Assessment on the lung injury of mice posed by airborne PM_{2.5} collected from developing area in China and associated molecular mechanisms: from histopathology to integrated transcriptome analysis

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Research

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Abstract

Background & aims

Some epidemiological investigations have revealed airborne fine particulate matter (PM_{2.5}) could induce adverse effects on respiratory system of human. However, the experimental evidences of the harmful effect of PM_{2.5} from mid-scale city in China and associated molecular mechanisms was still scarce. In this study, we aimed to evaluate the adverse effect on the lung system of PM_{2.5} collected from mid-city of China and elucidate the underlying molecular mechanism through mRNA-seq and microRNA-seq integrated analysis.

Methods

We exposed male Balb/C mice to PM_{2.5} collected from Baoji city, China for 8 weeks ($298.52 \pm 25.86 \mu\text{g}/\text{m}^3$) using a whole-body exposure system. Micro-CT and histopathological analyses were performed to determine the morphology and histopathology changes of lung tissues induced by PM_{2.5}. Transcriptome (both mRNA and microRNA) sequencing and the immunohistochemistry assay were performed to reveal the associated underlying mechanisms. The contents of PAHs absorbed in the PM_{2.5}, as well as the pearson correlation index between it and the target genes and microRNA were examined.

Results

Obvious lung injury including pulmonary dysfunction, immunological responses, and fibrosis were observed from the PM_{2.5} exposure group. The content of leucocyte of lung tissue was significantly increased. PM_{2.5} mainly induced immune pathways including B cell receptor signaling and cell adhesion molecules (CAMs). The expression levels of the associated genes and microRNA were verified using RT-qPCR. The protein expression of CD19, CD81, and PIK3CD involved into B cell receptor signaling significantly increased in exposure group. The concentrations of benzo(a)pyrene (BaP) showed a marked correlationship with the genes and microRNA.

Conclusions

PM_{2.5} from the mid-scale city of China caused adverse effects on lung system of mice, which was much stronger than the mega city, indicating the health risk of the air pollution in the developing area should be paid more attention. The toxicity of PM_{2.5} may associated with immune system response. This work for the first time provided a new sight into the molecular mechanism of PM_{2.5} toxicity on lung system using transcriptomics integrated analysis and help further to develop effective measures to prevent air pollution associated lung disease, especially in the developing area in China.

Background

Air pollution has been recognized may pose marked threat on human health, especially the respiratory and cardiovascular system[1, 2]. Fine particulate matter with aerodynamic diameter less than 2.5 μm (PM_{2.5}) as one of the important components of ambient air pollution can easily attach to the pulmonary epithelial, and then induce adverse lung injury[3]. Accumulating evidences has revealed that short- or long-term exposure to PM_{2.5} may increase the risk of various respiratory illness for human, including idiopathic pulmonary fibrosis (IPF)[4],

asthma[5], and chronic obstructive pulmonary disease (COPD)[6]. Otherwise, patients with lung-diseases would be two or three times the risk of death than normal population[7]. Hence, World Health Organization cites PM_{2.5} pollution as fifth leading risk factor for mortality and economic loss. In China, ambient PM_{2.5} was responsible for 1.2 million premature death as the forth healthy risk factor[8, 9]. From the public health view of point, it was imperative to comprehensively elucidate the causal relationship between PM_{2.5} pollution and lung injury.

There have been several publications about the adverse effects of PM_{2.5} to respiratory system using mice as model animal with oropharyngeal aspiration (OPA) or intranasal administration. Balb/c mice exposed to PM_{2.5} (2.5–20 µg for each mice) through OPA for 21 days showed obvious collagen deposition around small airway[10]. Acute OPA of PM_{2.5} extract to mice may induce greater lung neutrophilia and inflammation[11]. C57BL/6 mice exposed to PM_{2.5} (3 mg/kg) by OPA can adversely affect prenatal lung development in the offspring[12]. C57BL/6J mice showed significant inflammation and incipient fibrosis symptom after directly intranasal administration with PM_{2.5} (100 µg/day) for 4 weeks[13]. The obvious fibrous cap thickness observed from apoe^{-/-} mice exposed to PM_{2.5} by intranasal instillation[14]. Generally, direct administration by PM_{2.5} suspension may under- or overestimate the healthy risk. There were only few investigations about the whole-body inhalational exposure. Yuan et al. (2020) found that C57BL/6 mice whole-body exposed to PM_{2.5} (59.77 µg/m³) sampled from Beijing, China exhibited severe lung injury and fibrosis[11]. They also demonstrated that treatment with local PM_{2.5} polluted air at Beijing, China would strikingly induce lung oxidative stress and injury in mice for 3 weeks and 6 months, respectively[15, 16]. Zhou et al. (2019) exposed C57BL/6 mice to PM_{2.5} collected from Shijiazhuang, China, and found that significant increase in circulating white blood cells and inflammation in lungs of mice from exposure groups[17]. All the investigations were preformed to estimate the adverse effects on lung by PM_{2.5} in Beijing-Tianjin-Hebei region, China. The underlying mechanism have not been comprehensively elucidated.

