Polystyrene nanoplastic exposure enhances LPS-induced lung inflammation in mice by inducing M1 polarization of macrophages via ROS/JAK/STAT signaling pathway

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Abstract

Nanoplastics (NPs) are an emerging environmental pollutant. NPs can spike various tissues results to oxidative stress and tissue damage in organisms. While recent studies have reported a relationship between nanoparticles and respiratory system injury, the specific mechanism of NP exposure-induced lung damage remains to be explored. In the present study, C57BL6 male mice were treated intraperitoneal injection of PS-NPs and/or LPS. The relevant indicators were detected by HE staining, western blotting and qRT-PCR. RAW264.7 was pretreated with JAK2 inhibitor (AG490) to verify whether the JAK2/STAT3 signaling pathway is involved in PS-NPs exposure enhances LPS-induced pulmonary inflammatory response. We found decreased antioxidant capacity in mice lungs, activation of the JAK2/STAT3 pathway, and the expression levels of macrophage M1 marker genes increased (including CD16, CD86, and MCP1), while macrophage M2 marker genes (including CD206, PPARγ, and Arg1) expression levels decreased, resulting in a macrophage M1/M2 imbalance. In addition, PS-NPs can increase the expression of inflammation-related factors IL-1β, TNF-α and IL-6. In in vitro experiments, we obtained similar results to in vivo experiments. More importantly, the JAK2 pathway inhibitor AG490 reversed ROS-induced changes in macrophage imbalance and inflammation in PS-NPs and LPS-exposed RAW264.7 cells. In conclusion, PS-NPs activated the ROS/JAK/STAT pathway, aggravated LPS-induced lung M1/M2 macrophage imbalance and promoted inflammatory responses. Our results enrich the toxic effects and related molecular mechanisms of NPs-induced lung inflammation, and provide new insights into the toxic effects of NPs on mammals.

Highlights

- PS-NPs exposure aggravates LPS-induced lung inflammation in mice
- PS-NPs exposure-induced lung inflammation is caused by M1/M2 macrophage imbalance
- M1/M2 macrophage imbalance induced by PS-NPs exposure is regulated by ROS/JAK/STAT axis

1. Introduction

According to statistics, up to 74% of plastic waste in the world is landfilled into the environment through various channels every year(Barnes et al. 2009). Most plastics will eventually break down into countless pieces of plastic(Kole et al. 2017). Among them, nanoplastics (NPs) are plastic fragments with a diameter of less than 100 nm, which widely exist in soil, ocean, and even some remote rivers or lakes are inevitably polluted by NPs, (such as the Arctic) (Guo et al. 2020, Wang et al. 2019b, Yin et al. 2021). It has been reported that NPs can be ingested and accumulated in aquatic organisms, mammals, etc. along the food chain(Lu et al. 2016, Song et al. 2020). Many works have confirmed that NPs can exist in the natural environment and in vitro conditions for a long time without obvious degradation, therefore, the contamination of NPs has attracted extensive public attention. Polystyrene (PS) is one of the most commonly used plastics(Xu et al. 2019), especially in packaging, electronics and personal care products. PS-NPs can penetrate through biofilms, penetrate into tissues, and accumulate in organs, thereby
bringing about toxicological effects. It has been demonstrated that PS-NPs have the ability to translocate the mucosal tissues of blood, lymphatic circulation, and secondary organs (Rubio et al. 2020), depending on the physicochemical properties of the particles, including size, chemical structure, and surface charge (Fournier et al. 2020). PS-NPs may cause tissue dysfunction or damage where they persist, potentially leading to oxidative stress, metabolic disturbances, neurotoxicity, and immune dysfunction (Barboza et al. 2018, Limonta et al. 2021, Qiao et al. 2019). Lu et al. have demonstrated that exposure to PS-NPs results in an imbalance of intestinal homeostasis and impaired immune cytotoxicity, as well as a dysbiosis of the gut microbiota. For example, exposure to NPs (100 µg/L and 1000 µg/L) leads to impaired gut homeostasis and immune cytotoxicity (Lu et al. 2018). PS-MPs are deposited in the microglia of the brain after oral entry into the body, inducing microglial immune activation, and eventually leading to apoptosis (Kwon et al. 2022). Overall, PS-NPs have been found to induce hepatotoxicity, reproductive toxicity, and nephrotoxicity (Hirt & Body-Malapel 2020, Zheng et al. 2021). Environmentally exposed MPs enhance macrophage uptake, which upon activation can lead to inflammation-induced lesions (Merkley et al. 2022). Exposure to high concentrations of PS-NPs (100 and 200 µg/mL) resulted in increased reactive oxygen species (ROS) and damage to lysosomes in macrophages. In addition, some studies have shown that NPs also have obvious toxic effects on lung tissue (Vranic et al. 2013). The presence of MPs/NPs can be detected in human lungs (Amato-Lourenço et al. 2021). The overproduction of reactive oxygen species (ROS) that can be caused by the interaction between cells and NPs at the same time leads to oxidative stress, causes chronic inflammation and contributes to the development of lung disease (Amato-Lourenço et al. 2020). D. M. Brown's study found that PS-NPs enter the lung tissue of rats, and the increase in the number of neutrophils in the lungs leads to lung inflammation (Brown et al. 2001). Cheng's research has shown that exposure of lung epithelial cells to PS may cause the destruction of intercellular junction proteins, leading to lung barrier dysfunction and chronic obstructive pulmonary disease (COPD) (Dong et al. 2020). However, the mechanism of pulmonary toxicity of PS-NPs in mice in the context of systemic inflammation has not been elucidated in detail.

