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Chronic and low-level particulate matter exposure can sustainably mediate lung damage and alter CD4 T cells during acute lung injury

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ABSTRACT

Particulate matter (PM)2.5 is a common air pollutant known to induce damages in the respiratory, cardiovascular, and nervous systems. Previous study has shown that acute and high-level PM insult could significantly aggravate the severity of LPS-induced acute lung injury (ALI). However, humans typically experience more chronic and low-level PM, of which the effect on ALI is yet unclear. Here, we varied the concentration of PM from low, medium, to high, which was given to mice via intratracheal instillation for a short period of time. Compared to the saline-treated mice, mice with medium or high PM treatment presented significantly higher mortality rate, weight reduction, and bronchoalveolar lavage (BAL) protein concentration during ALI, while mice with low PM treatment did not demonstrate significant differences from saline-treated mice. However, when the PM was given for an elongated period of time, PM, even at the low level, significantly aggravated ALI severity. Furthermore, the PM-mediated changes were sustained even after PM withdrawal. We also examined the CD4 T cells in saline- or PM-treated mice. We found that, although PM did not significantly change the number of lung-infiltrating CD4 T cells, it significantly altered the composition of lung-infiltrating CD4 T cells, characterized by having a higher T-bet/Foxp3 ratio in the PM-treated group compared to the saline-treated group. Additionally, the Treg-mediated suppression was reduced in PM-treated mice. The effect of PM on CD4 T cells depended on the concentration of PM and the duration of the treatment, and was independent of the PM withdrawal. Overall, these results demonstrated that chronic and low-level PM was sufficient at aggravating ALI and altering pulmonary CD4 T cells, and the effect could be sustained even after PM withdrawal.

1. Introduction

Air pollution from anthropogenic sources is a major health threat, especially in developing countries where rapid industrialization is taking place and fossil fuels are the near-exclusive energy source (Xing et al., 2016). Particulate matter (PM)2.5 is a type of air pollution consisting of solid and liquid particles with an aerodynamic diameter of 2.5 μm or less. Examination of the human lung parenchyma revealed that exogenous particles could be retained in the lung in a size-dependent manner, and 96% of the retained particles belonged to PM2.5, making it a major class of lung pollutant (Churg and Brauer, 1997). A recent study estimated that in China alone, over 1.7 million people aged ≥ 65 years in 2010 had premature death related to PM2.5 exposure (Li et al., 2018). The respiratory, cardiovascular, and central nervous systems are all susceptible to PM2.5-induced damages (Block and Calderón-Garcidueñas, 2009; Chauhan, 2003; Kampfrath et al., 2011).

Acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) is a life-threatening complication that can develop in patients with pneumonia, sepsis, and traumatic injury. The reported mortality rate ranges between 11% and 87%, and is dependent on the patient's age, severity, and the preceding morbidity (Maca et al., 2017). The immune system is involved in almost every stage of ALI/ARDS, from induction, alveolar injury, resolution, to fibroproliferation (Johnson and Matthay, 2010; Parsons et al., 2005; Lin et al., 2018a). PM2.5 is shown to promote the release of interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α), both of which are implicated in ALI/ARDS pathogenesis

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(Johnson and Matthay, 2010; Baulig, 2007; Lin et al., 2018b). Lin et al. has demonstrated that PM2.5 instillation could directly induce ALI in wild-type as well as angiotensin-converting enzyme 2-knockout mice (Lin et al., 2018b). T regulatory (Treg) cells, on the other hand, can act as a sink of proinflammatory cytokines and downplay the extent of inflammation (Lin et al., 2018a). Treg cells can also promote the resolution of lung injury and inhibit fibroproliferation (D'Alessio et al., 2009; Garibaldi et al., 2013). However, it has been reported that mouse airway PM instillation had significantly aggravated the symptoms of LPS-induced experimental ALI, and impaired the function of Treg cells (Li et al., 2016). Transfer of Treg cells from untreated control mice to PM-treated mice was able to significantly reduce the severity of lung injury and improve the survival of the mice.

A major caveat of the previous investigation is that the PM concentration was significantly higher than that experienced by human residents of air-polluted areas, while the duration of PM instillation (2 weeks) was significantly shorter (Li et al., 2016). In addition, it is unclear whether PM2.5 reduction could revert the PM2.5-mediated damages. The effect of low but sustained PM2.5 treatment, as well as PM2.5 withdrawal, on the development of ALI is yet unclear. Hence, we present the current study to answer the above questions.

2. Materials and methods

2.1. Mice

Animal use, sample collection, and experimental procedures were approved by the ethics committee of East Hospital. All experiments used male C57BL/6 aged between 6 and 8 weeks old. These mice were obtained from the Jackson Laboratory and were housed inhouse pathogen-free facility.

2.2. PM administration and LPS challenge

The PM was prepared as described previously (Li et al., 2016). Briefly, mice were weighed and anesthetized using 150 μg/g ketamine and 13.5 μg/g acepromazine. PM was then instillated via the intratracheal route using a 20-gauge catheter at 20 μL per mice every 2 days. Control mice received 20 μL of sterile saline. For LPS challenge, 3.75 μg/g *Escherichia coli* LPS (Sigma-Aldrich) was given.

2.3. Bronchoalveolar lavage analysis

Bronchoalveolar lavage (BAL) was performed as previously described (D'Alessio et al., 2009). Briefly, the right trachea of each mice was cannulated using a 20-gauge catheter and was lavaged twice with 0.7 mL sterile saline. The cell was then pelleted from the BAL fluid via centrifugation at 600 *g* for 8 min at 4 °C. The total protein concentration was measured using the Modified Lowry Protein Assay Kit (Thermo Fisher Scientific) in the BAL supernatant.

