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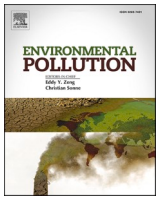

# Short-term exposure to fine particulate matter exposure impairs innate immune and inflammatory responses to a pathogen stimulus: A functional study in the zebrafish model☆

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ABSTRACT

Short-term exposure to fine particulate matter ($PM_{2.5}$) is associated with the activation of adverse inflammatory responses, increasing the risk of developing acute respiratory diseases, such as those caused by pathogen infections. However, the functional mechanisms underlying this evidence remain unclear. In the present study, we generated a zebrafish model of short-term exposure to a specific $PM_{2.5}$, collected in the northern metropolitan area of Milan, Italy. First, we assessed the immunomodulatory effects of short-term $PM_{2.5}$ exposure and observed that it elicited pro-inflammatory effects by inducing the expression of cytokines and triggering hyper-activation of both neutrophil and macrophage cell populations. Moreover, we examined the impact of a secondary infectious pro-inflammatory stimulus induced through the injection of *Pseudomonas aeruginosa* lipopolysaccharide (*Pa*-LPS) molecules after exposure to short-term $PM_{2.5}$. In this model, we demonstrated that the innate immune response was less responsive to a second pro-inflammatory infectious stimulus. Indeed, larvae exhibited dampened leukocyte activation and impaired production of reactive oxygen species. The obtained results indicate that short-term $PM_{2.5}$ exposure alters the immune microenvironment and affects the inflammatory processes, thus potentially weakening the resistance to pathogen infections.

## 1. Introduction

According to the World Health Organization (WHO), fine particulate matter -which refers to particulate matter with an aerodynamic diameter of ≤2.5 μm ($PM_{2.5}$)- is considered the leading environmental risk factor associated with a variety of diseases, such as cardiovascular disease, respiratory disease, cancer, and respiratory infections (WHO, 2022). These conditions all share a commonality: altered inflammatory and immune processes play a crucial role in their pathogenesis (Carugno et al., 2018; Nhung et al., 2017; Beelen et al., 2014; Burnett et al., 2018; Rush et al., 2017).

$PM_{2.5}$ enters the upper airway and can reach the bronchioles and alveoli and the PM particles with an aerodynamic diameter of ≤1 μm (i.e., $PM_1$) are even capable of entering the bloodstream (Xing et al., 2016; Yang et al., 2020). As a result, they can induce not only a local inflammatory response but also a systemic one (Li et al., 2022). Indeed, $PM_{2.5}$ exposure has been associated with increasing circulating levels of different biomarkers of inflammation, such as interleukins, fibrinogen, C-reactive protein (CRP), oxidative stress markers (e.g. 8-Iso-Prostaglanding F2α (8-iso-PGF2α), and activated innate immune cells such as neutrophils and macrophages (Garcia et al., 2023; Pietrogrande et al., 2023; Zhu et al., 2021). On the other hand, exposure to $PM_{2.5}$ has also been associated with local increases in inflammatory and oxidative stress biomarkers, along with the activation of infiltrating macrophages at the airway level (Guo et al., 2022; He et al., 2017; Laursen et al., 2023).

Emerging evidence suggests that chronic exposure to both $PM_{2.5}$ and $PM_{10}$ (i.e., PM with an aerodynamic diameter of ≤10 μm) may have

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different functional effects compared to acute exposure (Tang et al., 2020). In particular, chronic exposure to $PM_{2.5}$ and $PM_{10}$ may contribute to the development of low-grade systemic chronic inflammation, which plays a crucial role in the pathogenesis of non-communicable diseases, such as respiratory and cardiometabolic conditions (Chen and Hoek, 2020). On the other hand, acute exposure to high $PM_{2.5}$ and $PM_{10}$ concentrations may exacerbate pre-symptomatic conditions, worsen the symptoms of established diseases, or foster pathogen infections (Carugno et al., 2018; Lim et al., 2016; Nhung et al., 2017). In this regard, in infants, the respiratory syncytial virus and *Streptococcus* infections are associated with short-term $PM_{10}$ and $PM_{2.5}$ exposure (Carugno et al., 2018; Milani et al., 2022; Mishra et al., 2020; Silva, 2010; Zhi et al., 2022). Thus, $PM_{2.5}$ exposure seems to impair the host defense of the respiratory system, making the body more susceptible to infections (Yang et al., 2020). However, the mechanisms that alter the immune response to acute pathogen stimuli (e.g., bacterial or viral infections) in the context of short-term $PM_{2.5}$ toxicity remain unclear (Carugno et al., 2018; Milani et al., 2022; Mishra et al., 2020; Silva, 2010; Zhi et al., 2022).

To unravel the underlying molecular mechanisms, in the present study, we generated a zebrafish model of short-term exposure to a specific $PM_{2.5}$ collected in the northern metropolitan area of Milan, Italy and previously characterized (Rovelli et al., 2021). In this model, we assessed the immunomodulatory effects of $PM_{2.5}$ exposure and further examined the impact of a secondary infectious pro-inflammatory stimulus induced through the injection of *Pseudomonas aeruginosa* lipopolysaccharide (*Pa*-LPS) molecules. Zebrafish has emerged as a powerful functional model for environmental and toxicological studies (Duan et al., 2017a; Floris et al., 2021; McGrath and Li, 2008), including the evaluation of the toxicity of environmental pollutants (Chakraborty et al., 2016; Smoot et al., 2022). This model offers the advantage of reducing both time and costs compared to the mammalian models, while also aligning with the 3R principle (Geisler et al., 2017). In addition, zebrafish represents an ideal model for studying various aspects of innate immunity and inflammation. During the first month of life, zebrafish possesses only the innate immune system and inflammatory/innate immune processes occur similarly to mammals with conserved cell populations (Chatzopoulou et al., 2016; Forn-Cuní et al., 2017; Meeker and Trede, 2008; Novoa and Figueras, 2012; Rosowski, 2020; Trede et al., 2004). Taken together, these features enable a focus on innate immunity, which is particularly important in the initial response against environmental triggers (Paludan et al., 2021).

