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Direct effects of airborne $PM_{2.5}$ exposure on macrophage polarizations☆

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ABSTRACT

Background: Exposure of atmospheric particulate matter with an aerodynamic diameter less than 2.5 μm ($PM_{2.5}$) is epidemiologically associated with illnesses. Potential effects of air pollutants on innate immunity have raised concerns. As the first defense line, macrophages are able to induce inflammatory response. However, whether $PM_{2.5}$ exposure affects macrophage polarizations remains unclear.
Methods: We used freshly isolated macrophages as a model system to demonstrate effects of $PM_{2.5}$ on macrophage polarizations. The expressions of cytokines and key molecular markers were detected by real-time PCR, and flow cytometry. The specific inhibitors and gene deletion technologies were used to address the molecular mechanisms.
Results: $PM_{2.5}$ increased the expression of pro-inflammatory cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-6 (IL-6), interleukin-1β (IL-1β), tumor necrosis factor alpha (TNFα). $PM_{2.5}$ also enhanced the lipopolysaccharide (LPS)-induced M1 polarization even though there was no evidence in the change of cell viability. However, $PM_{2.5}$ significantly decreased the number of mitochondria in a dose dependent manner. Pre-treatment with NAC, a scavenger of reactive oxygen species (ROS), prevented the increase of ROS and rescued the $PM_{2.5}$-impacted M1 but not M2 response. However, mTOR deletion partially rescued the effects of $PM_{2.5}$ to reduce M2 polarization.
Conclusions: $PM_{2.5}$ exposure significantly enhanced inflammatory M1 polarization through ROS pathway, whereas $PM_{2.5}$ exposure inhibited anti-inflammatory M2 polarization through mTOR-dependent pathway.
General significance: The present studies suggested that short-term exposure of $PM_{2.5}$ acts on the balance of inflammatory M1 and anti-inflammatory M2 macrophage polarizations, which may be involved in air pollution-induced immune disorders and diseases. This article is part of a Special Issue entitled Air Pollution, edited by Wenjun Ding, Andrew J. Ghio and Weidong Wu.

1. Introduction

Airborne fine particles with an aerodynamic diameter equal to or less than 2.5 μm ($PM_{2.5}$) pose a serious threat to human health as their prevalence in the urban air. A number of epidemiological studies have shown statistical significant associations between exposure to respirable particles and elevated mortality, lung dysfunction and respiratory symptoms [1–3]. Ambient $PM_{2.5}$ alters innate lung immunity in multiple aspects, including altered mucociliary function, respiratory epithelial cell dysfunction and impaired alveolar macrophage phagocytosis [4,5]. It is well known that alveolar macrophages are the first line of defense in the lung and are essential in stimulating epithelial cells to produce pro-inflammatory mediators and clearing atmospheric particulates from the lung surface [6]. However, the molecular mechanisms involved in the relationship between PM and adverse health effects are not well understood. Nevertheless, it is believed that an association between inflammatory process and oxidative stress exists [7]. Inhalation or instillation of PM in animals and human promotes inflammatory responses characterized by cytokine release, increased oxidative stress, recruited neutrophils, as well as increased the expression of genes related to NF-κB activation, including tumor necrosis factors α (TNFα) and interleukin-6 (IL-6) [8].

Macrophages, with significant impact on protecting immunity and defense against immune-mediated pathological damage, orchestrate the initiation and resolution phases of both innate and adaptive immunity. Generally, macrophages can be polarized into two distinct phenotypes: the classically activated macrophages (M1) and alternatively

Abbreviations: PM, ambient airborne particulate matter; DCFH-DA, 2′, 7′-dichlorofluorescein diacetate; NAC, nacetylcysteine; MFI, mean fluorescence intensity; Arg1, arginase 1; Fizz1, found in inflammatory zone 1; Ym1, chitinase 3-like; MTT, methyl thiazolyl tetrazolium; ROS, reactive oxygen species; LPS, lipopolysaccharide; M1, classically activated macrophages; M2, alternatively activated macrophages; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-6, interleukin-6; IL-1β, interleukin-1 beta; TNF-α, tumor necrosis factor-alpha.

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activated macrophages (M2) [9]. In response to lipopolysaccharide (LPS), macrophages are considered to be M1 macrophages which are characterized by high antigen-presenting capacity and production of pro-inflammatory cytokines such as TNFα and IL-6 [10]. Consequently, M1 macrophages promote polarized type I immune response to mediate host defense against the infections of bacteria, protozoa and viruses. M2 macrophages, on the contrary, display anti-inflammatory function, by promoting adaptive Th2 immunity and regulate angiogenesis, tissue remodeling and wound healing [9]. Therefore, the balance of M1 and M2 macrophages is critical for host homeostasis.

To evaluate whether $PM_{2.5}$ acts directly on macrophage polarizations, we exposed freshly isolated mouse peritoneal macrophages to different concentrations of $PM_{2.5}$ in the inducing systems for macrophage polarizations. The cell viability, mitochondria number and the productions of cytokines were detected. We further determined the molecular mechanisms of $PM_{2.5}$ in modulating macrophage M1/M2 polarizations. The present studies offered important information for the direct actions of $PM_{2.5}$ on macrophage polarizations.

2. Materials and methods

2.1. Animals

C57BL/6 (B6) mice were purchased from Beijing University Experimental Animal Center (Beijing, China). Myeloid cell-specific mTOR conditional knockout mice ($LysM^{Cre}mTOR^{loxp/loxp}$, mTORKO) were obtained by crossing $mTOR^{loxp/loxp}$ mice with mice expressing Cre recombinase under the control of the Lysozyme promoter ($LysM^{Cre}$). All mice were maintained in a specific pathogen-free facility. All experimental manipulations were undertaken in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals, of the Institute of Zoology, Chinese Academy of Sciences (Beijing, China).

2.2. $PM_{2.5}$ sampling and preparation

$PM_{2.5}$ samples were collected persistently at Yuquan Road, Beijing, China from September to December 2012. $PM_{2.5}$ samples were collected on Teflon filters (diameter = 47 mm; Whatman, Piscataway, NJ, USA) for biological assay using a low volume sampler (42 I min^{-1}, URG, Chapel Hill, NC, USA). $PM_{2.5}$ samples on Teflon filters were extracted according to the method of Imrich et al. [11]. Briefly, Teflon filters were probe-sonicated for 1 min in ultra-pure (18.2 MΩ/cm) water, and then dried filters in the drying oven, equilibrated for 48 h and weighed on a microbalance. The extract particles were further diluted to 5 mg/ml. The Teflon filters were equilibrated in a condition of 30% relative humidity and 25 °C room temperature for over 48 h and then weighted on a high-precision microbalance (AG258; Mettler Toledo, Columbus, OH, USA) to measure the mass of collected $PM_{2.5}$. The physical and chemical characteristics of $PM_{2.5}$ were described in our recently published paper [12].