2.4. Tissue processing

Mice were sacrificed by exsanguination at inferior vena cava. Spleen was gently crushed and sieved through a sterile cell strainer (70 μm pore size; Falcon) to collect splenocytes. Lung was minced and incubated for 1 h in RPMI 1640 (Gibco) containing 2.4 mg/mL collagenase I and 20 μg/mL DNase (Sigma-Aldrich). The resulting slurry was then sieved through a sterile cell strainer (70 μm pore size) to obtain a single cell suspension. Lung-infiltrating lymphocytes were collected by centrifugation and aspiration at the 23%/70% Percoll interface.

2.5. CD4 T cell analysis

The CD4 T cells in the lung-infiltrating lymphocytes and the spleen were enumerated by counting $CD3^+CD4^+$ cells using standard flow cytometry. Briefly, FcγR III/II on the lymphocytes was blocked using anti-mouse CD16/CD32 (BioLegend). Anti-mouse CD3 and CD4 monoclonal antibodies (BioLegend) were then added for incubation at 4 °C for 30 min. CD4 T cells in the samples were identified, enumerated and sorted using a FACSAria instrument (BD). The sorted CD4 T cells were then treated with the RNeasy Mini kit (Qiagen) to collect total RNA. mRNA was transcribed into cDNA using iScript cDNA Synthesis kit (Bio-Rad). T-bet and Foxp3 expression levels were quantified using the TaqMan assay system according to the manufacturer's instructions.

A portion of CD4 T cells was cryopreserved at -150 °C in Recovery Cell Culture Freezing Medium (Gibco). The cells were then thawed, rested overnight, and sorted into $CD4^+CD25^-$ and $CD4^+CD25^+$ cells in a FACSAria instrument. The Treg suppression assay was performed according to a previously published protocol (Collison and Vignali, 2011). Briefly, $CD4^+CD25^-$ T cells were plated at 2.5×10^4 cells per well in a 96-well plate, and $CD4^+CD25^+$ T cells were added at various ratios as specified per experiment. Cells were stimulated with Human T-Activator CD3/CD28 beads (Thermo Fisher) for 72 h, and pulsed with 0.1 μCi H3-Thymidine (Amersham Biosciences) per well. The level of radioactive thymidine incorporation was measured in a direct beta counter.

2.6. Statistics

Data were presented as mean ± SD. when applicable. One-way ANOVA followed by Tukey's multiple comparisons was used to examine multiple groups on a single parameter, and two-way ANOVA followed by Tukey's multiple comparisons was used on two parameters. $P < 0.05$ was required for statistical significance. All tests were performed using Prism software version 7 (GraphPad).

3. Results

3.1. Weight reduction and bronchoalveolar lavage (BAL) protein concentration was positively associated with the concentration of PM2.5 treatment

Previous study demonstrated that ultrafine PM instillation could significantly aggravate murine LPS-induced ALI, characterized by higher protein concentration in bronchoalveolar lavage, stronger weight reductions, and higher mortality rate, accompanied with significant alterations in Treg cell frequency and function (Li et al., 2016). However, it is yet unclear whether low PM at an extended duration could produce the same effects. In the current study, we varied the concentration of PM from a low level of 10 μg/mL to a high level of 500 μg/mL. These PM-containing saline solutions or the pure saline control was given to mice via intratracheal instillation every two days, and the mice were challenged with LPS via the intratracheal route after 3 instillations. The outcome of mice was examined by the survival status, weight loss, and bronchoalveolar lavage (BAL) protein concentration every other day for a total of 6 days following LPS challenge (Fig. 1). The 10 μg/mL PM-pretreated mice presented similar survival, body weight change, and BAL protein concentration with the pure saline-pretreated mice, as no significant differences between the two groups were observed. However, the 50 μg/mL PM-pretreated mice and the 500 μg/mL PM-pretreated mice had markedly worse survival (Fig. 1A). They also presented more reductions in body weight and higher BAL protein concentrations (Fig. 1B and C). Interestingly, the BAL protein concentration in 500 μg/mL PM-pretreated mice was higher than the saline-pretreated mice at baseline (day 0). Together, these data demonstrated that higher PM concentration was associated with further weight reductions and higher BAL protein concentration.

