Supporting Information for

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

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Tables:

Figures:

Supporting Methods:

S1-1: ***WSF extraction, DCM fraction extraction:***

The WSF extraction was performed as follows: each $PM_{2.5}$ sample was cut into the pieces, dissolved in 5 mL of deionized water, and sonicated for 15 min. After treating three times, the suspension was filtered through 0.22-μm polytetrafluoroethylene syringe filters (Millipore, Billerica, MA, USA). Finally, samples were freeze-dried and dissolved in deionized water up to 50,000 PPM for storage liquid and they will be diluted to different concentrations in different experiments. A blank filter was treated using the same method as the method blank.

Dichloromethane (DCM) fraction of $PM_{2.5}$ extraction: The 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 core 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.

S1-2 : Inorganic chemical analyses

Water-soluble inorganic ions: A Metrosep C4-100 (Metrohm) column and Metrosep Supp 5 - 150 (Metrohm) column were used to separate the cations and anions, respectively. Buffer 1 (2 mmol/L H_2SO_4) and buffer 2 (3.2 mmol/L Na_2CO_3 and 1.0 mmol/L $NaHCO_3$) were used as the elution and balance buffers for the cations

and anions, respectively. The detection limits of all ions were 0.01 μg/mL. Fresh deionized water was injected thrice before each standard (Sigma-Aldrich, St. Louis, MO, USA) and sample injection to minimize cross-contamination.

Metal elements : The detection limits for the analyzed metals were as follows: Al (6 μg/L), Ti (6 μg/L), V (0.7 μg/L), Cr (0.9 μg/L), Mn (0.6 μg/L), Fe (9 μg/L), Co (0.3 μg/L), Ni (0.7 μg/L), Zn (9 μg/L), Cu (0.9 μg/L), As (0. 9 μg/L), Cd (0.6 μg/L), and Pb (0.7 μg/L) (Naimabadi et al., 2016).

S1-3: HULIS fractions analysis:

To separate HULIS from the WSF of $PM_{2.5}$, sample solutions were diluted to 20 mL and the pH was adjusted to 2. HULIS was separated using a 6-mL Oasis HLB column (Waters, Milford, MA, USA), in which 4 mL of methanol and 5 mL of deionized water (pH = 2) were added to activate the column, followed by the addition of the prepared sample. After adsorption, 2 mL of deionized water was added to the column to remove not absorbed organic and inorganic components therein. The column was freeze dried followed by the addition of 3 mL of 2% (v/v) ammonia/methanol (Sigma-Aldrich) to the column in order to elute the absorbed target compounds. The elution step was repeated once, the eluted solution was dried under a stream of liquid nitrogen and water was added to a constant volume up to 20 mL. The carbon content of HULIS was analyzed with a TOC analyzer.

S1-4: Cell treatment, DNA methylation and gene expression:

The treatment of cells:

A549 and Beas-2B cells were purchased from the Cell Bank of Type Culture

Collection of Chinese Academy of Sciences (Shanghai, China). A549 cells were cultured in RPMI 1640 with 10% FBS (v/v) at 37 °C in a 5% CO_2 atmosphere, and Beas-2B cells were cultured in high-glucose Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco, San Diego, CA, USA). All WSF samples were sonicated for about 20 seconds before use to expose cells.

Concentrations conversion:

In this study, the ability of IL-6 release induced by WSF samples between ten cities and four seasons was used to be compared, so WSF samples from same volume air, 10 m^3 air, was used to expose cells which make this comparison reasonable. Normally, $PM_{2.5}$ toxicity experiments prefer to use μg/cm^2 unite to express the exposure concentration in *in vitro* experiments, which is try to simulate human respiratory exposed $PM_{2.5}$. Because the A549 and Beas-2B were the adherent cells, the area of cells culture plate was recorded for calculate cell exposure concentrations in μg/cm^2 unite. The mass of $PM_{2.5}$ in 10 m^3 air was calculated using PM2.5 data (μg/m^3), then the cell exposure concentrations (μg/cm^2) of each sample was calculated as the rate of mass of $PM_{2.5}$ to cells area.

