

Effects of ozone exposure mediated by BEAS-2B cells on T cells activation: a possible link between environment and asthma

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Summary

Objective: To explore the possible link between ozone and asthma through analyzing Th1/Th2 differentiation of T cells following incubation with conditioned medium from the BEAS-2B cells exposed to ozone in vitro.

Method: Bronchial epithelial cell line, BEAS-2B, was cultured using an air-liquid interface culture system in a CO₂ incubator and exposed to 0 or 0.16 or 0.25 mg/m³ of ozone for 8 h. The amounts of IL-1 β , IL-6 and RANTES in the cell supernatant were detected. The cell culture supernatants were collected and used as conditioned medium in the next experiment. T cells from children recruited were incubated with conditioned medium for 12 h. Activation rate of CD69 and Th1/Th2/Th17 differentiation were analyzed.

Results: BEAS-2B cells exposed to different ozone concentrations showed morphological changes. Cells exposed to 0.16 and 0.25 mg/m³ ozone produced higher amounts of IL-1 β , IL-6 and RANTES than that in the control group. Children with allergic asthma had upregulated expression of genes related with asthma, including CCL2, CCR4, CXCL2, CYSLTR1, IL12RB2, IL13RA2, IL18R1, IL1B, IL8, IL8RB and TNFSF13. CD69 expression in T cells was significantly elevated irrespective of ozone exposure in children with allergic asthma. Following ozone exposure, in asthmatic children group, expression levels of cytokines of Th1 cells were collectively higher than those from Th2 cells. Ozone-exposed conditioned media could slightly increase all the Th1, Th2 and Th17 cytokines in T cells from allergic asthmatic children.

Conclusions: Our results suggested that Th1 cells activation might be predominant over Th2 activation upon ozone exposure in asthmatic children, which might help to clarify the mechanisms of asthma related to environmental factors like ozone. (*Asian Pac J Allergy Immunol* 2014;32:25-34)

Key words: bronchial epithelial cell; ozone; gene chip; Th1/Th2 differentiation

Introduction

The incidence of asthma is currently considered to be related with environmental factors. Epidemiological and laboratory studies have shown that air pollution, especially ozone exposure, exacerbates asthma symptoms, leading to increased asthma morbidity and mortality.¹ Studies have shown that ozone exposure can increase serum IgE levels in adult asthma patients.² Romieu et al.³ found that there is a significant positive correlation between ozone concentration and malondialdehyde content in breath condensate of asthmatic children. The pathogenesis of asthma is not fully understood,

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which might be regulated by extensive T helper cells including Th1, Th2, and Th17 cells.⁴ ‘Th2 hypothesis in allergy’ proposes that the Th1/Th2 (helper T-cells) imbalance, skewed towards a Th2 response, contributes to asthma pathogenesis [5]. Th1/Th2 differentiation is a key regulator of the immune responses. Anomalies or defects in Th1/Th2 differentiation can lead to diseases such as chronic infection, autoimmune disease or allergy. Thus, understanding Th1/Th2 differentiation has become a major research focus. Cytokines are considered to be the most important factors involved in Th cell differentiation and asthma incidences.⁶ Hence we studied the cytokine profiles of T cells that were exposed to ozone in order to identify the nature of the immune responses, which can be either Th1, Th2 or Th17. Th2 cells orchestrate inflammation in asthma through the secretion of a series of cytokines, particularly IL-4, IL-13, IL-5, IL-6 and IL-10, which are the major factors regulating IgE production by B cells and are required for optimal Th2 differentiation. In contrast, Th1 cells secrete IFN- γ , IL-2 and TNF- α , which mediate cellular immunity, cytotoxic T lymphocyte differentiation and activation, macrophage activation, and inhibition of B cells. Indeed, the increased and abnormal expression of cytokines in airway cells is one of the major targets of corticosteroid therapy, which is by far the most effective means of treatment for asthma. Surprisingly, adoptively transferred Th1 cells, though reduces airway eosinophilia, fail to reduce airway hyperresponsiveness (AHR) and potentiate cellular inflammation in the lungs.⁷ In agreement with this, the Th1-type cytokine IFN- γ has no significant effect in antigen-induced AHR, at least in some mouse models of asthma.⁸ The above evidence suggests that dysregulation in the immune system that leads to allergy and asthma cannot be explained completely by the ‘Th2 hypothesis in allergy’. Thus, further work is needed to determine the effect of *in vitro* exposure to ozone on the direction of Th1/Th2 differentiation in T cells collected from children with allergic asthma and to explain the relationship between ozone exposure and the occurrence and aggravation of asthma.

