

## Research Article

# Pre-exposure to Fine Particulate Matters may Induce Endotoxin Tolerance in a Mouse Model

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Exposure to low or moderate doses of Lipopolysaccharides (LPS) renders the host tolerance to a subsequent lethal dose of LPS, which is termed as endotoxin tolerance. It is characterized as the decrease in production of pro-inflammatory cytokines and the increase in production of anti-inflammatory mediators in response to subsequent LPS challenge. Whether other environmental factors also trigger endotoxin tolerance remains unclear. Both epidemiologic and experimental studies have provided a link between particulate matters and human health. Here, we speculated on the effect of fine particles priming on endotoxin tolerance in a mouse model.

**Keywords:** Lipopolysaccharides; Fine particulate matters; Survival curves; Heat-shock response

**Abbreviations**

PM: Particulate Matter; TLR: Toll-Like Receptor; LPS: Lipopolysaccharides; IL: Interleukin; HSP: Heat-Shock Protein

**Introduction**

The inhalation of toxic ambient particles is a worldwide public health problem; both epidemiologic and experimental studies have provided compelling evidence supporting the association between Particulate Matter (PM) and human diseases, including mortality and hospital admissions [1], cardiovascular diseases [2, 3], type 2 diabetes [4,5], asthma and chronic obstructive pulmonary disease [6,7], and non-alcoholic fatty liver disease [8]. Inflammatory response has been implicated as the key mechanism of PM-mediated healthy problems. Current evidence suggests that inhaled particles trigger innate immune signals in the lung through interacting with Toll-Like Receptors (TLRs), releasing cytokines into circulation and causing systemic inflammatory response [9]; and that direct penetration of leachable components such as reactive oxygen species and stable organic compounds into circulation also contributes to systemic inflammatory response [10].

Ambient particle pollution is a mixture of microscopic solids and liquids droplets suspended in air; it consists of a number of components, including acids, organic chemicals, metals, soils or dust particles, and allergens. According to its aerodynamic diameter, PM is classified into coarse (10 to 2.5  $\mu\text{m}$ ;  $\text{PM}_{10}$ ), fine (<2.5  $\mu\text{m}$ ;  $\text{PM}_{2.5}$ ), and ultrafine (<0.1  $\mu\text{m}$ ;  $\text{PM}_{0.1}$ ) particulate matters. The size of particles is directly linked to their potential for causing health effects. It is believed that fine particulate matters pose the greatest health problems, because they hold the potential to get and deposit deep into the lung, and may even penetrate into the bloodstream. PM composition and size together influence its adverse effects on public health [11,12].

Endotoxin, also known as Lipopolysaccharides (LPS), is a structural component of the gram-negative outer membrane. Leukocytes recognize LPS via TLR4 in the presence of myeloid

differentiation factor 2, triggering a powerful immune reaction [13]. This inflammatory response is tightly regulated and can show different forms, depending on the dose. Exposure to low or moderate doses of LPS renders the host tolerance to a subsequent lethal dose of LPS, which is termed as endotoxin tolerance. It is characterized as the decrease in production of pro-inflammatory cytokines such as tumor necrosis factor  $\alpha$ , Interleukin (IL)-6 and IL-1 $\beta$ , and the increase in production of anti-inflammatory mediators such as IL-10 in response to a second LPS challenge [14, 15]. The alteration of cytokine profile protects LPS-primed hosts against a normally lethal dose of subsequent LPS challenge. Nevertheless, whether other environmental factors also trigger endotoxin tolerance remains unclear. Here, we speculated on the effect of  $\text{PM}_{2.5}$  priming on endotoxin tolerance in a mouse model.

**Materials and Methods****Animal care**

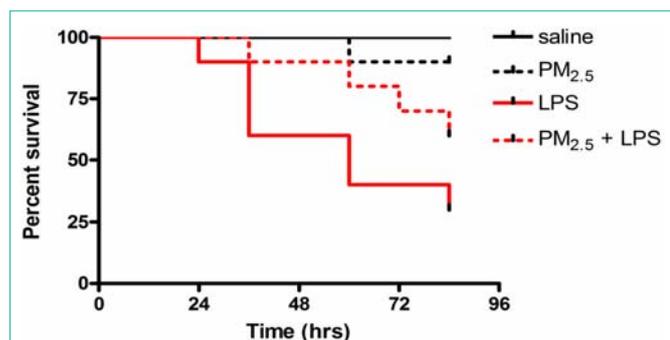
C57BL/6 mice (6-8 weeks old) were obtained from Jackson Laboratories (Bar Harbor, ME). Animals were maintained at 21°C and exposed to a 12-h light, 12-h dark cycle with free access to water and food. The protocols and the use of animals were approved by and in accordance with the Ohio State University Animal Care and Use Committee.