DNA methylation PCR array:

The DNA methylation PCR array, which included 22 tumor-related genes (*APC*, *CADM1 (TSLC1)*, *CDKN1C (p57Kip2)*, *CDKN2A*, *CDKN2B (p15INK4b)*, *FHIT*, *CDH1*, *CDH13*, *DLC1*, *OPCML (aOBCAM)*, *FHIT*, *MLH1*, *PRDM2 (RIZ1)*, *RASSF1*, *RASSF2*, *TCF21*, *CXCL12*, *APBA1*, *MGMT (AGT)*, *CYP1B1*, *PAX5*, and *MTHFR*) exhibiting DNA methylation dysregulation, might be associated with various lung diseases, was used to screen for genes targeted for DNA methylation after exposure to the WSF of $PM_{2.5}$.

RASSF2 and CYP1B1 gene methylation:

The Sequenom MassARRAY platform (Sequenom) was used to perform the quantitative methylation analysis of the *RASSF2* and *CYP1B1* genes. The primers were designed using Sequenom software (http://www.epidesigner.com) and described in detail in our previous paper[1] (Supplemental Table 1). We examined a 568-bp fragment of *CYP1B1* (GRch38, Chr2, 38074550–38075117) and a 539-bp fragment of *RASSF2* (GRch38, Chr20, 4823013–4823551); these included 65 CpG sites and 58 CpG sites, of which 48 and 30 CpG sites were detected, respectively. These two fragments are involved in CpG islands considered to participate in gene expression. The DNA methylation level of each CpG site was expressed as the mean of the methylated sites ratio to the sum of methylated and unmethylated sites, and DNA methylation degree of the two genes was calculated as the average of all sites.

LINE-1 and iNOS gene methylation:

Genomic DNA was bisulfited using a bisulfite conversion kit (Zymo Research, Orange, CA, USA). The DNA methylation level of *iNOS* has been reported to be negatively related to gene expression[2]. *LINE-1* gene methylation was expressed as the methylated sites divided by the sum of methylated and unmethylated sites, and the relative DNA methylation was the degree of methylation compared with that of blank samples, which was considered as 100%.

Gene expression:

Briefly, total cellular RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and 2 μg of total RNA from each sample was reverse-transcribed into cDNA using a Prime-Script™ RT Reagent Kit (TaKaRa, Dalian, China). The levels of the target gene mRNA transcripts relative to control β-actin were characterized by quantitative PCR using the cDNA template, specific primers (Excel Table S1), and

SYBR® Premix Ex Taq™ (TaKaRa) on an AB 7500 Fast System (Applied Biosystems, Foster City, CA, USA). Supplementary Table 1 lists the sequences of the primers used in this experiment. The levels of the target mRNA transcripts relative to control β-actin were analyzed with the $2^{\Delta\Delta Ct}$ method and expressed as fold changes.

Supplemental Tables and Figures:

Table S1. The primers used in this study.

MassARRAY	Gene Name
	cyp1b1
Forward primer (5′–3′)	aggaagagagGTTGTTTTATAGTGGTGTTGAATGG
Reverse primer (5′–3′)	cagtaatacgactcactatagggagaaggctCAATACCTCAAAAACTTATCCAAAAT
	rassf2
Forward primer (5′–3′)	aggaagagagGGTTGAAAAAGGTTAAGGGGTT
Reverse primer (5′–3′)	cagtaatacgactcactatagggagaaggctCAATACCTCAAAAACTTATCCAAAAT
Pyrosequencing	
	LINE-1
Forward primer (5′–3′)	TTTTGAGTTAGGTGTGGGATATA
Sequencing primer (5′–3′)	AGTTAGGTGTGGGATATAGT
Reverse primer (5′–3′)	Biotin-AAAATCAAAAAATTCCCTTTC
Sequence analyzed	CTCGTGGTGCGCCGTTTTTTAAGCCGTCGGAAAGCGC
	iNOS
Forward primer (5′–3′)	AATGAGAGTTGTTGGGAAGTGTTT
Sequencing primer (5′–3′)	TAAAGGTATTTTTGTTTTAA
Reverse primer (5′–3′)	Biotin-CCACCAAACCCAACCAAACT
Sequence analyzed	C/TGATTTTC/TGGGTTTTTT
Real-time PCR	
	cyp1b1
Forward primer (5′–3′)	TGAGTGCCGTGTGTTTCGG
Reverse primer (5′–3′)	GTTGCTGAAGTTGCGGTTGAG

	rassf2
Forward primer (5′–3′)	GCACTCTGGCTGTAACCTGG
Reverse primer (5′–3′)	CACTAGGCGTCCTCACATTGC
	IL-6
Forward primer (5′–3′)	ACTCACCTCTTCAGAACGAATTG
Reverse primer (5′–3′)	CCATCTTTGGAAGGTTCAGGTTG
	IL-8
Forward primer (5′–3′)	TTTTGCCAAGGAGTGCTAAAGA
Reverse primer (5′–3′)	AACCCTCTGCACCCAGTTTTC

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Table S2. Concentrations of 24h $PM_{2.5}$ from 10 large cities in China collected during 1 year.