Methods

Subjects

Children diagnosed with allergic asthma (n = 3, age is 5 years old, 9 years old and 12 years old) were recruited from our outpatient unit at the

Department of Pediatrics, the Second Affiliated Hospital of Sun Yat-sen University, China. Allergic asthma was diagnosed, according to the criteria of allergic asthma by Global Initiative for Asthma (GINA). The control group comprised of three children who had no allergic disease history, family history of allergies, asthma history, recent history of prescription drugs and were of similar age as that of the children with allergic asthma. Written informed consent was obtained from individual children's parents, and experimental protocols were approved by the Institution Research Board of our university.

Cell culture in air-liquid interface culture system

Human bronchial epithelial cell line, BEAS-2B (#CRL-9609, LOT: 6805252, 10⁵ cells/ml, ATCC, USA) were cultured in 2ml of LHC-9 (Biosource, Carlsbad, CA, USA) complete medium in a ventilated petri dish for 24 hours. The transwell nested Petri dishes inserted with polyethylene terephthalate (PET) membrane (#3450, 0.4 μ m membrane pore size, 24mm nested diameter, CORNING, USA) was pre-equilibrated with LHC-9 for one hour, in order to improve the efficiency of cell adhesion. After pre-equilibrium, the media was removed, 1.5 ml of BEAS-2B cell suspension was added to the nested compartment and 2.5 ml of fresh medium was added to the lower plate.

Production of ozone

Ozone, produced by physical electrode ozone generator (W00327-3R, Shanghai Scientific Instrument Factory, China), was mixed with activated carbon-filtered air and pumped into the cell incubator. Ozone concentration in the incubator was monitored in real time by UV absorption ozone analyzer (8810 type, Beijing Rayleigh Environmental Monitoring Instruments, China) and maintained within a 5% variation by regulating the flow of carbon-filtered air and ozone.

BEAS-2B cell exposure

The transwell transparent nested Petri dishes were incubated at 37°C in 5 % CO₂ incubator in the presence or absence of ozone. Ozone concentrations were 0 (control), 0.16 and 0.25 mg/m³ and exposure time was 8 hours as previously reported.⁹ Following ozone exposure, cells and their supernatants were separated. Cells were observed by transmission electron microscopy for ultrastructure, and part of the supernatant was collected for analyzing IL-1 β , IL-6 and RANTES amounts by enzyme-linked immunosorbent assay (ELISA) kits (R&D systems, Minneapolis, MN, USA), according to the



manufacturer's instructions. The sensitivity of the IL-1 β , IL-6 and RANTES ELISA kits was 0.3 pg/ml. Another part of supernatants were collected as conditioned medium and stored at -20°C.

Microarray analysis

Human blood samples were collected in EDTA-treated tubes from individual children with allergic asthma or healthy children. All samples were processed within two hours of collection. Total RNA was extracted using TRIzol reagent (Invitrogen™, Biosource, Carlsbad, CA, USA) and labeled using the TrueLabling-AMP™ Linear RNA Amplification kit (#GA-10, SABiosciences, USA) to synthesize labeled probe. Hybridization was completed using Oligo® GEArray Human Asthma Microarray (#OHS-067, SABiosciences, USA) at the Shanghai KangChen Bio-tech Inc.

Detection of T cell activation rate

Mononuclear cells in blood samples were prepared by Ficoll gradient centrifugation. T cells were purified using the T cell enrichment column (#HTCC525, R and D systems Inc, Minneapolis, USA), according to the manufacturer's instruction. The purified T cells with a purity of > 95% were used for the following experiments. The purified T cells at 1×10^6 cells/ml were cultured for 12h hours with 2% fetal bovine serum (FBS) medium containing different proportions of the conditional media from the BEAS-2B cells exposure to ozone or without exposure, which was reported in our previously research.⁹ T cells and supernatants were separated by centrifugation. T cell samples were incubated with 20 μ l of FITC mouse anti-human CD69 (#555748, BD Biosciences, USA) at 4°C in the dark for 30 min. The T cells were washed twice with PBS buffer and analyzed. T cells activation was measured according to the expression level of CD69. Activation rate of T cells were calculated as ratio value CD69+ cells over all cells tested by flow cytometry (FACS Calibur, BD, USA). The supernatants were harvested for next experiment.

Th1/Th2 cytokine profile

The Th1/Th2 cytokine profile of T cells was determined by RayBio® Human Th1/Th2/Th17 Antibody Array (#AAH-TH17-1-8, SABiosciences, USA) at the Shanghai KangChen Bio-tech Inc. The array detects 34 cytokines, chemokines and growth factors simultaneously. Array membranes were

processed following the manufacturer's recommendations. The resulting images were analyzed using Scanalyze software. In order to compare the luminescence intensity among samples, the luminescence of the background staining was subtracted from that of the sample and normalized to the data of the positive controls on the same membrane. To ensure the reliability of the results, each analysis was repeated once. Among the 34 different cytokines analyzed, we selected eight cytokines to assess Th1/Th2 status. IFN- γ , IL-2 and TNF- α were chosen to represent Th1 cytokines and IL-4, IL-5, IL-10, IL-13, IL-6 were chosen to represent Th2 cytokines.

