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Myeloid-Specific SIRT6 Deletion Protects Against Particulate Matter ($PM_{2.5}$)-Induced Airway Inflammation

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Purpose: Particulate matter ($PM_{2.5}$) is a common risk factor for airway inflammation. Alveolar macrophages play a critical role in airway inflammation. Sirtuin 6 (SIRT6) is a class Ill histone deacetylase that exerts an anti-inflammatory effect in airway diseases. However, the role of SIRT6 on PM2.5-induced airway inflammation in macrophages remains unclear. We aimed to determine whether SIRT6 protects against $PM_{2.5}$-induced airway inflammation in macrophages.

Methods: The effect of SIRT6 on $PM_{2.5}$-induced airway inflammation was assessed by using THP1 cells or bone marrow-derived macrophages (BMDMs) exposed to $PM_{2.5}$ in vitro and myeloid cell-specific SIRT6 conditional knockout mice (*Sirt6$^{fl/fl}$-LysMCre*) in vivo.

Results: PM2.5 increased SIRT6 expression in THP1 cells, but SIRT6 gene silencing decreased PM2.5 induced inflammatory cytokines in THP1 cells. Moreover, the expression of SIRT6 and inflammatory cytokines was also decreased in BMDMs with myeloid-specific deletion of SIRT6 after stimulation of $PM_{2.5}$. In vivo, *Sirt6$^{fl/fl}$-LysMCre* mice substantially decreased airway inflammation in response to $PM_{2.5}$ exposure.

Conclusion: Our results revealed that SIRT6 promotes the $PM_{2.5}$-induced airway inflammation in macrophages and indicated that inhibition of SIRT6 in macrophages may represent therapeutic strategy for airway disorders induced by airborne particulate pollution.

Keywords: particulate matter, SIRT6, macrophage, lung inflammation

Introduction

Airborne particulate matter ($PM_{2.5}$) pollution exposure is a major risk factor for global public health and known to cause many adverse health effects.[1] Epidemiological studies have documented that there is a close correlation between $PM_{2.5}$ exposure and increased incidence of respiratory diseases, such as asthma and chronic obstructive pulmonary disease.[2] $PM_{2.5}$ with aerodynamic diameter ≤ 2.5μm ($PM_{2.5}$) is a leading contributor to pulmonary inflammation because its major target organ is lung and can penetrate deep into the alveolar regions. $PM_{2.5}$-induced pulmonary inflammation might be seen as a critical process in mediating systemic adverse effects.[3] Therefore, it is urgently needed to explore the molecular mechanisms responsible for $PM_{2.5}$-induced airway inflammation.

As a fundamental component of the respiratory immune system, alveolar macrophages (AMs), which reside at the boundary between the human body and outside world, are the most abundant macrophage in the lung. They clear dead cells and foreign antigen or airborne particles through the release of anti-inflammatory cytokines and the high phagocytic activity. Thus, AMs play a critical role in host defense, tissue homeostasis and control of airway

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inflammation.[4–6] However, the underlying mechanisms of AMs in regulation of $PM_{2.5}$-induced airway inflammatory remain unknown.

Acetylation is a widely occurring epigenetic modification of proteins that are involved in diverse biological processes.[7,8] In recent years, the physiological functions of the sirtuin deacetylase family (SIRT1-SIRT7) have been studied. Among the seven sirtuins, SIRT6 plays a leading role in regulating aging, inflammation, cancer and metabolic homeostasis.[9] Accumulating evidences have revealed that SIRT6 widely participates in respiratory diseases, such as allergic airway inflammation,[10] acute respiratory distress syndrome (ARDS),[11] chronic obstruction pulmonary diseases,[9] and lung fibrosis.[12] Moreover, SIRT6 regulated macrophage polarization by activating the AMPK pathway and subsequent autophagy.[13,14] Although AMs are the first responders to ambient air pollution exposure in the airway, the possible role of SIRT6 in airway inflammation of macrophages following $PM_{2.5}$ exposure remains unclear.

In the present study, we sought to determine the underlying mechanisms of SIRT6 in the regulation of $PM_{2.5}$-induced airway inflammation in macrophages. Our findings demonstrated that myeloid-specific SIRT6 deletion protects against $PM_{2.5}$-induced airway inflammation and might suggest that inhibition of SIRT6 could prevent airway disorders induced by $PM_{2.5}$.

