Inflammation response of water-soluble fractions in atmospheric fine particulates: a seasonal observation in 10 large Chinese cities

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Abstract

Spatiotemporal trends in pro-inflammatory (interleukin (IL)-6 and IL-8) release after exposure to the water-soluble fractions (WSFs) of $PM_{2.5}$ sampled in 10 large Chinese cities over 1 year were investigated. Chemical components (water-soluble ions, metal(loid) elements, water-soluble organic carbon (WSOC), humic-like substances (HULIS), and endotoxins) in $PM_{2.5}$ samples were measured, and the molecular structure of WSOC was also analyzed by nuclear magnetic resonance. Changes in DNA methylation and gene expression of candidate genes were also evaluated to explore the potential mechanisms. $PM_{2.5}$ from southern cities induced lower pro-inflammatory responses than those from northern cities. Seasonal differences in toxicity were noted among the cities. IL-6 was significantly correlated with HULIS (as the main fraction of WSOC with oxygenated carbohydrate structures characteristic), Pb and endotoxin. Furthermore, DNA methylation and gene expression changes in *RASSF2*, and *CYP1B1* were related to pro-inflammatory secretion. Certain components of $PM_{2.5}$, rather than $PM_{2.5}$ mass itself, determine the pro-inflammatory release. In particular, HULIS, which originated from primary biomass burning and residual coal combustion, and secondary organic aerosols, appear to be the key component in $PM_{2.5}$ to induce human health risk.

Keywords: HULIS; NMR; $PM_{2.5}$; Spatiotemporal; DNA methylation

1. Introduction

Fine particulate matter ($PM_{2.5}$) causes respiratory and cardiovascular disease[1]. Inflammatory activity is considered to be the first biological reaction to $PM_{2.5}$ exposure, which could induce these diseases[2]. As such, *in vitro* human cell culture models have been used widely to evaluate the ability of $PM_{2.5}$ to trigger pro-inflammatory activity[3]. It has been reported that the water-soluble fraction (WSF) which accounts for the major proportion of $PM_{2.5}$[4], induces more abnormal biological outcomes than water-insoluble $PM_{2.5}$ components[5,6].

Identifying the potential fractions and components of $PM_{2.5}$ with the ability to induce pro-inflammatory activity change is critical in atmospheric research, and several studies have assessed the relationships between chemicals in PM and toxicity *in vitro*. For example, metal (loid) s (e.g., Cr, Al, Si, Ti, Fe, and Cu), ions (K^+ and NH_4^+), and polycyclic aromatic hydrocarbons (PAHs) showed significant relationships with toxicological outcomes in studies done in Finland[7], Mexico[3], and Italy[8]. However, the WSF of $PM_{2.5}$, aside from water-soluble ions and metal (loid)s which can be measured individually, contains numerous organic compounds (which are not easy to be fully identified in the WSF), which probably make a significant contribution to pro-inflammatory activity[9]. For instance, humic-like substances (HULIS), consisting of high-molecular-weight organic compounds and represent the main fraction of water-soluble organic carbon (WSOC) in $PM_{2.5}$, can induce reactive oxygen species (ROS), as shown by the dithiothreitol (DTT) assay[9,10]. Therefore, identification of the major types of organic matter and the molecular groups therein, represents an alternative approach to comprehensively determine pro-inflammatory organic components. Nuclear magnetic resonance (NMR) spectroscopy is considered as

a method to explore the molecular structural characteristics of organic components within a matrix[11].

In China, about 83% of people are currently living in the areas with $PM_{2.5}$ concentrations exceeding the Chinese Ambient Air Quality Standard (35 μg/m^3), and 1.37 million premature adult mortalities in 2013 may be attributable to air pollution[12]. However, there are limited data on the relationship between $PM_{2.5}$ components and pro-inflammatory activity in China[13].

The mechanisms linking $PM_{2.5}$ exposure to pro-inflammatory release have been not fully understood[14]. DNA methylation could be altered by environmental factors and can median this impact on a phenotype and disease[15]. Indeed, DNA methylation is thought to be related to air pollution toxicity due to the significant relationships between the changes in DNA methylation of several genes with PM exposure in several epidemiological studies[14,16]. Moreover, the modifiable characteristics of DNA methylation most likely render protective measures, and could be applicable for new drug development[17].

In this study, $PM_{2.5}$ samples from 10 large cities in China were collected during 1 year (2013–2014) and the WSFs of pooled $PM_{2.5}$ samples were used to evaluate their ability to induce pro-inflammatory activity in the human cell models. In addition, we characterized the inorganic and organic components of the WSF of $PM_{2.5}$. Organic components (i.e., WSOC and HULIS) were quantified and the structural characteristics of WSOC were qualified using NMR. Furthermore, the relationships between the components of $PM_{2.5}$ and their molecular structural characteristics were evaluated, and the DNA methylation mechanism were also explored, since this may help identify novel therapeutic targets against $PM_{2.5}$ exposure.

2. Methods

2.1 $PM_{2.5}$ sampling, organic fraction extraction, and WSF extraction

$PM_{2.5}$ samples were collected from 10 urban cities in China (Beijing (BJ), Shanghai (SH), Guangzhou (GZ), Nanjing (NJ), Wuhan (WH), Taiyuan (TY), Chengdu (CD), Lanzhou (LZ), Guiyang (GY), and Xinxiang (XX)) during spring (SP), summer (S), autumn (A), and winter (W). Detailed descriptions of the sampling sites, sampling methods, and protocols are given in our previous paper[18]. Densely inhabited districts in the cities were selected for sampling. And sampling sites were set up on rooftops approximately 15−20 m above ground level. $PM_{2.5}$ were collected using 20.3 × 25.4 cm prebaked (5 h at 450 °C) Whatman quartz microfiber filters (QFFs). Samples were collected during October 22 2013 to November 13 2013 for autumn (total 22 samples), December 30 2013 to January 20 2014 for winter (total 20 samples), March 30 2014 to April 20 2014 for spring (total 22 samples), and June 26 2014 to August 24 2014 for summer (total 28 samples), respectively. Each piece of filter was recorded the sampling time and rate, which was used to calculate the volume of sampling air. During each season, 24-h integrated $PM_{2.5}$ samples were collected. And a circle with 2.75 cm radius was cut from each piece of filters and then was pooled into a single sample for each season. Thus, 38 samples were used in the subsequent experiments (except GY, where only SP and S samples were collected).