Macrophages are a key line of defense against microorganisms, toxic substances, and environmental pollutants (Kong & Gao 2017). Macrophages activated by exogenous chemicals are classified into two major macrophage subsets based on the expression of cell surface markers, production of specific factors, and biological activity: classically activated pro-inflammatory macrophages (M1) and alternatively activated anti-inflammatory macrophages (M2) (Chen et al. 2019). M1 macrophages secrete mediators that promote inflammation, pro-inflammatory cytokines monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor (TNF)-α, interleukin (IL)-6, etc., thus exerting a powerful pro-inflammatory effect, bactericidal and anti-pathogenic effects. M2 phenotype macrophages express anti-inflammatory cytokines with anti-inflammatory, tissue repair and fibrosis-promoting effects, characterized by upregulation of arginase-1 (Arg-1), CD206 and PPARγ. The balance of M1/M2 can reflect the balance state of the organization. Multiple studies have shown that M1 macrophages predominate in inflammation-related diseases. Previous studies have shown that many environmental pollutants promote the occurrence of inflammation by affecting the M1/M2 balance of macrophages (Qiao et al. 2019). For example, cadmium (Cd) can cause an imbalance of M1/M2 in the porcine adrenal and
increase the release of pro-inflammatory cytokines(Yao et al. 2021). Elisabeth Wigenstam(Wigenstam et al. 2016) reported that after inhaling selenium disulfide, M1 macrophages are activated, not only causing systemic inflammation, but also causing lung inflammation.

Cytokines that promote phenotypic polarization of macrophages signal primarily through the JAK/STAT pathway, leading to the activation of transcription factors that determine M1/M2 polarization(Lescoat et al. 2020). Members of the STAT protein family activated by JAKs are also key transcription factors regulating M1/M2 polarization in macrophages(Albrahim et al. 2020). Members of the STAT protein family activated by JAK are also key transcription factors that regulate the polarization of macrophages M1/M2. In the LPS-induced lung injury model, STAT3 promotes the polarization of M1 macrophages(Zhao et al. 2016). In RAW264.7, bisphenol A (BPA) can promote the phosphorylation of STAT3 through the phosphorylation of JAK1 and JAK2 to significantly induce a pro-inflammatory response(Huang et al. 2019). Bisphenol F (BPF) promotes macrophage polarization to the pro-inflammatory M1 subtype by activating the ER-JAK2/STAT3/SOCS3 signaling pathway(Shi et al. 2020). ROS is an important mediator of the activation of pro-inflammatory signaling pathways(Hamed et al. 2020) and can interact with multiple signaling pathways such as JAK/STAT and MAPK(Phull et al. 2018). The presence of cysteine residues in JAK and the rapidly phosphorylated and dephosphorylated nature of STAT make this signaling vulnerable to oxidative stress(Paithankar et al. 2021). Excess ammonia can activate inflammation through oxidative stress and JAK/STAT, MAPK and other pathways, and induce cardiac inflammatory injury(Wang et al. 2020). Similarly, oxidative stress-JAK2/STAT3 signaling pathway is also involved in PM2.5-induced inflammation in human bronchial epithelial cells(Xu et al. 2020b). ROS can also be released from activated macrophages, triggering an inflammatory cascade that induces lung injury and aggravates a series of pro-inflammatory responses. Macrophages are essential for the initiation and resolution of inflammation caused by pathogens or tissue damage(Bashir et al. 2016). Therefore, we speculated that M1/M2 imbalance might play an important role in PS-NPs promoting LPS-induced lung inflammation in mice, and further explored the role of ROS/JAK/STAT pathway in PS-NPs toxicity mechanism.

The cytotoxic effect of NPs can penetrate the biofilm barrier and lead to oxidative stress and apoptosis in the intestine, liver, kidney, heart tissue, etc., and increase the incidence of chronic diseases after long-term exposure. Multiple studies have shown that NPs can modulate macrophage function and increase ROS production and lipid peroxidation(Fuchs et al. 2016). The oxidative metabolic state in lung macrophages is critical for lung homeostasis. Therefore, we speculate that M1 macrophage polarization may play an important role in the mechanism of PS-NPs triggering lung toxicity. Intraperitoneal injection of LPS is a widely used model to study chronic lung injury. We duplicated the PS-NPs and/or LPS-exposed mouse intraperitoneal injection model in vivo, and RAW264.7 were employed for finding specific mechanism due to PS-NPs and/or LPS exposure. To this end, we detected changes in pathological results, oxidative stress indicators, and the expression levels of JAK/STAT pathway, macrophage polarization and inflammatory factors. This study will provide technical reference and theoretical basis for the study of NPs exposure on LPS-induced lung toxicity, and enrich the relevant molecular mechanisms of NPs-induced lung inflammation.
2. Materials And Method

2.1 Characterization of PS-NPs

PS-NPs were purchased from Wuxi Rigor Technology Co., Ltd. (China): with the sizes of 100 nm, 2.5wt%.
The morphology of PS-NPs was measured by transmission electron microscopy (TEM) (HT7800, Japan) after diluting PS-NPs to 100 µg/mL with serum-free Dulbecco's Modified Eagle Medium (DMEM) medium.