2.3. Reagents

Anti-mCDF4/80-PE-Cy5, anti-mTNFα-PE, anti-mIL-6-PE were purchased from BD Biosciences PharMingen (San Diego, CA). Bacterial lipopolysaccharide (LPS; *E. coli* 055:B5) were purchased from Sigma-Aldrich (St Louis, MO, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2′, 7′-dichlorofluorescein diacetate (DCFH-DA) and nacetylcysteine (NAC) were obtained from Sigma-Aldrich. Mito-tracker green was obtained from Beyotime Biotechnology (Jiangsu, China). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Gibco (Grand island, NY, USA).

2.4. Flow cytometry

IL-6 and TNFα expression in the macrophages were detected using the BD Cytofix/Cytoperm Plus with GolgiPlug intracellular staining kits (BD Biosciences PharMingen, San Diego, CA, USA). The macrophages were stimulated with LPS (100 ng/ml) in 48-well plates for approximately 6 h [13]. During the last 4–6 h of culture, 1 ml aliquots of cells were pulsed with 1 ml BD GolgiPlug containing brefeldin A (BDBiosciences PharMingen). The macrophages were collected and washed once with FACS buffer. After incubation with the anti-FcR mAb (2.4G2) and FITC-conjugated anti-mouse F4/80 mAb in the dark at 4 °C for 30 min, the cells were washed once with staining buffer and then fixed and permeabilized with 500 ml BD Cytofix/Cytoperm solution at 4 °C in the dark for 20 min according to the manufacturer's instructions. Next, the cells were stained with either 0.25 mg PE-labeled anti-IL-6, TNFα mAb for 30 min at 4 °C in the dark and washed three times, and 10 000 $F4/80^+$ cells were then analyzed by FCM.

2.5. Cell preparation

Primary mouse peritoneal macrophages were obtained from the peritoneal exudates of 4–6-week old mice [14]. The peritoneal exudate cells were washed twice with PBS solution and then adjusted to 5×10^5 cells/ml in DMEM cultured for 3–4 h at 37 °C and 5% CO_2. The non-adherent cells were removed by washing with warm PBS. The purification of macrophage was analyzed by FCM (Beckman, CA), using a mouse macrophage marker $F4/80^+$. The adherent cells constituted more than 90% of $F4/80^+$ macrophages.

2.6. Bronchoalveolar lavage (BAL) preparation

BAL fluid was performed after $PM_{2.5}$ exposure according to the method of Haque et al. [15]. In brief, the lungs were lavaged in situ 3 times with 1.5 ml cold PBS. Recovered BAL fluid was immediately cooled to 4 °C and centrifuged (1700 rpm, 5 min). The BAL were washed twice with PBS solution and then adjusted to 5×10^5 cells/ml in DMEM cultured at 37 °C in a 5% CO_2 humidified atmosphere for 3–4 h. The cells were rinsed twice with warm PBS and to remove the non-adherent cells. The purification of macrophage was more than 90% as measured by a FCM.

2.7. Arginase assay

The arginase activity assay was performed as previously described [16,17]. Briefly, the cells were lysed in 0.1% Triton X-100. Tris–HCl was then added to the cell lysates at a final concentration of 12.5 mM, and $MnCl_2$ was added to obtain a 1 mM final concentration. The arginase was activated by heating for 10 min at 56 °C, and the L-arginine substrate was added at a final concentration of 250 mM. The reactions were incubated at 37 °C for 30 min and halted by the addition of H_2SO_4/H_3PO_4. After the addition of a-isonitrosopropiophenone and heating for 30 min at 95 °C, the urea production was measured as the absorbance at 540 nm, and the data were normalized to the total protein content.

2.8. MTT reduction assay

Cell viability was assessed by methyl thiazolyl tetrazolium (MTT). Briefly, after indicated treatment, cells were incubated with 100 μl of 0.5 mg/ml MTT solution for 1 h at 37 °C. Equal volume of dimethyl sulfoxide (DMSO) was added to dissolve the formazan converted from MTT. The absorbance was quantified at 490 nm using a microplate reader (MX30000, USA). The cell viability was finally expressed as a percentage of untreated cells.

2.9. Reactive oxygen species assay

The level of intracellular ROS generation was determined using the oxidative conversion of DCFH-DA to fluorescent compound

dichlorofluroescin (DCF). Briefly, peritoneal macrophages (5×10^5 cells) were treated with 50 μg/ml $PM_{2.5}$ for 6 h with or without LPS. The cells were collected with cold PBS. The fluorescence intensity was detected by flow cytometry and emission wavelengths of 488 nm.

2.10. Western blot assay

Macrophages were cultured in DMEM medium with 10% FBS in 12-well plates. Cells were treated with IL-4 (100 nM) or LPS (100 ng/ml) for the indicated time. After stimulation, cells were washed once in warm PBS, lysed in RIPA buffer (50 mM Tris–HCl, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, pH 7.4) with protease and phosphatase inhibitor cocktails (Sigma) for 10 min on a rocker at 4 °C. Protein concentration was determined using a BCA assay. Protein samples were analyzed on SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred ontopolyvinylidene fluoride (PVDF) membranes (Millipore, CA). Each PVDF membrane was blocked with TBST (100 mM Tris–HCl, 150 mM NaCl, 0.05% Tween20, pH 7.5) with 5% non-fat dried milk for 1 h, then incubated with primary antibodies overnight at 4 °C on a shaker [18]. The appropriate HRP-coupled secondary antibody was then added and was detected through chemiluminescence (Millipore). β-actin was used as a protein loading control.

2.11. Quantitative PCR analysis

Total RNA was isolated with TRIzol (Invitrogen), and cDNA was synthesized with M-MLV superscript reverse transcriptase according to the manufacturer's instructions. Real-time PCR kit (SYBR Premix Ex TaqTM, DRR041A) was purchased from Takara Bio Inc. All the PCR was performed on CFX96 (Bio-Rad) [19]. To determine the relative level of mRNA, the mRNA expression levels of each gene were normalized to the expression level of the endogenous control gene hypoxanthine phosphoribosyl transferase (HPRT). Each test was done at least in triplicate. Primers used for the amplification were listed in Table 1.