Two $PM_{2.5}$ samples from Guangzhou City in the winter of 2013 was collected using a $PM_{2.5}$ sampler. Combustion of corn stalks was collected through a sampling system[19]. Coal combustion was done with a high-efficiency stove with a $PM_{2.5}$ dilution sampling system[19].

The WSF fraction was extracted from $PM_{2.5}$ samples by sonication using deionized water. The extract was filtered through a 0.22 μm filter, freeze dried, and dissolved in deionized water. More details are presented in S1-1.

The dichloromethane (DCM) fraction was extracted from $PM_{2.5}$ samples collected in GZ in 2013 (GZ

W1 and GZ W2). $PM_{2.5}$ samples were collected from the combustion products of corn stalks, coal, and vehicle exhaust. The filters were then extracted with DCM using pressurized liquid extraction (ASE300; Dionex Corp., Sunnyvale, CA, USA) for 2 days. Finally, the extracts were gently evaporated and dried under nitrogen gas and reconstituted with dimethyl sulfoxide to various concentrations.

2.2 Inorganic chemical analyses

Ion chromatograph (883 Basic IC Plus; Metrohm, Herisau, Switzerland) was used to analyze Six cations (Li^+, Na^+, NH_4^+, K^+, Mg^{2+}, and Ca^{2+}) and seven anions (F^-, Cl^-, Br^-, NO_2^-, PO_4^-, NO_3^-, and SO_4^{2-}), and ICP-AES (VISTA-MPX; Varian, Palo Alto, CA, USA)[6] was used to measure the 13 metal (loid) elements. Details are shown in the SI. The result of each chemical are expressed as $\mu g/m^3$, and the blank filter was used as the blank, whose chemicals concentrations were subtracted.

2.3 Organic fraction measurement

The WSOC was measured with a total organic carbon (TOC) Analyzer (Sievers M9; GE, Milwaukee, WI, USA). All samples were measured in triplicate and the average of the three values was used. HULIS fractions were separated from 38 WSF samples using 6 mL Oasis HLB column (Waters, Milford, MA, USA), and details were presented in the SI. Endotoxins were analyzed using kinetic chromogenic Limulus amebocyte lysate assay[3] (Genscript. USA). Results of WSOC and HULIS were expressed as $\mu g/m^3$, and endotoxins was expressed as EU/ml, and the blank filter was used as the blank, whose chemicals concentrations were subtracted.

2.4 NMR analysis

The proton NMR (^1H NMR) spectra were recorded on an AVANCE III 400 spectrometer (Bruker, Billerica, MA, USA) with an operating frequency of 400.13 MHz. Spectra acquisition was performed with

a contact time of 2.27 s and the zg30 pulse program. The recycle delay was 2 s and the proton 90° pulse length was 8.87 μs. About 200 scans per spectrum were collected. A 1.0 Hz line broadening weighting function and baseline correction were applied. For the NMR analysis, the solid extracts of the samples were dissolved in D2O. Functional groups in the NMR spectra were identified based on the chemical shift (δ H) relative to that of the water (4.7 ppm). Each spectrum was then manually phase- and baseline-corrected with the chemical shift for the 38 water-soluble samples. The spectral regions of δ 0.7–1.9 ppm for aliphatic compounds, δ 1.9–3.2 ppm for unsaturated compounds, δ 3.3–4.5 ppm for carbohydrate compounds, and δ 6.7–8.3 ppm for aromatic hydrogens were based on a previous $PM_{2.5}$ study[11]. The area of the blank sample was subtracted from each sample, and the NMR value of each functional group was expressed as the proportion of each functional region area to the total area of the four function groups.

2.5 Cell treatment, inflammatory activity analysis, DNA methylation and gene expression analysis

The treatment of A549 and Beas-2B cells were shown in the SI. For comparing the ability of pro-inflammatory release of different $PM_{2.5}$ samples, cells were exposed to the 16.8–90.9 μg/cm^2 of $PM_{2.5}$ all collected from 10 m^3 of air for 3 days. Cells were harvested after the exposure, and the genomic DNA of the cells was extracted for the DNA methylation test. RNA was also extracted for the gene expression assay[21], and the cell supernatant was used for interleukin (IL)-6 and IL-8 assays using human IL-6/IL-8 Quantikine ELISA kits (R&D Systems, Minneapolis, MN, USA). A blank filter with same area as the sample filter from 10 m^3 air was used as the control.

DNA methylation PCR array: The Xinxiang spring (XXSP) sample, which induced the third highest IL-6 response among the samples, was selected for the PCR array (Lung Cancer DNA Methylation PCR Array; Qiagen, German) for screening candidate genes.

DNA methylation: *RASSF2* and *CYP1B1* gene methylation was performed using Sequenom MassARRAY method, and *LINE-1* and *iNOS* gene methylation were analyzed using the pyrosequencing method [21]. Details were shown in the SI.

The gene expression assay was performed as described in our previous work[21]. And primers are showed in Table S1.

2.6 Statistical analysis

We used the Kruskal–Wallis test to analyze for differences in water-soluble ion/element, WSOC, NMR, HULIS, $PM_{2.5}$, IL-6, IL-8, DNA methylation, and gene expression levels among the samples from all four seasons and 10 cities. Principal component analysis (PCA) was performed on the PM components. The associations between each chemical (or contributions) and cytokine production were evaluated using Pearson correlation analysis for normally distributed data and Spearman correlation analysis for non-normally distributed data. Relationships were considered to be significant when $p < 0.05$. The data were analyzed with SPSS software (ver. 20.0; IBM Corp, Armonk, NY, USA) (provided by the Chinese Center for Disease Control and Prevention). All biology experiments were carried out four times and three time in chemicals experiments, and the average and median data was used.