$PM_{2.5}$ concentration[a]	Median	Mean±SD	*p value
TY	331.70	303.15±34.35	$p = 0.003$
LZ	249.35	270.92±30.41	
XX	243.60	236.2±47.91	
GY	206.93	206.93±13.9	
BJ	202.56	189.2±22.8	
WH	161.45	187.74±43.58	
CD	138.34	156.81±39.01	
NJ	105.12	99.73±29.33	
SH	82.00	86.39±36.05	
GZ	81.94	80.73±25.03	
Spring	140.37	181.64±57.44	$p = 0.184$
Summer	128.16	132.8±62.03	
Autumn	156.63	165.2±45.47	
Winter	205.06	229.63±47.11	

*Kruskal–Wallis test. [a]μg/m^3.

TY, Taiyuan; LZ, Lanzhou; XX, Xinxiang; GY, Guiyang; BJ, Beijing; WH, Wuhan; CD, Chengdu; NJ, Nanjing; SH, Shanghai; GZ, Guangzhou

Table S3. Characteristics of HULIS and the NMR groups in the WSFs of $PM_{2.5}$.

	Median	Mean±SD	*p* value among seasons	*p* value among cities
HULIS	0.45	1.25±1.56	0.032	0.013
NMR1	40.450	44.67±19.407	0.003	0.021
NMR2	29.305	35.594±17.399	0.003	0.025
NMR3	53.750	60.523±28.273	0.036	0.009
NMR4	6.910	9.932±8.055	0.001	0.177

*μg/m^3. # Kruskal–Wallis test. HULIS, humic-like substances; NMR, nuclear magnetic resonance.

Table S4. PCA results of the water-soluble inorganic ions, water-soluble elements, HULIS, non-HULIS, endotoxin, and NMR1-4. Five factors were obtained, which accounted for 76.28% of the total variance. Values below 0.5 are not shown.

	PCA1	PCA2	PCA3	PCA4	PCA5
Na	.947				
Cl	.945				
NMR1	.887				
NMR2	.869				
K	.812				
NMR3	.795				
Zn	.783				
No_3^-	.775				
NH_4^+	.763		-.537		
Mg	.760		.558		
Mn	.756				
NMR4	.744				
SO_4^{2-}	.741				
Endotoxin	.719				
Co	.623	.569			
Ti	.600				
Cu	.578	.624			
Cr	.577	.521			
Ca	.518		.517		
As	.513			.545	
Al		.541			
V				-.559	
Fe		.715			
Ni				-.676	
Cd				.636	
Pb		.586	.589		
HULIS					-.676
Non-HULIS					.551