Materials and Methods

Animal Studies

The *LysMcre* mice were kindly donated by Dr G. Feng (University of California at San Diego, CA, USA). Using *Sirt6*$^{fl/fl}$ mice, obtained from Jax Lab., we developed Myeloid cell-specific *Sirt6* conditional knockout mice (*Sirt6*$^{fl/fl}$*-LysMCre*) by crossing *Sirt6*$^{fl/fl}$ mice with the *LysMCre* mice. The primer sequences for the *LysMCre* and *Sirt6* genes are shown in Table 1. A specific pathogen-free environment was maintained in the Laboratory Animal Center of Guangdong Medical University for all mice. A mouse model for short-term exposure to $PM_{2.5}$ was established according to a previous study.[8] Standard reference airborne $PM_{2.5}$ was purchased from National Institute of Standards and Technology (NIST) Company. Briefly, $PM_{2.5}$ was suspended and sonicated in saline at 100 μg PM (in 50 μL saline) per day by intratracheal instillation for 3 days. The same volume of saline was given to control mice. Experiments were conducted under protocols using experimental procedures and anesthesia methods according to the ethical review of laboratory animal welfare People's Republic of China National Standard GB/T 35892–2018. Experiments were also conducted under protocols using experimental procedures and anesthesia methods approved by the Animal Ethical Committee of Guangdong Medical University (No. GDY2003027).

Cell Cultures

Isolation and culture of BMDMs were carried out similarly to previous study.[7] Briefly, from mice aged 6–8 weeks, BM cells were harvested from femurs and tibiae with PBS. To promote differentiation of bone marrow-derived macrophages, BM cells were cultured in DMEM medium supplemented with 10% FBS (vol/vol) and 10 ng/mL recombinant murine M-CSF for 7 days.

The human monocyte-derived macrophages cell line, THP1 cells were purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China). THP-1 cells were cultured in RPMI-1640 medium supplemented with 10% FBS (vol/vol) and 20 nM PMA over 48 h as primary human macrophages. Water-saturated atmosphere containing 5% CO_2 was routinely maintained at 37°C for the cell cultures above. $PM_{2.5}$, which contains polycyclic aromatic hydrocarbons, was purchased from National Institute of Standards and Technology (NIST) Company. We suspended and sonicated $PM_{2.5}$ in PBS or saline at a final concentration of 2 μg/μL (mass/vol), harvested BMDMs and THP-1 cells were treated with four concentrations of $PM_{2.5}$ (25, 50, 75, 100 μg/mL) for 24 h or with $PM_{2.5}$ (100 μg/mL) for four times (3, 6, 12, 24 h).

Collection of Bronchoalveolar Lavage Fluid (BALF)

BALF was performed according to our previous study.[7] In brief, the lungs were instilled with 0.8 mL PBS after exsanguination. Cytospin slides were prepared by Wright-Giemsa staining, and cell counts in BALF were counted under the microscope in a blinded method.

Table 1 Sequence of Primers Were Used in This Study

Gene	Primer Sequence (5'-3')
SIRT6 (h)	F: CCCACGGAGTCTGGACCAT
	R: CTCTGCCAGTTTGTCCCTG
IL-6 (h)	F: CCTGAACCTTCCAAAGATGGC
	R: TTCACCAGGCAAGTCTCCTCA
TNF-α (h)	F: AGCTGGAGAAGGGTGACCGA
	R: CACAGGGCAATGATCCCAAAG
CXCL1 (h)	F: ACAGTGTGTGGTCAACATTTCTCAT
	R: GCCAGAAACACTGTAAAACTACCAT
CXCL2 (h)	F: CACAGTGTGTGGTCAACATTTCTC
	R: CTCTCTGCTCTAACACAGAGGGA
GAPDH (h)	F: TGTTGCCATCAATGACCCCTT
	R: CTCCACGACGTACTCAGCG
Sirt6(m)	F: ATGTCGGTGAATTATGCAGCA
	R: GCTGGAGGACTGCCACATTA
IL-6 (m)	F: TCTATACCACTTCACAAGTCGGA
	R: GAATTGCCATTGCACAACTCTTT
TNF-α(m)	F: CTGAACTTCGGGGTGATCGG
	R: GGCTTGTCACTCGAATTTTGAGA
Cxcl1(m)	F: CTGGGATTCACCTCAAGAACATC
	R: CAGGGTCAAGGCAAGCCTC
Cxcl2(m)	F: TGTCCCTCAACGGAAGAACC
	R: CTCAGACAGCGAGGCACATC
β-actin(m)	F: AGAGGGAAATCGTGCGTGAC
	R: CAATAGTGATGACCTGGCCGT
LysMCre-WT(m)	TTACAGTCGGCCAGGCTGAC
LysMCre-Mutant(m)	CCCAGAAATGCCAGATTACG
LysMCre-Common(m)	CTTGGGCTGCCAGAATTTCTC
Sirt6 gene deletion(m)	F: AGTGAGGGGCTAATGGGAAC
	R: AACCCACCTCTCTCCCCTAA

Abbreviations: h, Human; m, Mouse.

RT-PCR Analysis

Total RNA of lung tissue or cells was isolated using the Trizol reagent (Invitrogen). Reverse transcription was performed via PrimeScript RT reagent Kit with gDNA Eraser (TAKARA) according to the manufacturer's instructions. Quantitative PCR was carried out with SYBR Green PCR Master Mix (TAKARA). The primers are presented in Table 1.

Western Blot

Cells were lysed in RIPA buffer. Proteins in lysates of cells were separated by SDS-PAGE. Western Blot (WB) analyses were performed as described with antibody to SIRT6 (Santa Cruz), antibody to GAPDH, Tubulin and β-actin (Beyotime Biotechnology, China).

Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of mouse IL-6, TNF-α, CXCL1, and CXCL2 in lung homogenate were measured using ELISA kit (Elabscience).

siRNA Studies

siRNA for knockdown of *Sirt6* was synthesized by GenePharma, and the sequence (5'-3') was as follows: UCCAUCACGCUGGGUACAUTT. siRNA was transfected to cells using lipofectamine®3000 (Invitrogen), according to the manufacturer's protocols with the following minor modifications. After 4–6 h of siRNA transfection, the transfected cells were exposed to $PM_{2.5}$.

Statistical Analysis

Results are presented as mean with SEM. We used the GraphPad Prism software (version 8.0, San Diego, CA) for all calculations and graphing. Comparisons between two groups were made using the Student's *t*-test or Mann–Whitney *U*-test. One-way ANOVA was used for comparisons between more than two groups. Statistical significance was defined as a value of *P* less than 0.05.

Results

SIRT6 Expression is Increased in Macrophages Following $PM_{2.5}$ Exposure

To address the possible role of SIRT6 in $PM_{2.5}$-induced airway inflammation, we first assessed SIRT6 expression in the lung tissue of mice exposed to $PM_{2.5}$. A mouse model for short-term exposure to $PM_{2.5}$ was established as described in method. We found that the expression of SIRT6 was significantly increased in lung tissue of mice following $PM_{2.5}$ exposure compared with those in control groups (Figure 1A and Figure 1). To identify whether SIRT6 is macrophage specific, we first examined the expression of SIRT6 in THP1 derived macrophages exposed to $PM_{2.5}$. We found that