3. Results and Discussion

3.1 Characteristics of $PM_{2.5}$ samples

$PM_{2.5}$ characteristics: $PM_{2.5}$ concentrations were higher in W and SP than in S and A, but the difference was not significant ($p = 0.184$). Significant differences in $PM_{2.5}$ concentrations were observed among the cities ($p = 0.003$, Table S2). Coastal cities had the lowest concentrations, which may results from more rainwater in coastal cities leading to more atmospheric sedimentation, and also more air flow to

174 the sea comparing to insider cities may also contribute to this result.

175 *Characteristics of inorganic compounds:* Br^-, PO_4^{3-}, and NO_2^- were found in very low concentrations
176 and were subsequently disregarded. Northern cities had higher water-soluble ion contents than southern
177 cities (p = 0.016) and the average concentration followed the order: NJ > TY > XX > LZ > BJ > SH >
178 GZ > WH > CD > GY. Similarly, water-soluble metal (loid) elements, which accounted for about 0.75 ±
179 0.67% of $PM_{2.5}$, showed significant differences among the cities (p = 0.004).

180 Most ions/elements showed higher concentrations in W and A and lower concentrations in SP and S (p
181 < 0.05), except for Pb, which had higher concentrations in SP and S than A and W. A similar trend in Pb
182 was reported in Mexico City[3]. Based on the current use of electric vehicles, more Pb may leak from Pb
183 batteries in China under higher temperatures in SP and S, which might have contributed to the higher Pb
184 ion concentrations in these seasons. However, more data were needed to explore if battery was the main
185 reason, and other factors might be also contributed to this trend since the concentration of Pb was higher in
186 SP than S. The higher rainfall and temperature in SP and S may have lowered the Cl^-, NO_3^-, Na^+, K^+, Ca^{2+},
187 and Mg^{2+} concentrations in $PM_{2.5}$.

188 *Organic compound characteristics:* WSOC accounted for 2.93 ± 2.83% of $PM_{2.5}$ (m/m), and the
189 concentration of blank filter was 0.0102 μg/m^3. The molecular structural characteristics of WSOC were
190 studied using NMR. Based on the recommended method, δ^1H 0.7–1.9 ppm (NMR1; *H-C*, representing
191 compounds including protons from methyl, methylene, and methyne groups), δ^1H 1.9–3.2 ppm (NMR2;
192 *H-C-C*=, including protons bound to the carbon in the α-position adjacent to a double-bond in allylic,
193 carbonyl, or imino (H-Cα-C=O or H-Cα-C=N) groups and protons in secondary and tertiary amines), δ^1H
194 3.3–4.5 ppm (NMR3; *H-C-O*, compounds with protons bound to oxygenated saturated aliphatic carbon
195 atoms in alcohols, polyols, esters, and organic nitrate), and δ^1H 6.7–8.3 ppm (NMR4; *Ar-H*, including

196 protons bound to aromatic carbon) were taken to represent different types of non-exchangeable organic
197 hydrogens when analyzed with liquid particulate matter samples[11]. All groups showed significant
198 differences between the cities and seasons (Kruskal–Wallis test; Table S3), except NMR4 among the cities.
199 The HULIS fractions had high aliphatic (NMR1) and carbohydrate (NMR3) structural characteristics
200 [11]. HULIS components were therefore further extracted from the WSFs of $PM_{2.5}$ samples and quantified.
201 The average HULIS concentration was 1.54 ± 1.64 μg/m^3, and concentration of blank filter is 0.0191μg/m^3.
202 Moreover, the proportion of HULIS to WSOC was between 80 ± 1.66%, suggesting HULIS as the main
203 component of WSOC.
204 *3.2 Response of IL-6 and IL-8 to the WSF of $PM_{2.5}$*
205 First, we compared the ability to induce IL-6 release in A549 cells between the WSF and
206 dichloromethane (DCM)-soluble fraction of five $PM_{2.5}$ samples, and the results indicated that cells exposed
207 to the WSF released more IL-6 than those exposed to the DCM-soluble fraction (Figure S1). During Figure
208 S1, three different $PM_{2.5}$ samples represented three typical sources of pollution: biomass (corn stalk),
209 residential coal combustion (coal), and vehicle exhaust particles (vehicle). $PM_{2.5}$ normally had the highest
210 concentrations in winter, so these five samples were chosen to explore the ability of pro-inflammatory
211 release of WSF fraction in $PM_{2.5}$ than that of DCM fraction. The results may possibly be because the WSF
212 constituted more than 39% of the $PM_{2.5}$ mass. Specifically, the water-soluble inorganic ions, WSOC, and
213 water-soluble metal (loid) elements accounted for about 35%, 3%, and 1% of the $PM_{2.5}$ mass, respectively,
214 while the DCM-soluble fraction accounted for only about 0.1% of the $PM_{2.5}$ mass[4]. Similarly, the WSF
215 were also more likely to induce DNA damage or cytotoxicity than organic compounds in $PM_{2.5}$ and PM_{10}
216 from Mexico City[5] and Iran[6]. Therefore, we assessed the pro-inflammatory activity of the WSF of $PM_{2.5}$
217 from 10 large cities in China. The average response was 191.39 ± 70.13% and 112.70 ± 19.31% for IL-6