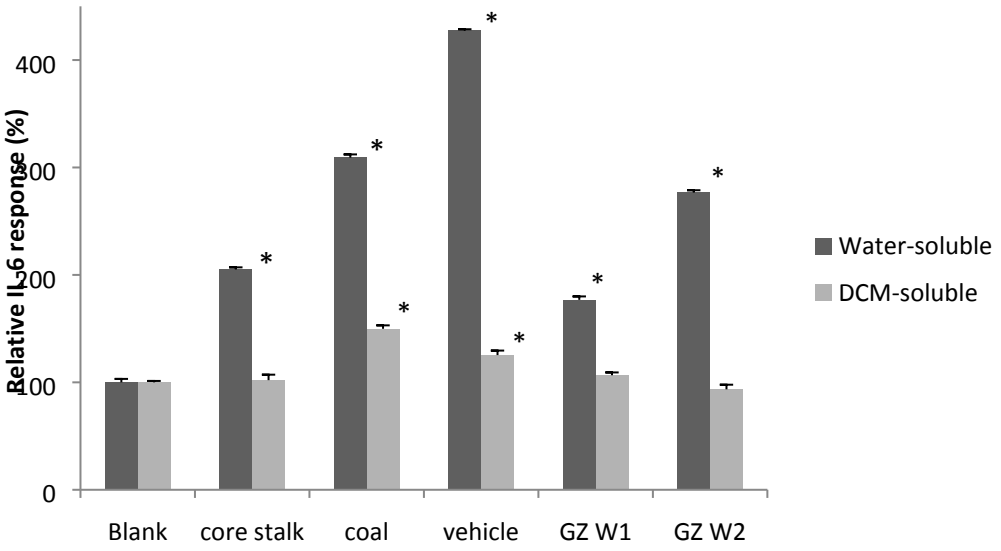

Figure S1. IL-6 response in A549 cells exposed to the dichloromethane (DCM) and WSF of $PM_{2.5}$ samples from the same volume of air. GZ W1 and GZ W2 are the $PM_{2.5}$ from Guangzhou sample 1 and sample 2 in the winter of 2013, respectively; core stalk and coal are $PM_{2.5}$ from the combustion of core stalks and coal, respectively; and vehicle represents $PM_{2.5}$ from vehicle emissions. Cell were exposed in WSF from same volume of 10 m^3 air for 3 days. *$p < 0.05$, ANOVA. Each experimental sample was compared with its respective sample blank, which was considered as 100%.

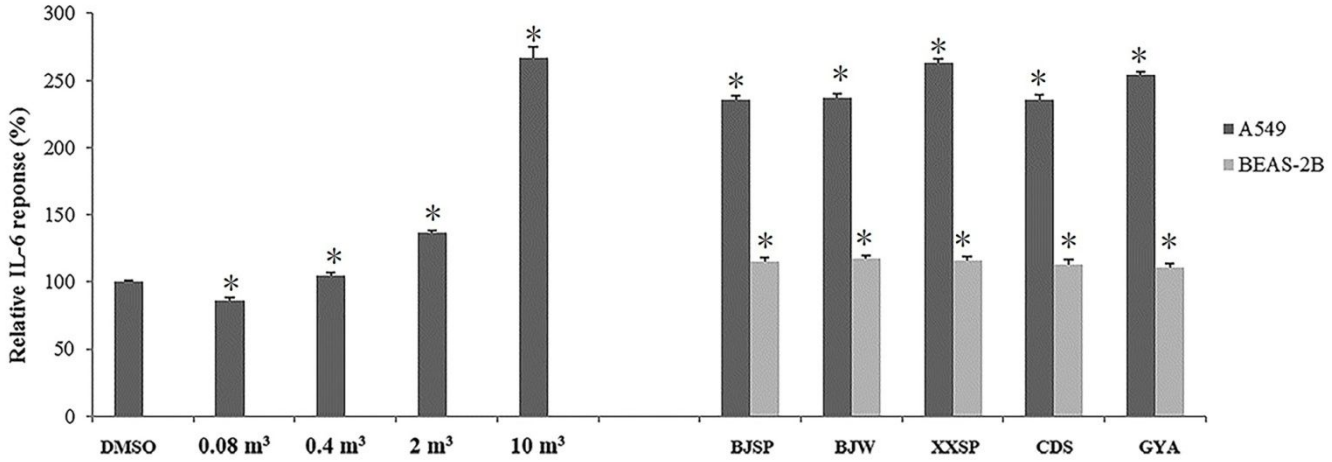

Figure S2. Confirmation of $PM_{2.5}$ induced IL-6 release. Left: Relative IL-6 response at various WSF concentrations of the Xinxiang spring (XXSP) samples. A549 cells were exposed to various WSF concentrations (from $PM_{2.5}$ of 0.08-10 m^3 air) of the XXSP $PM_{2.5}$ for 3 days, and IL-6 levels were assessed. Right: Relative IL-6 response in Beas-2B and A549 cells exposed for 3 days to the WSFs of five $PM_{2.5}$ samples derived from 10 m^3 of air. Blank samples were used as the control, which was considered as 100%. *$p < 0.05$, ANOVA. The concentration of IL-6 in blank sample is 22.38±0.13 pg/ml in left figure, 43.26±0.56 pg/ml in right figure for A549 cells, and 3.67 ±0.89 ng/ml for Beas-2B cells.