## 2. Materials and methods

### 2.1. Zebrafish husbandry

Zebrafish (*Danio rerio*) wild type strains AB (Wild type, Wilson lab, University College London, UK) and transgenic larvae for neutrophils *Tg BAC(mpx:EGFP)$^{i114}$* (known as *Tg(mpx:EGFP)*) (Renshaw et al., 2006) and macrophages *Tg(mpeg1:mcherry)* (Ellett et al., 2011), were maintained according to international (EU Directive, 2010/63/EU) and national guidelines (Italian decree No 26 of the 4$^{th}$ of March 2014) and raised at 28.5 °C on a 14 h light/10 h dark cycle, in the fish facility at the University of Milan, Via Celoria 26, 20133 Milan, Italy (Aut. Prot. N. 295/2012-A – December 20, 2012). Embryos were collected by natural spawning, staged according to Kimmel et al. (1995), and raised at 28.5 °C in E3 embryo/larvae growth medium (E3; Instant Ocean, 0.1% Methylene Blue) in Petri dishes, according to established techniques. Embryonic ages were expressed in h post fertilization (hpf) and days post fertilization (dpf). In the present study, exclusively embryos/larvae up to 120 hpf were used. To prevent pigmentation, from 24 hpf, 0.003% 1-phenyl-2-thiourea (PTU, Sigma Aldrich, St Luis, MO, USA) was added to the E3. Embryos were washed, manually or chemically dechorionated as described above, and anesthetized with 0.016% tricaine (Ethyl 3-aminobenzoate methanesulfonate salt; Sigma Aldrich) in E3, before observations, microinjection, tailfin amputation, and image acquisitions. For imaging analysis, unless otherwise stated, embryos were fixed for 2 h in 4% paraformaldehyde (PFA, Sigma) in Phosphate Buffer Saline (PBS, Sigma) at RT and then rinsed twice in PBS solution.

### 2.2. $PM_{2.5}$ sampling and preparation

The $PM_{2.5}$ used for the toxicological experiments in zebrafish larvae was collected during different sampling campaigns over three months between the end of winter and the beginning of spring 2019 (February–April 2019) at an outdoor urban background site in Como, a medium-sized provincial town in the Lombardy region (Northern Italy), belonging to the northern metropolitan area of Milan (about 40 km away). More in detail, the $PM_{2.5}$ sampling device consisted of a high-volume air sampler (Air Flow $PM_{2.5}$-HVS, Model 600/AFPM2501K, AMS Analitica, Pesaro, Italy) operated at a constant flow rate of 500 L/min (min) and equipped with polytetrafluoroethylene (PTFE)-coated glass fiber filters (PTFE coated borosilicate glass fiber substrate filters, 150-mm diameter, MTL/GP 150, Minneapolis, USA); a total of eight $PM_{2.5}$ samples (i.e., eight collected filters) were obtained from eight sampling campaigns of 24–96 h each. The filter substrates were then individually subjected to a sequential sonication procedure (four sequential 30-min cycles in a cold-water bath, $T < 5$ °C) using ultrapure water as the extraction medium, to allow the particles' dissolution and recovery from the collection substrates. All the obtained $PM_{2.5}$ suspensions were subsequently combined to get a final $PM_{2.5}$ pooled extract, that was properly filtered and purified using hydrophilic nylon net filters with a mesh opening of 10 μm-size (10-μm Nylon Net Filters, 25-mm diameter, Merck Millipore Ltd.) to remove the glass fibers released during extraction. Following extraction and filtration, the $PM_{2.5}$ final solution was used to prepare several homogeneous sub-samples containing approximately 0.7–1.2 mg of a dried $PM_{2.5}$ residue each. All the $PM_{2.5}$ aliquots were finally stored at 4 °C until use for the in vivo experiments. Any further details concerning the optimized methodological approach for the collection, extraction, and chemical characterization of the local $PM_{2.5}$ was previously detailed in Rovelli et al. (2021). Just before use, each dried $PM_{2.5}$ aliquot was dissolved in the E3 embryo medium to reach a stock concentration of 700 μg/mL. To further dissolve completely the dried material, after the addition of the E3 medium, the glass vial was vortexed 2 times for 30 s and then the resulting PM suspension was subjected to two sequential sonication processes at 30 kHz (30 s each) to obtain a clean homogeneous $PM_{2.5}$ suspension of a straw-yellow color. Stereomicroscopic observation of the suspension was performed to rule out aggregates or parts of the undissolved sample.

### 2.3. Embryo treatments with $PM_{2.5}$

Embryos at 30 hpf were manually dechorionated, if necessary, and divided into groups of 30–35. Each group was transferred into a 6-well plate in a total volume of 4 mL of E3 plus PTU added with $PM_{2.5}$ suspension at a final concentration of 15, 30, 45, 75, or 100 μg/mL per well. Control unexposed embryos were kept in E3 plus PTU without the addition of $PM_{2.5}$ suspension. To avoid particle precipitation, immediately after the addition of $PM_{2.5}$ suspension, the plate was shaken for 1 min, and then the embryos were incubated at 28.5 °C for the indicated time. At 18 h post-exposure (hpe) (2 dpf) embryos were observed under a stereomicroscope to assess the mortality rate and to check for the precipitation of PM inside the well. For short-exposure setting experiments, embryos were thoroughly washed three times with E3 plus PTU, transferred into clean Petri dishes, and incubated at 28.5 °C in E3 plus PTU. Subsequently, 3 dpf larvae were observed and washed as stated above and live/dead and morphological analysis was performed under a stereomicroscope. For survival rate analysis, larvae were scored as dead when heartbeat was absent, necrosis effects were present and movement in response to touch was absent, as previously described (Cafora et al.,

2020).

### 2.4. Generation of inflammatory stimuli in zebrafish larvae

To generate an acute inflammatory stimulus, zebrafish larvae at 3 dpf (exposed or not to $PM_{2.5}$) were microinjected with 5 mg/mL of the lipopolysaccharide of *Pseudomonas aeruginosa* (*Pa*-LPS) (derived from strain ATCC 27316, Sigma Aldrich). For gene expression experiments, to obtain systemic delivery, 2 nL of *Pa*-LPS were microinjected into the duct of Cuvier (therefore into the circulation) of larvae, as previously described (Cafora et al., 2020b). To conduct expression analyses, total RNA was obtained from a minimum of 15 whole larvae that underwent systemic injection, collected 8 h after the injection. For leukocyte recruitment experiments, *Tg(mpx:EGFP)* and *Tg(mpeg1:mcherry)* larvae were locally injected with 1 nL of *Pa*-LPS suspension intramuscularly, as previously described (Kwon et al., 2021; Phan et al., 2018). *Pa*-LPS was delivered into the skeletal muscle of the trunk region, in the area within the second and the fifth somite before the yolk extension. 10–12 larvae were injected for each condition. As a control, larvae were systemically/locally injected with PBS. Systemically/locally injected larvae were incubated at 28.5 °C in E3 plus PTU for the indicated time until the immune response analysis. To generate zebrafish sterile local inflammatory stimulus, 3 dpf larvae (exposed or not to $PM_{2.5}$) were amputated of a portion of the tailfin with the use of a sterile scalpel blade, without damaging the circulatory loop, as previously described (Cafora et al., 2020a; Chatzopoulou et al., 2016). The properly amputated larvae were selected for the assay and incubated at 28.5 °C in E3 plus PTU. After 6 h post-amputation (hpa) leukocyte recruitment in the caudal area of the tailfin was assessed.