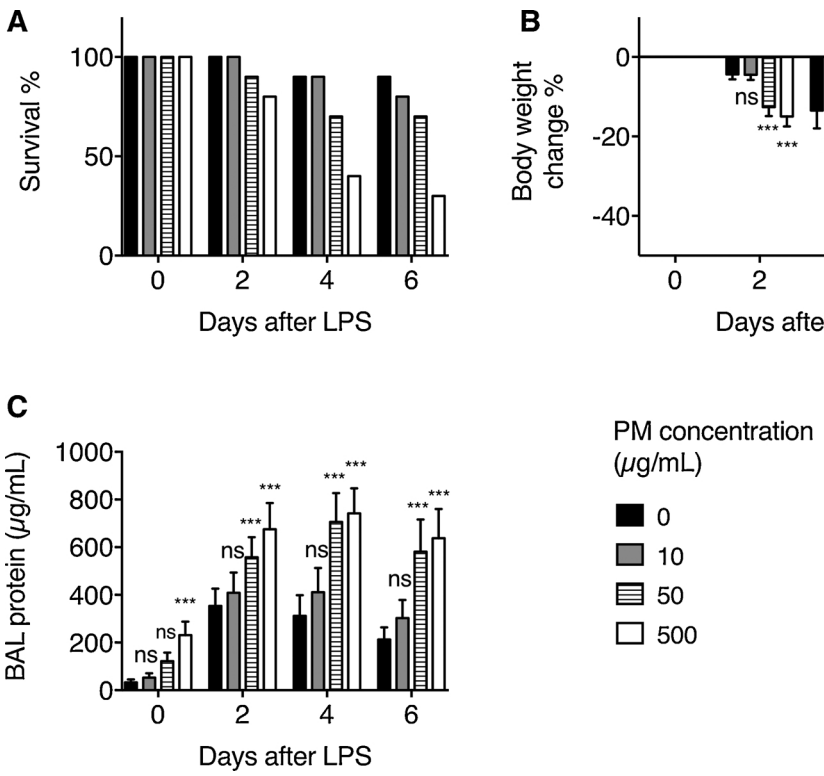


Fig. 1. ALI severity after intratracheal saline or PM instillations. Sterile saline were infused with 0–500 μg/mL PM and were given to mice (10 per group) via intratracheal instillation every other day. After 3 instillations, mice were challenged with intratracheal LPS at day 0. (A) Percentage survival of mice after LPS challenge. (B) Percentage reduction in body weight relative to day 0 measurements. (C) BAL protein concentration. Two-way ANOVA followed by Tukey's multiple comparisons. Mean ± SD. Asterisks indicate the differences between pure saline, 10 μg/mL PM, 50 μg/mL, and 500 μg/mL. ns, not significant. $^{**}P < 0.01$. $^{***}P < 0.001$.

3.2. Low PM concentration at longer durations significantly aggravated the severity of ALI

Human residents of air-polluted areas are usually subjected to PM for longer periods of time. To evaluate the effect of the duration of PM2.5 treatment, we gave to the mice (10 per group) 3–18 instillations of pure saline or saline containing a low concentration of 10 μg/mL PM. The mice were challenged with intratracheal LPS after the final intratracheal instillation, and the survival status, weight loss, and BAL were evaluated at day 4, which was the time point with the strongest weight reduction and the highest BAL protein concentration (Fig. 1B and C). We found that mice with more PM instillations, albeit at a low PM concentration of 10 μg/mL, had succumbed to death more easily than mice with fewer PM instillations (Fig. 2A). The reduction in body weight was comparable between PM- and saline-pretreated mice at 3 and 6 instillations (Fig. 2B). However, at 9 and 18 instillations, the body weight reduction was significantly more severe in PM-pretreated mice than in saline-pretreated mice (Fig. 2B). The same tendency was observed in terms of the BAL protein concentration (Fig. 2C).

Together, these results demonstrated that although a low level of PM at shorter durations did not aggravate ALI, the same low level of PM at longer durations could significantly worsen ALI.

3.3. PM induced aggravation of ALI was sustained after PM withdrawal

In the above investigations, LPS challenge was performed immediately after PM instillation. However, it is yet unclear whether PM instillation could still aggravate ALI, if the mice were allowed to rest in the absence of PM instillations prior to LPS challenge. In this experiment, mice were pretreated with 3 instillations of pure saline, or low (10 μg/mL) or medium (50 μg/mL) levels of PM. The mice were then for 0–28 days before LPS challenge. The weight reduction and bronchoalveolar lavage (BAL) were evaluated at day 4 after LPS challenge. We found that the weight reduction and BAL protein concentration were comparable in saline-pretreated or 10 μg/mL PM-pretreated mice, but was significantly higher in 50 μg/mL PM-pretreated mice (Fig. 3A and B). This tendency was conserved regardless of the resting time, although the body weight reduction and the BAL protein concentration were lower when the mice were rested for longer periods (Fig. 3A and B).

To examine the effect of time on PM pretreatment, the mice were treated with 18 instillations of saline or 10 μg/mL PM mice. These mice were then allowed to rest for 0–28 days before LPS challenge. The weight reduction and the BAL protein level were consistently higher in 10 μg/mL PM-pretreated mice, regardless of the resting time (Fig. 4A and B). Longer resting time did not reduce the extent of weight reduction, but slightly reduced the BAL protein level in 10 μg/mL PM-pretreated mice (Fig. 4B).

Together, these results demonstrated that the effect of PM was sustained even after PM withdrawal.

3.4. PM induced profound changes in the composition of pulmonary CD4 T cells

PM treatment was shown to elevate the Th1/Treg ratio (Li et al., 2016). In addition, the Th1/Treg ratio was positively associated with the reduction in body weight. Here, we investigated the effect of various PM treatment condition on the pulmonary and splenic composition of CD4 T cells. In pure saline-pretreated mice, little CD4 T cell infiltration in the lung was observed prior to LPS challenge (Fig. 5A, left panel). In 10 μg/mL PM-pretreated mice, though some pulmonary infiltration of CD4 T cells could be seen, the amount of CD4 T cell infiltration was not significantly different from that in pure saline-pretreated mice. The 50 μg/mL and 500 μg/mL PM-pretreated mice, however, presented significantly higher CD4 T cell infiltration in the lung before LPS challenge. At day 4 after LPS challenge, significant pulmonary infiltration of CD4 T cells were observed all murine groups (Fig. 5A, right panel). While the pure saline-pretreated and 10 μg/mL PM-pretreated mice presented similar levels of CD4 T cell infiltration, the 50 μg/mL and 500 μg/mL PM-pretreated mice presented significantly higher CD4 T cell infiltration. Saline or PM-pretreatment did not significantly affect the number of CD4 T cells in the spleen (data not