2.12. Statistical analysis

All data are presented as the mean ± SD. The differences between the mean values of two groups were determined by Student's t-test. Associations between the different variables were examined by one-way ANOVA, followed by post hoc comparisons using the Tukey's multiple paired comparison test. A value of p less than 0.05 was considered as statistically significant.

Table 1
Primers used in the present study.

Genes	Primer sequence (5′→3′)
Argainse1	Forward primer: CCAGAAGAATGGAAGAGTCAGTGT
	Reverse primer: GCAGATATGCAGGGAGTCACC
Ym1	Forward primer: CAAGTTGAAGGCTCAGTGGCTC
	Reverse primer: CAAATCATTGTGTAAAGCTCCTCTC
FIZZ	Forward primer: CTGCCCTGCTGGGATGACT
	Reverse primer: CATCATATCAAAGCTGGGTTCTCC
GM-CSF	Forward primer: CTTTTGTGCCTGCGTAATGAG
	Reverse primer: AGTCAGCGTTTTCAGAGGG
IL-1β	Forward primer: TGGGAAACAACAGTGGTCAGG
	Reverse primer: CCATCAGAGGCAAGGAGGAA
IL-6	Forward primer: AACCGCTATGAAGTTCCTCTC
	Reverse primer: AATTAAGCCTCCGACTTGTGAA
TNFα	Forward primer: GAGTGACAAGCCTGTAGCC
	Reverse primer: CTCCTGGTATGAGATAGCAAA
HPRT	Forward primer: AGTACAGCCCCAAAATGGTTAAG
	Reverse primer: CTTAGGCTTTGTATTTGGCTTTTC

3. Results

3.1. Effects of $PM_{2.5}$ exposure on inflammatory response in macrophages

To evaluate whether $PM_{2.5}$ has the ability to directly induce inflammation in macrophages, peritoneal macrophages were exposed to 0, 25, 50 and 100 μg/ml $PM_{2.5}$ respectively (Fig. 1A). After 6 h of exposure, morphological changes of macrophages were analyzed by a microscopy. Compared with the control group, macrophages exposed to $PM_{2.5}$ appeared to be swelling and remarkably particle inclusion and with vacuoles formation. With increasing concentrations of $PM_{2.5}$, the above alterations of macrophages were more clearly. At the presence of $PM_{2.5}$, the numbers of activated macrophages were significantly increased in a dose-dependent manner as observed by microscope. To determine the effect of $PM_{2.5}$ exposure on the cell viability of macrophages, we measured the viability by MTT assay. Exposure to $PM_{2.5}$ for 6 h did not reduce the cell viability of freshly isolated peritoneal macrophages, but instead slightly elevated the cell viability (Fig. 1B). When macrophages were exposed to $PM_{2.5}$ for 6 h, the mRNA levels of pro-inflammatory cytokines like GM-CSF, IL-6, IL-1β and TNFα were significantly increased in dose-dependent manner ($p < 0.05$, Fig. 1C). These data suggest that $PM_{2.5}$ exposure directly promoted inflammatory response of primary macrophages.

3.2. Effect of $PM_{2.5}$ exposure on M1 macrophage polarization

To determine the impact of $PM_{2.5}$ exposure on M1 polarized macrophages, freshly isolated peritoneal macrophages was pre-incubated with or without $PM_{2.5}$ (50 μg/ml) and then activated by LPS which induces M1-like macrophage polarization. Consistently, $PM_{2.5}$ exposure alone induced the mRNA expressions of GM-CSF, IL-6, IL-1β, and TNFα without LPS (Fig. 2A, Suppl. Fig. 1A). However, with different concentrations of LPS (0.1, 1, 10, 100, 1000 ng/ml), $PM_{2.5}$ dramatically increased GM-CSF, IL-6, IL-1β and TNFα mRNA expressions compared with LPS stimulation alone for 6 h ($p < 0.01$, Fig. 2A). Meanwhile, the exposure of $PM_{2.5}$ (0, 1, 10, 25, 50, 100 μg/ml) with LPS dramatically increased GM-CSF, IL-6, IL-1β and TNFα mRNA expressions in macrophages compared with PM2.5 stimulation alone for 6 h (Suppl. Fig. 1A). Moreover, combined treatment of $PM_{2.5}$ and LPS induced a time-dependent elevated levels of GM-CSF, IL-6, IL-1β and TNFα mRNA expressions than LPS or $PM_{2.5}$ treatment alone ($p < 0.01$, Fig. 2B). Consistent with the mRNA results, the data of flow cytometry and ELISA analysis further confirmed that $PM_{2.5}$ exposure markedly enhanced IL-6 and TNFα productions (Fig. 2C, D, Suppl. Fig. 1B). Similar to the response of freshly isolated peritoneal macrophages, $PM_{2.5}$ exposure also increased the inflammatory response to LPS of alveolar macrophages, as indicated by the increased GM-CSF, IL-6, IL-1β and TNFα mRNA expressions (Fig. 2E). Compared with the untreated control cells, $PM_{2.5}$ exposure did not decrease the cell viability in the presence or absence of LPS (Suppl. Fig. 1C). Thus, $PM_{2.5}$ exposure significantly increases macrophage inflammatory response and enhances LPS-induced M1 macrophage polarization.

3.3. Effect of $PM_{2.5}$ exposure on mitochondria in macrophages

Mitochondria, which regulate cellular energy homeostasis and cell death, are essential organelles [20]. As a selective form of autophagy, mitophagy removes damaged or excessive mitochondria, is essential for keeping cellular energy functions and homeostasis [21]. Accumulating evidence suggests that mitophagy has a crucial role in the immune responses regulation [22]. To determine whether $PM_{2.5}$ exposure have influence on mitochondria, we labeled mitochondrial by Mito-tracker green which passively diffuse across the plasma membrane. As the exposure doses of $PM_{2.5}$ increasing, the percentages of $F4/80^+$ Mito-tracker$^+$ macrophages were similar as determined by flow cytometry assay (Fig. 3A). However, the mean fluorescence intensity (MFI) of

