of Treg cells (Woodfolk 2007; Nguyen et al. 2008). A more recent study showed that PAHs exposure might alter methylation patterns of Foxp3 gene involved in immune regulation (Hew et al. 2015). These changes of methylated Foxp3 might lead to inhibited protein expression (Mao et al. 2015). In the present study, the mRNA of Foxp3 obtained from PBMCs was used to test the expression of mRNA of Foxp3. Results showed that subjects living in Lintao had a lower level of Foxp3 gene expression compared to those living in Lanzhou, suggesting a lower expression of mRNA Foxp3 influenced by the high concentration of PAHs in Lintao.

TGF-β is a key cytokine mediator in immune tolerance. It is secreted by many immune cells. Part of its function is to adjust the expression of Foxp3 in human $CD4^+$ T cells and promote $CD4^+CD25^-$T cell transformation to Treg cells (Chen and Konkel 2010). Apart from this, it also plays an important role in the regulating effect of Treg cells (Hew et al. 2015). Huter et al. found that the level of TGF-β was associated with the level of Foxp3 mRNA in asthma, suggesting that the low level of Foxp3 mRNA might be influenced by hyposecretion of TGF-β (Huter et al. 2008). Our data demonstrated the expression of TGF-β having a similar trend as the level of Foxp3 gene expression, both being lower among children living in Lintao where PAH ambient level was much higher than that of Lanzhou.

IL-35 is a new member of IL-12 family cytokines. It is mainly secreted by Treg cells and is a major cytokine of immune negative regulation (Chaturvedi et al. 2011; Whitehead et al. 2012). However, it does not directly induce the production of Foxp3 (Collison et al. 2010). Collison and his colleges identified that IL-35 had a similar function as TGF-β in promoting the production of Treg cells. Therefore, the expression of IL-35 in Treg cells may be associated with the downstream production of Foxp3 (Collison et al. 2007). Given the similarity of people living in these two cities such as in lifestyle, dietary habits, and ethnicity, we found differences in gene expressions not only of Foxp3 and TGF-β but also of IL-35 between two groups, which strongly indicated the potential influence of PAH exposure on immune parameters among healthy children from these cities. But the specific cellular mechanism remains to be further studied.

The number of Treg cells in the function of immune system

No significant difference was observed in the Treg cells number (as percentage of $CD4^+$ T cells) between healthy children living in Lanzhou and Lintao. A possible explanation is that exposure to ambient PAHs might influence the production of proteins at mRNA levels without leading to the significant changes in the number of Treg cells. The miRNAs regulate the expression of protein-coding genes. Approximately 700 miRNAs have been found in mammals. They play an important role in biological functions (Bartel 2004; Griffiths-Jones et al. 2006; Shimon et al. 2008). It has been reported that one of these miRNAs, miR-155, is highly expressed in Treg cells and contributes to the Treg cells development, but is not essential for Treg cells function (Cobb et al. 2006; Zheng et al. 2007). We found significant difference in Foxp3 mRNA

expression but not in Treg cells numbers. We speculate that micRNAs might have played a role in this phenomenon.

The exact mechanism warrants further study.

Limitations

This study focused on PAH exposure from ambient air pollution. As a pilot study, the small sample size may have limited statistical power in detecting exposure effects. The cross-sectional design also limited the ability to determine the cause of immunological effects. We noticed the differences in parental smoking rate and household fuel use between two groups, which would potentially lead to overestimation of the effects induced by exposure to the ambient air pollution. These factors need to be considered in the future studies in order to better understand PAH exposure-induced immune impairments. Unbalanced social economic status might have influence on our results as well. Potential influence of dietary intake of PAHs could be evaluated by testing urinary PAH metabolites in the future research.

Conclusion

In this study, we examined the relationship between Treg cell function and exposure to ambient air pollution, specifically PAHs among healthy children living in Gansu, China. We observed repressed gene expressions of Foxp3 ($P < 0.05$), IL35 ($P < 0.05$), and TGF-β in the city with higher PAH pollution, suggesting an impairment of Treg cells function potentially associated with higher PAH exposure. To the best of our knowledge, this is the first study evaluating Treg cells' responses to the exposure of PAHs in ambient air pollution among healthy children in China. Mechanisms of the immune system coping with extremely high air pollution and consequent health effects of any potential immune compromises warrant further investigation.

Acknowledgments This study was supported by Chau Hoi Shuen Women in Science Program. The authors wish to thank the hospital staff for their assistance in blood sample collection and the children from both cities for their participation.