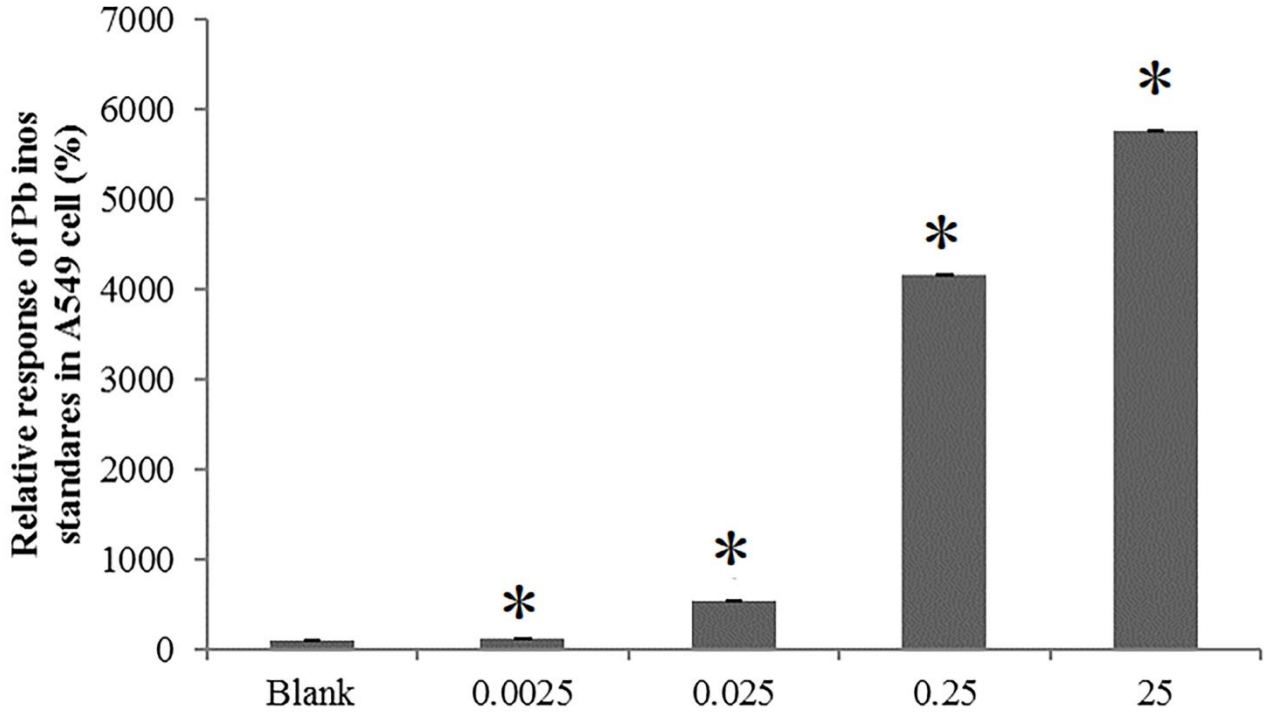

Figure S3. Relative IL-6 response in A549 cells exposed to various concentrations of Pb. A549 cells were exposed in Pb standard ions (0-25 ppm) for 3 days. Dimethyl sulfoxide-treated cells were used as the control, which was considered as 100%. *$p < 0.05$, ANOVA.