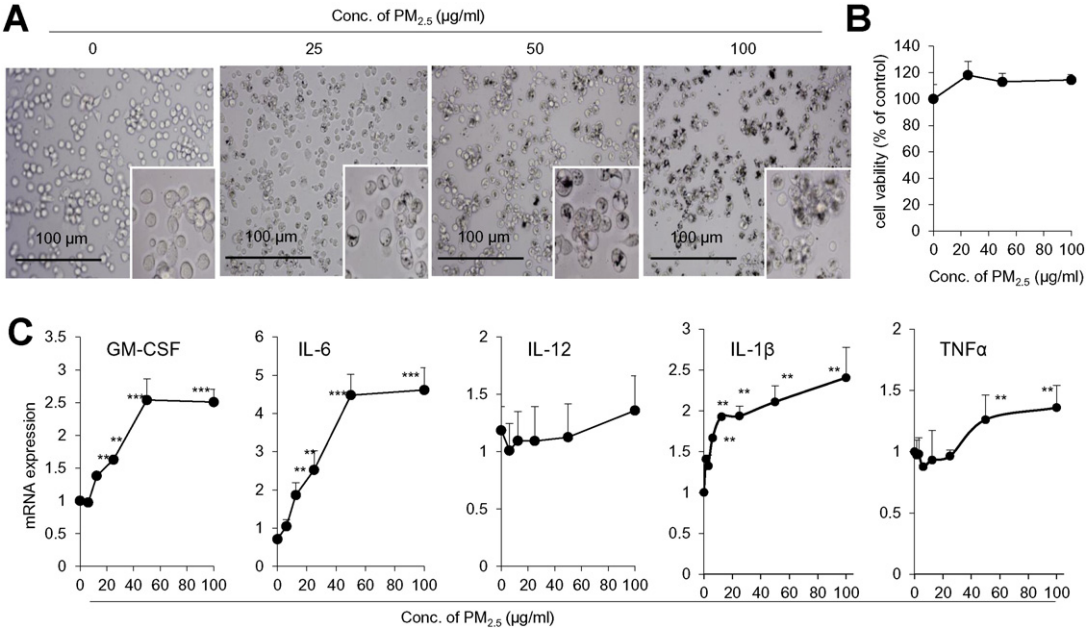

Fig. 1. Short-term exposure to $PM_{2.5}$ induces macrophage inflammatory response. (A) Morphological images of macrophages exposed to 0, 25, 50 or 100 μg/ml $PM_{2.5}$ for 6 h. (B) Cell viability of macrophages after 0, 25, 50 or 100 μg/ml $PM_{2.5}$ for 6 h. The cell viability was assayed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. (C) Macrophages were treated with 0, 1, 5, 10, 25, 50 or 100 μg/ml $PM_{2.5}$ for 6 h. GM-CSF, IL-6, IL-1β and TNFα mRNA expressions were determined by real-time PCR. Assays were performed more than three times. Data were shown as mean ± SD ($N = 4$). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{**}p < 0.001$ compared with the untreated control cells.

Mito-tracker^{+} macrophages with or without LPS stimulation was gradually decreased in a $PM_{2.5}$ dose dependent manner (Fig. 3B). The potential ability of $PM_{2.5}$ exposure to induce ROS generation was assessed by DCFH-DA intensity in freshly isolated peritoneal macrophages. The freshly isolated macrophages were exposed to 50 μg/ml $PM_{2.5}$ for 6 h as described above and the level of ROS was measured by flow cytometry assay. $PM_{2.5}$ significantly induced an increase in ROS generation (Fig. 3C, D), which is in agreement with the previous studies [21]. Therefore, $PM_{2.5}$ exposure induces mitochondria damage and subsequently results in the production of excessive ROS.

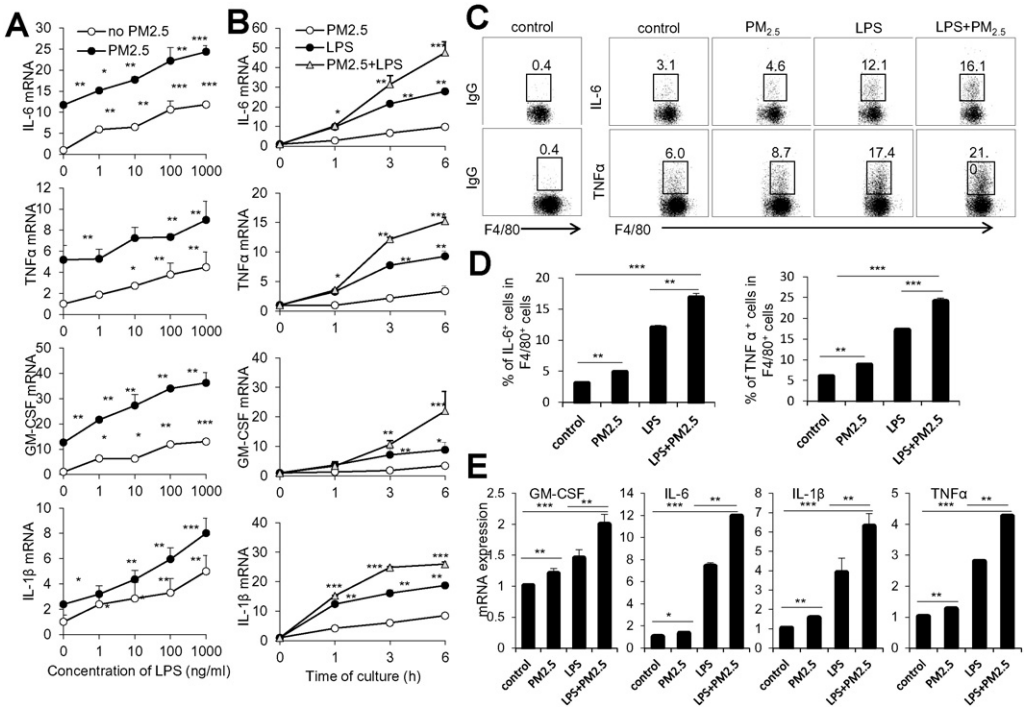

Fig. 2. $PM_{2.5}$ increases LPS-induced M1 polarization. (A) The freshly isolated macrophages were pretreated with or without 50 μg/ml $PM_{2.5}$ for 1 h and stimulated with 0.1, 1, 10, 100, and 1000 ng/ml LPS for 6 h. The mRNA expression of GM-CSF, IL-6, IL-1β and TNFα were determined by real-time PCR. (B) Gene expression of GM-CSF, IL-6, IL-1β and TNFα in macrophages after 50 μg/ml $PM_{2.5}$ treatment with or without LPS (1 μg/ml) for 1, 3 or 6 h were measured by quantitative real-time PCR. (C, D) Macrophages were exposed on $PM_{2.5}$ for 1 h and then stimulated with LPS for 6 h. TNFα and IL-6 expression were determined by intracellular staining flow cytometry. (E) Alveolar macrophages (AM) were isolated from BAL and exposed to 50 μg/ml $PM_{2.5}$ with or without LPS for 6 h. The GM-CSF, IL-6, IL-1β and TNFα were determined by real-time PCR. Data are shown as mean ± SD ($N = 4$). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the untreated control group.