Statistical analysis

Data were analyzed with the SPSS for windows (SPSS Inc. USA). Data were expressed as means \pm standard deviation (SD). The difference in CD69 expression between treatment groups was analyzed by chi-square test. The difference in the amounts of IL-1 β , IL-6 and RANTES was analyzed by the two-tailed Student's t-test. Significance was determined at $P < 0.05$.

Results

Ultrastructure of BEAS-2B cells under the transmission electron microscopy

BEAS-2B cells cultured without exposure to ozone exhibited a normal structure with intact cell membranes and microvilli. The nucleus had a normal shape with an intact nuclear membrane and nucleoli. Other cell organelles in the cytoplasm were also normal. Mitochondria were of expected number, exhibited mitochondrial crests and had microfilaments with clear structure (Figure 1. Aa). BEAS-2B exposed to 0.16 mg/m³ ozone had increased organelle density and deformed nuclear membrane. The mitochondria and endoplasmic reticulum occasionally exhibited an inflated and fuzzy membrane structure with fractured and fragmented mitochondrial crests or matrix vacuolization (Figure 1. Ab). BEAS-2B cells exposed to 0.25 mg/m³ ozone had a disordered cytoskeleton, expanded organelles, vacuolation, ruptured structures, and a fuzzy and fragmented nuclear membrane with karyopyknosis (Figure 1. Ac).

Ozone stimulates the production of inflammatory cytokines in BEAS-2B cells

The amounts of IL-1 β , IL-6, and RANTES in the supernatants of the cultured BEAS-2B were

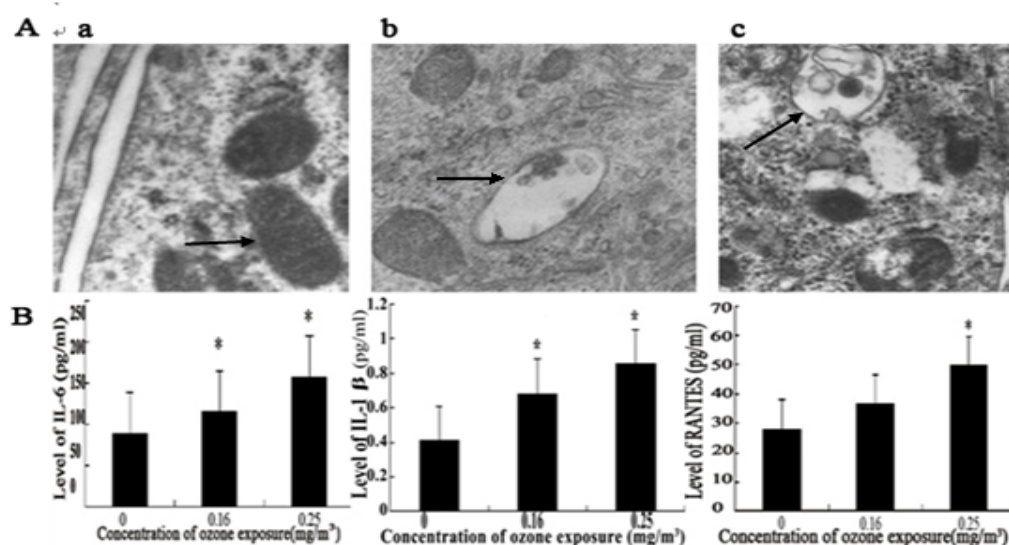


Figure 1. A. Ozone exposure leads to morphological changes of BEAS-2B cells *in vitro*. The cells were harvested and observed by transmission electron microscopy for ultrastructure. a. Representative ultrastructure images of BEAS-2B cells exposed to 0 mg/m³ ozone for 8 h in air-liquid interface (52000 magnification). As shown by arrows, mitochondria were of expected number, exhibited mitochondrial crests and had microfilaments with clear structure. b. Representative ultrastructure images of BEAS-2B cells exposed to 0.16 mg/m³ ozone for 8 h in air-liquid interface (21000 magnification). As shown by arrows, mitochondrial exhibited an inflated and fuzzy membrane structure with fractured and fragmented mitochondrial crests or matrix vacuolization. c. Representative ultrastructure images of BEAS-2B cells exposed to 0.25 mg/m³ ozone for 8 h in air-liquid interface (28500 magnification). As shown by arrows, cells had a disordered cytoskeleton, expanded organelles, vacuolation, ruptured structures, and a fuzzy and fragmented nuclear membrane with karyopyknosis.