### 2.5. Image acquisition and quantification of total/local leukocytes

To characterize neutrophil and macrophage activation following $PM_{2.5}$ exposure and/or post-inflammatory trigger, larvae from the *Tg(mpx:EGFP)* and *Tg(mpeg1:mcherry)* zebrafish transgenic reporter lines were fixed in 4% PFA in PBS for 2 h at RT. Single slice bright-field and fluorescent images of the lateral/dorsal side of larvae were sequentially acquired using an epi-fluorescence stereomicroscope (M205FA, Leica, Germany) equipped with a fluorescent lamp and a digital camera and mounting mCherry-filter (excitation 587 nm) and GFP-filter (excitation of 488 nm). Images were processed using Adobe software, merging different focal planes when necessary. Neutrophils and macrophages were quantified as $mpx^+$ and $mpeg1^+$ cell count, respectively. For $PM_{2.5}$-exposure experiments, quantification analysis was performed on the whole embryo (total leukocyte count), in the head region (dashed box) at 18 and 42 hpe, and 6 h after amputation (hpa) in the tailfin amputation model. Cell count was performed by computation using Fiji (ImageJ software, developer: Wayne Rasband) as described by Ellett and Lieschke (2012); Greco et al. (2022). To avoid including false positives due to autofluorescence of the yolk, the "subtract background" function with a proper "rolling ball" setting was used, as described also previously (Ellett and Lieschke, 2012; Greco et al., 2022). Cell count was performed with the "Find maxima" function. The mean and standard error of the mean (SEM) of cell count of at least two independent experiments were reported on graphs.

### 2.6. Analysis of reactive oxygen species (ROS) production in zebrafish larvae

ROS production was assessed in 3 dpf *Tg(mpx:EGFP)* larvae exposed or not to $PM_{2.5}$ and stimulated by intramuscular injection of *Pa*-LPS. ROS analysis 4 h post-injection (hpi) was performed using the DHE kit (dihydroethidium, Santa Cruz Biotechnology, US) as previously described (Phan et al., 2018). Quantification of neutrophils actively producing ROS ($mpx^+$ $DHE^+$ cell count) was extrapolated as a co-localization signal between red cells (ROS producers) related to green cells (neutrophils) through computation using Fiji, by using the "color threshold" function to extrapolate $mpx^+$ $DHE^+$ cells. The relative percent of neutrophils actively producing ROS was extrapolated by considering the ratio of $mpx^+$ $DHE^+$ cells and local neutrophil count ($mpx^+$ $DHE^+$/local $mpx^+$).

### 2.7. Gene expression analysis

mRNA expression levels were assessed by reverse transcription and real-time quantitative-PCR (RT-qPCR) assays. Total RNA was extracted from groups of 15–20 zebrafish whole larvae/condition using NucleoZOL reagent (Macherey-Nagel, Germany) according to the manufacturer's instructions. 1 μg of RNA was reverse-transcribed using the "ImProm II™ Reverse Transcription System" (Promega), according to the manufacturer's instructions. qPCRs were carried out in a total volume of 10 μl containing 1X iQ SYBR Green Super Mix (Promega) using the QuantStudio 5 Real-Time PCR System (Applied Biosystems, ThermoFisher Scientific). Thermocycling conditions were 95 °C for 10 min, 95 °C for 10 s (sec), and 55 °C for 30 s. All reactions were performed at least in triplicate for 40 cycles. The relative expression level of each gene was calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). For normalization purposes, *rpl8* and *β-actin* genes were used as internal reference genes. Primers used in the analyses are listed in Supplementary Table S1. The mean and SEM of at least three independent experiments were reported on graphs.

### 2.8. Statistical analysis

The statistical analyses were performed using GraphPad (Prism) software version 8.0.2 for Windows. The normal distribution of all datasets was guaranteed by the Kolmogorov-Smirnov or Shapiro-Wilk normality test. Outliers' data were excluded from analyses. In particular, the ROUT method on GraphPad Prism software was used, which allows to detect the outliers in a dataset while fitting a curve with nonlinear regression. This method was set with low aggressivity (i.e., stringency), to determine and discard just definitive outliers. Moreover, a manual evaluation of outliers' exclusion was also performed, to exclude as little data as possible from the analysis. Specific statistical tests were used to evaluate the significance of differences between experimental groups: unpaired two-tailed *Student's t-test* with Welch correction when comparing two groups, One-sample *t-test* when comparing a group with a hypothetical value (when control group = 1) or ordinary one-way analysis of variance (ANOVA) followed by Tukey post hoc correction for multiple comparisons. The results represented data derived from at least two (for cell count and area measurement analyses) or three (for qPCR molecular analyses) independent experiments and mean ± SEM was reported in the graphs. P-value$<$0.05 was defined to indicate statistically significant differences.

## 3. Results

### 3.1. Set up of zebrafish larvae exposure to $PM_{2.5}$

Embryos were exposed to $PM_{2.5}$ for 18 h, from 30 hpf to 48 hpf, and then the survival rate and morphology were analyzed at 42 hpe. Different concentrations of $PM_{2.5}$ were tested (i.e., 15, 30, 45, 75, and 100 μg/mL) (Fig. 1a). At 18 hpe no significant differences in embryo mortality were observed in embryos exposed to a concentration equal to or lower than 45 μg/mL in comparison to not-exposed control embryos. Similarly, at 42 hpe embryo mortality rate was not increased with concentrations of 15, 30, and 45 μg/mL, while the exposure to concentrations equal to or greater than 75 μg/mL resulted in a higher mortality rate (Fig. 1b). In addition, larvae that survived after the exposure to 75 μg/mL generally exhibited morphological alterations, with diffuse necrosis, yolk sac, pericardial edema, and muscle fiber disorganization (Fig. 1c). Accordingly, 45 μg/mL was set as the working