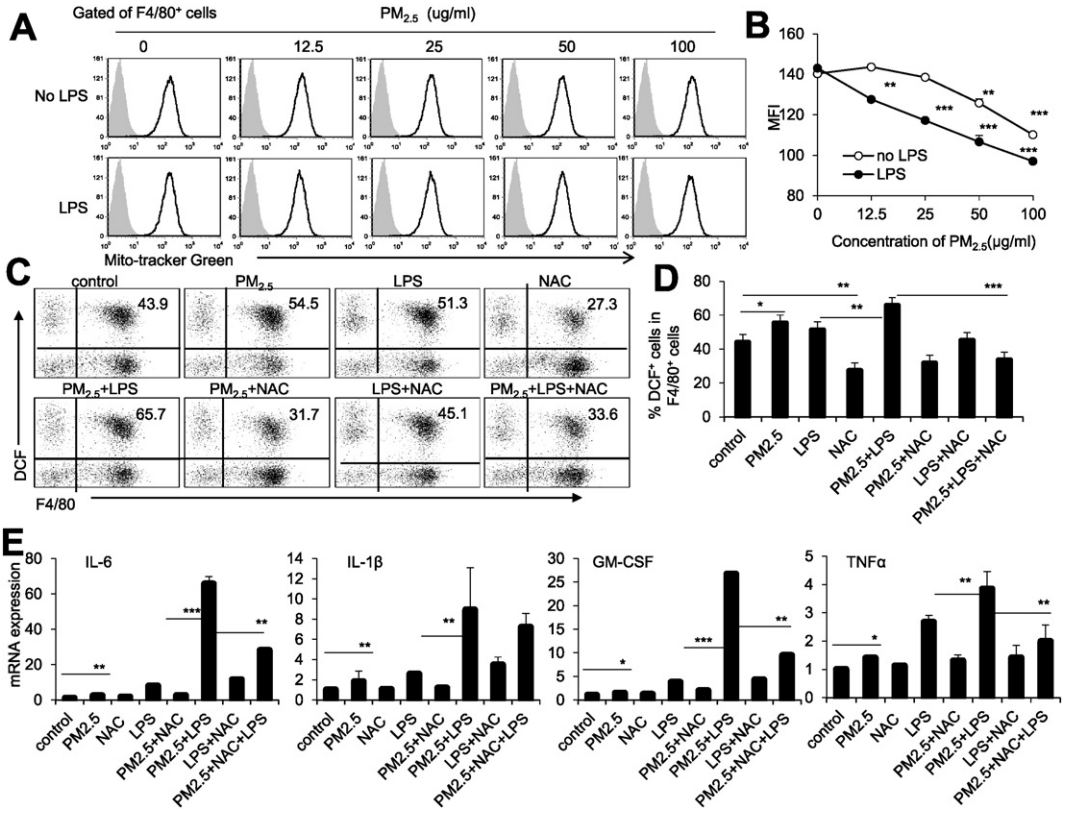

Fig. 3. Effect of $PM_{2.5}$ on mitochondria and ROS generation in macrophages. Peritoneal macrophages were freshly isolated and treated with 0, 25, 50 or 100 μg/ml $PM_{2.5}$ with and without LPS for 6 h, respectively. (A) The levels of Mito-tracker in $F4/80^+$ macrophages were determined by flow cytometry assay. (B) The median fluorescence intensity (MFI) of Mito-tracker in each sample was summarized. Data were shown as mean ± SD ($N = 4$). $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the untreated or the indicated groups. Experiments showing identical results were performed at least twice. Macrophages were exposed to 50 μg/ml $PM_{2.5}$ with or without LPS for 6 h, respectively. (C, D) The ROS levels were determined by measuring the oxidative conversion of DCFH-DA to DCF by flow cytometry assay. To investigate if the $PM_{2.5}$-induced inflammatory response was related to the decrease in the antioxidant defense, peritoneal macrophages were pretreated with 5 mM NAC for 1 h and then treated with 50 μg/ml $PM_{2.5}$ with or without LPS for 6 h. (E) The expression of GM-CSF, IL-6, IL-1β and TNFα were determined by real-time PCR. Assays were performed more than two times. Data were shown as mean ± SD ($N = 4$). $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the indicated group.

3.4. Effects of $PM_{2.5}$-induced ROS on inflammatory response in macrophages

In order to determine whether $PM_{2.5}$ exposure to induce ROS generation is involved in the enhanced inflammatory response in macrophages, we used a ROS scavenger NAC to address this issue. As shown in Fig. 3C–D, pretreatment with NAC (5 mM) significantly inhibited the ROS generation ($p < 0.01$, Fig. 3C, D). Meanwhile, NAC treatment obviously decreased the LPS + $PM_{2.5}$-induced GM-CSF, IL-6 and TNFα expressions compared with macrophages as determined by real-time PCR ($p < 0.01$, Fig. 3E). These data indicate that the release of inflammatory cytokines enhanced by $PM_{2.5}$ was ROS dependent.

3.5. Effect of $PM_{2.5}$ on IL-4-induced M2 macrophage polarization

IL-4-induced M2 macrophages display anti-inflammatory functions, promote adaptive Th2 immunity and regulate angiogenesis, tissue remodeling and wound healing in immune response [17]. The balance of M1 and M2 macrophages is critical for host homeostasis. To demonstrate the roles of $PM_{2.5}$ exposure in M2 macrophage polarization, we measured the molecular hallmakers of IL-4-induced M2 macrophages after macrophages were exposed to $PM_{2.5}$. $PM_{2.5}$ exposure significantly decreased arginase 1 (Arg1), found in inflammatory zone 1 (Fizz1), and chitinase 3-like (Ym1) mRNA expressions in IL-4-induced peritoneal macrophages, as determined by quantitative PCR (Fig. 4A). The decreased activity of arginase after $PM_{2.5}$ exposure also indicated that $PM_{2.5}$ exposure caused the deficiency of M2 polarization (Fig. 4B). Furthermore, IL-4-induced M2 macrophages express significantly less CD206 on cell surface after $PM_{2.5}$ exposure as detected by a flow cytometry (Fig. 4C, D). Consistent with the protein level, the presence of $PM_{2.5}$ significantly decreased IL-4-induced CD206 mRNA expression in M2 macrophages (Fig. 4E). These data collectively indicated that $PM_{2.5}$ exposure significantly impaired M2 polarization.