B. Ozone exposure stimulates the production of IL-1 β , IL-6 and RANTES in BEAS-2B cell *in vitro*. Data are expressed as mean \pm SD of each group of cells from three independent experiments. * ozone exposure group Vs control group, $p < 0.05$.

analyzed by ELISA to determine the biological activity of conditioned media (Figure 1B). BEAS-2B cells cultured without exposure to ozone had very low levels of IL-1 β , IL-6 and RANTES. BEAS-2B cells cultured with 0.16 mg/m³ ozone had increased levels of IL-1 β , IL-6, and RANTES. The levels of IL-1 β , IL-6 and RANTES were further significantly increased in the supernatants of the cells exposed to 0.25 mg/m³ ozone ($P < 0.05$). This indicated that *in vitro* ozone exposure stimulated the production of inflammatory cytokines in BEAS-2B cells and that the supernatants could be used as conditioned medium.

Asthma-related genetic characteristics in patients

The 1.7 fold cut-off point was chosen in accordance with reference values provided in the literature.¹⁰ A high cut-off point will miss important changes.¹¹ The microarray profile of T cells from children with and without allergic asthma is provided in Table 1. Compared with the gene

expression profile of control children, eleven genes were up-regulated in allergic asthma children by at least 1.7 fold. These genes include CCL2, CCR4, CXCL2, CYSLTR1, IL12RB2, IL13RA2, IL18R1, IL1B, IL8, IL8RB and TNFSF13.

Activation of T cell

CD69 belongs to the C-type lectin receptor family, and is the earliest to be expressed as a surface antigen upon T cell activation. CD69 expression in T cells was significantly elevated after incubation with conditioned medium irrespective of ozone exposure in children with allergic asthma when compared to that from nonallergic control children (Figure 2). The higher CD69 expression in T cells indicated that T cells were activated and that there is a difference in T cell activation between nonallergic children and allergic asthmatic children.

Table 1. List of up-regulated genes in children with asthma that met the criteria of $P < 0.05$ and fold change of ≥ 1.7 .

Position	GeneBank	Symbol	Description	Ratio
8	NM_002982	CCL2	Chemokine (C-C motif) ligand 2	1.99
20	NM_005508	CCR4	Chemokine (C-C motif) receptor 4	1.81
35	NM_002089	CXCL2	Chemokine (C-X-C motif) ligand 2	2.05
37	NM_006639	CYSLTR1	Cysteinyl leukotriene receptor 1	2.78
57	NM_001559	IL12R β 2	Interleukin 12 receptor, beta 2	2.10
60	NM_000640	IL13R α 2	Interleukin 13 receptor, alpha 2	2.10
65	NM_003855	IL18R1	Interleukin 18 receptor 1	1.94
68	NM_000576	IL1 β	Interleukin 1, beta	2.43
82	NM_000584	IL8	Interleukin 8	1.73
84	NM_001557	IL8R β	Interleukin 8 receptor, beta	1.87
107	NM_003808	TNFSF13	Tumor necrosis factor (ligand) superfamily, member 13	1.91

Th1/Th2 cytokine profile

The Human Th1/Th2/Th17 array is sensitive enough to detect cytokines up to pg/ml level. Data in Table 2, indicated that in the absence of ozone exposure, T cells from allergic asthmatic children had higher Th2 cytokines, particularly IL-4 (1.64 fold), IL-5 (1.25 fold), IL-10 (6.39 fold), IL-13 (1.90 fold) and lower Th1 cytokines, particularly (IFN- γ (0.84 fold) and IL-2 (0.71 fold) than that from nonallergic children. This suggests that T cells from allergic asthmatic children, when incubated with conditioned medium in the absence of ozone exposure, are skewed towards Th2 differentiation, while T cells from nonallergic children are skewed

towards Th1 differentiation, which is consistent with the 'Th2 hypothesis in allergy'.