Evidences have unveiled that air pollution was much more serious in economically developing levels cities than the economically developed mega-cities in China[18–20]. However, the assessment on the healthy risk of PM_{2.5} pollution in this mid-scale cities was scarce. An increase of 10 µg/m³ PM_{2.5} was associated with 15–27% increase in lung cancer mortality[21]. Moreover, the annual mean concentration of PM_{2.5} in mid-scale cities in China was marked higher than the limitation suggested by WHO as 10 µg/m³[19, 22]. Hence, its warranted to investigate the consequences posed by PM_{2.5} in this region to pulmonary system using “real-world” exposure system.

High-throughput sequencing is a precise method to measure global gene expression profiles by RNA-sequencing including mRNA and microRNA. Recently, this tool has been used to illuminate the associated molecular mechanism of cytotoxicity induced by PM_{2.5}[23, 24]. There was only one publication about the potential mechanism of lung injury posed by airborne PM_{2.5} at Beijing, China using transcriptome analysis[25]. Meanwhile, some evidences suggested that microRNA deregulation was likely to be associated with the respiratory diseased induced by PM[26, 27]. Hence, the integrated analysis between mRNA and microRNA to elucidate the underlying mechanisms of the lung injury posed by PM_{2.5} was imperative.

In summary, we for the first time established a whole-body exposure system to investigate the adverse effects posed by PM_{2.5} sampled from Baoji (33.35°-35.06° N, 106.18°-108.03° E), an inland city located in the mid-west part of China, using mice for 40 days. The objectives are to evaluate: (1) histopathological changes of lung tissue; (2) morphological changes of lung; (3) underlying mechanisms using mRNA-seq, microRNA-seq, and immunohistochemical analysis; (4) the potential biomarkers to lung injury posed by PM_{2.5}; (4) the dominant components in PM_{2.5} associated with lung injury.

Results

The contents of PAHs

Seventeen US. EPA priority PAHs were detected with the sum concentrations of $570.48 \pm 143.82 \text{ ng/m}^3$. As showed in Figure. S3, 5-ring PAHs accounted for 42% of the total. Benzo(b/k) fluoranthene (BbkF) was the most abundant PAH with concentration at $140.00 \pm 21.97 \text{ ng/m}^3$, followed by benzo(a)pyrene (BaP) ($57.50 \pm 10.37 \text{ ng/m}^3$), and dibenzo(a,e)pyrene (DBP) ($52.67 \pm 9.29 \text{ ng/m}^3$). The molecular distributions were consistent to the previous reports for the samples from Baoji, China[18].

PM_{2.5}-induced lung injury in mice

Micro-CT were performed to determine the potential effect on the lung morphology induced by PM_{2.5} *in vivo* after the exposure before the histopathology assay. The tomograms showed higher density and confluent opacity of the mice lung tissues from exposure groups (Figure. 1d and e), indicating the remarkable pulmonary inflammation induced by PM_{2.5}, while no morphological changes were observed in the control (Figure. 1a and b). Moreover, we created the 3D reconstruction of the lung tissue of the mice to estimate the pulmonary function injury posed by PM_{2.5}. As shown in Figure.1c and f, the effective pulmonary function from exposure group markedly shrunk compared to the control group. The same results about the higher lung density and deteriorated PF were found by previous publication through intranasally administrating[10, 13, 28]. However, the adverse effects found in this work were much more obvious than the previous results, while the exposure concentration ($289.52 \text{ } \mu\text{g/m}^3$) was much lower than that it (equal to $400\text{--}1543 \text{ } \mu\text{g/m}^3$) in the previous work[10, 13], indicating exposure way may markedly impact the assessment on the toxicity of PM_{2.5} on the respiratory system. Meanwhile, the PM_{2.5} particles used previously were obtained from Shanghai, China, one of the megacities located in Yangtze River Delta, which suggested that the hazard level of the PM_{2.5} collected from the developing area in this work may be higher than the developed city.

As shown in Figure. 2, obvious alveolar intervals thicken, inflammatory cell infiltration, and alveolar structure damage (Figure. 2b, e, h) were observed from the pulmonary alveoli from exposure group compared with the vacuolated thin-walled of alveolar cavity as the intact structure shown in the control (Figure. 2a, d, g). The marked bronchiolar epithelial hyperplasia and endoluminal inflammatory cells infiltration were shown in the lung of PM_{2.5}-treated mice (Figure. 2c, f, i), while simple ciliated columnar epithelium of bronchia in lung was characterized as normal pulmonary structure observed in control mice (Figure. 2a, d, g). The results found in this work were consistent to the previous investigations[15, 17, 27, 29]. Noted that the pulmonary histopathological injury was irreversible, the long-term exposure to PM_{2.5} may pose severe threat on the

respiratory system to human beings since the pollution is persistent. Since the prevalence of pulmonary fibrosis (PF), a critical lung interstitial disease, was significantly associated with long-term exposure to PM_{2.5}[1], we determined lung fibrosis in mice from control and exposure groups using Masson trichrome stain. Obvious lung fibrosis increase was observed from exposure group (Figure. 2k) compared with the control (Figure. 2j), and the statistical results showed 2.56-fold ($p < 0.05$) increase between the exposure and control group (Figure. 2l).