and IL-8 compared to the control samples, respectively, and the concentrations of blank filter were 43.26±0.56 pg/ml of A549 cells, and 3.67 ±0.89 ng/ml for Beas-2B cells. Using the XXSP sample, we observed a dose-dependent relationship in both A549 cells (Figure S2) and Beas-2B cells (Figure S2), which confirmed that the increase in IL-6 levels in human lung cells was caused by exposure to the WSF of $PM_{2.5}$. Interestingly, the samples from different cities showed similar trends in terms of their ability to induce IL-6 and IL-8 with a greater response for IL-6 than IL-8 under the same exposure time (p = 0.045 for IL-6, p = 0.049 for IL-8, Table 1, Figure 1). This followed the order GY > XX > CD > BJ > TY > LZ > NJ > GZ > WH > SH for IL-6. The GY, XX and CD samples elicited the greatest response, whereas the GZ, WH, and SH samples exploited the lowest responses (Table 1). Generally, the WSF of $PM_{2.5}$ in northern cities in China induced more IL-6 than that in southern cities, neglecting the GY city due to missing spring and summer samples (Figure 1). This could be because southern China has higher temperature and more rainwater than northern China, leading to less biomass burning for heating, more gas usage for cooking and less incomplete combustion, which can all result in more secondary organic aerosol[22]. In addition, more rainwater results in more atmospheric washout, which with more air flow to the sea, attribute to better air quality in southern China.

Significant variations among the seasons were observed for the IL-6 response, but not the IL-8 response (p = 0.03 for IL-6, p = 0.57 for IL-8, Table 1). In addition, different locations showed different trend for the IL-6 response. For instance, colder seasons samples in BJ, SH, GZ, LZ, and CD were more potent in inducing IL-6 than those from warmer seasons, while in four cities, TY, XX, NJ and WH, SP and S samples were more potent in inducing IL-6 than samples from A and W (p<0.05, Figure 1). These differences in toxicity among cities can be used for better understanding the overall state of $PM_{2.5}$ pollution in China.

3.3 Components of PM2.5 associated with pro-inflammatory cytokine release

IL-6 and PCA: No significant correlation was found between mass of $PM_{2.5}$ and IL-6 or IL-8. PCA of water-soluble inorganic ions, and metal (loid) elements, HULIS, non-HULIS, endotoxin, and NMR1-4 were performed; 5 factors accounting for 76% of the total variance, were identified (Table S4).

PCA1 mainly included Na^+, Cl^-, NMR1, NMR2, K^+, NMR3, Zn, NO_3^-, NH_4^+, Mg^{2+}, Mn, NMR4, SO_4^{2-}, Endotoxin, Co, TI, Cu, Cr, Ca, and As ions; it explained 43% of the total variance, which may be from secondary inorganic aerosol and sea salt. PCA2 mainly included Al, Cr, Fe, Co (negative), Cu, and Pb; it accounted for 12% of the total variance, which may be from industrial sources. PCA3 included NH_4^+ (negative), Ca^{2+}, Mg^{2+}, and Pb; it represented 9% of the total variance, which may be from road dust sources and formation of NH_4^+ may be from NOx catalytic unit of motor vehicle. PCA4 included V (negative), Ni (negative), As, and Cd; it accounted for 8% of the total variance, which may represent ship emissions. PCA5 included HULIS (negative) and non-HULIS; it explained 5% and may represent primary biomass burning, residual coal combustion and secondary organic aerosols.

A correlation analysis between pro-inflammatory and the PCA factors showed that there is only a significant correlation between PCA3 (included NH_4^+, Pb, Ca^{2+}, and Mg^{2+}) and IL-6 ($r = 0.370$, $p = 0.022$, Table 2) confirmed by linear regression ($B = 0.370$, $p = 0.022$) and multiple linear regression analyses ($B = 0.370$, $p = 0.026$; Table 2). These results suggest that Pb, Mg, and Ca^{2+} were the main components of the WSF of $PM_{2.5}$ that induced IL-6 release, whereas NH_4^+ may have exerted a negative influence on this process. A linear regression analysis between IL-6 and each ion/element was performed, in which only Pb and IL-6 were significantly correlated ($B = 0.337$, $p = 0.039$; Table 2). Similarly, the Pb standard induced IL-6 in a dose-dependent manner (Figure S3) in A549 cells. These results suggest that Pb may be a non-negligible component of $PM_{2.5}$ for IL-6 induction.

In cells exposed to $PM_{2.5}$ from Mexico City, Ca^{2+} showed similar correlation with pro-inflammatory responses with our results[3]. Moreover, an epidemiological study reported that acute exposure to Pb of $PM_{2.5}$ was associated with negative health effects[23]. To date, several water-soluble ions and metal (loid) elements from $PM_{2.5}$ collected in different areas have exhibited a relationship with the pro-inflammatory response. For example, K^+ showed contrasting effects on the inflammatory response to $PM_{2.5}$ from Milan[8] and North Carolina[24], while Si and Al in $PM_{2.5}$ from these two areas exhibited similar effects. In addition, Fe ions in PM_{10} collected in Milan[26] induced a pro-inflammatory response in cells, while the inverse result was reported for $PM_{2.5}$ and PM_{10} in Mexico City[3]. Overall, PM from different areas contains similar components but in different proportions, leading to regional variations in toxicity due to complex reactions between components. Therefore, understanding the interactions among individual components of PM is important for identifying the primary toxic components of $PM_{2.5}$.

IL-6 and endotoxins: As expected, a significant correlation was noticed between IL-6 and endotoxins (r = 0.363, p = 0.025), and the linear regression relationship confirmed this correlation (B = 1.859, p = 0.025). Our results also confirmed the idea that endotoxins in $PM_{2.5}$ are important for toxicity[3].

IL-6 and organic compounds: We observed a slight but not significant correlation between IL-6 and WSOC (r = 0.295, p = 0.072, Pearson correlation analysis), and not IL-8 and WSOC (r = 0.030, p = 0.875, Spearman correlation analysis). IL-6 and HULIS were significantly correlated (r = 0.322, p = 0.049; Table 2), and the linear regression analysis yielded similar results (B = 0.322, p = 0.049; Table 2) in A549 cells. Moreover, samples with higher proportions of HULIS induced greater IL-6 release in Beas-2B cells (Figure S4). Finally, the HULIS and non-HULIS fractions were extracted from six $PM_{2.5}$ water-soluble samples. Under the same exposure concentrations, the ability of these fractions to induce IL-6 release followed the order HULIS > WSF > non-HULIS (Figure S4). These results suggest that HULIS might be

the main fraction in $PM_{2.5}$ that induced cytokine release. This is the first report of a relationship between HULIS in $PM_{2.5}$ and the cytokine response in a human cell line.