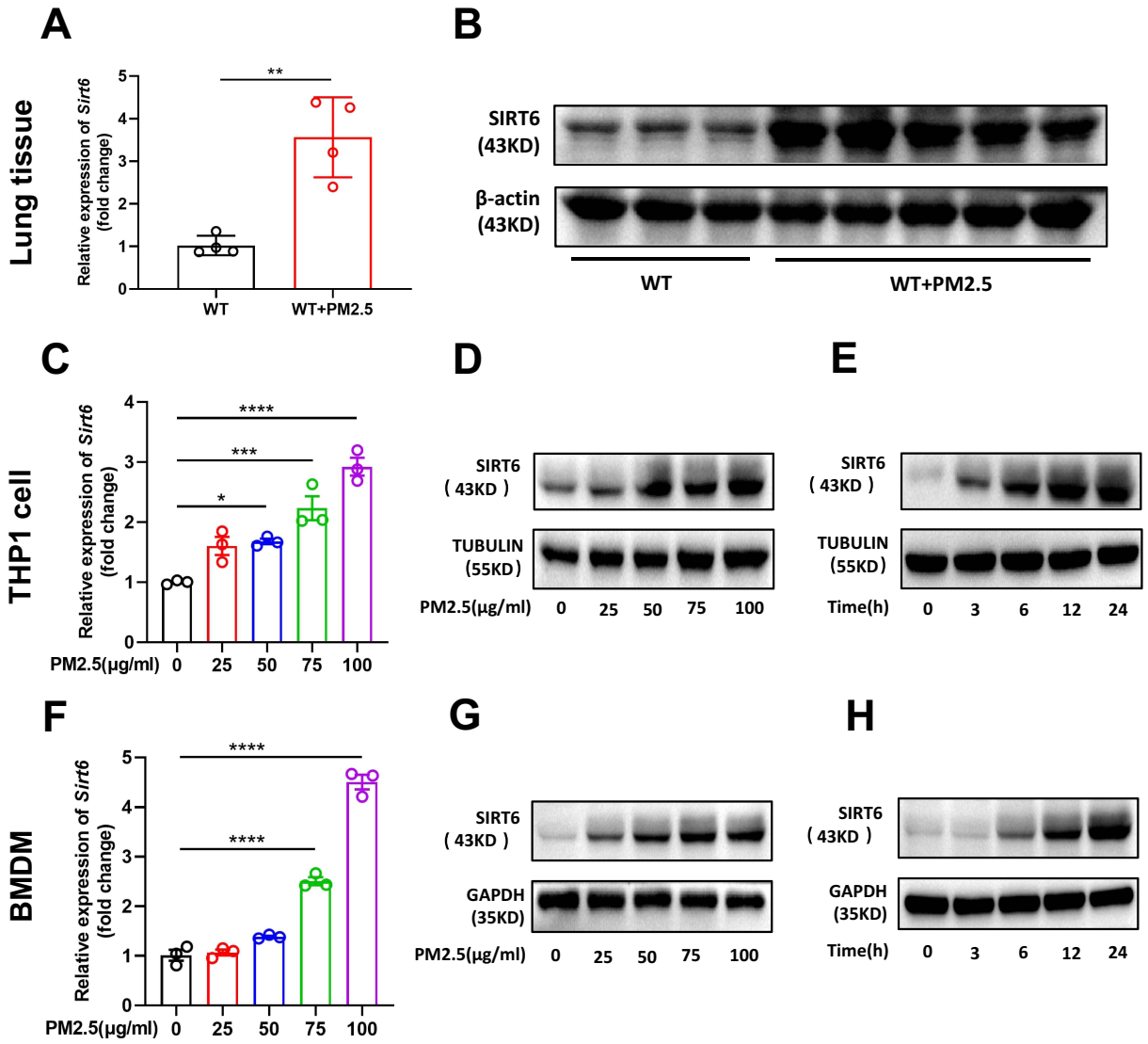

Figure 1 $PM_{2.5}$ exposure induced SIRT6 expression in macrophages. The expression of SIRT6 in lung tissues of mice was analyzed by RT-PCR (**A**) and Western blot (**B**). THP1 Cells were exposed to $PM_{2.5}$ at indicated times or concentrations, SIRT6 expression was analyzed by RT-PCR and Western blot (**C**–**E**). BMDMs were exposed to $PM_{2.5}$ at indicated times or concentrations, SIRT6 expression was analyzed by RT-PCR and Western blot (**F**–**H**). Data are presented as the mean ± SEM of three independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.001, and *****P*<0.0001.

Abbreviations: PM, Particulate matter; SIRT6, Sirtuin 6; BMDMs, bone marrow–derived macrophages.

treating THP1 cells with $PM_{2.5}$ resulted in a dose- or time-dependent increase of SIRT6 (Figure 1C–). Furthermore, we generated bone marrow–derived macrophages (BMDMs) from *Sirt6*$^{fl/fl}$ mice. Consistent with the above findings, the expression of SIRT6 was significantly increased in $PM_{2.5}$-induced BMDMs in a dose- and time-dependent manner (Figure 1F–). Collectively, these findings suggested that macrophages are involved in SIRT6-mediated airway inflammation induced by $PM_{2.5}$.

Inhibition of SIRT6 Attenuated $PM_{2.5}$-Induced Inflammatory Cytokines in vitro

Previous studies showed that $PM_{2.5}$ induces inflammatory cytokine secretion. In the present study, we also found that mRNA levels of tumor necrosis-alpha (TNF-α), interleukin 6 (IL6), C-X-C ligand 1 (CXCL1), and CXCL2 were significantly increased in $PM_{2.5}$-induced THP1 cells and BMDMs (Figure 2). We further investigated the role of SIRT6 gene silencing in THP1 cells on PM2.5-induced inflammatory cytokines (Figure 3A and Figure 3). SIRT6 gene silencing further increased $PM_{2.5}$ induced inflammatory cytokines compared with the control cells that received control siRNA (Figure 3C–).

To further confirm this effect, we generated mice with myeloid conditional deletion of *Sirt6* (*Sirt6*$^{fl/fl}$*-LysMCre*). The genotyping results were determined by WB and RT-PCR analysis (Figure 4A and Figure 4). Representative genotyping results as shown in Supplementary Figure 1A. Moreover, double staining of macrophage (F4/80) and SIRT6 was also performed. Compared with the *Sirt6*$^{fl/fl}$ mice, the expression of SIRT6 in macrophages was significantly decreased in PM2.5-exposed *Sirt6*$^{fl/fl}$*-LysMCre* mice (Supplementary Figure 1B and C). BMDMs were isolated from *Sirt6*$^{fl/fl}$*-LysMCre* and *Sirt6*$^{fl/fl}$ mice. Consistent with the above findings, we found that the levels of IL-6, TNF-α, CXCL1, and CXCL2 were significantly decreased in *Sirt6*-deficient BMDMs compared with the control group (Figure 4C–). Collectively, these findings imply that the loss of SIRT6 in macrophages attenuated $PM_{2.5}$-induced inflammatory cytokine.