2.2 Animal groups and model establishment

All procedures used in this study were approved by the Northeastern Agricultural University Institutional Animal Care and Use Committee under approved protocol number SRM-11. Adult C57BL/6 male mice (age: 8–12 wk, weight: 25–30 g) used in this study were purchased from Cyagen Biosciences (China). 24 mice were randomly divided into four groups: the control group, the PS group, the LPS group, and the LPS + PS group. Mice in PS group and LPS + PS group were intraperitoneally injected with 5 µg/g PS-NPs, once every 2 days for 2 consecutive weeks. LPS (10 mg) was purchased from Biotopped (Bei Jing, China). 8 h before sacrifice, mice in the LPS group and the LPS + PS group were intraperitoneally injected with 20 µg/g LPS. All mice were allowed free access to tap water and food pellets. After 2 weeks, all the mice in the 4 groups were euthanized, and the lung tissues were collected and fixed with 4% paraformaldehyde (PFA) or stored at -80°C.

2.3 H&E staining

To evaluate the effect of PS-NPs on mouse lung tissue, we fixed lung tissue with 4% paraformaldehyde. After washing and dehydration, transparent, wax dipping, embedding, and other steps to make into paraffin blocks. The prepared tissue wax blocks were cross-sectioned into 5 µm slices and mounted on glass slides; histopathological damage was assessed using hematoxylin and eosin staining, respectively.

2.4 Cell culture and treatment

RAW264.7 cell lines were purchased from Procell and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Meilunbio, China) with 10% FBS (Procell, USA) and 1% penicillin/streptomycin in humidified atmosphere at 37°C with 5% CO₂. The PS-NPs and/or LPS group cells were seeded in 6-well plates, and then PS-NPs (100 µg/mL) (Li et al. 2019)and/or LPS (0.1 µg/mL) (Matsebatlela et al. 2015) were added to each well for 24 hours each. In addition, 30 nM AG490(Okugawa et al. 2003) (AbMole, USA) cells were added for 1 hour pretreatment before treatment in one of the PS + LPS groups.

2.5 Oxidative stress measurement

The lung tissue was taken and added with normal saline to make tissue homogenate, centrifuged at 3000 rpm for 20 min, and the supernatant was taken. Protein concentrations were determined with Coomassie brilliant blue using a protein quantification (TP) (Nanjing Jiancheng Bioengineering Institute, China) kit following the manufacturer's instructions. Total superoxide dismutase (T-SOD), malondialdehyde (MDA),
glutathione (GSH), catalase (CAT) assay kits (Nanjing Jiancheng Bioengineering Institute, China) were used according to the manufacturer’s instructions for the detection of antioxidant indicators.

2.6 Measurement of ROS

RAW264.7 discarded the upper medium of each well of 24 plates, and used ROS detection kit (Nanjing Jiancheng Bioengineering Institute, China) to measure the amount of ROS production in each group of cells. 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) was diluted at 1:1000 with fresh serum-free medium to a final concentration of 10 µmol/L, and each well was added 300 µL of diluted DCFH-DA. Incubate in a 37°C cell incubator for 20 min, then wash the cells in each well 3 times with 100 µL of cold PBS buffer to adequately remove DCFH-DA that did not enter the cells. Finally, the intensity of fluorescence before and after stimulation was detected using a fluorescence microscope. The fluorescence intensity indicates the content of ROS.

2.7 Total RNA isolation and qRT-PCR

The total RNA was extracted in mouse lung tissue using the classic Trizol extraction method. The concentration and purity of RNA were measured with NanoDrop at 260/280 nm. The first-strand cDNA was synthesized using the qRT-PCR synthesis kit (Bioeasy Technology, China) according to the manufacturer's instructions. Gene expression levels were detected via qRT-PCR with the Light Cycler® 480 System (Roche, Basel, Switzerland). The sequences of mRNAs are detailed in Tables 1. Relative levels of mRNA were calculated by the $2^{-\Delta\Delta Ct}$ method using β-actin as an internal control.
<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequence</th>
</tr>
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<tbody>
<tr>
<td>CD16</td>
<td>Forward 5′-TCGACGTTGGAGACTCTGCTA-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse 5′GCCCGAGTCTTGATTCGAT-3′</td>
</tr>
<tr>
<td>CD86</td>
<td>Forward 5′-AACAGGGAAGAAAGCGGAACAC-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse 5′TCCACTGCCCCAGTTTTTTGA-3′</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Forward 5′-AGATGCAGTTAACGCCGCAC-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse 5′-AGACCTTAGGCGAGATGCAG-3′</td>
</tr>
<tr>
<td>CD206</td>
<td>Forward 5′-GTCAGAACAGACTGCGTGGA-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse 5′-AGGGATCGCCTGTTTTCCAG-3′</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Forward 5′-GACGCGGAAGAAGAGACCTG-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse 5′-GACGCGGAAGAAGAGACCTG-3′</td>
</tr>
<tr>
<td>Arg1</td>
<td>Forward 5′-CTTGCAGACGTAGACCCCTG-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse 5′-CTTCCTTCCAGCAGGAGTGC-3′</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Forward 5′-AGGCACTCCCCAAAGATG-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse 5′-CCACTTGGTGTTTGTGAGTG-3′</td>
</tr>
<tr>
<td>IL-6</td>
<td>Forward 5′-CAACGATGATGCACCTTGAGA-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse 5′-TGTGACTCCAGCTATCTTTGG-3′</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Forward 5′-ACTGGGCA TCAAGGGCTACA-3′</td>
</tr>
<tr>
<td></td>
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<tr>
<td>IFN-γ</td>
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<td></td>
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<td>JAK2</td>
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<td></td>
<td>Reverse 5′-CACAGGCCTATACACCACAGC-3′</td>
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<tr>
<td>STAT3</td>
<td>Forward 5′-TGTGACGACCTTGATGC-3′</td>
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<td></td>
<td>Reverse 5′-TGCCCCAGATGGCCAAAGAT-3′</td>
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<tr>
<td>β-actin</td>
<td>Forward 5′-AGTGTGAGCTGGATGCAGTCT-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse 5′-AGCTAGCAGTCCCGCTA-3′</td>
</tr>
</tbody>
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2.8 Protein extraction and western blot analysis
Total protein was extracted by Western and IP (Beyotime Biotechnology, China) cell lysis buffer supplemented with PMSF protease inhibitor. The supernatant was obtained by centrifugation at 12000 rpm. The protein concentration was identified using the BCA assay kit (WANLEI, China). Protein was subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions on 10% and 12% gels. Then, the gels transferred to PVDF membranes at 200 mA for 30–75 min in Tris-glycine buffer containing 20% methanol. Then seal the membrane with 5% skimmed milk at 37°C for 2 h. Then incubate the membrane overnight with the diluted primary antibody (1: 1000); all the antibodies except for CD16(Bioss), CD206(Boster), and IL-1β(Bioss), were purchased from WANLEI Biotechnology Co., Ltd. (Shenyang, China). Following incubation with horseradish peroxidase- (HRP-) conjugated goat anti-rabbit IgG (1: 8000, Bioss), the band were detected using the SH-523 system. Grayscale analysis of the results was performed using ImageJ software.