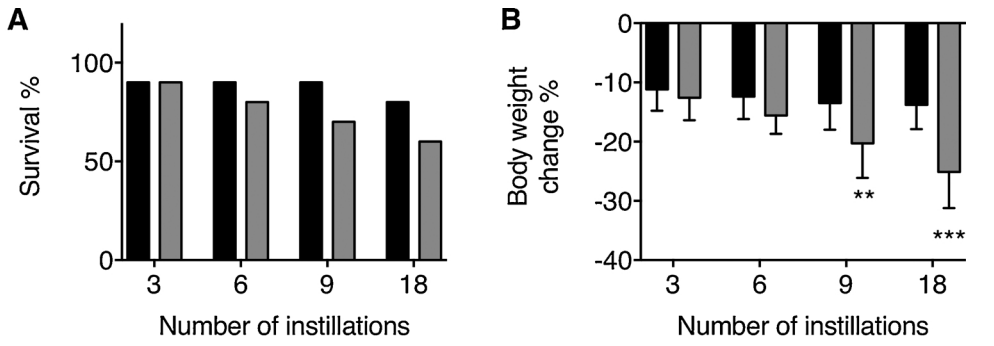
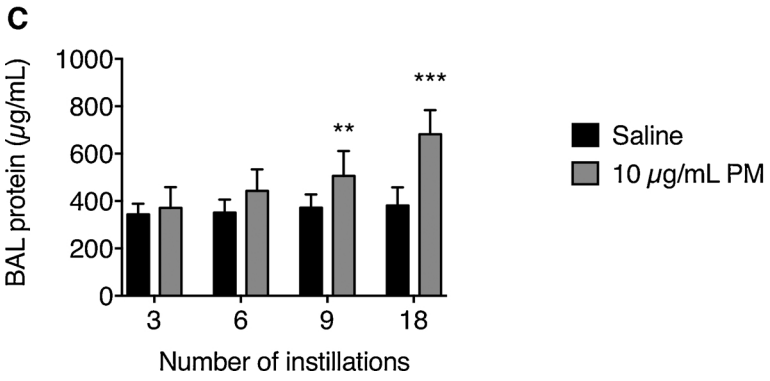


Fig. 2. ALI severity after various numbers of PM instillations. Mice (10 per group) received pure saline or saline with 10 μg/mL PM via the intratracheal instillation every other day, for a total of 3–18 instillations. The mice were then challenged with intratracheal LPS at day 0, and (A) the percentage survival, (B) the percentage reduction in body weight, and (C) the BAL protein concentration were measured at day 4. Two-way ANOVA followed by Tukey's multiple comparisons. Mean ± SD. **P < 0.01. ***P < 0.001.



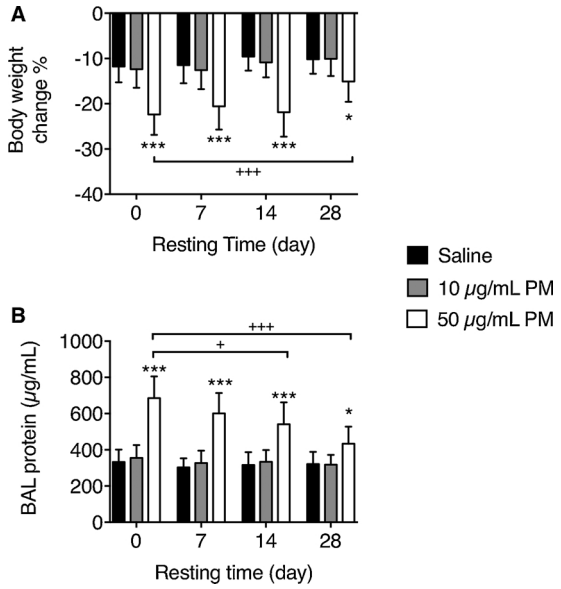


Fig. 3. ALI severity in PM-pretreated mice rested before LPS challenge. Mice pretreated with 3 instillations of pure saline or 10 μg/mL or 50 μg/mL PM were rested for 0–28 days, before intratracheal LPS challenge. The (A) percentage reduction in body weight and (C) BAL protein concentration were measured4 days after LPS challenge. Two-way ANOVA followed by Tukey's multiple comparisons. Mean ± SD. Asterisks indicate the differences between pure saline, 10 μg/mL PM, and 50 μg/mL, and plus signs indicate the differences between 0, 7, 14, and 28 resting days. */$^{+}$P < 0.05. ***/$^{+++}$P < 0.001.

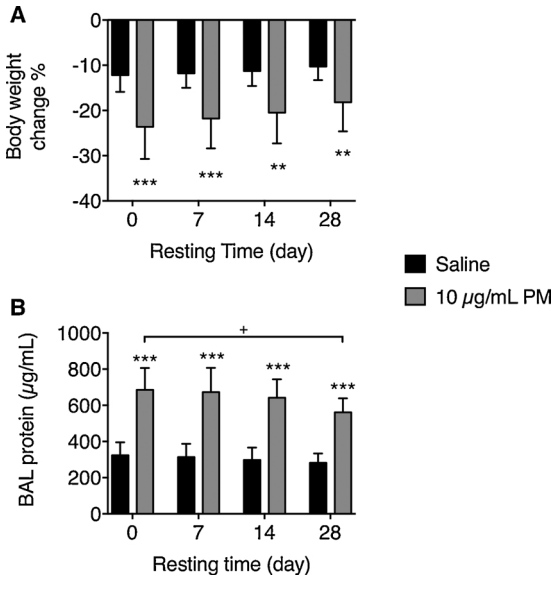


Fig. 4. ALI severity in long-term 10 μg/mL PM-pretreated mice rested before LPS challenge. Mice pretreated with 18 instillations of pure saline or 10 μg/mL PM were rested for 0–28 days, before intratracheal LPS challenge. The (A) percentage reduction in body weight and (C) BAL protein concentration were measured4 days after LPS challenge. Two-way ANOVA followed by Tukey's multiple comparisons. Mean ± SD. Asterisks indicate the differences between pure saline and 10 μg/mL PM, and plus signs indicate the differences between 0, 7, 14, and 28 resting days. $^{+}$P < 0.05. **P < 0.01. ***P < 0.001.

shown).