To further elucidate the immune response activated through PM_{2.5} exposure in mice respiratory system, the relative abundances of leucocytes CD45+, as well as the epithelial cells (CD45-/CD31-/CD326+) in lung tissue of mice were determined using flow cytometry analysis. As shown in Figure. 3, the relative abundance of leucocytes in exposure group were significantly higher than it in the control (Figure. 3a-c). However, there was no statistical difference of the epithelial cell abundances between the PM_{2.5}-treated and control groups (Figure. 3d-f). The results indicated that immune system response may be the dominate mechanism for the effects by PM_{2.5}, which was parallel to previous works[30, 31]. However, the bronchiolar epithelial hyperplasia cannot be manifested using the relative abundance of the cells, which may ascribe to the interference of the overwhelming quantity advantage of the leucocytes. Further experiments (e.g., single-cell sequencing) will be performed to elucidate the underlying mechanism of the difference occurred in epithelial cells.

PM_{2.5}-induced changes of gene expression profile in mice lung

To investigate the underlying molecular mechanism of the lung injury posed by PM_{2.5}, RNA-sequencing was performed to analyze the whole-genome expression profiling changes of the mice lung tissues with or without exposed to PM_{2.5}. As shown in the Figure. S4, there was 1607 DEGs (1159 up-regulation and 448 down-regulation) identified in comparison of control and exposure groups (Volcano Plot). The top 8 up-regulated KEGG enriched pathway including B cell receptor signaling pathway, p53 signaling pathway, and T cell receptor signaling pathway, and the top 10 down-regulated KEGG enrichment including cell adhesion molecules (CAMs), calcium signaling pathway, and malaria were characterized (Table S3), which was parallel to the previous study about the mice exposed to PM_{2.5} collected from Beijing, China[25]. Meanwhile, the top 10 up- and down-regulated genes were listed in Table S4.

PM_{2.5}-induced changes of microRNA expression profile in mice lung

According to the microRNA-sequencing analysis, a total of 70 microRNA (53 up-regulation and 17 down-regulation) were identified in comparison of control and exposure groups, and the fold changes were shown in Figure. S5. As shown in Table S5, the top 20 KEGG pathway enrichment determined by KEGG annotation including GnRH signaling pathway, HIF-1 signaling pathway, and MAPK signaling pathway were identified. The similar results of the KEGG pathway (i.e., HIF-1 signaling pathway, Insulin signaling pathway, and MAPK signaling pathway) were found by previous investigation about the PM_{2.5} collected from Shijiazhuang, Hebei, China using microRNA microarray analysis[27]. The top 10 up- and down-regulated microRNA were listed in Table S6.

Integrated analysis between the gene expression and microRNA

To further elucidate the molecular mechanisms, the integrated analysis of mRNA and microRNA expression data were performed. A total of 3024 mRNA (1625 up-regulated and 1399 down-regulated) and 100 microRNA (59 up-regulated and 41 down-regulated) were identified in comparison of control and exposure groups (Figure. S6a). The KEGG pathway (top 20) involved into inflammation and immune pathways, including cell adhesion molecules (CAMs) ($p = 0.0068$), B cell receptor signaling pathway ($p = 0.019$), primary immunodeficiency ($p = 0.078$), were significantly enriched (Figure. S6b and Table S7), which was consistent to the KEGG annotations associated with mRNA expression levels, but was robust through integrated analysis. GO annotations found that the biology process (BP) including immune system process, regulation of biological quality, and regulation of immune system process were significantly enriched (Figure. S6c).

To further validate the causal relationship between the enriched KEGG pathway and the lung injury posed by $PM_{2.5}$. Nine representative pathways involved into the immune system, including B cell receptor signaling pathway, cell adhesion molecules (CAMs), antigen processing and presentation, were chosen (Figure. 4a). The input genes associated with the enriched KEGG pathway annotated by integrated analysis were showed in Table S7. As showed in Figure. 4b, we selected 27 genes to explore the mechanism for the pathway in $PM_{2.5}$ -exposed lung tissue. Among these genes (showed in Table 1), *Cd72*, *Cd81*, *Cd19*, *Pik3cd*, and *Ppp3cc* were involved into B cell receptor signaling pathway, while *Cd80*, *Cd22*, *H2-M2*, *H2-T24*, and *H2-T3* were involved into cell adhesion molecules (CAMs). *Tapbp* and *Klrc1*, *Prf1*, and *Jak3* and *Tnfrsf13c* were involved into antigen processing and presentation, graft-versus-host disease, and primary immunodeficiency, respectively. *Angpt2*, *Pdgfra*, *P2ry1*, and *Igfl* were associated with Rap1 signaling pathway. *Ptpn7* and *Fgf22* were related to MAPK signaling pathway. *Rasa1* and *Lamc1* were involved into Ras signaling pathway and small cell lung cancer, respectively. Gene *Met*, *Ralgds*, and *Cd8b1* were identified involved into multiple pathways. The associated microRNAs (total of 30) were mapped in Figure.4b and Table 1.

Table 1
Selected genes and microRNAs expression changes validated by RT-qPCR.