The upregulated and downregulated Th1/Th2 cytokines of T cells incubated with conditioned medium with or without 0.25 mg/m³ ozone exposure, were classified by the Hierarchical clustering method (Figure 3). Upregulated Cluster I cytokines of IFN- γ , IL-2 and TNF- α , representing Th1 cytokines, were not significant in asthmatic children as compared to nonallergic children. Cluster II cytokines of IL-4, IL-5, IL-10, IL-13, and IL-6, representing Th2 cytokines, and were expressed at low levels in both asthmatic children and nonallergic children. In asthmatic children group, expression levels of cytokines of Th1 cells were collectively higher than those from Th2 cells. Ozone-exposed conditioned media could slightly increase all the Th1, Th2 and Th17 cytokines in T cells from allergic asthmatic children. (Figure 4)

Discussion

Previous studies have shown that environmental pollution, such as increased ozone content, is one of the contributory factors for the development of asthma and inflammation of airway epithelium.¹² Ozone, a main component of photochemical smog, is an active oxidant. During formation of ozone-related asthma, several studies showed that activated dendritic cells,⁴ neutrophils, macrophages and Th7 cells⁴ and NKT cells¹³ might contribute to the heterogeneous asthma phenotypes.¹⁴ In our study, air-liquid interface cell culture system was used to simulate the exposure of bronchial epithelial cell to gaseous pollutants as reported earlier.¹⁵ This *in vitro* setup was also used to analyze the effect of cytokines produced by BEAS-2B cells on effector cell (T cell) by incubating T cells with conditioned medium from BEAS-2B cells. Transmission electron microscopy results confirmed the injury caused by ozone to bronchial epithelial cells. Yang et al.¹⁶ have shown that the degree of inflammation in the asthmatic airway epithelial cells is controlled through IL-1 β . IL-6 initiates the acute phase immune response. RANTES is an important chemokine of the CC family which can regulate the expression of CD28, CD40L and IL-12R by T cells. Our data indicated that low levels of ozone exposure for a short period induced inflammatory cytokines, and the conditioned medium had biological activity, which could subsequently activate T cells, thereby potentially driving the pathogenic process of asthma in children.



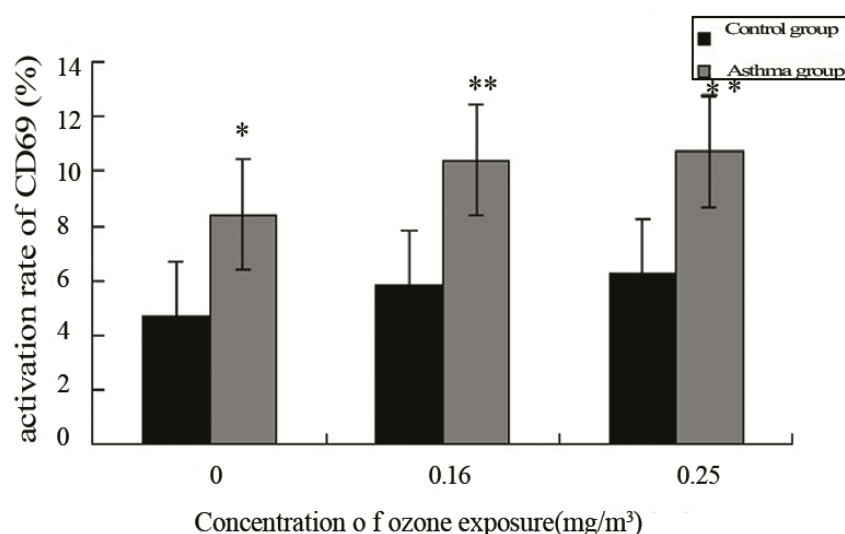


Figure 2. The ozone simulated airway epithelial inflammatory cytokines enhanced further T-cell activation in vitro. BEAS-2B cells were cultured in complete medium and exposed to 0.16 mg/m³ and 0.25 mg/m³ ozone for 8 hours. Additional BEAS-2B cells were cultured in the same condition without ozone exposure and used as controls. Their supernatants were harvested and used as the conditioned media. Peripheral blood T cells were isolated from asthmatic children (n=3), and healthy controls (n=3), and cultured with complete medium containing the conditioned medium from the BEAS-2B cells exposed to ozone or the cells without ozone exposure for 12 hours. Subsequently, the T cells were harvested and the activation rate of CD69 in T cells were analyzed by flow cytometer. Data are expressed as mean ± SD. * *P* < 0.01 ** *P* < 0.05, asthma group Vs control group.

Differential gene expression of T cells from children with allergic asthma and control children identified 17 genes that are expressed differentially between the two groups. IL-1 β , IL-8, and chemokines, which were upregulated in this study, are involved in chemotaxis and activation of eosinophils, basophils, mast cells and neutrophils, which induce airway allergic inflammation. Wohlfahrt et al.¹⁷ compared peripheral CD4⁺ lymphocytes in subjects with asthma and normal control subjects using cDNA microarray techniques, and found evidence for up-regulation of several other gene families in asthma, including those for chemokines, chemokine receptors, fibroblast growth factors. Our data confirmed this allergic feature to be present in asthmatic children and not in nonallergic children. Therefore, comparison of these two sets of children's T cell response to conditioned medium exposed to ozone will identify the impact of genetic and environmental factors on asthma.