2.9 Statistical analysis

Statistical analysis and graph generation were performed for all data using SPSS version 23.0 software and GraphPad Prism version 8.0 software. The software showed a normal distribution and passed equal variance testing. All results were expressed as the mean ± SEM. One-tailed Student’s t-tests and one-way ANOVA with Duncan’s new multiple range tests were used. $p < 0.05$ was determined as a statistically significant difference.

3. Result

3.1 PS-NPs exposure exacerbates LPS-induced lung inflammation in mice

As shown in Fig. 1A, the SEM images show the particle size distribution of the PS-NPs, where it was observed that they had the same shapes and smooth spherical surfaces. The average diameter of the PS-NPs is 90.9 ± 16.3 nm, which is consistent with the size defined by nanoplastics (Gigault et al. 2018), which can be used for subsequent experiments. To visualize the toxic effects of PS-NPs on an LPS-induced mouse lung inflammation model, the lungs of mice were histologically examined after intraperitoneal injection of PS-NPs and/or LPS. We examined the pathological changes in the lungs of mice after PS-NPs and/or LPS treatment. H&E staining of lung tissue confirms our conjecture. As shown in Fig. 1B, we found that in the control lungs, the alveolar structure was clear and the alveolar septa were intact. In contrast, after PS-NPs treatment, the alveoli were destroyed, the alveolar walls were slightly thickened, and there was a small amount of inflammatory cell infiltration in the septa. The alveoli in the LPS group were collapsed and infiltrated by inflammatory cells. After LPS + PS treatment, the alveolar walls collapsed and adhered, the number of alveoli was reduced, the alveolar septa had obvious congestion and edema, and a large number of inflammatory cells infiltrated. All observations confirmed that continuous PS-NPs stimulation aggravated the structural disruption and tissue damage in the lungs of LPS-treated mice.
After confirming that PS-NPs and/or LPS exposure caused histological damage to mouse lung tissue, we further assessed the expression levels of inflammation-related genes in mouse lung (Fig. 1C-D). We detected the mRNA and protein levels of genes related to inflammation. The results showed that compared with the control group, the expression levels of IL-1β, TNF-α, and IL-6 were further increased in the PS group ($p < 0.05$). The expression levels of IL-1β and TNF-α in the LPS and PS co-exposed groups were significantly higher than those in the LPS or PS-NPs group alone ($p < 0.05$). Similarly, in the LPS + PS group, the protein expression level of IL-6 was twice that of the LPS group ($p < 0.05$), indicating that PS-NPs had a promoting effect on the inflammatory response. Taken together, these results suggest that PS is able to induce lung inflammation and exacerbate LPS-induced lung inflammation in mice. These results indicated that the model of PS-NPs aggravating LPS-induced lung injury was successfully established.

### 3.2 PS-NPs exposure aggravates LPS-induced inflammation in RAW264.7

Likewise, we examined the effect of PS-NPs on the toxic effects of LPS in RAW264.7. In the results of this study, the mRNA and protein expression levels associated with inflammation in RAW264.7 cells were the same as those in the in vivo experiments. The result shows, compared with the control group, the gene expressions of IL-1β, IL-6, and TNF-α were significantly up-regulated in the PS group, the LPS group, and the LPS + PS group ($p < 0.05$) (Fig. 1E); Moreover, the protein and mRNA levels of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α in the LPS + PS group were significantly higher than those in the LPS group alone ($p < 0.05$), and similar observations were obtained at the protein level. (Fig. 1F). These results suggest that PS is able to induce inflammation in RAW264.7 and exacerbate LPS-induced lung inflammation.