To assess the composition of pulmonary CD4 T cells, the expression levels of T-bet, the Th1-specific transcription factor, and Foxp3, the Treg-specific transcription factor, were examined in CD4 T cells from the lung and spleen samples from PM-pretreated mice. In pure saline and 10 μg/mL PM-pretreated mice, the T-bet/Foxp3 ratios were below 1 and were comparable before LPS challenge (Fig. 5B, left panel). 50 μg/mL and 500 μg/mL PM pretreatment significantly increased the T-bet/Foxp3 ratio. At day 4 after LPS challenge, the T-bet/Foxp3 ratio was below 1 in pure saline-pretreated mice, approximately 1 in 10 μg/mL PM-pretreated mice, and significantly higher in 50 μg/mL and 500 μg/mL PM-pretreated mice (Fig. 5B, right panel). On the other hand, no significant differences between pure saline-pretreated or PM-pretreated mice were observed in the composition of splenic CD4 T

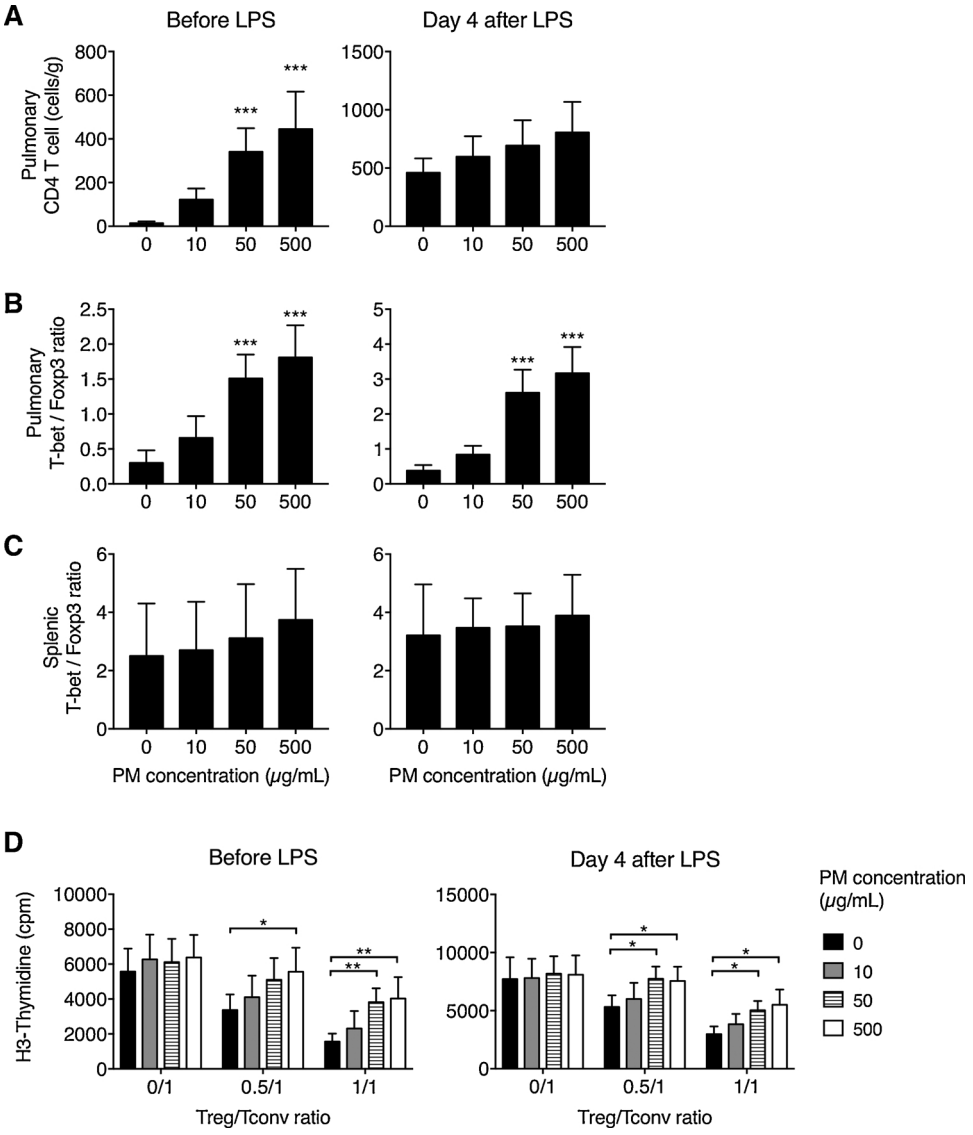


Fig. 5. Number and type of lung-infiltrating CD4 T cells before and after LPS challenge. Mice (10 per group) were given pure saline or various concentrations of PM via intratracheal instillation every other day. After 3 instillations, half of the mice (5 per group) were sacrificed, while the other half were challenged with intratracheal LPS, and sacrificed 4 days later. (A) The amount of CD4 T cells in the lung tissue. (B) The ratio of T-bet/Foxp3 transcription levels in pulmonary CD4 T cells. (C) The ratio of T-bet/Foxp3 transcription levels in splenic CD4 T cells. One-way ANOVA followed by Tukey's multiple comparisons. (D) $CD4^+CD25^+$ Treg cells and $CD4^+CD25^-$ Tconv cells from pulmonary tissues were co-incubated at ratios indicated in the x-axis, and were stimulated for 72 h using anti-CD3/CD28 beads. The T cells were then pulsed with tritiated thymidine (H3-Thymidine) for 6 h, and the proliferation potential was measured using thymidine incorporation. Two-way ANOVA followed by Tukey's multiple comparisons. Mean ± SD. *P < 0.05. **P < 0.01. ***P < 0.001.

cells, either before or after LPS challenge (Fig. 5C).