3.6. $PM_{2.5}$ impairs M2 macrophage polarization partially through mTOR-dependent pathway

To assess whether JNK, ERK1/2, p38 MAPK, STAT6, PI3K AKT, and ROS signaling pathways were involved in the $PM_{2.5}$-induced reduction of M2 polarization, we pre-treated macrophages with the inhibitors of PI3K (Ly294002), ERK1/2 (PD98059), JNK (SP600125), and ROS (NAC) with the previously determined doses [18,23] for 1 h respectively before exposure to $PM_{2.5}$. The expressions of Arg, Ym1, Fizz and CD206 in IL-4-induced M2 macrophages were determined by real-time PCR (Fig. 5). Pretreatment with these inhibitors respectively could not remarkably reverse the decreased M2 polarization caused by $PM_{2.5}$ exposure. These data suggested that these signal pathways were not detectably involved in $PM_{2.5}$ exposure-inhibited M2 polarization.

It is well known that STAT6 plays a central role in IL-4-induced M2 macrophage polarization [17,24]. In the present study, $PM_{2.5}$ exposure increased p-STAT6 activity during M2 macrophage induction by IL-4 (Fig. 6A, B), indicated that STAT6 is not required for $PM_{2.5}$-induced reduction of M2 polarization. Activation of ERK was predominately observed in IL-4-induced M2 macrophage polarization. However, inhibitor of ERK1/2 (PD98059) could not rescue the impaired M2 polarization (Figs. 6A, 5B). Previous studies demonstrate mTORC1-mediated attenuation of M2 polarization [17,25] and ribosomal protein S6

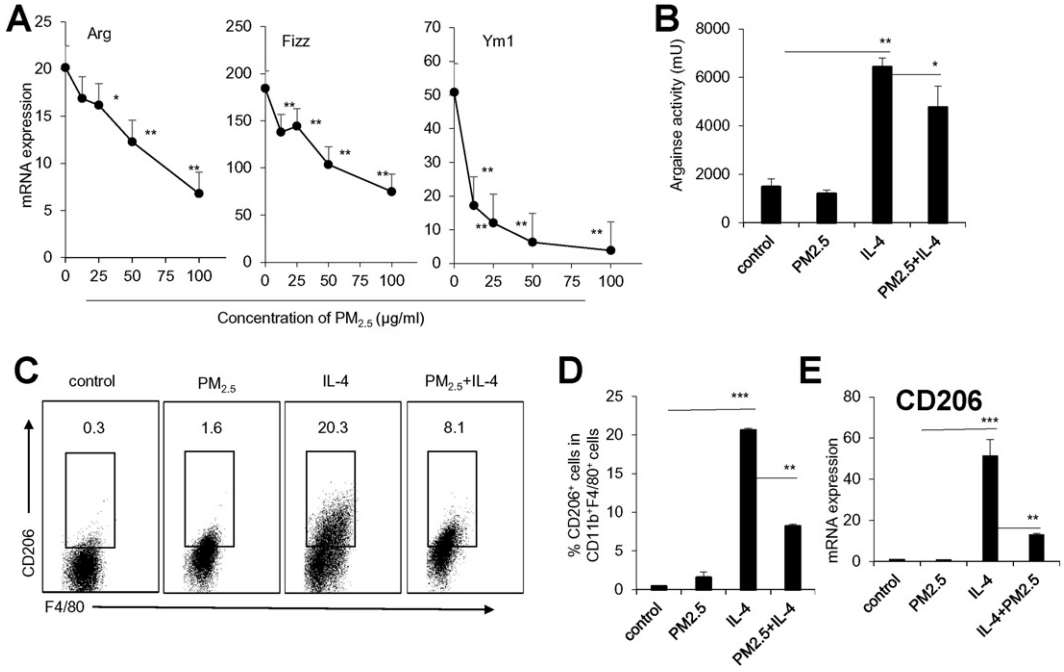

Fig. 4. $PM_{2.5}$ decreases IL-4-induced M2 macrophages. (A) The Arg, Fizz, Ym1 mRNA expressions in peritoneal macrophages were determined by real-time PCR after pretreated with $PM_{2.5}$ for 1 h and stimulated with IL-4 for 48 h. (B) Peritoneal macrophages were pretreated with $PM_{2.5}$ for 1 h and stimulated with IL-4 for 48 h. The activity of arginase was determined by fluorescence. (C) Florescence-activated cell sorting (FACS) analysis of CD206 level with the exposure of $PM_{2.5}$ in IL-4-induced M2 macrophages. (D) The peritoneal macrophages were treated with $PM_{2.5}$ for 1 h and stimulated with IL-4 for 48 h. Flow cytometry analysis of $CD206^+$ cells in $CD11b^+F4/80^+$ cells. (E) The mRNA expression of CD206 was determined by real-time PCR. Experiments were done more than two times. Data were shown as mean ± SD ($N = 4$). $^{**}p < 0.01$ compared with the indicated group.

(rpS6) as an essential component of the ribosomeis inducible phosphorylated following mTOR activation [26–29]. To clarify the intracellular pathways involved in $PM_{2.5}$ exposure-impacted M2 polarization, we blocked mTOR activity by its inhibitor rapamycin and found that the treatment of rapamycin could partially rescue $PM_{2.5}$ exposure-impaired M2 differentiation by real-time PCR (Fig. 6C). To address the