T cells play a central role in the pathogenesis of asthma, and there is evidence for increased T cell activation in patients with acute asthma.¹⁸ Leckie et al.¹⁹ found that CD69 was expressed in T cells of induced sputum from asthma patients. Our study found that the level of CD69 in T cells from asthma

Table 2. Level of cytokines from T lymphocytes of allergic asthmatic children and control children after incubation with conditioned medium without ozone exposure.

cytokine		Control children	Asthmatic children	Ratio*
		1	2	
Th1 cytokines	IFN- γ	0.24±0.032	0.20±0.065	0.84
	TNF- α	0.20±0.056	0.24±0.095	1.20
	IL-2	0.23±0.035	0.16±0.038	0.71
Th2 cytokines	IL-4	0.063±0.025	0.10±0.054	1.64
	IL-5	0.038±0.0089	0.047±0.021	1.25
	IL-6	0.063±0.0009	0.056±0.030	0.89
	IL-10	0.0027±0.0011	0.018±0.0076	6.39
	IL-13	0.010±0.0017	0.027±0.0057	1.01

*Cytokines expression changes are shown with variable 2/1 ratios.

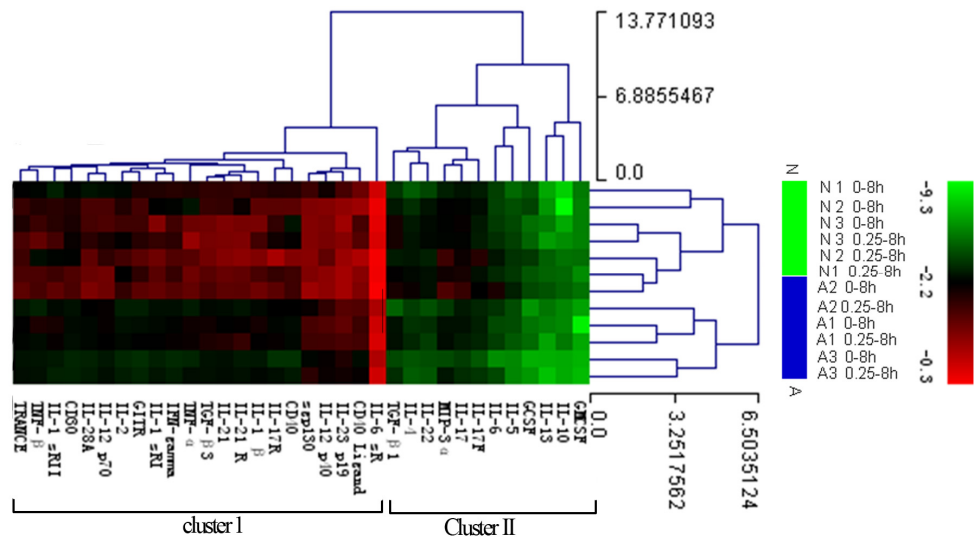


Figure 3. Hierarchical clustering of Human Th1/Th2/Th17 Array. Data are in matrix format, with columns representing cytokines and rows representing each sample. Red, high expression; green, low expression; black, no significant change in expression level between the control and sample. "N" represents nonallergic children and "A" represents allergic asthmatic children. Upregulated Cluster I cytokines of IFN- γ , IL-2 and TNF- α , representing Th1 cytokines, were not significant in asthmatic children as compared to nonallergic children. Cluster II cytokines of IL-4, IL-5, IL-10, IL-13, and IL-6, representing Th2 cytokines, and were expressed at low levels in both asthmatic children and nonallergic children. In asthmatic children group, expression levels of cytokines of Th1 cells were collectively higher than those from Th2 cells.

children were significantly higher than that of control children ($p < 0.05$), which is supported by earlier research results.²⁰ Although the difference in CD69 expression of T cells incubated with different ozone exposure conditioned medium was non-statistically significant, the CD69 expression on T cells increased with increasing concentrations of ozone. The results suggested that T cells were more activated in children with asthma than in the nonallergic children, and the amount of activation positively correlated with the ozone concentration.

Th1/Th2 differentiation following T cell activation is directly related to the type of inflammatory response in asthma and is the focus of this study. T cells differentiate into Th1/Th2 cells. The two sets form a complex and orderly network characterized by antagonistic mechanisms and self-promotion and regulate immune responses. Th1 cells (secreting IFN- γ , IL-2 and TNF- α) lead to cell-mediated responses, whereas Th2 cells (secreting IL-4, IL-10, IL-5, IL-6) mediate humoral immune responses, which are involved in the pathogenesis of asthma and the formation of airway inflammation.²¹ Cytokines expression changes, shown in table 2, indicated that after T cells were incubated with

conditioned medium without ozone exposure, T cells from allergic asthmatic children were skewed towards Th2 differentiation, while T cells from nonallergic children were skewed towards Th1 differentiation, which is consistent with 'Th2 hypothesis in allergy'.