HULIS represents a complex class of organic macromolecular compounds, including aromatic and polyacidic molecules. It is reported that HULIS from atmospheric aerosol samples induced ROS in a cell-free DTT assay[10], which is the only direct evidence of HULIS toxicity against reversible redox sites. However, it has been proposed that HULIS is associated with inflammatory and fibrotic lung disease based on the complex host Fe, which initiates inflammation pathways and subsequent fibrosis[26]. In this study, using human cell line models, the correlation analysis results indicated that HULIS contributed to pro-inflammatory release, and the extracted HULIS fraction results (Figure S4) provided direct evidence confirming this hypothesis. Based on Fourier-transform ion cyclotron resonance mass spectrometry and dual carbon isotope analysis, we previously revealed that primary emissions (i.e., biomass burning and residual coal combustion) and secondary organic formation were important sources of HULIS[27,28]. Therefore, these two sources should be targeted by air pollution control countermeasures.

Here the relationship between the molecular groups and IL-6, NMR1 and NMR3 were significantly correlated with IL-6 release (NMR1 and IL-6: $r = 0.358$, $p = 0.028$; NMR3 and IL-6: $r = 0.333$, $p = 0.041$; Table 2). This suggested that organic species in $PM_{2.5}$ with aliphatic (NMR1, δ^1H 0.7–1.9 ppm) and carbohydrate (NMR3, δ^1H 3.3–4.5 ppm) structural characteristics might contribute to the IL-6 response. Meanwhile, compounds with characteristics of NMR2 (δ^1H 1.9–3.2 ppm) and NMR4 (δ1H 6.7–8.3 ppm) might represent nontoxic components[11]. Figure 2 presents the spectra of the WSFs of NJ and TY from S and W. IL-6 concentrations were higher in cells exposed to the NJW and TYW samples, and higher NMR1 and NMR3 peaks were observed in these two samples compared with the NJS and TYS samples, respectively.

306 Shima reported that hydroxyl and carbonyl functional groups in the n-hexane-insoluble fraction of
307 diesel exhaust particles may have been responsible for the inflammatory response in a rat alveolar
308 epithelial cell line (SV40T2)[29]. In our previous paper, we reported that hydroxyl groups had an important
309 role in the liver tumor-promoting effect of triclosan[20]. Therefore, hydroxyl functional groups in the WSF of
310 $PM_{2.5}$ may have played an important role in IL-6 release in this study. This is the first report of the
311 structural characteristics of the toxic fractions of the WSF of $PM_{2.5}$ from 10 large cities in China.
312 Five cities samples (BJ, SH, GZ, LZ, and CD) elicited higher IL-6 response in colder seasons than
313 those of warmer seasons. On the other hand, lower IL-6 release in colder seasons was observed in four
314 cities (TY, XX, WH, and NJ) samples. Generally, in all samples, our data showed that HULIS, Pb, and
315 endotoxins components may be reasons for the IL-6 release for the significant correlation, and NMR1 and
316 NMR3 structural groups also play important roles to pro-inflammatory response. For different cities,
317 different factors may contribute to pro-inflammatory release. For example, for BJ, SH, GZ, and LZ cities,
318 higher HULIS, PCA3, NMR1, and NMR3 may contribute to higher IL-6 release in colder seasons, and for
319 CD city, HULIS, PCA3, NMR1 may lead to this trend. For WH, TY, and XX cities, higher IL-6 release
320 potent in warmer seasons may results from higher NMR3/NMR1 and Pb, however, for NJ city, higher Pb
321 ions may lead to higher IL-6 release in warmer seasons. Similar results of these two different trends were
322 reported through epidemiology and *in vitro* experiments data. For example, $PM_{2.5}$ from BJ city samples
323 showed lower inflammatory release in *in vivo* experimental data[30]. For the second trend, epidemiology data
324 reported that PM from warmer season showed stronger association with respiratory mortality in different
325 parts of the world[31], and with daily mortality in Canada[32] and U.S.[33]. *In vitro* experiments reported that PM
326 collected during S had a greater ability to induce pro-inflammatory activity than samples collected during
327 W in Finland[7], Mexico City[3] and Milan[8]. It is clear that different sampler sites have different seasonal

differences in inflammatory, and different components are the main reasons for seasonal difference.

Our data showed that IL-8 production was dispersed compared to IL-6 production with no significant correlation with $PM_{2.5}$ constitutes. This difference between IL-6 and IL-8 could be because of complex interactions between $PM_{2.5}$ components and modulatory interaction between IL-6 and IL-8[3]. Similar results showed that IL-8 has no significant changes after exposure to various combinations of immunomodulatory in monocytes, while significant IL-6 release in this cell was seen[33].

A549 cells are a type of lung cancer cells, and Beas-2b cells are normal lung epithelial cells, which are commonly used as the cell model of lung exposure at present. In this experiment, A549 cells were mainly used, and Beas-2B cells were used to verify some conclusions, such as verifying the ability of $PM_{2.5}$ to induce inflammatory cytokines, HULIS was more easily to induce inflammation comparing the ability of non-HULIS, and the ability of $PM_{2.5}$ in northern China induced higher IL-6 than that in southern China. Two cells showed similar trends, while in addition, it was also found that the $PM_{2.5}$ induced change trend was more obvious in A49 cells (Figure S2) than that in Beas-2B cell, which needed further experiments to confirm.