KEGG pathway	mRNA	Q-RT-PCR	Transcriptomics	microRNA	RT-qPCR	Transcriptomics
B cell receptor signaling pathway	<i>Cd72</i>	2.50 ± 0.19	2.44 ± 0.28	149-5p	0.78 ± 0.017	0.50 ± 0.033
				328-3p	0.85 ± 0.10	0.57 ± 0.054
	<i>Cd81</i>	0.36 ± 0.032	0.39 ± 0.024	24-3p	0.89 ± 0.078	0.59 ± 0.063
	<i>Cd19</i>	4.87 ± 0.26	2.87 ± 0.33	466i-5p	0.84 ± 0.16	0.40 ± 0.081
	<i>Pik3cd</i>	2.03 ± 0.13	1.65 ± 0.18	203-5p	1.22 ± 0.15	2.79 ± 1.01
				7a-5p	1.58 ± 0.23	2.15 ± 0.93
	<i>Ppp3cc</i>	1.63 ± 0.47	1.66 ± 0.21	92a-1-5p	1.52 ± 0.32	2.93 ± 1.33
Cell adhesion molecules (CAMs)	<i>Cd80</i>	1.68 ± 0.13	5.26 ± 0.65	674-3p	0.86 ± 0.086	0.61 ± 0.038
				1247-5p	0.48 ± 0.055	0.17 ± 0.011
	<i>Cd22</i>	2.18 ± 0.16	3.74 ± 0.59	3110-5p	1.97 ± 0.88	10.17 ± 1.81
	<i>H2-M2</i>	514.25 ± 0.36	17.37 ± 0.44			
	<i>H2-T24</i>	0.64 ± 0.036	0.30 ± 0.032			
	<i>H2-T3</i>	1.51 ± 0.059	3.96 ± 0.54	486a-3p	0.62 ± 0.11	0.57 ± 0.033
Antigen processing and presentation	<i>tapbp</i>	0.41 ± 0.070	0.42 ± 0.051			
	<i>Klrc1</i>	0.93 ± 0.034	0.19 ± 0.011	1981-5p	0.029 ± 0.011	0.49 ± 0.054
Graft-versus-host disease	<i>Prf1</i>	0.46 ± 0.077	0.12 ± 0.021			

B-Actin and GAPDH were the endogenous control for gene, U6 was the endogenous control for microRNA.

KEGG pathway	mRNA	Q-RT-PCR	Transcriptomics	microRNA	RT-qPCR	Transcriptomics
Primary immunodeficiency	<i>Jak3</i>	3.50 ± 0.23	2.14 ± 0.19	3074-5p	0.75 ± 0.14	0.59 ± 0.061
				351-3p	0.85 ± 0.14	0.13 ± 0.012
	<i>Tnfrsf13c</i>	2.02 ± 0.38	5.03 ± 0.66	676-3p	0.57 ± 0.11	0.51 ± 0.031
Rap1 signaling pathway	<i>Angpt2</i>	0.39 ± 0.042	0.47 ± 0.022			
	<i>Pdgfra</i>	0.39 ± 0.018	0.50 ± 0.035	222-3p	0.69 ± 0.094	0.61 ± 0.050
				15a-5p	1.09 ± 0.29	1.73 ± 0.32
				344-3p	1.63 ± 0.38	2.09 ± 0.41
	<i>P2ry1</i>	0.45 ± 0.029	0.32 ± 0.015	205-5p	1.40 ± 0.59	3.74 ± 1.11
	<i>lgf1</i>	1.78 ± 0.16	4.86 ± 0.98			
MAPK signaling pathway	<i>Ptpn7</i>	1.68 ± 0.18	5.42 ± 0.88			
	<i>Fgf22</i>	1.53 ± 0.16	-			
Ras signaling pathway	<i>Rasal1</i>	1.75 ± 0.14	10.03 ± 1.33	27a-3p	0.75 ± 0.050	0.58 ± 0.044
				329-5p	1.31 ± 0.15	2.96 ± 1.32
Small cell lung cancer	<i>Lamc1</i>	0.35 ± 0.027	0.42 ± 0.051	138-5p	1.23 ± 0.17	3.76 ± 1.85
				211-5p	1.16 ± 0.19	8.83 ± 1.93
PI3K-Akt signaling pathway	<i>Rxra</i>	0.58 ± 0.057	0.64 ± 0.041	486b-3p	0.47 ± 0.18	0.57 ± 0.046
Rap1 signaling pathway/MAPK signaling pathway/Ras signaling pathway/PI3K-Akt signaling pathway	<i>Met</i>	0.56 ± 0.037	0.34 ± 0.029	449a-5p	2.92 ± 0.64	5.10 ± 1.87
<i>B-Actin</i> and GAPDH were the endogenous control for gene, U6 was the endogenous control for microRNA.						

KEGG pathway	mRNA	Q-RT-PCR	Transcriptomics	microRNA	RT-qPCR	Transcriptomics
				143-5p	0.87 ± 0.19	0.55 ± 0.029
Rap1 signaling pathway/Ras signaling pathway	<i>Ralgds</i>	1.35 ± 0.14	1.65 ± 0.24	6538	0.89 ± 0.24	
				449c-5p	1.50 ± 0.37	7.82 ± 1.88
				3074-2-3p	0.86 ± 0.17	0.48 ± 0.033
Cell adhesion molecules (CAMs)/Antigen processing and presentation/Primary immunodeficiency	<i>Cd8b1</i>	1.40 ± 0.38	3.55 ± 0.38			
<i>B-Actin</i> and GAPDH were the endogenous control for gene, U6 was the endogenous control for microRNA.						