To verify whether the Treg compartment in pulmonary T cells after PM-pretreatment was impaired, in cryopreserved pulmonary T cell samples, we isolated $CD4^+CD25^+$ Treg cells and $CD4^+CD25^-$ T conventional (Tconv) cells using flow cytometry sorting. The $CD4^+CD25^+$ Treg cells were then coincubated with $CD4^+CD25^-$ Tconv cells at various ratios, and the effect of $CD4^+CD25^+$ Treg cell-mediated suppression on the proliferation of $CD4^+CD25^-$ Tconv cells was examined. Without $CD4^+CD25^+$ Treg cells (0/1 Treg/Tconv ratio), the $CD4^+CD25^-$ Tconv cells from 0, 10, 50, and 500 μg/mL PM-pretreatments presented similar levels of proliferation (Fig. 5D). $CD4^+CD25^+$ Treg cells reduced the proliferation of $CD4^+CD25^-$ Tconv cells in a dose-dependent manner, but in 50 μg PM and 500 μg/mL PM-pretreated mice, the $CD4^+CD25^+$ Treg cells were less effective, both before and after LPS (Fig. 5D).

Together, these data demonstrated that PM pretreatment could significantly increase the number and type of lung-infiltrating CD4 T cells before LPS challenge, in a manner that depended on the concentration of the PM. After LPS challenge, although the pure saline-pretreated and medium/high PM-pretreated mice both presented high pulmonary infiltration of CD4 T cells, the T-bet/Foxp3 ratio was significantly higher in medium/high PM-pretreated mice than in saline-pretreated mice. Moreover, the pulmonary Treg cells from medium/high PM-pretreated mice were less effective at suppressing Tconv proliferation.

3.5. Low PM concentration at longer durations significantly altered the composition of pulmonary CD4 T cells

We also examined the pulmonary and splenic CD4 T cells in mice with longer durations of 10 μg/mL PM instillation. Before LPS challenge, 10 μg/mL PM at 3 instillations did not alter the amount of lung-infiltrating CD4 T cells, but at more instillations, 10 μg/mL PM significantly increased the amount of lung-infiltrating CD4 T cells (Fig. 6A, left panel), as well as increased the T-bet/Foxp3 ratio (Fig. 6B, left panel). After LPS challenge, the pulmonary infiltration of CD4 T cells was comparable in pure saline- and 10 μg/mL PM-pretreated mice, regardless of the number of instillations (Fig. 6A, right panel). However, the T-bet/Foxp3 ratio was significantly higher in mice pretreated for long durations of 10 μg/mL PM (Fig. 6B, right panel). In addition, the $CD4^+CD25^+$ Treg cells from mice with longer PM pretreatment were less effective at suppressing Tconv proliferation than the $CD4^+CD25^+$ Treg cells from mice with shorter PM pretreatment (Fig. 6C). No significant differences in the number and the composition of splenic CD4 T cells were observed between pure saline-pretreated and PM-pretreated mice (data not shown).