Author contribution Sa Liu, Junling Wang, and S. Katharine Hammond conceived and designed the study. Panhong Gou, Zhonghui Ye, and Yueli Yao carried out the data collection. Panhong Gou, Xiaoru Chang, and Patton Khuu Nguyen conducted the labor test. Panhong Gou analyzed the data and developed the initial draft of the paper. All authors reviewed and provided input to the writing, editing, and finalization of the paper.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

References

Arnold J, Zimmerman B, Li M, Lairmore MD, Green PL (2008) Human T-cell leukemia virus type-1 antisense-encoded gene, Hbz, promotes T-lymphocyte proliferation. Blood 112:3788–3797

Baccarelli A, Wright RO, Bollati V, Tarantini L, Litonjua AA, Suh HH, Zanobetti A, Sparrow D, Vokonas PS, Schwartz J (2009) Rapid DNA methylation changes after exposure to traffic particles. Am J Respir Crit Care Med 179:572–578

Bartel DP (2004) MicroRNAs genomics, biogenesis, mechanism, and function. Cell 116:281–297

Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, Kelly TE, Saulsbury FT, Chance PF, Ochs HD (2001) The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nat Genet 27:20–21

Chaturvedi V, Collison LW, Guy CS, Workman CJ, Vignali DA (2011) Cutting edge: human regulatory T cells require IL-35 to mediate suppression and infectious tolerance. J Immunol 186:6661–6666

Chen W, Konkel JE (2010) TGF-beta and 'adaptive' Foxp3(+) regulatory T cells. J Mol Cell Biol 2:30–36

Cobb BS, Hertweck A, Smith J, O'Connor E, Graf D, Cook T, Smale ST, Sakaguchi S, Livesey FJ, Fisher AG, Merkenschlager M (2006) A role for Dicer in immune regulation. J Exp Med 203:2519–2527

Cohen AC, Nadeau KC, Tu W, Hwa V, Dionis K, Bezrodnik L, Teper A, Gaillard M, Heinrich J, Krensky AM, Rosenfeld RG, Lewis DB (2006) Cutting edge: decreased accumulation and regulatory function of CD41 CD25(high) T cells in human STAT5b deficiency. J Immunol 177:2770–2774

Collison LW, Workman CJ, Kuo TT, Boyd K, Wang Y, Vignali KM, Cross R, Sehy D, Blumberg RS, Vignali DA (2007) The inhibitory cytokine IL-35 contributes to regulatory T-cell function. Nature 450:566–569

Collison LW, Chaturvedi V, Henderson AL, Giacomin PR, Guy C, Bankoti J, Finkelstein D, Forbes K, Workman CJ, Brown SA, Rehg JE, Jones ML, Ni HT, Artis D, Turk MJ, Vignali DA (2010) IL-35-mediated induction of a potent regulatory T cell population. Nat Immunol 11:1093–1101

Fragale A, Gabriele L, Stellacci E, Borghi P, Perrotti E, Ilari R, Lanciotti A, Remoli AL, Venditti M, Belardelli F, Battistini A (2008) IFN regulatory factor-1 negatively regulates CD4+CD25+ regulatory T cell differentiation by repressing Foxp3 expression. Immunology 181:1673–1682

Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ (2006) miRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acids Res 34:D140–D144

Gupta RS, Springston EE, Warrier MR, Smith B, Kumar R, Pongracic J, Holl JL (2011) The prevalence, severity, and distribution of childhood food allergy in the United States. Pediatrics 128:E9–E17

Hew KM, Walker AI, Kohli A, Garcia M, Syed A, McDonald-Hyman C, Noth EM, Mann JK, Pratt B, Balmes J, Hammond SK, Eisen EA, Nadeau KC (2015) Childhood exposure to ambient polycyclic aromatic hydrocarbons is linked to epigenetic modifications and impaired systemic immunity in T cells. Clin Exp Allergy 45:238–248

Hill JA, Benoist C, Mathis D (2007) Treg cells: guardians for life. Nat Immunol 8:124–125

Hori S, Nomura T, Sakaguchi S (2003) Control of regulatory T cell development by the transcription factor Foxp3. Science 299:1057–1061

Huter EN, Punkosdy GA, Glass DD, Cheng LI, Ward JM, Shevach EM (2008) TGF-b-induced Foxp3+ regulatory T cells rescue scurfy mice. Eur J Immunol 38:1814–1821

IARC (1987) Overall Evaluations of Carcinogenicity. IARC monographs on the evaluation of carcinogenic risk of chemicals to humans, Supplement 7.