The RT-qPCR analysis was performed to validate the results from transcriptomics. As showed in Table 1, most of the genes and microRNAs we selected showed similar results to those of the transcriptomics. Gene *Cd72*, *Cd19*, *Pik3cd* involved into B cell receptor signaling pathway were significantly up-regulated from exposure group, while gene *Cd81* was markedly down-regulated (Figure. 4c). The mRNA transcript levels of gene *Cd22* and *H2-M2* related to CAMs strikingly increased, and the levels of *H2-T24* decreased markedly (Figure. 4c). In addition, the associated microRNA involved into B cell receptor signaling pathway including 203-5p, 7a-5p, and 92a-1-5p were significantly up-regulated in PM_{2.5}-exposed group. The obvious down-regulations of microRNA 149-5p, 328-3p, 466i-5p, and 24-3p were observed in exposure group (Figure. 4d). Among the CAMs, we found that the microRNA 674-3p, 486a-3p, and 1247-5p increased remarkably, while 486a-3p was significantly down-regulated in exposure group (Figure. 4d).

The protein expression of CD19, CD81, and PIK3CD involved into the B cell receptor signaling pathway was examined to confirm the changes of the associated genes by immunohistochemistry. As showed in Figure. 5, PM_{2.5} exposure significantly increased CD19 (Figure. 5b), CD81 (Figure. 5e), and PIK3CD (Figure. 5h) expression levels compared with the control (Figure. 5a, d, g). Semiquantitative analysis showed that the fold changes of the expression of CD19, CD81, and PIK3CD from PM_{2.5}-exposure group were 6.948-, 2.646-, and 2.724- compared with the expression in the control, respectively (Figure. 5c, f, i, $p < 0.05$).

Discussion

Some evidences have suggested that the adverse effects on lung tissue posed by PM_{2.5} maybe associated with the immune and inflammatory response[11, 15, 32]. Hence, B cell receptor signaling pathway, as the important pathway responsible for immune response[33], enriched by integrated analysis was foreseeable, which was also illuminated by previous study using RNA-sequencing[25]. The transcriptional levels of the target genes involved in this pathway were also significantly up- or down-regulated by PM_{2.5}. Among them, *Cd72*, type II

transmembrane protein coding gene belonging to C-type lectin family[34], was elucidated play an important role in controlling the magnitude of B cell responses[35], and then responsible for the immune system homeostasis regulation[36]. The overexpression of *Cd72* found in this work from PM_{2.5}-exposed group may promote B cell survival and proliferation, and enhance release of CD23, and then activate the immune response[37–39]. In addition, anti-CD72 monoclonal antibodies (mAbs) can activate CD19 tyrosine phosphorylation[40]. The similar result was observed in this work (the up-regulation of *Cd19* induced by PM_{2.5}). It was reported that mutation of gene *pik3cd* was associated with the prevalence of systemic lupus erythematosus, which is a typical immune system disease[41]. Hence, the marked increase of the mRNA level of *pik3cd* in lung tissue from exposure group might related to the immune response induced by PM_{2.5}. Meanwhile, the associate microRNA of *Cd72* 149-5p and 328-3p were significantly down-regulated by PM_{2.5}. It has reported that the two microRNA may be responsible for the lung inflammatory and fibrotic pathology in mice[42]. The microRNA (203-5p and 7a-5p) related to *Pik3cd* were markedly up-regulated in mice lung from exposure group. Based on the KEGG and GO annotations, the microRNA 203-5p was involved into the B cell receptor signaling pathway (Figure. S7a). Therefore, the increase of the expression levels of gene *Cd72*, *Cd19*, and *Pik3cd* involved into B cell receptor signaling pathway may contribute to the lung injury including inflammation and fibrosis induced by PM_{2.5}.

Surprisingly, we firstly found the most significantly enriched KEGG pathway of DEGs through integrated analysis was cell adhesion molecules (CAMs), which was identified to mediate the process of cell recruitment and homing, and play an important role in the inflammatory process[43]. Once the inflammatory process of pulmonary immune system was triggered by PM_{2.5}, the up-regulation of adhesion molecules genes occurred in endothelial and immune cells to mediate leukocyte adhesion and then migrating to inflammation sites[44]. From the results of RT-qPCR quantification and transcriptomics analysis, the significant upswing of the expression levels of genes *Cd22* and *H2-M2* were observed from PM_{2.5}-treated group. The gene *Cd22*, a immunoglobulin superfamily cell-surface molecule that serves as an adhesion receptor for sialic acid-bearing ligands[45], was elucidated to active B cells and regulate antigen receptor signaling in vitro[46]. Similarly, the associated microRNA 3110-5p for *Cd22* were significantly stimulated from exposure group. Thus, our finding indicated that CAMs may be a key pathway responsible for the adverse effects posed by PM_{2.5} to respiratory system. According to the KEGG and GO annotations (Figure. S7b), the genes *Cd22* and *H2-M2* can be used as feasible biomarkers.

Furthermore, the target genes involved in the insignificant ($P > 0.05$) enriched KEGG pathways (i.e., antigen processing and presentation, graft-versus-host disease, primary immunodeficiency, rap1 signaling pathway, MAPK signaling pathway, ras signaling pathway, small cell lung cancer, and PI3K-Akt signaling pathway) were also significantly up- or down-regulated by PM_{2.5}, suggesting these pathways related to immune response of lung tissue should also be concerned for the further investigation.