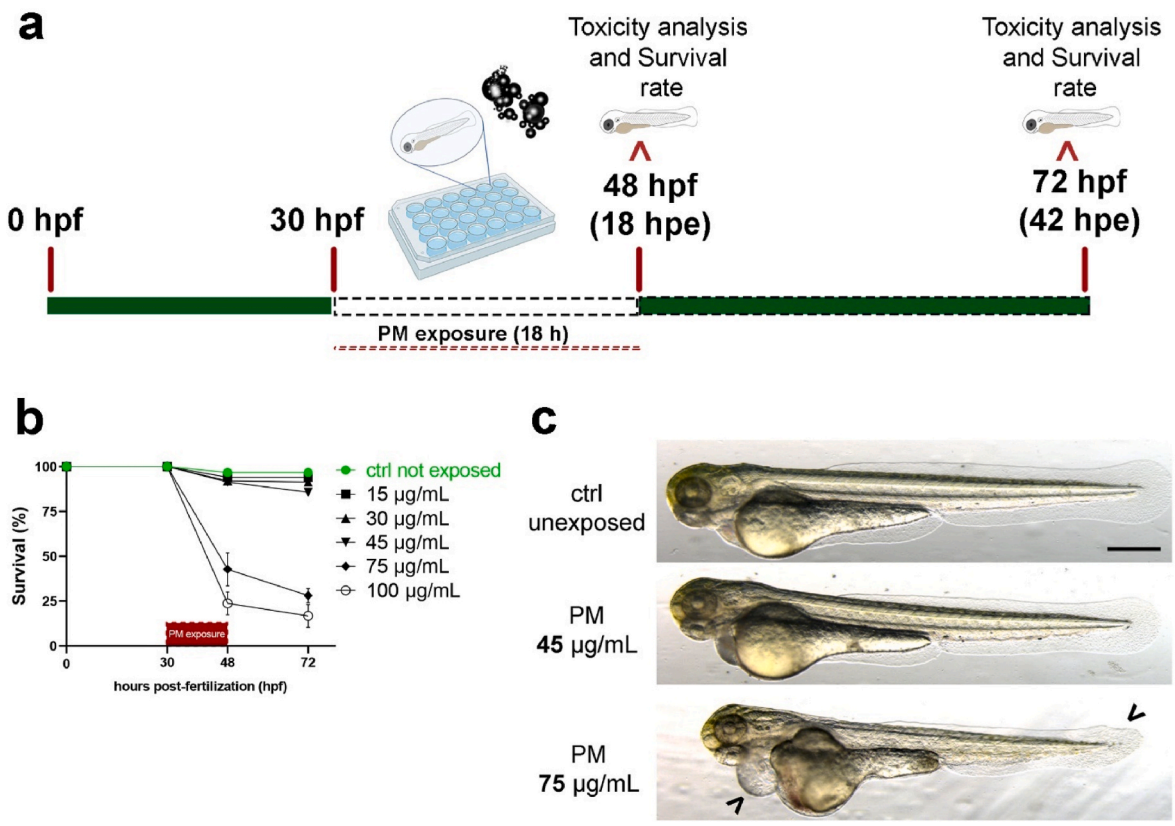


**Fig. 1.** Effects of PM on survival rate and morphology of zebrafish larvae. **(a)** Schematic representation of PM exposure setting. **(b)** Survival rate at 48 and 72 hpf (18 and 42 hpe, respectively) reported for different concentrations of $PM_{2.5}$; n = 30 (three biological replicates) **(c)** Representative image of 72 hpf larvae after exposure to different concentrations of PM; black arrowhead indicates morphological alterations of pericardial edema and tailfin. Scale bar = 400 µm.

concentration and 18 h as the exposure time limit for the following innate immune response analyses.

### 3.2. $PM_{2.5}$ exposure elicits leukocyte activation and inflammatory markers overexpression

To investigate the effects of $PM_{2.5}$ exposure on innate immune response, zebrafish larvae at 42 hpe were assessed for neutrophils and macrophage activation, as well as for expression of inflammatory genes (Fig. 2a). *Tg(mpx:EGFP)* and *Tg(mpeg1*:mcherry) zebrafish transgenic reporter lines, with green fluorescent neutrophils and red fluorescent macrophages respectively, were used to dissect the response of the two myeloid populations. Embryos exposed to $PM_{2.5}$ were analyzed and imaged under fluorescence stereomicroscope and leukocytes were counted. Both whole embryo and head region quantifications were performed to evaluate differential activation of leukocytes in sensitive regions (i.e., mouth and branchial arches) (Fig. 2b–e). The whole embryo average number of $mpx^+$ neutrophils/embryo increased at 42 hpe in a $PM_{2.5}$ concentration-dependent manner (i.e., controls, 87.1; 15 µg/mL, 110.7, *p-value = 0.0145*; 30 µg/mL, 111.0, *p-value = 0.0128*; 45 µg/mL, 119.9, *p-value < 0.0001*) (Fig. 2c). We investigated also neutrophil migration by measuring their recruitment to the head region of the larva after 48 hpe. An increase in neutrophil concentration in the head region was observed in larvae exposed to the higher concentration of $PM_{2.5}$ (45 µg/mL) compared to control embryos (*p-value = 0.0004*). Moreover, although not statistically significant, we also observed a tendency of neutrophil concentration in the head region for 15 and 30 µg/mL PM exposure concentrations (Fig. 2d). However, the ratio of the neutrophil count in the head over the whole embryo (i.e., head/total, %) did not show differences in larvae exposed to all concentrations of PM (Supplementary Figure S1 a). Considering macrophage activation, $mpeg^+$ macrophages were quantified at 42 hpe in *Tg(mpeg1:mcherry)* larvae, but no differences were observed in whole embryo count assay after exposure to 45 µg/mL of $PM_{2.5}$ (Fig. 2 f). On the contrary, the presence of macrophages in the head region increased in larvae exposed to 45 µg/mL of $PM_{2.5}$ compared with controls (i.e., 33.17 vs 39.40, *p-value* = 0.463) (Fig. 2 g), and, consequently, their relative percentage (i.e., 32.8% vs 36.8%, *p*-value = 0.361) (Figure S1 b).

We further investigated at the molecular level the above-described altered leukocyte activation. For this purpose, we assessed in parallel experiments the gene expression levels of pro-inflammatory cytokines and markers of endothelial activation on the whole larvae, exposed or not to 45 µg/mL. The mRNA expressions of *cxcl8,* the primary neutrophils chemotactic factor, and of the key pro-inflammatory cytokine *il-1β* were increased (*p-value = 0.0199* and *0.0456,* respectively) in PM-exposed larvae. Also, $PM_{2.5}$ exposure increased the expression of the pro-inflammatory cytokines *il-6* and *tnf-α* by about 6 and 3.5 times respectively (*p-value = 0.0017* and *0.0266,* respectively) in comparison to unexposed controls (Fig. 3 a-d), suggesting the induction of a robust pro-inflammatory state. Interestingly, also the expression levels of all analyzed endothelial markers were increased in PM-exposed embryos, in particular, *endothelin1* (*edn1*) (*p-value = 0.0057*), *endoglin* (*eng*) (*p-value = 0.0011*) and *vcam-1a* (*p-value = 0.0187*) (Fig. 3 f, h, i). Besides, *selectin* E (*sele*) and *icam1* mRNA expression levels showed a tendency of increased gene expression, although without statistical significance (Fig. 3 e, g).