Kavouras IG, Tsapakis KP, Lagoudaki M, Stephanou E, Von EG, Baer D, Oyola P (2001) Source apportionment of urban particulate aliphatic and polynuclear aromatic hydrocarbons (PAHs) using multivariate methods. Environ Sci Technol 35:2288–2294

Li W, Wang C, Wang H, Chen J, Shen H, Shen G, Huang Y, Wang R, Wang B, Zhang Y, Chen H, Chen Y, Su S, Lin N, Tang J, Li Q, Wang X, Liu J, Tao S (2014) Atmospheric polycyclic aromatic hydrocarbons in rural and urban areas of northern China. Environ Pollut 192: 83–90

Lin SS, Huang Y, Wang CY, Ren AG (2016) Polycyclic aromatic hydrocarbons exposure and birth defects. Chin J Prev Med 50:563–568

Mao J, Zhao MT, Whitworth KM, Spate LD, Walters EM, O'Gorman C, Lee K, Samuel MS, Murphy CN, Wells K, Rivera RM, Prather RS (2015) Oxamflatin treatment enhances cloned porcine embryo development and nuclear reprogramming. Cell Reprogram 17:28–40

Marson A, Kretschmer K, Frampton GM, Jacobsen ES, Polansky JK, MacIsaac KD, Levine SS, Fraenkel E, von Boehmer H, Young RA (2007) Foxp3 occupancy and regulation of key target genes during T-cell stimulation. Nature 445:931–935

Miller RL, Ho SM (2008) Environmental epigenetics and asthma: current concepts and call for studies. Am J Respir Crit Care Med 177:567–573

Moorman JE, Akinbami LJ, Bailey CM, Zahran HS, King ME, Johnson CA, Liu X (2012) National surveillance of asthma: United States, 2001–2010. Vital Health Stat 35:1–58

Nadeau K, McDonald-Hyman C, Noth EM, Pratt B, Hammond SK, Balmes J, Tager I (2010) Ambient air pollution impairs regulatory T-cell function in asthma. J Allergy Clin Immunol 126:845–852 e810

Nguyen KD, Fohner A, Booker JD, Dong C, Krensky AM, Nadeau KC (2008) XCL1 enhances regulatory activities of CD4+CD25+ CD127-T cell in human allergic asthma. J Immunol 181:5386–5395

Noth EM, Lurmann F, Northcross A, Perrino C, Vaughn D, Hammond SK (2016) Spatial and temporal distribution of polycyclic aromatic hydrocarbons and elemental carbon in Bakersfield, California. Air Qual Atmos Health 9:899–908

Perera F, Tang WY, Herbstman J, Tang D, Levin L, Miller R, Ho SM (2009) Relation of DNA methylation of 5′-CpG island of ACSL3 to transplacental exposure to airborne polycyclic aromatic hydrocarbons and childhood asthma. PLoS One 4:e4488

Perez L, Declercq C, Iniguez C, Aguilera I, Badaloni C, Ballester F, Bouland C, Chanel O, Cirarda FB, Forastiere F, Forsberg B, Haluza D, Hedlund B, Cambra K, Lacasana M, Moshammer H, Otorepec P, Rodriguez-Barranco M, Medina S, Kunzli N (2013) Chronic burden of near-roadway traffic pollution in 10 European cities (APHEKOM network). Eur Respir J 42:594–605

Pleil JD, Vette AF, Johnson BA, Rappaport SM (2004) Air levels of carcinogenic polycyclic aromatic hydrocarbons after the World Trade Center disaster. Proc Natl Acad Sci U S A 101:11685–11688

Sakaguchi S, Wing K, Yamaguchi T (2009) Dynamics of peripheral tolerance and immune regulation mediated by Treg. Eur J Immunol 39: 2331–2336

Shimon S, Tomoyuki Y, Takashi N, Masahiro O (2008) Regulatory T cells and immune tolerance. Cell 133:775–787

Takeuchi Y, Nishikawa H (2016) Roles of regulatory T cells in cancer immunity. Int Immunol 28:401–409

Truyen E, Coteur L, Dilissen E, Overbergh L, Dupont LJ, Ceuppens JL, Bullens DM (2006) Evaluation of airway inflammation by quantitative Th1/Th2 cytokine mRNA measurement in sputum of asthma patients. Thorax 61:202–208

Verhasselt V, Milcent V, Cazareth J, Kanda A, Fleury S, Dombrowicz D, Glaichenhaus N, Julia V (2008) Breast milk-mediated transfer of an antigen induces tolerance and protection from allergic asthma. Nat Med 14:170–175

Wang M, Zheng S, Wang S, Tao Y, Shang K (2012) A time-series study on the relationship between gaseous air pollutants and daily hospitalization of respiratory disease in Lanzhou City. J Hyg Res 41:771–775

Whitehead GS, Wilson RH, Nakano K, Burch LH, Nakano H, Cook DN (2012) IL-35 production by inducible costimulator (ICOS)–positive regulatory T cells reverses established IL-17–dependent allergic airways disease. J Allergy Clin Immunol 129:207–215.e205

Woodfolk JA (2007) T-cell responses to allergens. J Allergy Clin Immunol 119:280–294

Yamagiwa S, Gray JD, Hashimoto S, Horwitz DA (2001) A role for TGF-beta in the generation and expansion of CD4+CD25+ regulatory T cells from human peripheral blood. J Immunol 166:7282–7289

Zheng Y, Josefowicz SZ, Kas A, Chu TT, Gavin MA, Rudensky AY (2007) Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. Nature 445:936–940