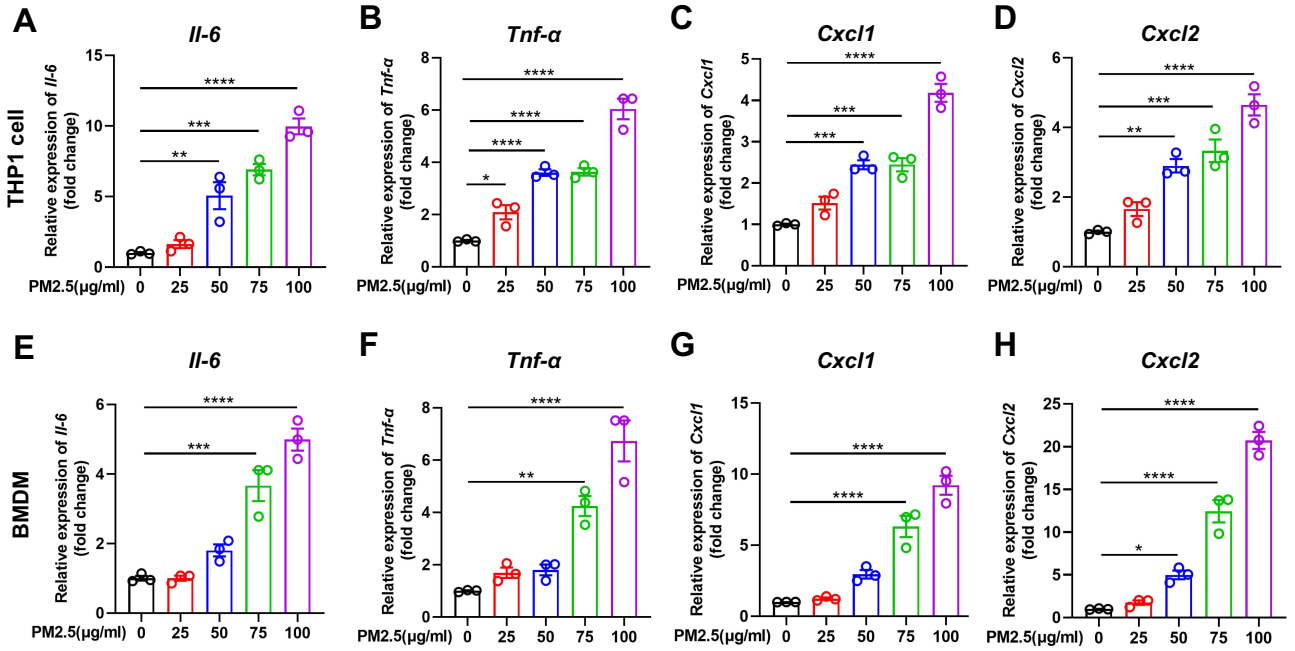

Figure 2 $PM_{2.5}$ induced inflammatory cytokines production in macrophages. THP1 cells and BMDMs were treated with $PM_{2.5}$ for 4 hours at indicated concentrations (25, 50, 75, 100μg/mL). The mRNA expression of *Il-6, Tnf-α, Cxcl1*, and *Cxcl2* were increased in THP1 cells (**A**–**D**) and BMDMs (**E**–**H**) was analyzed by RT-PCR. Data are presented as the mean ± SEM of three independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.001, and *****P*<0.0001.
Abbreviations: PM, Particulate matter; BMDMs, bone marrow–derived macrophages; Il-6, interleukin 6; Tnf-α, tumor necrosis-alpha; Cxcl, C-X-C ligand.