3.4 Mechanisms of IL-6 induction via exposure to the WSF of $PM_{2.5}$

The DNA methylation inhibitor 5-aza-2´-deoxycytidine (AZA) heightened the ability of the WSF to induce IL-6 release (Figure S5, SI). Therefore, DNA methylation may be a pathway by which WSF exposure upregulated pro-inflammatory cytokines in A549 cells, although the extent of the increase or decrease of major disease-related genes was unknown. Using lung DNA methylation chip, we found only methylation of *CYP1B1* and *RASSF2* differed significantly (p = 0.021 for *CYP1B1*, and $p=0.036$ for *RASSF2*, ANOVA) from the control (Figure S6). Therefore, we selected *CYP1B1* and *RASSF2* for further experiment.

The MassARRAY assay showed that DNA methylation of *CYP1B1* was significantly decreased compared with blank samples (Table 3) in cells exposed to $PM_{2.5}$ samples, meanwhile, *RASSF2* showed hypermethylation in the CpG islands in its promoter area (Table 3), and DNA methylation of blank filter exposed cells was 30.4% and 10.5% for *CYP1B1* and *RASSF2* genes. Reverse transcription PCR revealed increased *CYP1B1* expression and decreased *RASSF2* expression (Table 3). Both *CYP1B1* and *RASSF2* mRNA expression showed negative correlations with DNA methylation (*CYP1B1*: r = -0.402, p = 0.014, Pearson correlation analysis; *RASSF2*: r = -0.325, p = 0.032, Pearson correlation analysis). In addition, significant correlations were found between *CYP1B1* mRNA and IL-6, respectively (*CYP1B1*: r = 0.286, p = 0.049, Pearson correlation analysis), but not *RASSF2* and IL-6 (r = -0.305, p = 0.062, Pearson correlation analysis), or between the DNA methylation levels of either gene and IL-6. Overall, the results suggested that DNA methylation changes in the promoter areas of the *RASSF2* and *CYP1B1* genes might have induced abnormal expression of these two genes, in turn contributing to the increase in IL-6 release in cells exposed to the WSF of $PM_{2.5}$.

Hypomethylation of the *iNOS* gene promoter has been reported to have a significant down-regulating effect on $PM_{2.5}$[35]. We assessed this relationship *in vitro* using pyrosequencing to analyze *iNOS* promoter gene methylation[20]. The results showed that *iNOS* exhibited DNA hypomethylation (80.26 ± 38.08 %; Table 3) and but no significantly correlated with IL-6 release (r = 0.311, p = 0.057, Pearson correlation analysis). Also, there was no significant relationship between *iNOS* mRNA and other chemical components. We also assessed DNA methylation of *LINE-1* to clarify global DNA methylation changes in cells exposed to the WSF of $PM_{2.5}$[20], however, no significant difference was observed between WSF samples exposed cells and the control (100.34 ± 2.63%).

The mechanism of pro-inflammatory cytokine induction are important of PM toxicity, however, there

372 are few reports on it. *CYP1B1* encodes a cytochrome P450 enzyme that is abundant in airway epithelium,
373 and an upregulation of gene expression of this gene was reported in both cells and mice exposed to PAHs
374 and PM[36]. Meanwhile, *RASSF2* is a potential tumor suppressor gene that might promote apoptosis and cell
375 cycle arrest[37], and has shown hypermethylation and downregulated expression in various cancer tissues[37].
376 Thus, further work is needed to clarify the pathway of the pro-inflammatory response involving these two
377 genes.

378 Furthermore, IL-6 had a more significant relationship than gene expression/DNA methylation with
379 the components of $PM_{2.5}$. For example, *CYP1B*1 mRNA levels were not correlated with the PCA factors,
380 HULIS, or NMR groups, while IL-6 showed a significant correlation with PCA3, HULIS, NMR1, and
381 NMR3. This might be because gene expression or DNA methylation of *CYP1B1*, *RASSF2*, and *iNOS* may
382 only partially contribute to pro-inflammatory cytokine release. For example, DNA methylation may
383 regulate gene expression, thereby mediating pro-inflammatory release after $PM_{2.5}$ stimulation.
384 Nevertheless, other factors might be involved in this pathway, and data on pro-inflammatory cytokine
385 release can be used to evaluate the $PM_{2.5}$ toxicity, while gene expression and gene modification data can be
386 advised to elucidate the mechanisms of toxicity in *in vitro* studies.

387 Additional studies are needed to further clarify the toxicity of organic-extracted fractions of $PM_{2.5}$
388 from different cities and seasons in China, to confirm whether the trends in toxicity are similar to those of
389 the WSF of $PM_{2.5}$. Our results showed that organic compounds might contribute more than other
390 compounds in the WSF of $PM_{2.5}$ to toxicity. HULIS was confirmed as the major toxic component of
391 pro-inflammatory cytokine release; however, the individual compounds of HULIS were not evaluated in
392 terms of their toxicities. Therefore, future work should identify such compounds (e.g., using
393 high-performance liquid chromatography tandem mass spectrometry or Fourier-transform mass

spectrometry) to further clarify the chemical characteristics of the NMR3 fractions. Free radicals occur on the surface and inside of $PM_{2.5}$, which will seize the free radicals on biomacromolecules, may lead to inflammation, cell damage and a series of biological toxicity[38,39]. Further work is needed to explore the relationship between free radicals and pro-inflammatory release. As another limitation of this study, we pooled every 30 samples to yield the amount of $PM_{2.5}$ required for the toxicity experiments, which reduced the total number of samples. Therefore, future studies should include more samples to perform positive matrix factor analysis and clarify the relationship between $PM_{2.5}$ sources and toxicity, which is important for the development of air pollution control countermeasures.

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Conflict of interest

The authors declare no conflict of interest.

Supporting Information

Table S1: The primers used in this study; Table S2, S3: Chemical characteristics of WSFs; Table S4: PCA results of the water-soluble inorganic ions, water-soluble elements, HULIS, non-HULIS，endotoxin，and NMR1-4; Figure S1-5: IL-6 response of dichloromethane (DCM)

and WSF of $PM_{2.5}$ samples, different concentration of $PM_{2.5}$, different HULIS fractions, different concentrations of Pb, and DNA Methylation inhibitor; Figure S6: Relative DNA methylation of 22 tumor-related genes in A549 cells exposed to the WSF of $PM_{2.5}$.