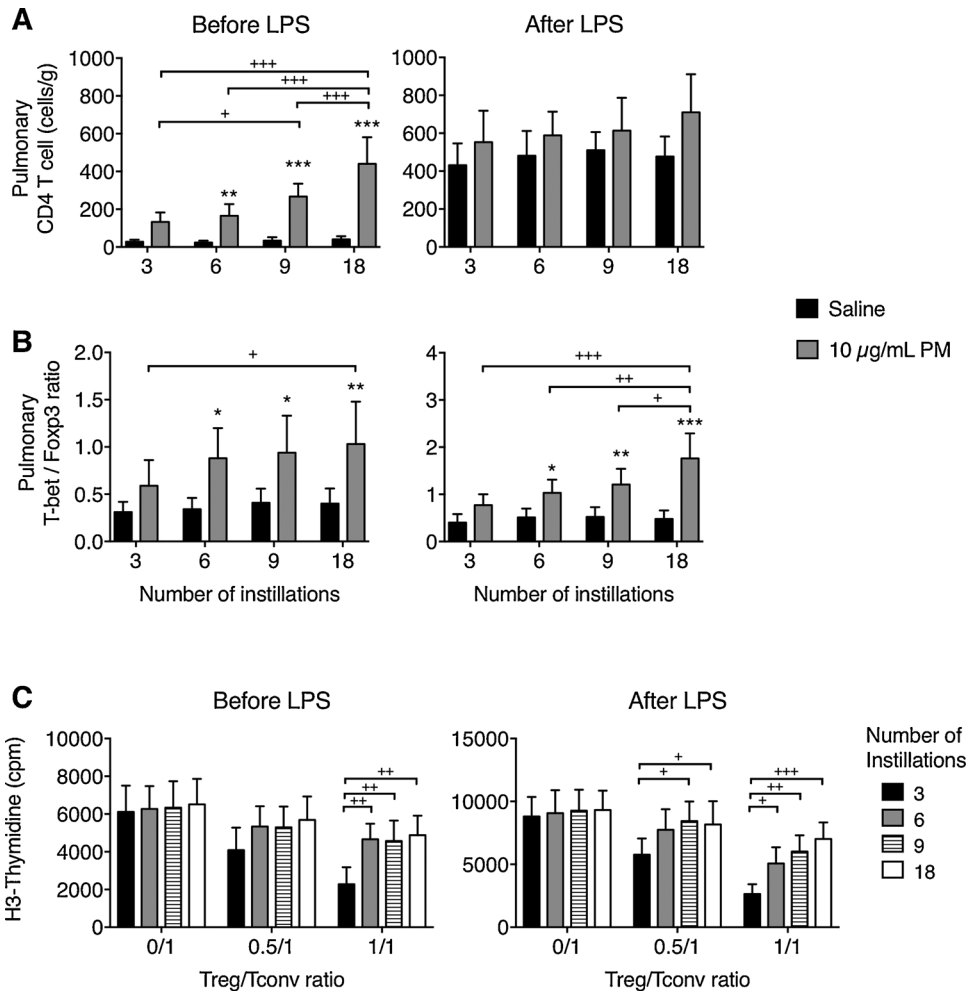


Fig. 6. Pulmonary CD4 T cells in mice pretreated for longer durations of PM. Mice (10 per group) were given pure saline or 10 μg/mL PM via intratracheal instillation every other day, for 3–18 instillations. Half of the mice (5 per group) were sacrificed, while the other half were challenged with intratracheal LPS, and sacrificed 4 days later. (A) The amount of CD4 T cells in the lung tissue. (B) The ratio of T-bet/Foxp3 transcription levels in pulmonary CD4 T cells. (C) $CD4^+CD25^+$ Treg cell-mediated suppression of $CD4^+CD25^-$ Tconv cell proliferation. Cells were isolated from 10 μg/mL PM-pretreated pulmonary tissues, coincubated at various ratios, and stimulated for 72 h using anti-CD3/CD28 beads. The proliferation potential was measured using thymidine incorporation. Two-way ANOVA followed by Tukey's multiple comparisons. Mean ± SD. Asterisks indicate the differences between pure saline and 10 μg/mL PM, and plus signs indicate the differences between 3, 6, 9, and 18 instillations. $*/^+P < 0.05$. $**/^{++}P < 0.01$. $***/^{+++}P < 0.001$.

3.6. Alteration in pulmonary CD4 T cells could be sustained after PM withdrawal

Subsequently, the number and composition of CD4 T cells were examined in mice that received PM but subsequently rested without PM. With the exception that the pulmonary T-bet/Foxp3 ratio in 50 μg/mL PM-pretreated mice after 28 resting days was lower, the number and the composition of pulmonary CD4 T cells, as well as the impairment in $CD4^+CD25^+$ Treg-mediated suppression of Tconv proliferation, were maintained according to the initial PM treatment levels and were largely independent of the resting time (Fig. 7A–C). Again, no change in splenic CD4 T cell number and composition was detected with varying resting time (data not shown).

Together, these data demonstrated that the PM-mediated effects on pulmonary CD4 T cells were sustained even after PM withdrawal.

4. Discussion

Multiple epidemiological studies have identified an association between PM2.5 levels and increased incidences of illness in the respiratory and the cardiovascular systems (Imrich et al., 2000; Collison and Vignali, 2011; Salvi et al., 1999). ALI/ARDS is a life-threatening complication that can develop in patients with airway diseases as well as in patients with sepsis, pancreatitis, and multiple trauma (Rubenfeld and Herridge, 2007). The effect of PM2.5 on the risk of developing ALI/ARDS and on the progression of established ALI/ARDS should thus be investigated. A previous report showed that PM treatment induced a more severe form of ALI in mice, with significant increase in the Th1/Treg ratio in lung-infiltrating lymphocytes and impaired Treg function, characterized by an inability to reduce inflammation during adoptive transfer (D'Alessio et al., 2009; Li et al., 2016). However, the previous report used a high concentration (500 μg/mL) for a short period of time (2 weeks), while human patients tend to experience PM at lower concentrations for longer durations. To address these shortcomings, we further investigated the effect of PM in ALI using a variety of conditions. The results demonstrated that the PM-induced damage is dependent on both the level of PM and the duration of PM treatment. When the duration of the treatment was conserved, the extent of damage was positively associated with the concentration of PM, and when the concentration of PM was conserved, longer treatment predicted higher severity. Notably, while low (10 μg/mL) concentration of PM did not aggravate ALI severity at shorter durations, it significantly aggravated ALI severity at longer durations. Furthermore, the PM-mediated aggravation of ALI could persist after the PM treatment was withdrawn. Mechanistically, we observed that PM treatment significantly altered the pulmonary, but not splenic, CD4 T cells, together with a reduction in Treg-mediated suppression. These PM-induced alteration of pulmonary CD4 T cells also depended on the concentration and duration of PM treatment. In addition, the alteration of pulmonary CD4 T cells was maintained even after the PM treatment was withdrawn.

This study has several limitations and requires further investigations. First, the progression of ALI was measured in terms of weight change and BAL protein concentration. However, the physiology of the lung, such as the partial pressure of oxygen and carbon dioxide, were not examined. Second, the expression of cytokines and the localization