### 3.3. Innate immune response in zebrafish larvae exposed to $PM_{2.5}$ and to a second inflammatory trigger

The effects on the innate immune response of a second inflammatory trigger were assessed on zebrafish larvae exposed to 45 µg/mL of $PM_{2.5}$. As shown in Fig. 4a, to generate an acute infectious inflammatory trigger after $PM_{2.5}$ exposure, we induced a local/systemic infectious stimulus in

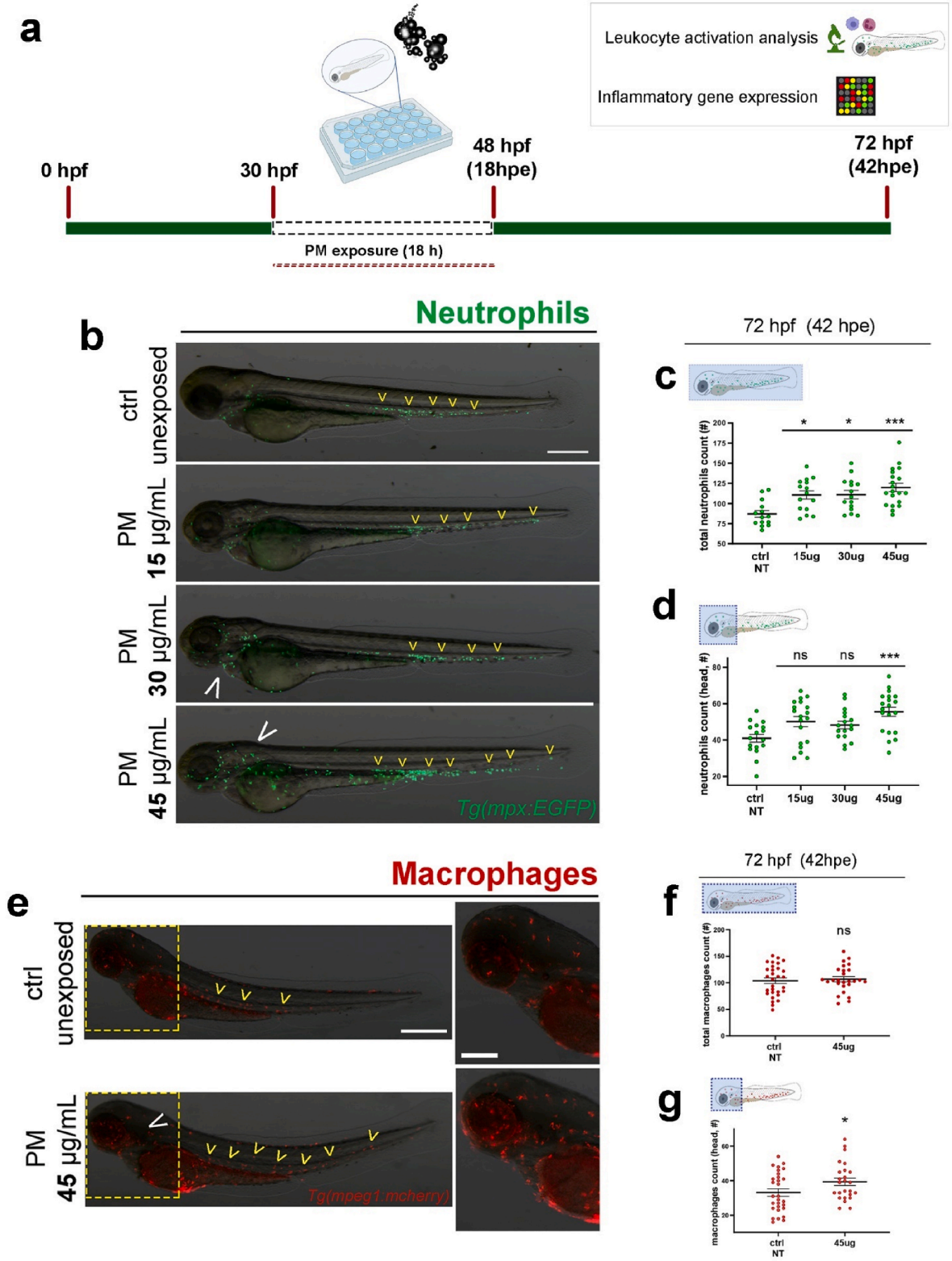


**Fig. 2.** Effects of $PM_{2.5}$ exposure on innate immunity activation. **(a)** Schematic representation of model setting. **(b–d)** Effects of $PM_{2.5}$ exposure on neutrophil activation **(b)** Representative panel of 72 hpf Tg(*mpx:EGFP*) larvae (42 hpe) exposed to different concentrations of $PM_{2.5}$. White arrows indicate neutrophil concentration in the head region and yellow arrows in the trunk Scale bar = 400 μm. **(c)** Quantification of total neutrophils, ctrl n = 14; 15 μg n = 15; 30 μg n = 17; 45 μg n = 20. **(d)** Quantification of neutrophils in the head region (dashed box, ctrl n = 17; 15 μg n = 19; 30 μg n = 15; 45 μg n = 20). **(e–g)** Effects of $PM_{2.5}$ exposure on macrophage activation. **(e)** Representative panel of 72 hpf Tg(*mpeg1:mcherry*) larvae exposed to 45 μg/mL of $PM_{2.5}$ or unexposed. The white arrow indicates macrophage concentration in the head region and the yellow arrows in the trunk region Scale bars = 400 μm (left) and 200 μm (right). **(f)** Quantification of total macrophages ctrl n = 30; 45 μg n = 26. **(g)** Quantification of macrophages in the head region (dashed box). Results are presented as mean ± SEM of two biological replicates. Statistical significance was assessed by unpaired *t*-test with Welch's correction: *$p < 0.05$; ns = not significant. Each dot represents a single individual. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