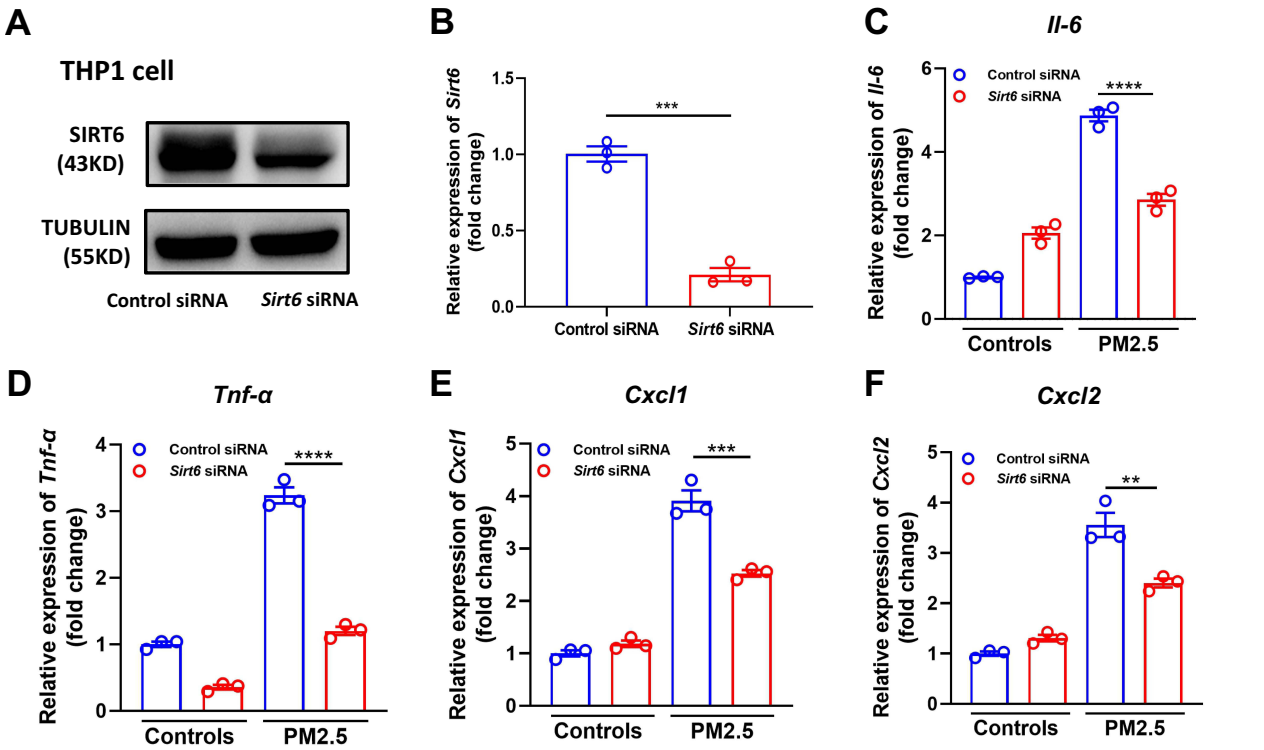

Figure 3 Knockdown of SIRT6 reduced $PM_{2.5}$-induced inflammatory cytokines in THP1 cells. THP1 cells were transfected with control siRNA and *Sirt6* siRNA for 24 h, and then were treated with $PM_{2.5}$ at 100 μg/mL for 4 h. SIRT6 expression was measured using Western blot (**A**) and RT-PCR (**B**). The mRNA expression of *Il6, Tnf-α, Cxcl1*, and *Cxcl2* in THP1 cells were determined using RT-PCR (**C**–**F**). Data are presented as the mean ± SEM of three independent experiments. ***P*<0.01, ****P*<0.001, and *****P*<0.0001.
Abbreviations: PM, Particulate matter; Il-6, interleukin 6; Tnf-α, tumor necrosis-alpha; Cxcl, C-X-C ligand.

Sirt6$^{fl/Fl}$*-LysMCre* Mice Attenuated $PM_{2.5}$-Induced Airway Inflammation

Next, we further elucidate the role of SIRT6 in $PM_{2.5}$-induced airway inflammation in vivo. As described in method, a mouse model for short-term exposure to $PM_{2.5}$ was established using *Sirt6*$^{fl/fl}$ littermates and *Sirt6*$^{fl/fl}$*-LysMCre* mice. As described in Figure 5A, compared with $PM_{2.5}$-induced *Sirt6*$^{fl/fl}$ mice, SIRT6 expression in lung tissue was significantly decreased in *Sirt6*$^{fl/fl}$*-LysMCre* mice after PM2.5 exposed. The number of total cells, macrophage, neutrophil, lymphocytes and eosinophil were significantly decreased in BALF of PM2.5-induced *Sirt6*$^{fl/fl}$*-LysMCre* mice compared with those in *Sirt6*$^{fl/fl}$*-LysMCre* mice after PM2.5 exposed (Figure 5B–). Moreover, airway inflammation was significantly decreased in *Sirt6*$^{fl/fl}$*-LysMCre* mice exposed to $PM_{2.5}$ (Figure 5H and Figure 5). Compared with the *Sirt6*$^{fl/fl}$ mice, both mRNA and protein expression of IL-6, TNF-α, CXCL1, and CXCL2 in the lung tissue were significantly decreased in PM2.5-exposed *Sirt6*$^{fl/fl}$*-LysMCre* mice (Figure 5J and Figure 5).

Discussion

In the present study, we showed that SIRT6 expression was increased in $PM_{2.5}$-induced BMDMs, but *Sirt6*-deficient BMDMs decreased cytokine secretion when treated with $PM_{2.5}$. Moreover, mice with myeloid cells specific knockdown of SIRT6, display decreased airway inflammation. Collectively, our data suggested that inhibition of SIRT6 in macrophages may represent therapeutic strategy for $PM_{2.5}$-induced airway inflammation.

Sirtuin family, type III histone deacetylase (HDAC), consists of seven members. They participate in aging, inflammation, cancer and metabolic homeostasis by regulating cell cycle, cell differentiation, cell metabolism, cell senescence and death. Previous study revealed that SIRT6 might act as a double-edged sword in respiratory inflammatory diseases. SIRT6 has been found to play a protective role in allergen-induced inflammation by inhibiting IL-4-associating TH2 immune response, attenuating inflammatory cell recruitment, decreasing mucin production.[10,15] To identify whether SIRT6 is macrophage specific, we first examined the expression of SIRT6 in