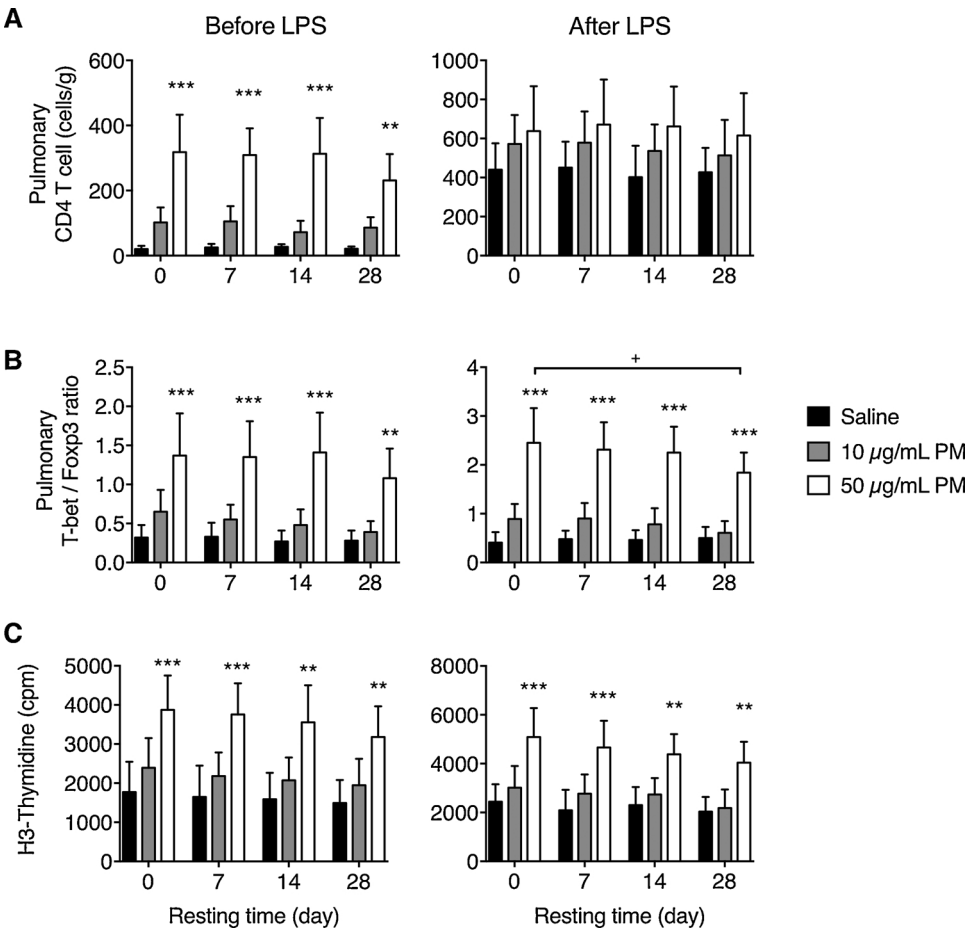


Fig. 7. Pulmonary CD4 T cells in long-term 10 μg/mL PM-pretreated mice rested before LPS challenge. Mice (10 per group) pretreated with 3 instillations of pure saline or 10 μg/mL or 50 μg/mL PM were rested for 0–28 days. Half of the mice (5 per group) were sacrificed, while the other half were challenged with intratracheal LPS for 4 days before sacrifice. (A) The amount of CD4 T cells in the lung tissue. (B) The ratio of T-bet/Foxp3 transcription levels in pulmonary CD4 T cells. (C) $CD4^+CD25^+$ Treg cells and $CD4^+CD25^-$ Tconv cells were isolated from 0, 10, or 50 μg/mL PM-pretreated pulmonary tissues, and were coincubated at 1/1 ratio. Following 72 h-stimulation using anti-CD3/CD28 beads, the proliferation potential was measured using thymidine incorporation. Two-way ANOVA followed by Tukey's multiple comparisons. Mean ± SD. Asterisks indicate the differences between pure saline, 10 μg/mL PM, and 50 μg/mL, and plus signs indicate the differences between 0, 7, 14, and 28 resting days. $^+P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$.

of various subsets of immune cells in the lung tissues were not examined. And third, we discovered sustained alterations to pulmonary CD4 T cells in PM-treated mice, but these changes were only observed in the lung, but not in the spleen. How these changes could be sustained remains unclear. Previous studies have shown exposure to air pollution could change the DNA methylation in the exposed individuals (Baccarelli et al., 2009; Miller and Ho, 2008). Interestingly, demethylation of Foxp3, which enables stable Foxp3 expression, could significantly improve the resolution of ALI (Singer et al., 2015). Hence, PM instillation might have changed DNA methylation patterns in immune cells, but we have not performed experiments to investigate this possibility. For better understanding of the role of inflammation in PM-mediated effects, further experiments, including lung function assessments, histological examination of the harvested lung tissue, and gene methylation and expression in pulmonary immune cells, should be further investigated.

Many questions regarding the effect of PM should be investigated. We found that PM treatment did not significantly increase the number of CD4 T cells infiltrating the lung during LPS-induced ALI, or significantly alter the splenic CD4 T cells. However, PM treatment significantly altered the composition of pulmonary CD4 T cells. The reason for this lung-specific effect is unclear. PM treatment possibly affected the trafficking of certain CD4 T cell subsets. Alternatively, the pulmonary environment during PM treatment may favor the development of some but not other CD4 T cell subsets. Previous investigations have demonstrated that PM could directly promote the proinflammatory polarization of macrophages, which in turn promote T cell inflammation (Zhao et al., 2016; Ma et al., 2017). Interestingly, the airway contains residential or recruited alveolar macrophages, which play critical roles in regulating the inflammatory response during ALI/ARDS (Herold et al., 2011a). Currently, it is known that macrophages can release IL-1 receptor antagonist (IL-1RA), which blocks IL-1β-mediated upregulation of adhesion molecules and downregulate the infiltration of immune cells (Herold et al., 2011b). Macrophages may also eliminate infiltrating neutrophils via inducing apoptosis or direct phagocytosis (McGrath et al., 2011; Mukaro V and Hodge, 2011). Whether Also, it was previously shown that alloreactive Th1 cells could exacerbate lung injury, associated with the activation of alveolar macrophages and the induction of chemokines (Clark et al., 1998; Dixon et al., 2017). Whether PM treatment could alter alveolar macrophage function, and whether a positive feedback mechanism exists between Th1 cells and macrophages to induce further damage, should be addressed in future studies.

Conflict of interests

The authors declare no conflict of interests.

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