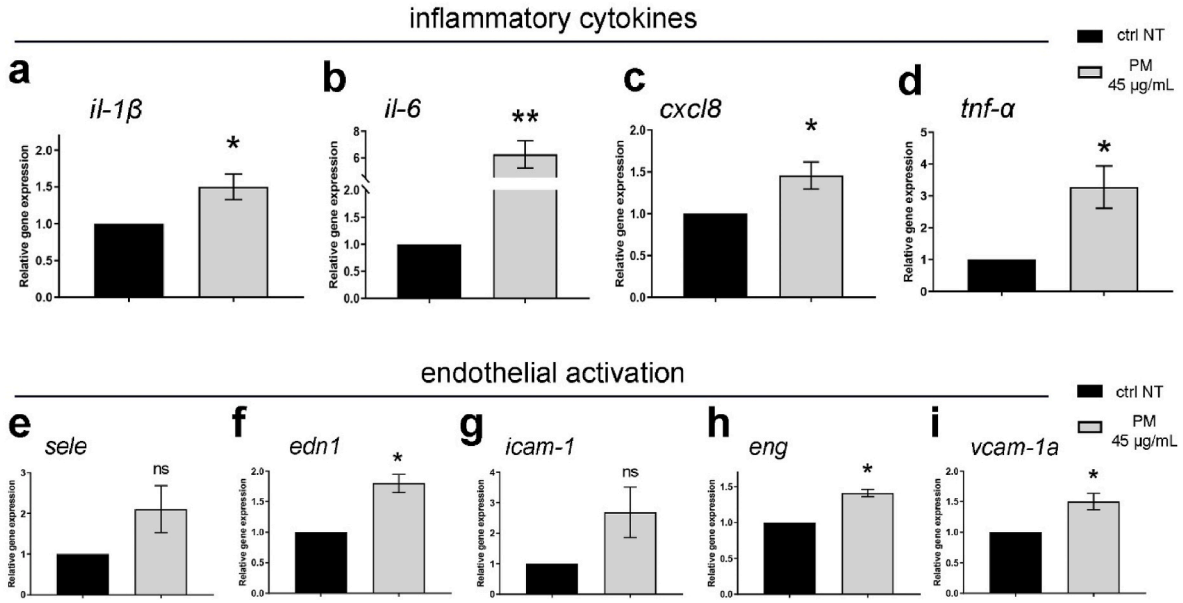


**Fig. 3.** Immunomodulatory effects of $PM_{2.5}$ exposure on inflammatory markers expression. **(a–d)** Relative expression of pro-inflammatory cytokines genes. **(e–i)** Relative expression of activated endothelial genes. Results are presented as mean ± SEM of three independent experiments (n = 15 embryos/experimental category for each replicate). Statistical significance was assessed by unpaired *t*-test: **p < 0.01; *p < 0.05; ns = not significant.

72 hpf larvae (at 42 hpe) by microinjecting 2 nL of bacterial LPS derived from *P. aeruginosa* (*Pa*-LPS) intramuscularly.

Leukocyte migratory stimulation was assessed at 4 h post LPS injection. LPS injection triggered a strong local inflammation including the migration of leukocyte populations, as shown in images of Fig. 4b. Larvae pre-exposed to $PM_{2.5}$ showed a reduced migration of neutrophils toward the injection site in comparison with not-exposed larvae (i.e., 29.0 vs 23.2, *p-value* = 0.0196) (Fig. 4c). Similarly, macrophage migration was reduced by $PM_{2.5}$ pre-exposure as the mean number of $mpeg^{+}$ cells at the site of infection was higher in not-exposed larvae in comparison to those pre-exposed (i.e. 4.6 vs 3.6, *p*-value = 0.0444) (Fig. 4d). Moreover, the effects of $PM_{2.5}$ exposure were analyzed on a sterile inflammatory trigger. At 6 h post-amputation (hpa) of the tailfin in 3 dpf larvae, both neutrophils, and macrophages migrated toward the wounded area, but exposure to $PM_{2.5}$ reduced their migration capacity (Fig. S2).

Furthermore, we assessed the effects on the inflammatory gene expression in larvae systemically injected with *Pa*-LPS suspension, which induces a quick and strong inflammatory stimulus, as already reported (Cafora et al., 2019; Rojas and Shiau, 2021). As controls, for both PM-exposed and unexposed larvae, PBS was systemically injected. Consistently with the effect on the migration of leukocytes, the relative expression level of the pro-inflammatory cytokines was lower in PM-exposed larvae (Fig. 4 e-k and S2). Due to the large variability of the immune response to infectious stimulus among experiments, only *tnf-α* and *cox2b* levels showed significant differences (Fig. 4 h,k; *p-value* = 0.0499 and 0.0492 respectively), while the relative expression levels of *il-1β il-6 cxcl8* and *cox2a* resulted lower but without statistical significance in comparison with untreated controls (Fig. 4 e,f,g,j). However, considering the ratio of the expression levels of unexposed over PM-exposed larvae (ctrl unexposed/PM exposed) normalized on the value of the control (i.e., PBS) of the same category, *il-1β* relative expression resulted in more than double in unexposed (i.e., ratio = 2.28, *p-value* = 0.0352), *cxcl8* almost doubled (i.e., ratio = 1.81, *p-value* = *0.0217*), and *tnf-α* more than tripled (i.e. ratio = 3.2, *p-value* = *0.0449*) (Fig. S3 g, h). Also, ROS production-related marker *cox2a* was lower in larvae upon $PM_{2.5}$ exposure (*p-value* = *0.0237*) (Fig. S3). On the other hand, although not significantly different, the relative level of the anti-inflammatory cytokine *il-10* resulted conversely higher in PM-exposed larvae (Fig. 4 i and S3 e).

### 3.4. ROS production in response to infectious stimuli is impaired by exposure to $PM_{2.5}$

To investigate whether the effects of $PM_{2.5}$ exposure may impair the oxidative burst in response to a second infectious stimulus, *Pa*-LPS intramuscular-injected *Tg(mpx:EGFP)* larvae pre-exposed or not with $PM_{2.5}$ were assessed for intracellular ROS production through DHE staining, a cell-permeable probe that fluoresces (red) when it reacts with superoxide within the cell (Fig. 5). Live larvae were stained with DHE, and neutrophils and ROS local quantification were performed (Fig. 5 a). Larvae treated with *Pa*-LPS exhibited a ROS signal while in LPS-injected larvae pre-exposed to $PM_{2.5}$ the $DHE^{+}$ cell count was significantly decreased(i.e., 6.6 vs 9.3 *p-value* = *0.0342*), suggesting a dampened ROS-mediated response (Fig. 5 b). In addition, considering the average number of neutrophils actively producing ROS/larva (i.e., $mpx^{+}DHE^{+}$ cell count) no marked differences between the two categories were observed (Figure S4 a). However, even though recruited neutrophils at the injection site were less in pre-exposed larvae as expected by previous analyses (please, see Fig. 4 b, c), they were able to produce the same amount of ROS with respect to controls. Indeed, the relative amount of $mpx^{+}$ $DHE^{+}$ cells normalized on the number of neutrophils locally recruited at the site of infection resulted from a significant increase, of about 4.7% (i.e., unexposed controls = 13.5% vs PM-exposed = 18.2%, *p-value* = *0.0412*) (Figure S4 b).