A number of investigations have demonstrated that long-term exposure to PAHs increased risk of developing lung cancer[47–49]. CCA was performed using concentrations of PAHs as environmental factor. As showed in Figure. S8, the top three PAHs canonically correlated to target genes and microRNA were BaP, DBP, DBA, and BaP, DBP, benzo[b]fluorene (BF), respectively, indicating the four PAHs not the one with top concentration maybe the main impact factors. Among them, BaP was traditionally used as the surrogate marker for the toxicity of

PAHs mixtures[50]. DBP has been evaluated 100 times carcinogenic potency than BaP[50]. The potency value of dibenzo[a, h]anthracene (DBA), which was estimated to be associated with human cancer[51], was reported to be up to 10 times than Bap[52]. To further explore the covariation between the PAHs and genetic indicators, Pearson's index was determined. Obvious covariable relationship was observed between the target genes including *Cd72*, *Cd19*, *Pik3cd*, *Cd22*, *H2-M24*, and associated microRNA, and the PAHs detected from PM_{2.5} (Figure. S9, $p < 0.05$). This result suggested the target genetic indicators we selected showed a significant covariation with environmental pollutants, and then they may be used as the biomarkers to indicate the healthy risk posed by these types of pollutants.

From the public health point of view, PM_{2.5} samples used in this work were collected from Baoji, the mid-scale city located at the developing area. The main components of PAHs detected from the PM_{2.5} were similar to the mega-city (e.g., Beijing, Nanjing, et.al), while the concentrations of them, especially for Bap, DBA, DBP were statistically higher than the developed area[53–55]. Noted the concentrations of PM_{2.5} in all regions in China were very high than the threshold proposed by WHO (35 µg/mL) or more permissive limits adopted by China (75 µg/mL) in spite of the general decrease trends observed recent years[56]. Meanwhile, energy structure was much different between the mid-scale city and the mega-city in China[57], which directly result in the variation of the occurrence levels of the organic pollutants (PAHs) derived from the fossil fuel combustion[58]. Combined with the obviously adverse effects of the PM_{2.5} collected from Baoji city on the respiratory system found in this work, the healthy risk posed by PM_{2.5} at the mid-scale city of China should be paid more attention.

Conclusion

This work for the first time estimated the lung injury posed by PM_{2.5} collected from Baoji, a representative mid-scale city of China. We also elucidated the underlying mechanisms through integratedly analyzing the profiles of mRNA-seq and microRNA-sEq. Obvious lung injuries including pulmonary dysfunction, inflammatory response, pulmonary fibrosis were observed in the lung tissues of mice from exposure group. As revealed by KEGG annotations, the main pathway induced by PM_{2.5} was immune system associated pathway, especially B cell receptor signaling pathway and cell adhesion molecules (CAMs). Moreover, the expression levels of the key genes and microRNAs involved into the pathway, as well as the associated protein expression were verified. The results from this work may provide deeper insight into the mechanisms for the pulmonary toxicity posed by PM_{2.5} in the developing area. The more adverse effects on lung tissue posed by PM_{2.5} from mid-scale city of China than it from the mega city suggested the potential health risk of PM_{2.5} in developing area should be more concerned.

Methods

PM_{2.5} particle collection and analysis

Ambient PM_{2.5} were collected on quartz filter (2 µm pore size) by a High-Volume Ultrafine Particle Sampler (Ju Kang Technology, China) at a 5 L/min flow rate from Baoji, shaanxi, China, maintained for 24 h. PM_{2.5} was extracted described previously[59]. Briefly, the filters were chopped into small fragments, and then shaken for 20 min after treated by ultrasonic in ice for 30 min in a 50-mL centrifuge tube. The process was repeated for

three times. The mixture was centrifuged at 8000 rpm for 10 min, and then the precipitate was transplanted into a new tube and dried by lyophilization. The powder was stored at -80 °C until experiment. The concentrations of seventeen EPA priority polycyclic aromatic hydrocarbons (PAHs) bonding on the PM_{2.5} were determined by gas chromatography coupled with a mass spectrometer (GC-MS, Agilent GC 6890, MS 5973, USA) following the previous publication[60]. The details were shown in supporting information. Instrumental and method detection limits were showed in Table S1.

Animals and whole-body inhalation

Male Balb/C mice at 8 weeks of age with body mass of 20 ± 2 g were purchased from Chengdu Dashuo Company (Chengdu, Sichuan, China) and acclimated for a week before exposure in SPF level animal house of West China Hospital. Subsequently, twenty mice were divided randomly into two groups ($n = 10$ for each group) exposed to either filtered air (control) or concentrated PM_{2.5} air (exposure) in a “real-world” exposure system for 8 h/day (9:00 a.m. to 17:00 p.m.), 6 days/week, for 8 weeks (November 25st, 2019 to January 20th, 2020). The exposure system comprises two chambers: (1) ambient air was inhaled into the chamber filtered by a Teflon filter to eliminate all the fine particles as the control group; (2) the concentrated PM_{2.5} regenerated using a aerosol generator (HRH-7886, Beijing Huironghe Technology Co., Ltd., China) was inhaled into the chamber filtered by a swirler to remove the PM with an aerodynamic diameter greater than 2.5 μm as the exposure group. The exposure schematic was showed in Figure.S1.