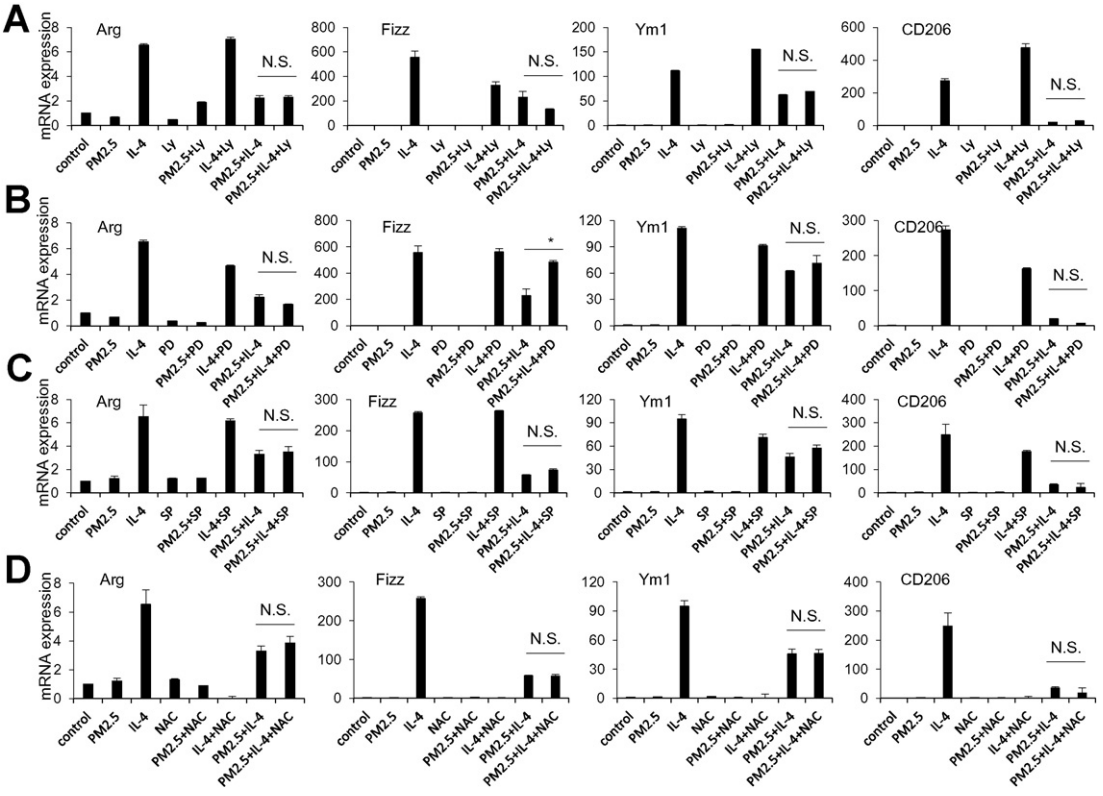

Fig. 5. JNK, PI3K, ERK and ROS signaling pathways are not involved in the effect of $PM_{2.5}$ on M2 polarization. The Arg, Fizz, Ym1 and CD206 mRNA expressions in peritoneal macrophages were determined by real-time PCR after pretreated with (A) Ly294002 (1 μM), (B) PD98059 (50 μM), (C) SP600125 (10 μM) and (D) NAC for 1 h respectively and stimulated with IL-4 and $PM_{2.5}$ for 48 h. Experiments were done more than two times. Data were shown as mean ± SD ($N = 4$).

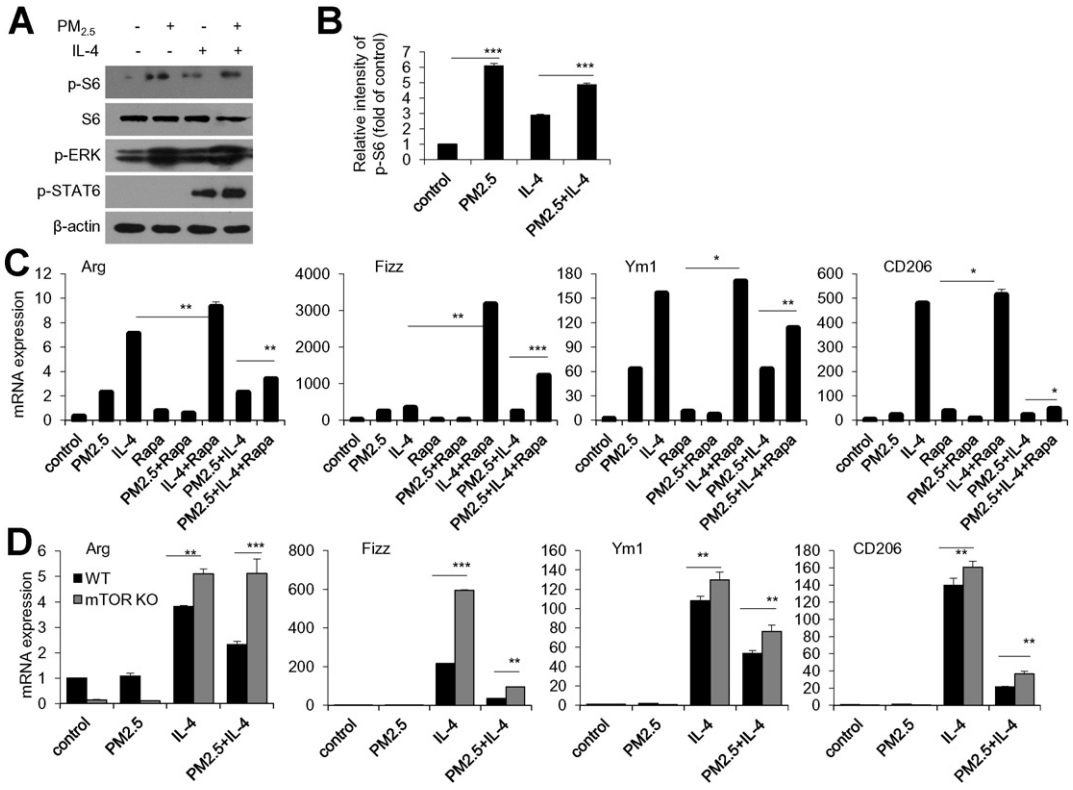

Fig. 6. The mTOR-dependent signaling pathway is involved in the effect of $PM_{2.5}$ on M2 polarization. (A) Peritoneal macrophages were exposed to 50 μg/ml $PM_{2.5}$ for 30 min and stimulated with IL-4 for 60 min. Cell lysates were blotted for p-S6, p-ERK1/2, p-STAT6, respectively. Experiments were done more than two times. (B) The ratio of p-S6/S6 in western blots. (C) The peritoneal macrophages were treated with rapamycin (100 nM) for 1 h and then treated with $PM_{2.5}$ and IL-4 stimulation for 48 h, respectively. The mRNA expression levels of Arg, Fizz, Ym1 and CD206 were determined by real-time PCR. (D) The WT and mTOR-deficient macrophages were treated with $PM_{2.5}$ and IL-4 stimulation for 48 h. The mRNA expression levels of Arg, Fizz, Ym1 and CD206 were determined by real-time PCR. Data were shown as mean ± SD ($N = 4$). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the indicated group.

effect of mTOR, we employed mice with a myeloid-specific deletion of mTOR (Suppl. Fig. 2). We observed that genetic deletion of mTOR macrophages could partially but significantly rescue $PM_{2.5}$ exposure-impacted M2 polarization (Fig. 6D, Suppl. Fig. 3). Thus, $PM_{2.5}$-induced mTOR activation is partially involved in the impaired M2 macrophage polarization.