## 4. Discussion

Acute exposure to fine particulate matter is linked to an increased risk or exacerbation of various conditions, such as pathogen infections. Therefore, it is necessary to understand the biological mechanisms involved in these processes. For this purpose, we generated a zebrafish functional model to assess the effects of short-term $PM_{2.5}$ exposure. In recent years, there has been a growing emphasis on using alternative model organisms like zebrafish, nematodes, and fruit flies, which have proven to be more suitable for toxicity studies on inhalable pollutants compared to traditionally used mammalian models. Zebrafish has emerged as a valuable model due to its skin epithelial responses, which

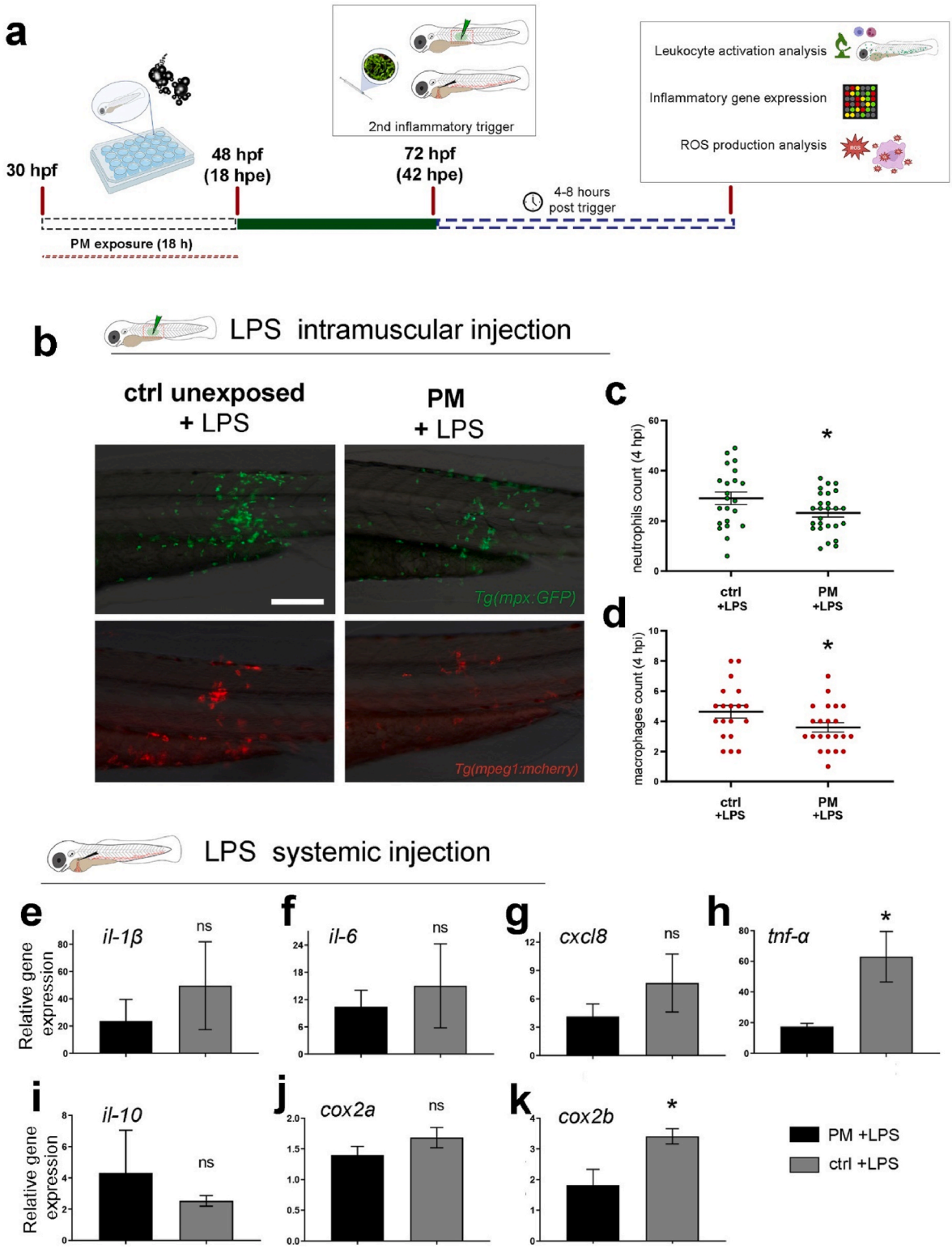


**Fig. 4.** Effects of a second pro-inflammatory infectious stimulus, after $PM_{2.5}$ exposure. **(a–d)** Intramuscular acute infectious stimulus. **(b)** Representative panel of PM-exposed and unexposed 72 hpf larvae at 4 hpi of LPS. Scale bar = 100 μm. **(c)** $mpx^+$ neutrophils count (ctrl + LPS n = 21; PM + LPS n = 26). **(d)** $mpeg^+$ macrophages count at 4 hpi in the trunk region (dashed box). Each dot represents a single individual. **(e–k)** Relative expression of inflammatory genes. Larvae exposed or not to 45 μg/mL of $PM_{2.5}$. Relative gene expression levels were normalized on the value of the control related to the same category (physiological solution injected larvae) (ctrl + LPS and PM + LPS). Results are presented as mean ± SEM of three biological replicates (n = 15 embryos/experimental category for each replicate). Unpaired *t*-test with Welch's correction: *$p < 0.05$; ns = not significant.