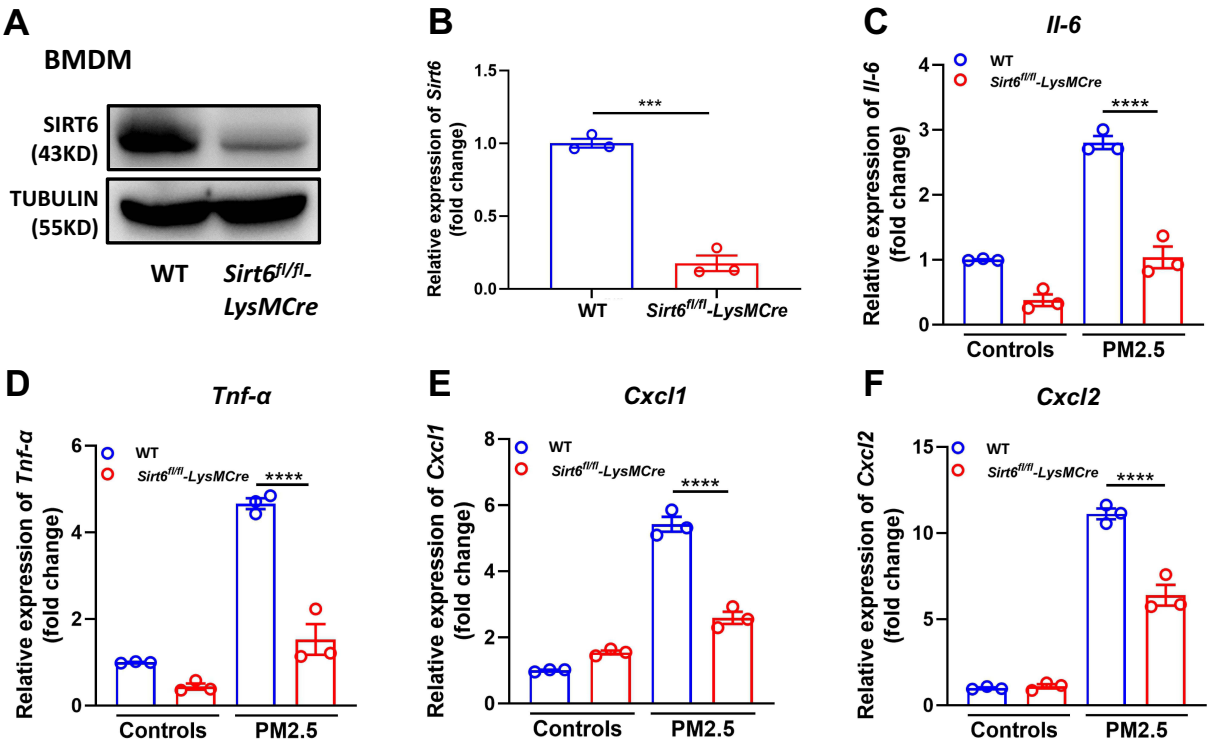

Figure 4 *Sirt1*-deficient BMDMs attenuated $PM_{2.5}$-induced inflammatory cytokines. Genotyping was assessed in BMDMs from *Sirt6*$^{fl/fl}$ and *Sirt6*$^{fl/fl}$*-LysMCre* mice using Western blot (**A**) and RT-PCR analysis (**B**). BMDMs were treated with $PM_{2.5}$ at 100 μg/mL for 4 h to measure the levels of *Il6*, *Tnf-α*, *Cxcl1*, and *Cxcl2* using RT-PCR (**C**–**F**). Data are presented as the mean ± SEM of three independent experiments. ***P<0.001 and ****P<0.0001.

Abbreviations: PM, Particulate matter; Il-6, interleukin 6; Tnf-α, tumor necrosis-alpha; Cxcl, C-X-C ligand.

THP1 derived macrophages exposed to $PM_{2.5}$. Furthermore, we generated bone marrow–derived macrophages (BMDMs) from *Sirt6*$^{fl/fl}$ mice. The expression of SIRT6 was significantly increased in $PM_{2.5}$-induced BMDMs and THP1 cells in a dose- and time-dependent manner. Double staining of macrophage (F4/80) and SIRT6 was also performed and confirmed that SIRT6 gene knockout is macrophage-specific. We found that inflammatory cytokines were decreased in BMDMs with myeloid-specific deletion of SIRT6 after stimulation of $PM_{2.5}$. In vivo, *Sirt6*$^{fl/fl}$*-LysMCre* mice substantially decreased airway inflammation in response to $PM_{2.5}$ exposure. According to previous studies, we found that the profiles of sirtuin deacetylase family in the myeloid cells are different in different diseases. Some sirtuin such as SIRT1, SIRT4, SIRT5, and SIRT7protect again airway inflammation, hepatocellular carcinoma development, pro-inflammatory response, or mycobacterial clearance in macrophages.[7,16–18] However, some sirtuin such as SIRT2 and SIRT3 promote chronic staphylococcal infection or inflammasome activation in macrophages.[19,20] Therefore, the exact functions and mechanisms of sirtuin members in different diseases need to be further elucidated.

Limitations

Our study does have some limitations. First, the use of simulated PMs in our study was purchased from National Institute of Standards and Technology (NIST) Company, which do not represent airborne PMs that contain more chemicals. Although we found that PMs could induce airway inflammation in vivo and in vitro experiments, it is true that the use of simulated PMs in our study does not represent airborne PMs that contain more chemicals. Second, macrophages are highly phagocytic that may affect the experimental results in our study. Therefore, the role of SIRT6 on $PM_{2.5}$-induced airway inflammation in macrophages should be interpreted cautiously due to limitations in our study.