During the exposure, the mice were fed commercial mouse chow and distilled water *ad libitum*, the humidity was kept at 40–60%, and the temperature was controlled at 22 ± 2 °C in the cages with 12 h/12 h light-dark cycle. Meanwhile, the concentration of the PM_{2.5} was on-line monitored with a concentration monitor (DUSTTRAKTM II-Model 8530, TSI Instrument, Shoreview, MN). The PM_{2.5} concentrations were lower than detection level in control chamber, and $298.52 \pm 25.86 \mu\text{g}/\text{m}^3$ (the same magnitude with the PM_{2.5} levels in Baoji air) in exposure chamber, respectively. Animal studies were performed in accordance with the principles of laboratory animal care (NIH publication no. 85 – 23, revised 1985) and with approval by the Animal Ethical and Welfare Committee of West China Hospital (No. 2019272A). The experiment timeline was showed in Figure.S2. According to the criteria provided previously, the daily ventilation volume of adult male Balb/C mice is 0.0864 m^3 [61]. Previous investigation has revealed that 75% of PM_{2.5} particles would enter and touch alveoli through inhalation[62]. Hence, the daily exposure dose of PM_{2.5} for one mouse was: $298.52 \mu\text{g}/\text{m}^3 \times 0.0864 \text{ m}^3 \times 0.75 = 19.34 \mu\text{g}$.

Lung imaging

After exposure, eight mice (each group 4 mice) were randomly selected and anesthetized with isoflurane for an in vivo lung micro-CT analysis. Images were obtained using Small-animal Micro-CT (Quantum GX, PerkinElmer). Parameters used for scanning were showed below: scanning field of view 50 mm, voxel size 50 μm , scanning time: 4 min. After reconstructed by NRecon software, the tomograms were analyzed by Micro-CT/Small-animal imaging data analysis system (Analyze 12.0, PerkinElmer).

Lung tissues preparation and histopathology assay

After exposure, the mice were anesthetized by pentobarbital sodium. Blood samples were collected from the cardiac vein. Serum was obtained by centrifuged at 3000 rpm for 15 min with no anticoagulant added to detect

the routine blood indexes. The lung tissue of each mice was immediately separated. After washed by pre-cooled phosphate buffered saline (PBS) for 3 times, the left lung tissues were divided into two parts. One part was used for flow cytometry analysis, and the other was fixed with 10% formaldehyde solution immediately and then embedded in paraffin. Paraffin-embedded tissues (60 °C) were sectioned consecutively with a thickness of 5 µm. The slices were then deparaffinized and stained with hematoxylin and eosin (H&E) or Masson trichrome. More than five replicates per lung tissue each mouse were observed with an optical microscope to determine the histopathology from exposure group. The right lung tissues were kept at -80°C for further analysis.

Flow cytometry analysis

The flow cytometry analysis was performed as previously described. Briefly, one part of the left lung tissues were minced on ice, and then digested by 0.1% Type I and Type II Collagenase in 4 ml of the PBS system, in 37 °C in the incubator, shaking incubation for 1 h. After digestion completely, cell suspension was filtered through 50 µm filter and centrifuged at 500 rpm for 5 min, 4 °C. Cell pellets were resuspended by 100 µL PBS and staining with 1 µL Ms CD45 PE 30-F11 (BD Pharmingen), Ms CD326 APC G8.8 (BD Pharmingen), Alexa Fluor 488 anti-mouse CD31 (Biolegend) and incubated at 4 °C for 30 min. Leucocytes were defined by CD45 + cells, and epithelial cells were defined by CD45-/CD31-/CD326 + cells. The measurements were performed by MoFlo Astrios EQ (BECKMAN COULTER). The percentage of different cells were analyzed by Flowjo_V10 Software.

RNA extraction, sequencing and analysis

Total RNA, including microRNA, was extracted from lung tissues by Trizol (Invitrogen, USA) according to manufacturer's protocol. RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA), integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA), and concentration was measured using Qubit® RNA Assay Kit in Qubit®2.0 Fluorometer (Life Technologies, CA, USA). Library construction and mRNA and microRNA sequencing were performed on an Illumina Novaseq platform. The detailed protocols were showed in supporting information.

The paired-end RNA reads were aligned to the reference genome using Hisat2 v2.0.5 software. The gene expression level was quantified by feature Counts v1.5.0-p3. The microRNA reads were quality controlled using miRBase20.0 as reference, modified by software miredeep2. The potential microRNA secondary structures were obtained using srna-tools-cli. MicroRNA expression levels were estimated by TPM (transcript per million). Differential expressions of RNA and microRNA between exposure and control group were analyzed using the DESeq2 R package (1.16.1) and DESeq R package (1.8.3), respectively. The resulting P-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. Corrected P value of 0.05 was set as the threshold for significantly differential expression by default.

The integrated mRNA and microRNA expression data were used to predict microRNA targets genes by Target Scan Mouse V 7.1 and miRDB V5 databases. According to the inhibition principle of microRNA on mRNA, the significantly down-regulated microRNA and significantly up-regulated mRNA, as well as the combination of significantly up-regulated microRNA and significantly down-regulated mRNA were selected as target gene pairs. Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) enrichment analysis of differently expressed genes was determined by clusterProfiler R package. P value less than 0.05 were considered as significant enrichment.