Hierarchical clustering analysis of all samples was based on the similarity in the expression pattern of all cytokines. Samples were separated into two main groups, the nonallergic children cluster and the asthma children cluster. Each distinctive cytokine cluster was identified by delineation using a hierarchical clustering dendrogram. Hierarchical clustering analysis confirms that in asthmatic children group, expression levels of cytokines of Th1 cells were collectively higher than those from Th2 cells. Randolph et al.²² found that injecting allergens induce Th1 cells migration to the airway, thereby exacerbating airway eosinophilic inflammation. Th1 cells injected into the asthma model cannot alleviate the airway inflammation and AHR. Research has also found that allergic specific Th2 cells are not involved in the incidences or exacerbation of asthma induced by infection, air pollution and exercise.²³ Another study found that

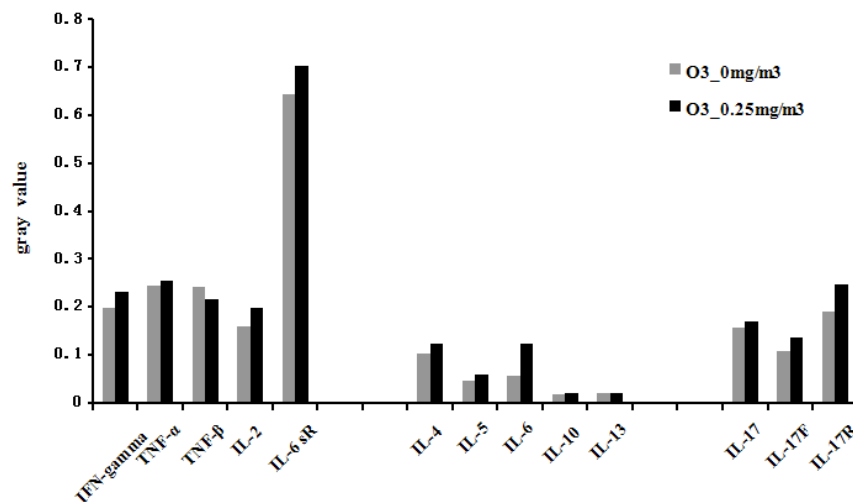


Figure 4. Cytokine profile of T cells from allergic asthmatic children incubated with condition media with or without 0.25 mg/m³ ozone exposure. Ozone-exposed conditioned media could slightly increase all the Th1, Th2 and Th17 cytokines in T cells from allergic asthmatic children.

targeting Th2 cells through IL-4, IL-5 or IL-4/IL-13 antibodies did not achieve the desired efficacy, and did not eliminate the airway hyperresponsiveness in asthma patients.²⁴ Therefore, we hypothesized that induction or exacerbation of asthma following environmental ozone exposure in allergic individuals may be associated with higher expression of Th1 cytokines, which is in conflict with current strategy of asthma clinical treatment using Th1-skewing pharmaceuticals.

Conclusions

In vitro BEAS-2B epithelial cells could be damaged and altered in cytokines expression by ozone exposure, which may further mediate the effects of ozone on the activation of T cells from asthmatic children. Our results suggested that Th1 cells activation might be predominant over Th2 activation upon ozone exposure in asthmatic children, which might help to clarify the mechanisms of asthma related to environmental factors like ozone.

Conflict of interest

All of the authors have no conflicts of interest in the manuscript.