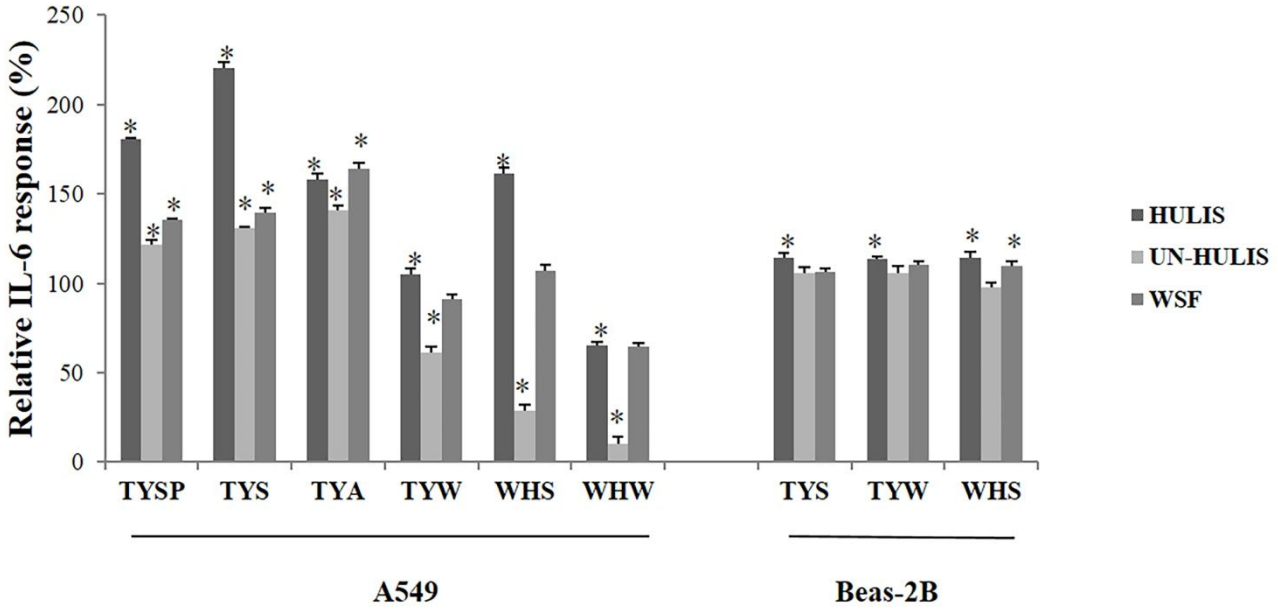

Figure S4. Relative IL-6 response of humic-like substances (HULIS), non-HULIS, and the WSFs of $PM_{2.5}$ samples. Samples included Taiyuan spring (TYSP), Taiyuan summer (TYS), Taiyuan Autumn (TYA), Taiyuan Winter (TYW), Wuhan summer (WHS), and Wuhan winter (WHW) $PM_{2.5.}$ A549 cells and Beas-2B cells were exposed at concentrations of 3.12 μg/cm^2. Quartz filter sample blanks underwent the same treatment as the HULIS, non-HULIS, and WSF extraction methods, and the IL-6 responses of the blanks were considered as 100%. * $p < 0.05$, ANOVA.