### 3.3 PS-NPs exposure promotes LPS-induced enhanced M1 polarization and M1/M2 imbalance in mouse lung macrophages

To investigate whether PS-NPs exposure has an effect on the polarization of lung macrophages and whether PS-NPs synergize with LPS in this regard. We selected M1 macrophage surface markers CD16, CD86 and MCP-1 for study. The results show, we found that compared with normal mice, the protein expression levels of M1 macrophage marker molecules CD16, CD86 and MCP-1 were significantly increased in PS-NPs or LPS-treated mice ($p < 0.05$) (Fig. 2A), which was the same as the changes in mRNA expression levels (Fig. 2B). This result indicated that the lung macrophages were polarized in the direction of M1. In addition, in the PS-NPs and LPS co-treatment group, the expression of M1-related marker molecules was significantly higher than that in the LPS alone treatment group, indicating that PS-NPs and LPS have the same effect, promoting the polarization of M1 macrophages. Meanwhile, compared with normal mice, the protein and mRNA levels of M2 macrophage markers CD206, PPARγ, Arg1 were decreased after PS-NPs and/or LPS treatment ($p < 0.05$) (Fig. 2C-D), which indicated that PS-NPs exposure would aggravate the M1/M2 imbalance in lung macrophages.
Through western blot analysis, we observed that the protein expression and mRNA levels of CD16, CD86, and MCP1 in RAW264.7 increased significantly after PS-NPs treatment ($p < 0.05$) (Fig. 2E, 3A). Compared with the LPS group, the expression level of the LPS + PS group also increased significantly, showing M1 Macrophage phenotype ($p < 0.05$). This result is consistent with the in vivo results that PS-NPs promoted LPS-induced polarization of M1 macrophages. On the other hand, M2 macrophage markers such as CD206, PPARγ, Arg1 did not change significantly or even slightly decreased ($p < 0.05$) (Fig. 3B-C). Overall, an M1/M2 imbalance appeared in RAW264.7 cells after PS-NPs and/or LPS treatment.

### 3.4 PS-NPs and LPS promote lung oxidative stress in mice

To illustrate the effect of PS-NPs on oxidative stress in the lungs of mice, we examined the activities of SOD and CAT and the concentrations of GSH and MDA in the lungs. As shown in Fig. 3D, after 2 weeks of exposure, the antioxidant index SOD and CAT activities in the lungs of all treatment groups were significantly decreased compared with the control group ($p < 0.05$), respectively. Compared with the control group, the concentration of GSH in the lungs of each exposure group decreased by the same level ($p < 0.05$). In addition, MDA levels in the lungs of the PS-NPs group were increased after 2 weeks of exposure ($p < 0.05$) compared to the observations in the control group. The levels of MDA in the lungs of the LPS + PS group were significantly higher than those of the groups exposed to PS-NPs or LPS alone ($p < 0.05$). These results suggest that PS-NPs exposure attenuated the antioxidant capacity of mouse lungs.

Similarly, PS-NPs exposure in RAW264.7 also induced oxidative stress in mouse lungs, we induced ROS generation using PS-NPs and/or LPS, respectively, in RAW264.7 cells (Fig. 4A). The results showed that ROS levels were significantly elevated in the PS group ($p < 0.05$). Moreover, the combined exposure of PS-NPs and LPS induced significantly higher ROS production than either PS-NPs or LPS alone ($p < 0.05$). Taken together, these results suggest that PS-NPs and LPS synergistically induce oxidative stress in RAW264.7 cells.

### 3.5 Activation of JAK2/STAT3 pathway in mouse lung tissue induced by PS-NPs and LPS

After we established that PS-NPs exposure could induce oxidative stress, we next examined the activation of a downstream pathway, namely the JAK/STAT pathway. To verify whether the JAK2/STAT3 pathway is involved in PS-NPs-induced lung inflammation, as shown in Fig. 4B and 4C, we first examined the expression of JAK2/STAT3-related genes and proteins in mouse lungs. Quantitative protein analysis showed that compared with the Control group, the phosphorylation levels of JAK2 and STAT3 in the PS group were increased by nearly 2-fold and 2.07-fold, respectively ($p < 0.05$) (Fig. 4C). Similarly, compared with the LPS group, the phosphorylation levels of JAK2 and STAT3 in the LPS + PS group were increased by nearly 1.31-fold and 1.54-fold, respectively ($p < 0.05$) (Fig. 4C). The mRNA levels of JAK2 and STAT3 in the PS-NPs and LPS groups were significantly increased, and were increased by about 1.4-fold and 1.56-fold compared with the Control group ($p < 0.05$) (Fig. 4B). The mRNA levels of JAK2 and STAT3 in the LPS + PS group were also significantly increased, about 2.4-fold higher than those of the LPS group, and
about 2.66-fold higher than those of the PS group ($p < 0.05$) (Fig. 4B). Our results suggest that the activation of the JAK/STAT pathway may be involved in the mechanism of PS-NPs' pulmonary toxicity in mice.