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References

1. Peel JL, Tolbert PE, Klein M, Metzger KB, Flanders WD, Todd K, et al. Ambient air pollution and respiratory emergency department visits. *J Epidemiology*.2005;16:164–74.
2. Rage E, Jacquemin B, Nadif R, Oryszczyn MP, Siroux V, Aquileral I, et al. Total serum IgE levels are associated with ambient ozone concentration in asthmatic adults. *Allergy*. 2009;64:40-6.
3. Romieu I, Barraza-Villarreal A, Escamilla-Nunez C, Almstrand AC, Diaz-Sanchez D, Sly PD, et al. Exhaled breath malondialdehyde as a marker of effect of exposure to air pollution in children with asthma. *J Allergy Clin Immunol* .2008;121:903-9.
4. Hollingsworth JW, Free ME, Li Z, Andrews LN, Nakano H, Cook DN. Ozone activates pulmonary dendritic cells and promotes allergic sensitization by a TLR4-dependent mechanism. *J Allergy Clin Immunol*. 2010;125:1167–70.
5. Deo SS, Mistry KJ, Kakade AM, Niphadkar PV. Role played by Th2 type cytokines in IgE mediated allergy and asthma. *Lung India*.2010;27:66-71.
6. Agnello D, Lankford CS, Bream J, Morinobu A, Gadina M, O’Shea JJ, et al. Cytokines and transcription factors that regulate T helper cell differentiation: new players and new insights. *J Clin Immunol*. 2003;23:147-61.
7. Hansen G, Berry G, Dekruyff RH, Umetsu DT. Allergen-specific Th1 cells fail to counterbalance Th2 cell-induced airway hyperreactivity but cause severe airway inflammation. *J Clin Invest* .1999;103:175–83.
8. Kumar RK, Herbert C, Webb DC, Li L, Foster PS. Effects of anti-cytokine therapy in a mouse model of chronic asthma. *Am J Respir Crit Care Med*. 2004;15:1043–8.
9. Song H, Tan W, Zhang X. Ozone induces inflammation in bronchial epithelial cells. *J Asthma*. 2011;48:79-83.



10. Brudler R, Hitomi K, Daiyasu H, Toh H, Kucho K, Ishiura M, et al. Identification of a new cryptochrome class. Structure, function, and evolution. *Mol Cell* .2003;11:59-67.
11. Yuen T, Wurmbach E, Pfeffer RL, Ebersole BJ, Sealfon SC. Accuracy and calibration of commercial oligonucleotide and custom cDNA microarrays. *Nucleic Acids Res* .2002;30:e48.
12. Salam MT, Islam T, Gauderman WJ, Gilliland FD. Roles of arginase variants, atopy, and ozone in childhood asthma. *J Allergy Clin Immunol* .2009;123:596-602.
13. Pichavant M, Goya S, Meyer EH, Johnston RA, Kim HY, Matangkasombut P, et al. Ozone exposure in a mouse model induces airway hyperreactivity that requires the presence of natural killer T cells and IL-17. *J Exp Med* .2008;250:385-93.
14. Kim HY, DeKruyff RH, Umetsu DT. The many paths to asthma: phenotype shaped by innate and adaptive immunity. *Nat Immunol* .2010;11:577-84.
15. Damera G, Zhao H, Wang M, Smith M, Kirby C, Jester WF, et al. Ozone modulates IL-6 secretion in human airway epithelial and smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* .2009;296:L674-83.
16. Yang Y, Bin W, Aksoy MO, Kelsen SG. Regulation of interleukin-1beta and interleukin-1beta inhibitor release by human airway epithelial cells. *Eur Respir J* .2004;24:360-6.
17. Wohlfahrt JG, Kunzmann S, Menz G, Kneist W, Akdis CA, Blaser K, et al. T cell phenotype in allergic asthma and atopic dermatitis. *Int Arch Allergy Immunol* .2003;131:272-82.
18. Georas SN, Guo J, De Fano U, Casolaro V. T-helper cell type-2 regulation in allergic disease. *Eur Respir J* .2005;26:1119-37.
19. Leckie MJ, Jenkins GR, Khan J, Smith SJ, Walker C, Barnes PJ, et al. Sputum T lymphocytes in asthma, COPD and healthy subjects have the phenotype of activated intraepithelial T cells (CD69+ CD103+). *Thorax* .2003;58:23-9.
20. Wilder JA, Collie DD, Bice DE, Tesfaigzi Y, Lyons CR, Lipscomb MF. Ovalbumin aerosols induce airway hyperreactivity in naive DO11.10 T cell receptor transgenic mice without pulmonary eosinophilia or OVA-specific antibody. *J Leukoc Biol* .2001;69:538-47.
21. van Oosterhout AJ, Motta AC. T h1/Th2 paradigm: not seeing the forest for the trees? *Eur Respir J* .2005;25:591-3.
22. Randolph DA, Stephens R, Carruthers CJ, Chaplin DD. Cooperation between Th1 and Th2 cells in a murine model of eosinophilic airway inflammation. *J Clin Invest* .1999;104:1021-9.
23. Pichavant M, Goya S, Meyer EH, Johnston RA, Kim HY, Matangkasombut P, et al. Ozone exposure in a mouse model induces airway hyperreactivity that requires the presence of natural killer T cells and IL 17. *J Exp Med* .2008;205:385-93.
24. Wenzel S, Wilbraham D, Fuller R, Getz EB, Longphre M. Effect of an interleukin 4 variant on late phase asthmatic response to allergen challenge in asthmatic patients: results of two phase 2a studies. *Lancet* .2007;370:1422-31.