4. Discussion

Exposure to excess airborne fine particles $PM_{2.5}$ has been epidemiologically associated with sudden deaths, respiratory, and cardiovascular diseases [30–32]. Previous studies have demonstrated that PM significantly affected pro-inflammatory cytokines secretion of M1 and anti-inflammatory response of M2 [33–35]. PM impaired the pro-inflammatory responses of monocyte-derived macrophages to pathogenic stimulation [36]. In this study, we found that $PM_{2.5}$ exposure induces pro-inflammatory response and further increased LPS-induced M1 polarization. Furthermore, $PM_{2.5}$ exposure did not affect the cell viability of macrophages under indicated concentration but significant decreased mitochondria amount in a single cell suggested that exposure to $PM_{2.5}$ for a short time had significant impacts on mitochondria activity. Consistently, $PM_{2.5}$ exposure induces ROS release in M1 polarized macrophages, but scavenging ROS with NAC significantly attenuated the expression of inflammatory cytokines in M1 macrophages. On the other hand, $PM_{2.5}$ exposure decreased the expression of Arg, Fizz, and Ym1 in IL-4-induced M2 macrophages. Moreover, pretreatment with mTOR inhibitor (rapamycin) but not ROS inhibitor (NAC) partially rescued the expression of Arg, Fizz, and Ym1 indicated that mTOR signaling pathway which was involved in $PM_{2.5}$-impaired M2 polarization. Collectively, we characterized that $PM_{2.5}$ exposure had different roles on M1/M2 macrophage polarizations with distinctive signal pathways.

Previous studies have demonstrated that chemical composition of $PM_{2.5}$ is involved in its toxicological effects, including both organic and inorganic fraction [37,38]. In our recent study, we have demonstrated that the high levels of Fe, Pb, Zn, Ti, Mn, acenaphthylene, acenaphthene, benzo fluoranthene, benzo pyrene and chrysene were major constituents of $PM_{2.5}$ which were harvested in Beijing [12]. Moreover, $PM_{2.5}$ particles also have higher compassion of water soluble inorganic ions such as SO_4^{2-}, NO_3^-. The constitutes of $PM_{2.5}$, including transition metals, water soluble inorganic ions, carbonaceous fractions and polycyclic aromatic hydrocarbons, caused oxidative stress as a common pathway for $PM_{2.5}$ exposure-induced oxidative damage although the mechanism underlying the correlation between $PM_{2.5}$ exposure and adverse effects has not been fully elucidated [38–40]. It has been hypothesized that $PM_{2.5}$-induced oxidative stress originates to produce ROS and stimulate ROS cellular generation directly. Oxidative damage pathway could be activated once excess ROS is generated and in turn produce cell or tissue damage [41]. Thus, the mechanisms which include cytotoxicity and inflammation have evolved to maintain cellular redox equilibrium and counter the potential health impacts of oxidative stress [42].

As a sensitive target, mitochondrion is easily affected by oxidative stress and environmental toxicants like $PM_{2.5}$ [43]. Elevated levels of pro-inflammatory mediators in M1-polarized macrophages play a central role in a polarized type I response, characterized by increased oxidative stress [44]. Apart from serving as an antimicrobial defense in macrophages, ROS are key factors in immune cell signaling [45]. Mitochondrial ROS are implicated in the pro-inflammatory responses of macrophages, meanwhile various treatments that induce mitochondrial ROS result in enhanced activation of inflammation transcription factors [46,47]. In the present study, we found that $PM_{2.5}$ exposure induced inflammatory response may be mediated partially by mitochondrial dysfunction and excess ROS generation.

Mitochondrion is a sensitive target of both oxidative stress and environmental toxicants like $PM_{2.5}$ [43,48]. $PM_{2.5}$ could induce mitochondrial damage in exposed individuals [49], and PM-induced effects on the respiratory tract may be mediated partially by mitochondrial dysfunction [50]. ROS is mainly produced by members of the NADPH oxidase family in the plasma membrane and mitochondria [51,52]. Consistent with previous results, our group's work showed that $PM_{2.5}$ exposure had influence on mitochondria and induced an increase in ROS generation. Nitric oxide (NO) is a multifunctional gaseous molecule and a highly reactive free radical. It is synthesized from L-arginine, NADPH and oxygen by NO synthase (NOS) [53]. Although we have detected the significantly increased expressions of pro-inflammatory cytokines like GM-CSF, IL-6, IL-1β and TNFα in macrophages after exposure to $PM_{2.5}$ in a dose-dependent manner, we failed to detect the detectable alteration of iNOS expression caused by $PM_{2.5}$.

The mechanistic target of rapamycin (mTOR) is a key nutrient/energy sensor and regulates a wide range of biological effects on adaptive responses. The activation of mTOR inhibits M2 macrophages polarization [25]. Our data also showed that M2 polarization was significantly enhanced after mTOR inhibitor treatment. Moreover, specific inhibition of mTOR by rapamycin or genetic deletion of mTOR gene also partially reversed the $PM_{2.5}$-attenuated M2 polarization, indicating that $PM_{2.5}$ exposure induced mTOR activation may be one of the signaling pathways which contribute to the alternatively activated M2 polarization.

In summary, our present studies demonstrated that $PM_{2.5}$ exposure has broken the balance of M1/M2 polarizations. $PM_{2.5}$ exposure causes mitochondria damage and then induces inflammatory response through a ROS dependent pathway, while $PM_{2.5}$ exposure decreases M2 polarization through mTOR signaling pathway. Understanding the signaling pathway implicated in $PM_{2.5}$-mediated inflammation provides new insights into the pathophysiology of diseases associated with airborne $PM_{2.5}$ pollution.

Competing financial interests

The authors have no competing financial interests

Transparency document

The Transparency document associated with this article can be found in online version.

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Appendix A. Supplementary data

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