### 3.6 Inhibition of JAK2 can reduce PS-NPs and LPS-induced macrophage polarization and inflammatory response

Finally, we verified whether the JAK2/STAT3 pathway is involved in PS-NPs-induced lung inflammation. As shown in Fig. 4D and 4E, in the in vitro experiments, the mRNA levels of both JAK2 and STAT3 were significantly increased in the PS and/or LPS groups compared with the Control group. Next, we validated the JAK2/STAT3 pathway using the JAK2 inhibitor AG490 and added AG490 to the LPS + PS-treated group. The results showed that compared with the LPS + PS group, the gene expression levels of JAK2 and STAT3 in the LPS + PS + AG490 group were significantly decreased ($p < 0.05$), the phosphorylation levels of JAK2 and STAT3 proteins were also significantly decreased, and the JAK2/STAT3 pathway was significantly affected (Fig. 4D). At the same time, the mRNA and protein of pro-inflammatory cytokines (including IL-1β, TNF-α and IL-6) and M1 macrophage-related markers (including CD16, CD86 and MCP1) in LPS + PS + AG490 group RAW264.7 The expression level was significantly lower than that in the LPS + PS group ($p < 0.05$), while the expression of M2 macrophage-related markers (including CD206, PPARγ and Arg1) was increased ($p < 0.05$) (Fig. 1E-F, 2E, 3A-C), which indicates that the JAK/STAT signaling pathway is highly positively correlated with M1 macrophage-related polarization markers and pro-inflammatory cytokines, but negatively correlated with M2 macrophage-related polarization markers. So the JAK2/STAT3 pathway is involved in PS-NPs promote LPS-induced M1 macrophage polarization and inflammatory response.

### 4. Discussion

As an emerging pollutant, nanoplastics have attracted more and more attention for their potential toxicity to aquatic organisms and mammals (Prata et al. 2020). PS-NPs aggravated the endocrine disrupting effect of triphenyl phosphate (TPhP) in zebrafish (He et al. 2021) and increased the developmental toxicity of 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47) (Wang et al. 2022), also able to increase (synergistically) heavy metal toxicity in fish (Santos et al. 2022). Many studies have detected the adsorption and accumulation of chemical pollutants on MPs in the surrounding environment, but relatively speaking, the more important toxicity mechanism of NPs is chemical toxicity (Machado et al. 2021). Lipopolysaccharide (LPS), a major component of the outer cell membrane of Gram-negative bacteria, can effectively induce systemic inflammatory responses. However, the mechanism of toxicity of PS-NPs and LPS to systemic tissues and organs in mice is still very limited. Intraperitoneal injection of LPS is a widely used model to study chronic lung injury. After intraperitoneal lipopolysaccharide (LPS) administration, the proportion of lung macrophages gradually increases during inflammation, preventing harmful substances from entering the lungs from the bloodstream (Sajti et al. 2020). Therefore, this study explored the toxicity mechanism of PS-NPs and/or LPS exposure to pulmonary macrophage polarization in pulmonary inflammation in mice. We selected C57BL6 male mice exposed to 100 nm PS-NPs and/or
LPS as an experimental model. Herein, we demonstrated that PS-NPs exposure can aggravate LPS-induced excessive oxidative stress, activate JAK/STAT signaling to promote M1 polarization of macrophages, which in turn secrete pro-inflammatory cytokines, promote the development of inflammation, and ultimately lead to lung injury. Furthermore, JAK2 inhibitor AG490 inhibition decreased JAK2 expression in RAW264.7, inhibited M1 macrophage polarization and downregulated proinflammatory factor levels. JAK2/STAT3 signaling pathway is an important pathway for PS-NPs to aggravate LPS-induced lung injury.

Previous studies have shown that NPs are easily inhaled by humans and other terrestrial animals and cause local biological responses in respiratory mucosa and lung tissue. PS-NPs (25 nm and 70 nm) were taken up in human alveolar epithelial A549 cells, triggering a TNF-α-related apoptotic pathway and arresting the cell cycle in S phase(Xu et al. 2019). The expression of inflammatory proteins (TGF-β and TNF-α) in lung tissue increased in a concentration-dependent manner of PS-NPs(3.75, 15, or 60 mg/kg body weight) inhalation exposure. Tracheal instillation of PS-MPs impairs airway epithelial barrier integrity and induces lung injury and inflammation after inhalation exposure(Fan et al. 2022). However, there are no reports on the toxic effects of PS-NPs in inflammatory environments. In this study, lung injury was induced by intraperitoneal injection of LPS, and LPS stimulation led to the induction of systemic inflammation, including lung, liver, spleen, and gut (Unpublished results), indicating that the inflammation model was successfully constructed. We found that co-exposure of PS-NPs and LPS synergistically induced an inflammatory response. Although many studies on nanoparticle toxicity have been conducted, more precise molecular biology studies are needed to explore the relevant mechanisms. Lung exposure to environmental pollutants generates ROS and promotes redox reactions in cells, leading to the production of oxidative stress and mediators of inflammation(Wen et al. 2021). Silica nanoparticles induce oxidative stress in dose-dependent manner indicated by induction of ROS generation, and membrane lipid peroxidation (LPO) in A549(Akhtar et al. 2010). One of the markers used to assess LPO in an organism is malondialdehyde (MDA) levels, and cadmium can induce oxidative stress by increasing ROS and MDA levels and reducing T-SOD and CAT activities(Chen et al. 2022). SOD systems are often considered to be the first line of defense in producing ROS. Superoxide radical (O$_2^-$) can be converted into H$_2$O$_2$ by SOD catalysis to prevent lipid peroxidation(Gottfredsen et al. 2013). The decrease in SOD activity may indicate inhibition of SOD enzymes for ROS production(Park et al. 2020). Therefore, to gain insight into the mechanism of PS-NPs toxicity to mouse lungs, we investigated changes in oxidative stress-related factors. Our results showed that PS-NPs induced up-regulation of MDA, down-regulation of SOD, and decreased levels of antioxidant defense systems. At the same time, many nanoparticles can induce cell death through increased ROS-mediated cell damage(Babaei et al. 2022). The uptake of NPs by tissue cells leads to the loss of cell membrane integrity, and the dysfunction of lysosomes and mitochondria increases the production of intracellular ROS(Li et al. 2019, Yang et al. 2009). Previous studies have shown that nanoplastics can promote the production of ROS in the intestines, liver, and kidneys of mice, and induce oxidative stress in macrophages(Choi et al. 2021, Liang et al. 2021, Lu et al. 2016). Similar to the above findings, our study shows that PS-NPs contribute to pulmonary oxidative stress by regulating the levels of MDA, GSH-PX, CAT, and SOD. At the same time, the ROS level of RAW264.7 after PS-NPs
treatment was also significantly increased. These observations confirmed that PS-NPs exposure induced damage through oxidative stress. This result is consistent with the findings of Yang et al. (Yang et al. 2021), who suggested that PS-NPs can disrupt redox balance and induce inflammation. A ROS-induced state of peroxidation caused by intracellular redox imbalance, leading to the production of superoxide (Valavanidis et al. 2013). The generated peroxides can damage the cell structure and affect the physiological functions of normal cells, which further induces changes in related signaling pathways (Hu & Palić 2020).