Supporting Methods: WSF extraction, DCM fraction extraction; Inorganic chemical analyses; HULIS fractions analysis; Cell treatment; DNA methylation and gene expression; PCA analysis.

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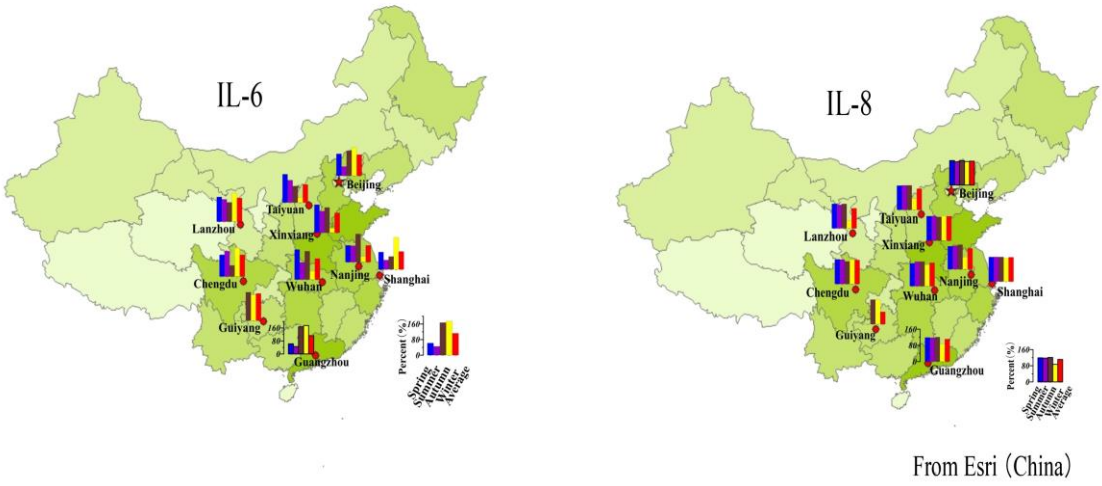

Figure 1. Relative interleukin (IL)-6 and IL-8 response in A549 cells exposed for 3 days to the water-soluble fraction (WSF) of fine particulate matter ($PM_{2.5}$) collected in 10 large cities in China during all four seasons. The WSFs were derived from the same volume of air (10 m^3) and the concentrations were 0.35–10.32 μg/cm^2. Blank samples that underwent the same sample treatment were used as the control, which was considered as 100%. Pictures were drawn using ArcGIS 10.2 software, and the base map of China was from http://www.arcgisonline.cn/arcgis/home/item.html?id=a2071f54e2434e0384b8ffab75a19771.

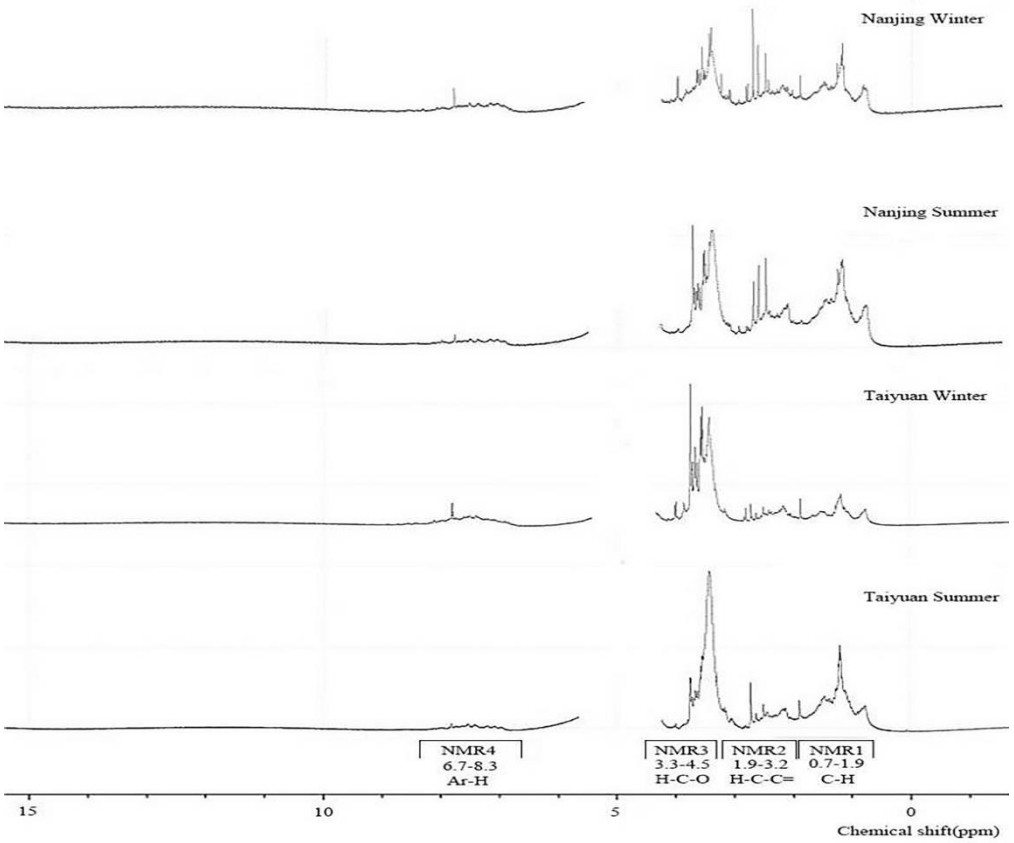

Figure 2. The proton nuclear magnetic resonance (^{1}H NMR) spectra of the WSFs of the Guangzhou summer (GZS), Guangzhou winter (GZW), Taiyuan summer (TYS), and Taiyuan winter (TYW) $PM_{2.5}$ samples. The region between δ^1H 4.0 and δ^1H 5.0 was ignored because of the residual signal of deuterium protium oxide. The four regions (NMR1–4) considered in this study, and the identified chemical groups, are shown in the figure.