**Intranasal exposure to  $\text{PM}_{2.5}$** 

Mice were exposed to  $\text{PM}_{2.5}$  by intranasal instillation, which is an effective and noninvasive technique in toxicity studies [16,17]. This instillation technique consists in deliver drop-wise the particle suspension or the vehicle to the nares using a micropipette, while the mouse is in a supine position. Animals were lightly anesthetized with 2% isoflurane and intranasally instilled with 20  $\mu\text{l}$  of free-particle saline or  $\text{PM}_{2.5}$  (0.5  $\mu\text{g}/\mu\text{l}$ ) saline, three times per week for eight weeks.

**Survival study**

Endotoxic shock was induced by peritoneal injection of LPS (20  $\mu\text{g}/\text{g}$ ; Escherichia coli serotype 055:B5; Sigma-Aldrich) and mice (n = 10) were monitored up to 84 hours. Survival curves were compared



**Figure 1:** PM<sub>2.5</sub> priming attenuates LPS-induced mortality in wild-type mice. Mice (n = 10) were exposed to 20 µl of free-particle saline or PM<sub>2.5</sub> (0.5 µg/µl) saline, three times per week for eight weeks by intranasal instillation. After exposure, endotoxin shock was induced by peritoneal injection of LPS (20 µg/g), and mice were monitored up to 84 hours for survival study. Survival curves were compared using Kaplan–Meyer log-rank test. All tests were conducted at the two-sided 5% significant level. LPS: Lipopolysaccharides; PM<sub>2.5</sub>: Fine particulate matters.

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## Results

All mice treated with saline without LPS injection survived; one mouse exposed to PM<sub>2.5</sub> without LPS injection died ( $p > 0.05$  vs. saline). LPS injection induced a significant decrease in survival rate ( $p < 0.01$  vs. saline); pre-exposure to PM<sub>2.5</sub> induced tolerance to death from a subsequent lethal LPS dose, however, these two survival curves were not significantly different ( $p > 0.05$  vs. LPS) (Figure 1).

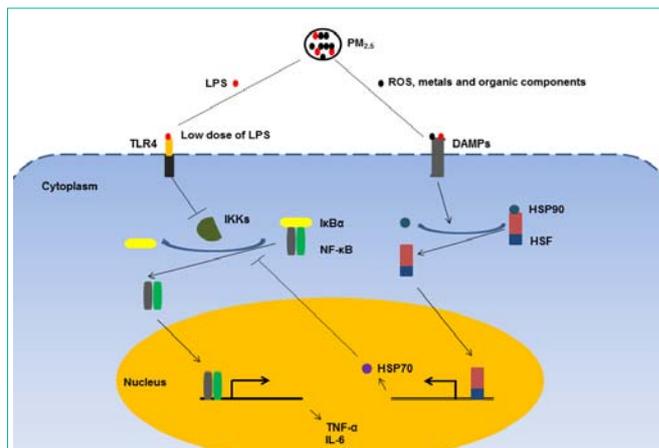
## Hypothesis and evaluation

Our preliminary data showed an evident trend of survival curves between PM<sub>2.5</sub>-exposure and PM<sub>2.5</sub> priming plus LPS treatment, suggesting that PM<sub>2.5</sub> priming may cause endotoxin tolerance in mice. To verify this hypothesis, a study with larger sample size is needed. Sample sizes are determined using a two-sided, 0.05-significance level log rank test with 80% statistical power and equal allocation. The log-rank test is a popular nonparametric test to compare the survival distributions of two groups. Let  $i = 1, i$  be the distinct times of observed events. For each time  $i$ , let  $N_{1i}$  and  $N_{2i}$  be the numbers of subjects at risk at the start of time  $i$  in the two groups. Define  $N_i = N_{1i} + N_{2i}$ . Let  $O_{1i}$  and  $O_{2i}$  be the observed numbers of events in the groups respectively at time  $i$ , and define  $O_i = O_{1i} + O_{2i}$ . Under the null hypothesis that the two groups have the same survival function, the distribution of the number of events in the first group has expected value  $E_i = \frac{O_i}{N_i} N_{1i}$  and variance  $V_i = \frac{O_i N_{1i} (N_i - N_{1i}) (N_i - O_i)}{N_i^2 (N_i - 1)}$ .