Quantitative real-time PCR analysis

To verify the data from the transcriptomics, 27 genes and 29 microRNA were chosen as candidates for RT-qPCR analysis. Primer sequences were listed in Table S2. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and *β-actin* genes were used as the endogenous control to monitor the quality of the target genes. MicroRNA U6 was used as the internal standard to monitor the quality of the target microRNA. The variations in the endogenous control expression were below 10% for all groups. Amplification was performed using SYBR Green PCR master mix (Applied Biosystems) according to the manufacturer's instructions. Gene and microRNA expressions were quantified using the $2^{-\Delta\Delta Ct}$ method suggested by Applied Biosystems (Foster City, CA, USA). The fold change between the exposure group and the control were calculated by the geometric mean of the relative expression normalized by the two housekeeping genes (*β-Actin* and GAPDH)[63].

Immunohistochemical analysis

Immunohistochemistry was performed according to standard procedures involving monoclonal antibodies against CD19 (ab245235, Abcam), CD81 (sc-166029, Santa Cruz Biotechnologies), and PIK3CD (sc-55589, Santa Cruz Biotechnologies).

Statistical analysis

The statistical program SPSS 18.0 (Chicago, IL, USA) was used to analyze all the collected data. Five or more replicates of each parameter were determined to eliminate the variability of the results. All the data were expressed as mean $\pm$ standard deviations (S.D). Two-tail student's T-test (95% confidence interval) was used to examine the significance of differences between control and exposure groups. Semiquantitative analysis on the results of Masson trichrome stain and immunohistochemistry was determined by Image-Pro Plus 6.0 software. The Pearson correlation test was performed for correlations between the biomarkers. Canonical correlation analysis (CCA) among the biomarkers were conducted using Canoco version 4.5. The statistic significant was defined as $p < 0.05$.

Abbreviations

PM_{2.5}

PM < 2.5 μ m in diameter

PIK3CD

phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta

PAHs

polycyclic aromatic hydrocarbons

BaP

benzo(a)pyrene

CAMs

cell adhesion molecules; IPF, idiopathic pulmonary fibrosis

COPD

chronic obstructive pulmonary disease

OPA

oropharyngeal aspiration

PBS

phosphate buffered saline

HE

hematoxylin and eosin

PCR

Polymerase Chain Reaction

RT-qPCR

Quantitative real-time Polymerase Chain Reaction

GAPDH

Glyceraldehyde 3-phosphate dehydrogenase

S. D

T. standard deviations

CCA

Canonical correlation analysis

BbkF

Benzo(b/k) fluoranthene

DBP

dibenzo(a,e)pyrene

CAMs

cell adhesion molecules

BP

biology process;

mAbs

monoclonal antibodies.

Declarations

Ethics approval and consent to participate

Animal studies were performed in accordance with the principles of laboratory animal care (NIH publication no. 85-23, revised 1985) and with approval by the Animal Ethical and Welfare Committee of West China Hospital (No. 2019272A).

Consent for publication

Not applicable.

Not applicable.Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Contributions

S.D performed the experiments and generated data. Z.W helped with the data collection and analysis. Y.Y and T.G helped data analysis. W.L are the guarantor of this work, had full access to all the data, and takes full responsibility for the integrity of data and the accuracy of data analysis. The author(s) read and approved the final manuscript.

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Figures

Figure 1

PM_{2.5} induced obvious pulmonary dysfunction in male Balb/C mice lung tissues. (a, b) Micro-CT images of mice lungs from the control; (c) 3D reconstruction of mice lung from the control; (d, e) Micro-CT images of mice lungs from exposure group; (f) 3D reconstruction of mice lung from exposure group.

Figure 2

PM2.5 induced pulmonary inflammation and fibrosis in male Balb/C mice lung tissues. (a-c) 100 $\times$ HE staining of mice lung tissue; (d-f) 200 $\times$ HE staining of mice lung tissue; (g-i) 400 $\times$ HE staining of mice lung tissue; (j, k) 100 $\times$ Masson trichrome staining of mice lung tissue; (l) semiquantitative analysis of fibrosis levels of mice lung tissue. Red rectangle: target zone of lung tissue; arrows: inflammatory infiltration and bronchial wall thickening of lung tissue. (100 $\times$, Bar: 100 μ m; 200 $\times$, Bar: 50 μ m; 400 $\times$, Bar: 25 μ m).

Figure 3

PM2.5 induced leucocyte proliferation in male Balb/C mice lung tissues. (a-c) relative abundance of leucocyte CD45+ in mice lung tissue; (d-f) relative abundance of epithelial cells CD45-/CD31-/CD326+ in mice lung tissue.

Figure 4

Some results of the transcriptomics analysis and the RT-qPCR. (a) different KEGG pathway annotated by DEGs between control and exposure groups and the selected KEGG pathway in mice lung tissue; (b) comparison of expression profile associated with the selected KEGG pathway in mice lung tissue and the selected genes and microRNA; (c, d) Relative expression (ΔC_t values) of genes and associated microRNA involved into B cell receptor signaling pathway and cell adhesion molecules (CAMs) by RT-qPCR assay.

Figure 5

The changes of protein expression of CD19, CD82, and PIK3CD induced by PM2.5 using immunohistochemistry analysis. (a-c) CD19; (d-f) CD81; (g-i) PIK3CD. (200×, Bar: 50 μm).

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