ROS have been shown to activate multiple signaling pathways leading to tissue damage including JAK/STAT, NF-κB, and MAPK (Rendra et al. 2019). ROS are essential for the production of inflammatory mediators such as NO, cytokines and chemokines through STAT1 activation (Yang et al. 2013). Amandine Charras et al. showed that ROS induces the expression of ICAM-1 and PD-L1 by activating STAT3 (Charras et al. 2019). The researchers demonstrated that JAK tyrosine kinases are activated by ROS to phosphorylate STAT, form homodimers or heterodimers, and translocate from the cytoplasm to the nucleus, where they bind specific regulatory sequences to activate or repress target gene expression transcription and expression (Valdembri et al. 2002, Yan et al. 2018). Studies have found that JAK signal transduction and STAT pathway are closely related to lung diseases. Increased levels of JAK correlate with enhanced K-RAS activity in human lung adenocarcinoma patient samples and promote the proliferation of K-RAS mutant lung adenocarcinomas. Additionally, we verified that PS-NPs could activate the JAK/STAT signaling pathway in mouse lungs to promote LPS-induced lung injury. To further confirm the above findings, we determined that the damage induced by PS-NPs in RAW264.7 could be initiated by the JAK/STAT pathway by examining the indicators of the JAK/STAT pathway. Our findings suggest that the activation of ROS/JAK/STAT plays an important role in the promotion of LPS-induced Macrophage M1 polarization by PS-NPs exposure. However, this study only proves that PS-NPs can regulate the expression of ROS/JAK/STAT, but whether ROS is a direct upstream gene of JAK/STAT, that is, whether ROS directly regulates the JAK/STAT pathway is unclear.

Macrophage polarization is associated with the occurrence and development of various diseases, and also produces various cytokines to affect other immune cells (Hu et al. 2019). Therefore, it is of great significance to study the mechanism of macrophage polarization. In this study, PS-NPs were exposed by intraperitoneal injection, so that PS-NPs rapidly accumulated in the abdominal cavity, absorbed into the blood, and acted on the tissues through the circulatory system. Macrophages readily detect external stimuli and homeostatic changes to prevent lesions and maintain general cellular functions, therefore, we focused on the injury mechanism of PS-NPs in LPS-induced inflammation by causing lung macrophage polarization. Classical M1 macrophages are activated to produce pro-inflammatory cytokines/chemokines and a range of cytotoxic molecules as cellular mediators of acute and chronic inflammation (Zhao et al. 2019), the activated M1 phenotype can be induced in vitro by LPS to express various pro-inflammatory factors, such as IL-1β, iNOS and CD16 (Zhang et al. 2019). MCP-1 is a chemokine that induces regulation of immune cell recruitment and monocyte/macrophage infiltration, and polychlorinated biphenyls (PCB) PCB 126 can elevate MCP-1 expression to promote macrophage polarization and inflammation (Wang et al. 2019a). Consistent with previous studies, our data showed
that PS-NPs could induce the expression of macrophage M1 polarization markers CD16, CD86 and MCP-1 and enhance the effect of LPS on macrophage M1 polarization. In addition, the expressions of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 were also significantly increased, and the collapse of alveolar spaces and the infiltration of inflammatory cells further confirmed that the lungs of mice were in a pro-inflammatory stage. These findings support the notion that PS-NPs further potentiate LPS-induced lung inflammation in mice through M1/M2 macrophage imbalance. Mouse leukemia cells of monocyte macrophage RAW264.7 cells are a well-established model of inflammation and oxidative stress and are used in many studies of pulmonary toxicology. Therefore, we examined the polarization direction of macrophages upon exposure to PS-NPs and/or LPS in vitro using RAW264.7 cells. Consistent with the above results, PS-NPs were able to induce ROS generation and enhance LPS-stimulated macrophage M1 polarization.