The log-rank statistic compares each observed event count to its expectation and is defined as

$$Z = \frac{\sum_{i=1}^l (O_i - E_i)}{\sqrt{\sum_{i=1}^l V_i}}$$

Under the null hypothesis, it follows approximately a standard normal distribution. Based on our preliminary study, 60% of mice survived after 84 hours in PM<sub>2.5</sub> priming plus LPS treatment group, while only 30% of mice survived in LPS treatment group. Assuming a constant hazard model and using a log rank test, we would need 40 mice in each group to detect the difference in survival curve. The



**Figure 2:** Proposed mechanisms of PM<sub>2.5</sub>-mediated endotoxin tolerance. PM<sub>2.5</sub> consists of a number of components, including acids, organic chemicals, metals, ROS and low dose of LPS. On the one hand, pre-exposure to PM<sub>2.5</sub> may prime animals with possible low dose of LPS and contribute to endotoxin tolerance via inhibition of IκBα degradation. On the other, LPS and other components such as ROS, metals and organic chemicals may activate heat-shock response via binding to DAMPs, up regulating the expression of HSP70. It has been shown that HSP70 interrupts NF-κB signaling and inhibits pro-inflammatory cytokine release by stabilizing the complex between NF-κB and its inhibitor IκBα. These signaling pathways may contribute to particle-mediated endotoxin tolerance. PM<sub>2.5</sub>: Fine Particulate Matters; LPS: Lipopolysaccharides; ROS: Reactive Oxygen Species; TLR4: Toll-Like Receptor 4; DAMPs: Damage-Associated Molecular Patterns; IKKs: IκB kinases; IκBα: κB inhibitor α; NF-κB: Nuclear Factor-κB; HSP: Heat-Shock Protein; HSF: Heat-Shock Factor; TNF-α: Tumor Necrosis Factor-α; and IL: Interleukin.

sample size calculation is conducted using SAS proc power (SAS 9.2, SAS Institute Inc. Cary, NC).

To verify our findings and mimic the effects of “real world” air pollution, we will repeat this experiment using Versatile Aerosol Concentrator and Enrichment System, which is used to generate ambient air pollution murine models for *in vivo* toxicological studies. This system has been well introduced by Chen Lab at New York University [18-20]. The system in our lab is quite similar. Mice (n = 40) will be exposed to either filtered air or concentrated ambient PM<sub>2.5</sub> for 6 hours/day, 5 days/week for 8 weeks. The exposure protocol and PM<sub>2.5</sub> compositions have been reported in our previous studies [4, 21-23]. After exposure, endotoxin shock will be induced as previously described.

How may PM<sub>2.5</sub> priming lead to endotoxin tolerance in mice? Based on our knowledge of this compound, we speculate that at several mechanisms PM<sub>2.5</sub> may actively regulate endotoxin tolerance (Figure 2). On the one hand, PM<sub>2.5</sub> is a mixture of various chemical and biological constituents, including a low dose of LPS [24]. In our experiment, pre-exposure to PM<sub>2.5</sub> may prime animals with low dose of LPS, preventing death from a subsequent lethal dose. On the other hand, PM<sub>2.5</sub> may induce endotoxin tolerance through regulation of heat-shock response. It has been shown that PM<sub>2.5</sub> exposure significantly increases the expression of Heat-Shock Protein (HSP) 70 [25,26]. HSP70, a prominent chaperone protein, functions individually or as part of larger heterocomplexes to maintain protein homeostasis in response to various stress stimuli [27]. In addition, HSP70 exhibits anti-inflammatory effect and inhibits the release of

pro-inflammatory cytokines, e.g. IL-6, IL-1 $\beta$  and tumor necrosis factor  $\alpha$ , through interaction with nuclear factor- $\kappa$ B complex [28-30].

### Potential implications

The adverse effects of PM air pollution have robustly been investigated since global air quality becomes worse, particularly in the developing countries [31,32]. Although PM-induced inflammation is implicated as one of potential mechanisms, the modulated effects of PM on immune system, particularly in response to subsequent infection, could be more complicated than we thought. If our hypothesis is to be confirmed, it may refresh our knowledge about this compound and its inflammatory effects.

Additionally, we want to highlight the use of log-rank test as a suitable tool to determine the sample size and evaluate the data fidelity for survival study. The most considerable advantage of this test is that it does not require the knowledge about the shape of the survival curve or the distribution of survival times in advance [33].

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