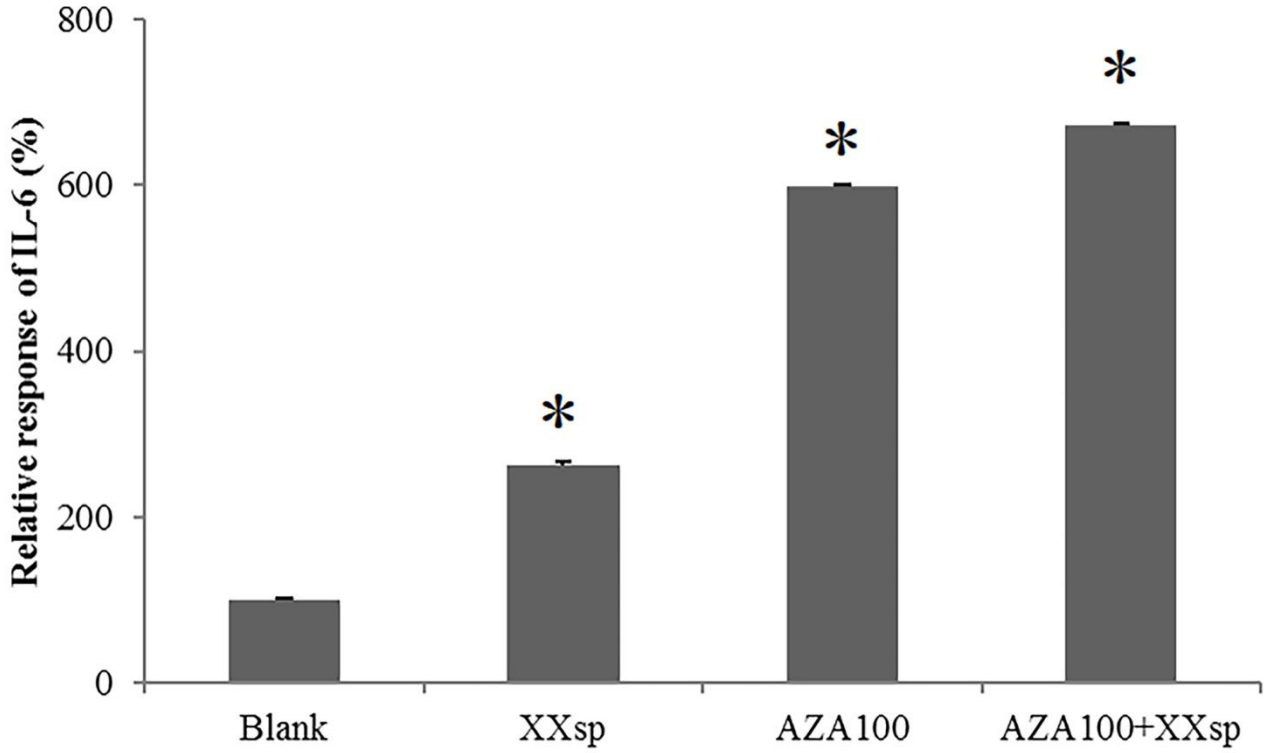

Figure S5. The DNA methylation inhibitor 5-aza-2′-deoxycytidine (AZA) enhanced $PM_{2.5}$-induced IL-6 release in A549 cells. A549 cells were treated with 100 μM AZA for 3 days and then exposed to the WSF of XXSP $PM_{2.5}$. The IL-6 levels in cells exposed to blank samples treated the same as the $PM_{2.5}$ samples, which were considered as 100%. *$p < 0.05$, ANOVA.

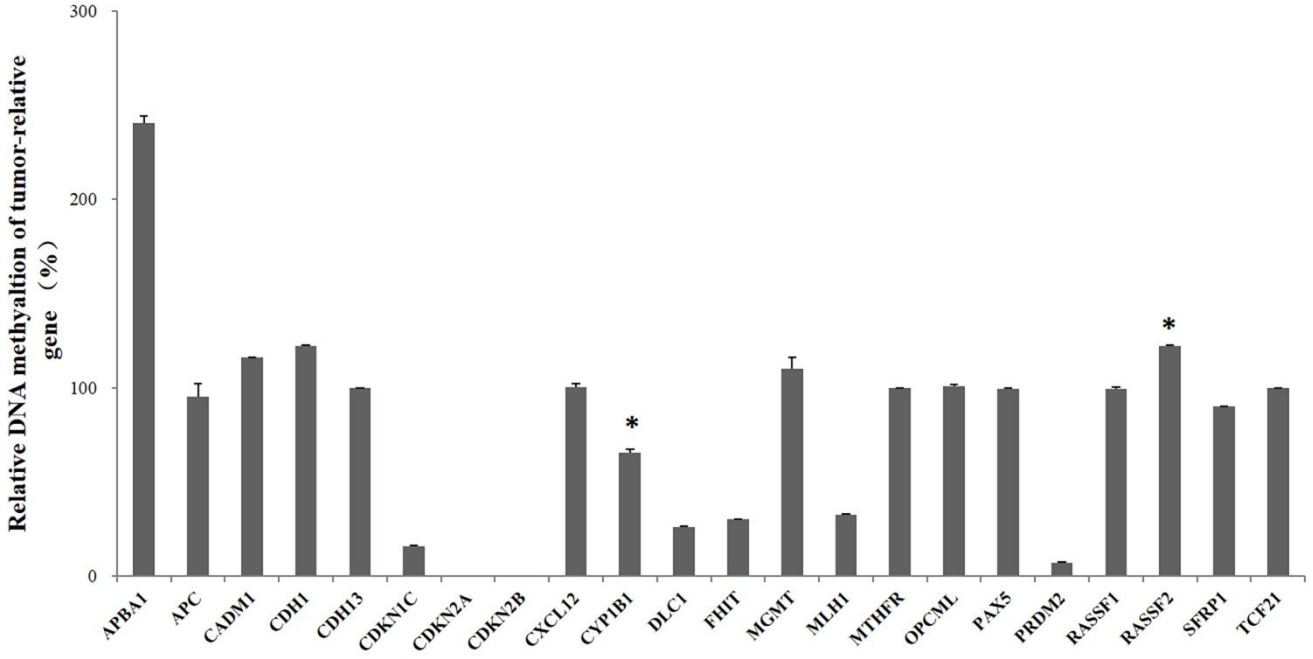

Figure S6. Relative DNA methylation of 22 tumor-related genes in A549 cells exposed to the WSF of Xinxiang Spring (XXSP) $PM_{2.5}$. Blank samples were used as the control, which was considered as 100%. *$p < 0.05$, ANOVA.

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