564 Table 1. Cytokine production in response to the WSF of $PM_{2.5}$ collected from 10 large
565 cities in China during 1 year, listed in descending order according to the median values.
566

	IL-6*			IL-8*	
	Median	Mean±SD		Median	Mean±SD
GY	249.0	249.0±2.791	GY	121.3	121.3±0.1356
XX	247.2	207.7±50.06	XX	121.1	120.1±1.932
CD	233.7	201.8±35.52	CD	120.5	118.5±3.820
LZ	233.2	221.6±30.2	TY	120.4	104.3±31.24
BJ	224.4	195.3±34.56	LZ	118.6	99.40±40.55
NJ	182.6	158.9±57.06	GZ	118.5	111.3±14.60
TY	173.3	147.4±54.65	BJ	118.5	118.2±2.621
GZ	136.4	141.8±70.33	WH	117.0	115.4±6.400
SH	132.9	132.4±56.49	NJ	114.8	102.9±27.58
WH	130.5	139.0±66.49	SH	78.87	84.22±28.40
	IL-6*			IL-8	
	Median	Mean±SD		Median	Mean±SD
Spring	235.8	218.7±28.00	Autumn	121.0	119.7±2.620
Summer	206.3	167.6±57.00	Summer	121.0	119.0±2.310
Winter	189.0	145.8±63.57	Spring	120.8	119.0±2.870
Autumn	170.6	174.7±35.30	Winter	111.0	94.34±33.49

567
568 *$p < 0.05$, Kruskal–Wallis test. Blank samples were used as the control, which was
569 considered as 100%. GY, Guiyang; XX, Xinxiang; CD, Chengdu; LZ, Lanzhou; BJ, Beijing;
570 NJ, Nanjing; TY, Taiyuan; GZ, Guangzhou; SH, Shanghai; WH, Wuhan; IL, interleukin
571
572

Table 2.

IL-6[a]		
	r	*p*
PCA3	0.370	0.022
HULIS	0.322	0.049
Endoxin	0.363	0.025
Pb	0.337	0.039
NMR1	0.358	0.028
NMR3	0.333	0.041
[b]linear analysis	B	*p*
PCA3	0.370	0.022
HULIS	0.322	0.049
Endoxin	1.859	0.025
Pb	0.337	0.039
[c]multiply linear analysis	B	*p*
PCA3	0.370	0.026

HULIS, humic-like substances; NMR, nuclear magnetic resonance.

Note: [a] Correlation analysis. [b] Multiple linear regression model, in which the five principal components (PCA1, PCA2, PCA3, PCA4, and PCA5) were independent variables.

580 Table 3: DNA methylation and expression of several genes in A549 cells exposed to the WSF of $PM_{2.5}$ samples.

City	*RASSF2* DNA methylation	*CYP1B1* DNA methylation	*iNOS* DNA methylation	*LINE-1* DNA methylation	*RASSF2* mRNA	*CYP1B1* mRNA	*iNOS* mRNA
BJ	126.1±36.86	88.89±22.78	97.56±20.36	99.55±1.680	92.90±34.56	220.4±114.2	124.9±56.32
SH	106.9±2.201	83.79±24.02	63.34±43.05	100.3±3.282	88.01±12.38	185.2±90.15	162.5±172.8
GZ	121.2±78.25	96.15±8.780	53.21±35.73	99.77±2.720	96.06±8.930	184.3±96.29	481.2±504.7
NJ	124.6±43.13	95.16±9.12	96.44±73.58	101.5±3.292	86.47±21.98	271.9±78.54	297.8±216.5
CD	103.8±38.07	93.12±19.46	81.93±18.48	100.6±1.653	89.08±48.52	227.7±128.3	156.6±65.73
WH	130.2±30.68	91.49±7.182	71.58±47.69	99.61±2.223	99.79±5.310	239.9±61.30	126.7±31.58
LZ	131.6±57.45	82.85±26.38	75.69±17.04	101.3±2.070	83.74±35.26	318.3±300.6	162.0±86.58
GY	142.8±21.53	82.11±7.200	54.11±74.76	97.23±3.710	82.25±22.30	253.4±128.6	156.9±80.92
TY	158.4±70.42	98.13±7.560	91.00±18.82	103.6±2.300	69.53±22.83	299.8±141.6	115.4±64.02
XX	107.0±13.41	94.30±12.42	104.6±12.87	98.56±1.441	87.83±28.72	260.4±120.1	155.2±110.1
Season							
spring	114.2±41.01*	95.33±3.73*	76.39±55.78*	99.84±2.350	87.09±19.67*	250.4±80.80*	186.2±120.7
summer	152.7±43.93*	88.81±8.82*	89.55±11.32*	101.6±1.690	83.93±32.76*	202.9±111.1*	149.9±69.09
autumn	104.4±26.11*	98.49±8.200*	100.6±19.28*	101.3±1.630	103.1±13.21*	175.3±68.06*	286.1±355.1
winter	129.6±52.75*	75.30±17.98*	54.98±37.92*	98.76±3.580	88.40±26.00*	350.6±170.9*	155.7±128.1
average	119.1±46.61*	92.02±16.07*	80.25±38.08*	100.3±2.63	89.76±23.62*	245.7±130.7*	195.9±205.0

581 *$p < 0.05$, ANOVA. BJ, Beijing; SH, Shanghai; GZ, Guangzhou; NJ, Nanjing; CD, Chengdu; WH, Wuhan; LZ, Lanzhou; GY, Guiyang; TY,
582 Taiyuan; XX, Xinxiang. Cells were exposed in WSF from 10 m^3 air. Blank samples that underwent the same sample treatment were used as the
583 control, which was considered as 100%, and DNA methylation and gene expression of each gene was expressed as relative DNA
584 methylation/gene expression comparing to control.