To further explore the molecular mechanism of PS-NPs toxicity, we investigated in vitro whether JAK/STAT is an essential pathway for PS-NPs to promote LPS-induced lung injury. The JAK/STAT signaling pathway is involved in various physiological processes, including immune function, cell growth, differentiation, and death (Chen et al. 2020), and it has been described as an important signaling axis in macrophage biology (Arora et al. 2018). Therefore, we examined whether PS-NPs could activate the JAK/STAT signaling pathway in mouse lung. In our study, changes in the gene levels of JAK2 and STAT3 and increased phosphorylation of JAK2 and STAT3 indicated the activation of the JAK/STAT pathway, while the JAK2-specific inhibitor AG490 significantly attenuated the phosphorylation of JAK2 and STAT3 in vitro. When the JAK2 inhibitor AG490 blocked the activation of the JAK/STAT pathway, the levels of M1 polarization marker molecules in the LPS + PS group were also inhibited to a certain extent, while the expression of M2 polarization marker molecules was alleviated to a certain extent. Our study showed that PS-NPs exposure enhanced LPS-induced inflammatory cytokine production in mouse lungs via activation of JAK/STAT signaling pathway. Many drugs modulate disease progression by inhibiting JAK-STAT signaling during immune responses and inflammation. baikalin alleviated I/R myocardial injury and reduced inflammation via JAK/STAT pathway (Xu et al. 2020a). Aloin attenuates LPS-induced inflammation by inhibiting ROS-mediated activation of the JAK-STAT signaling pathway (Ma et al. 2018). Cirsimarin downregulates phosphorylation of JAK/STATs and p38/MAPK, and interferon regulatory factor (IRF)-3 exerts anti-inflammatory effects (Han et al. 2018). Resokaempferol inhibits the inflammatory response of LPS- or IL-6-activated macrophages by blocking the activation of the JAK2/STAT3 pathway (Yu et al. 2016). Therefore, the JAK/STAT pathway may serve as an important therapeutic target for the inflammatory pathway of lung injury.

In conclusion, we found that PS-NPs exposure induced increased M1 macrophage polarization, which subsequently increased LPS-induced inflammatory responses. And we also found that the antioxidant capacity of mice was reduced, and the expression of JAK/STAT pathway was increased. Treatment with a JAK2 inhibitor (AG490) resulted in improved macrophage M1/M2 balance and inflammatory responses, which are critical for understanding the mechanisms of PS-NPs toxicity. The above results suggest that PS-NPs promote LPS-induced M1/M2 imbalance through the ROS/JAK/STAT pathway and increase
inflammation. The results obtained in this study provide new information about the potential risks of NPs exposure in mouse lungs.

**Declarations**

**Statements & Declarations**

**Ethics approval and consent to participate**

All procedures used in this study were approved by the Northeastern Agricultural University Institutional Animal Care and Use Committee under approved protocol number SRM-11.

**Consent for publication**

Not applicable

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The authors have no relevant financial or non-financial interests to disclose.

**Author contributions**


**Data Availability Statement**

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

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Figures
PS-NPs exposure aggravates LPS-induced lung inflammation in mice. (A) Microscopic characterization of PS-NPs (scale bar, 200 nm). (B) Histopathological changes of mouse lungs (scale bar, 20 μm). (C) Expression of protein (C) and mRNA (D) of inflammation-related genes in mouse lungs (n=3; the significant difference (p<0.05) between bars that do not share the same letter). (E) (F) The protein (E) and mRNA (F) expression of inflammation-related genes in RAW264.7 (n=3; the significant difference (p<0.05)
between bars that do not share the same letter). Results are presented as mean ± SEM. Statistical significance was obtained using one-way analysis of variance (ANOVA) or unpaired Student's t-test.

Figure 2

PS-NPs exposure promotes LPS-induced M1 polarization of mouse lung macrophages. (A) (B) Expression of M1 macrophage marker protein (A) and mRNA (B) in mouse lungs (n=3; the significant difference ($p < 0.05$) between bars that do not share the same letter). (C) (D) Expression of M2 macrophage marker
protein (C) and mRNA (D) in mouse lungs (n=3; the significant difference ($p < 0.05$) between bars that do not share the same letter). (E) Expression of M1 macrophage marker protein (E) in RAW264.7 (n=3; the significant difference ($p < 0.05$) between bars that do not share the same letter). Results are presented as mean ± SEM. Statistical significance was analyzed by one-way ANOVA or unpaired Student's t-test.
PS-NPs exposure promotes LPS-induced M1 polarization of RAW264.7. (A) The gene expression levels of M1 macrophage markers after PS-NPs and LPS treatment of RAW264.7 (n=3; the significant difference (p<0.05) between bars that do not share the same letter). (B) (C) The protein and gene expression levels of M2 macrophage markers after PS-NPs and LPS treatment of RAW264.7 (n=3; the significant difference (p<0.05) between bars that do not share the same letter). (D) Indicators of oxidative stress in the lungs of mice exposed to PS-NPs for 2 weeks (n=3; the significant difference (p<0.05) between bars that do not share the same letter). Results are expressed as mean ± SEM. Statistics Significance was analyzed by one-way ANOVA or unpaired Student's t-test.
Figure 4

ROS/JAK/STAT participate in PS-NPs-induced inflammation in RAW264.7. (A) Fluorescence microscopy and ImageJ analysis of ROS (green) generation in RAW264.7 (scale bar, 200 µm). (B) (C) The gene (D) and proteins (E) expression of JAK/STAT pathway-related genes in PS-NPs and/or LPS-exposed mice lung (n=3; the significant difference ($p<0.05$) between bars that do not share the same letter). (D) (E) The proteins (D) and gene expression (E) of JAK/STAT pathway-related genes in PS-NPs and/or LPS-exposed RAW264.7 (n=3; the significant difference ($p<0.05$) between bars that do not share the same letter).

Results are presented as mean ± SEM. Statistical significance was analyzed by one-way ANOVA or unpaired Student's t-test.

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