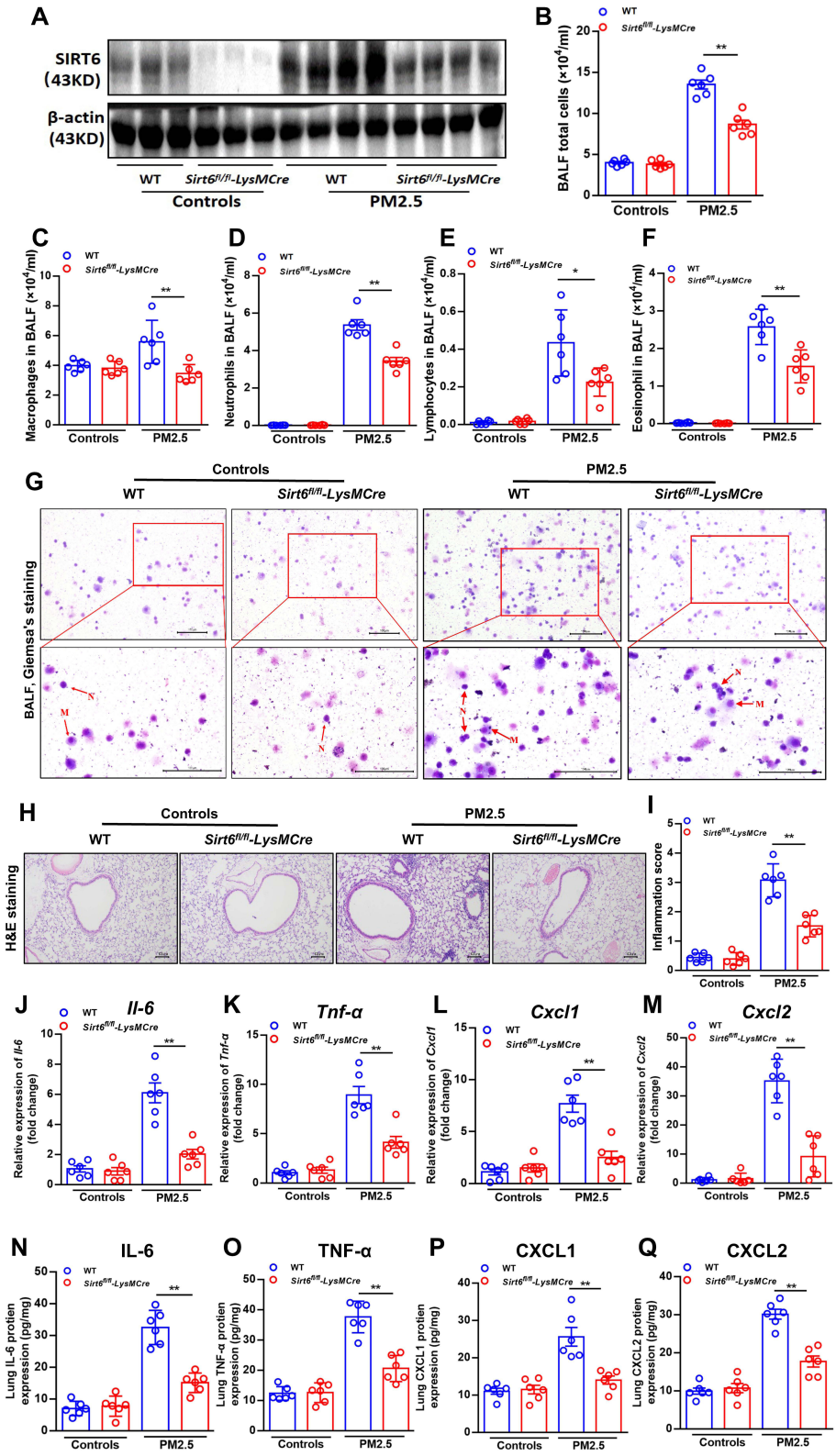

Figure 5 Conditional knockout of SIRT6 in macrophages attenuated $PM_{2.5}$-induced inflammatory responses in mice. The expression of SIRT6 in the lung tissue was assessed by Western blot (**A**). The number of total cells (**B**), macrophage (**C**), neutrophil (**D**), lymphocytes (**E**) and eosinophil (**F**) in the BALF were measured by Flow cytometry. Representative images of macrophages and neutrophils in the BALF were analyzed using Giemsa's staining (**G**, Scale bar: 100 μm). Representative images of lung sections with hematoxylin and eosin (H&E) staining (**H**, Scale bar: 100 μm). Semi quantification of inflammation expression in the lungs were performed using Image Pro 6.1 software (**I**). *Il6, Tnf-α, Cxcl1* and *Cxcl2* mRNA expression in lung homogenate were measured by QPCR (**J**–**M**). Protein concentrations of cytokines above in lung homogenate were measured by ELISA (**N**–**Q**). Data are presented as Mean ± SEM of three independent experiments (n=6 for each group). *P<0.05 and **P<0.01.
Abbreviations: BALF, Bronchoalveolar lavage fluid; Il-6, interleukin 6; Tnf-α, tumor necrosis-alpha; Cxcl, C-X-C ligand.

Conclusion

Collectively, our study indicated that SIRT6 promoted $PM_{2.5}$-induced airway inflammation in macrophages and suggested that inhibition of SIRT6 in macrophages might be a strategy for treating the airway inflammation induced by $PM_{2.5}$.

Acknowledgments

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Disclosure

The authors declare no competing interests.

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