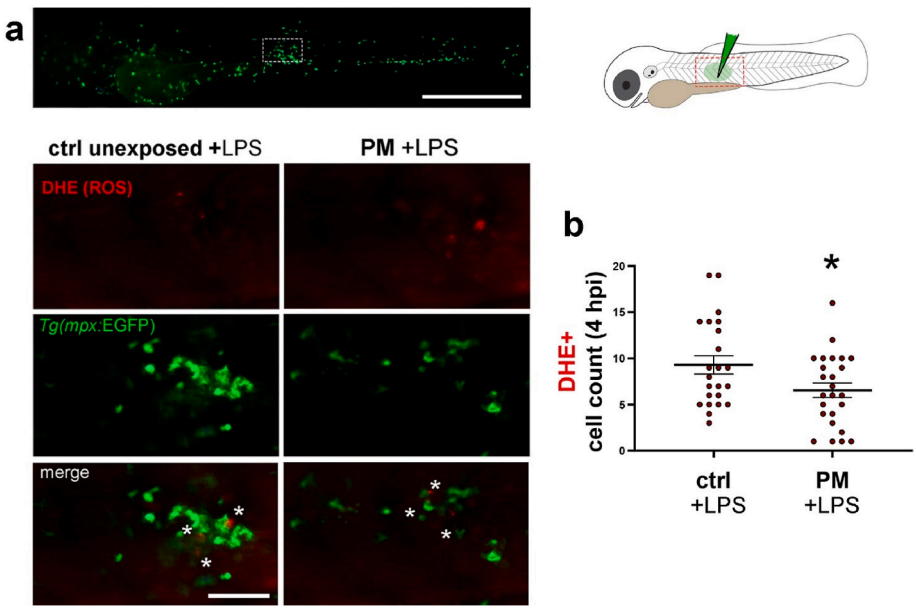


**Fig. 5.** Immunomodulatory effects of $PM_{2.5}$ exposure on ROS production after an acute infectious stimulus. **(a)** Lateral view of the whole injected-larva (scale bar = 500 μm) and schematic representation of the injection site; representative panels of PM-exposed and unexposed larvae following LPS injection: ROS DHE staining in red; $mpx^+$ neutrophils in green; merge panels; scale bar = 20 μm. **(b)** $DHE^+$ cells quantification in injection region (dashed box); Dots represent data for single individuals. Results are presented as mean ± SEM of two biological replicates (ctrl + LPS n = 23; PM + LPS n = 25). Unpaired *t*-test with Welch's correction: *$p < 0.05$; ns = not significant. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

can predict reactions in mammalian lung epithelium. Moreover, zebrafish gills exhibit structural and functional similarities to mammalian alveolar epithelium and respond to air pollutants similarly. (McLeish et al., 2010; Progatzky et al., 2016).

We exposed zebrafish larvae to a specific $PM_{2.5,}$ collected at an urban background site in the northern metropolitan area of Milan-Italy during the winter season. The possibility of using a particulate sample that accurately represents a specific spatial (local) and temporal (present-day) condition is a crucial point in the assessment of the actual PM-related health effects for specific exposure scenarios and exposed populations (Rovelli et al., 2021). Indeed, it is well known that the particulate composition and physicochemical characteristics may vary greatly with space and time (Cesari et al., 2018), thereby resulting in a potentially different PM toxicity (Mirowsky et al., 2013). As we thoroughly described elsewhere (Rovelli et al., 2021), the major contribution to the mass balance of the $PM_{2.5}$ that we utilized in the present study, was represented by the anionic component (34.0%), followed by cations (17.4%), organic and elemental carbon (14.1 and 0.5%, respectively). The elemental content was less than 0.5%, with Zn and Fe as the most abundant (87% of the total) among the characterized elements. In terms of relative contributions to the $PM_{2.5}$ mass, comparable results were found for anions (with a prevalence of nitrates and sulfates) and cations (mostly ammonium) within the town of Milan (Giannetto et al., 2005; Lonati et al., 2008), as well as for organic matter in other cities of northern Italy (Sandrini et al., 2014).

Previous studies have analyzed the inflammatory effects of PM using the zebrafish model (Chen et al., 2020; Kühnert et al., 2013; Zhang et al., 2018). However, it should be noted that in most of these studies, the authors analyzed the effects of urban $PM_{2.5}$ collected in East Asia, which has a different chemical composition compared to the European $PM_{2.5}$ used in the present study. Indeed, although it had a similar percentage content of organic carbon and inorganic ionic components, the PM used in the Asian studies generally contained higher levels of some toxicologically relevant metals, such as lead (10–20 folds), manganese (5–10 folds), cadmium (>100 folds) and chrome (3–5 folds) (Bekki et al., 2016; Chen et al., 2020; Dai et al., 2021; Duan et al., 2017a; Viertel et al., 2014; Zhang et al., 2018).

The exposure of zebrafish larvae to $PM_{2.5}$ was achieved through immersion in a solution containing 45 μg/mL of $PM_{2.5}$ for a short time period (i.e., 18 h). This exposure setting which did not lead to mortality or morphological alterations in the embryos, has been applied in previous toxicological studies employing zebrafish to assess the effects of airborne pollutants (Duan et al., 2017b; Jia et al., 2022; Liao et al., 2021; Zhang et al., 2021). The toxicity exhibited at concentrations higher than 45 μg/mL could be attributed to the deposition of $PM_{2.5}$ on the embryo surface, thereby impeding the exchange of molecules with the growth medium, including the uptake of oxygen.

Exposure to $PM_{2.5}$ elicited strong pro-inflammatory effects in zebrafish larvae and upregulated the pro-inflammatory markers *il-1β*, *il-6*, *tnf-α,* and *cxcl8*, according to what has been described in the context of human airway inflammation (Arias-Pérez et al., 2020; De Grove et al., 2018; Yang et al., 2021). Interestingly, *il-6* and *cxcl8* overexpression was reported to be a predictive marker of viral-induced respiratory symptoms (Arias-Pérez et al., 2020; Capistrano et al., 2016).

The increased expression of endothelial markers, such as *edn1, eng,* and *vcam-1a*, indicates endothelial involvement after short-term $PM_{2.5}$ exposure. This result confirms the epidemiological evidence of a strong association between PM exposure and endothelial dysfunction, which is linked to an increased risk of cardiovascular diseases (Kim et al., 2020; Mozzoni et al., 2022; Pope et al., 2016). Previous *in vitro* studies conducted on human endothelial cell lines have elucidated the mechanisms that link $PM_{2.5}$ exposure and endothelial dysfunction. The initial activation of the inflammatory response and the production of ROS, as we also observed in our functional zebrafish model, leads to cell damage through different mechanisms, such as apoptosis, autophagy, pyroptosis, ferroptosis, and the weakening of adhesion cell junctions. These events eventually result in an increased permeability of the endothelial cells, a condition further worsened by the fact that $PM_{2.5}$ reduces vascular repair capacity (Zhang et al., 2021).

We further investigated the immunomodulatory effects of short-term $PM_{2.5}$ exposure. To our knowledge, this is the first study addressing this aspect in vivo, while previous studies reported the activation of leukocytes and neutrophils after $PM_{2.5}$ exposure *in vitro* (Arias-Pérez et al., 2020; Rota et al., 2020). In zebrafish, we observed the activation of

neutrophils but not of macrophages, suggesting different responsiveness of the two cell populations to $PM_{2.5}$ exposure. We observed a tendency of neutrophil activation even at lower doses, indicating the enhancement of inflammatory response. However, given the observed overexpression of *tnf-α* at 42hpe, we could speculate that the pro-inflammatory M1-like setting is predominant, an effect reported in a recent work (Chen et al., 2020). Although not apparently hyperactivated, macrophages were more concentrated in the head region of larvae which is a region with structures more susceptible to exogenous agents, such as branchial arches and rudiment of gills and mouth (Kimmel et al., 1995).

We took advantage of *Pa*-LPS local/systemic injection models, to induce rapid acute inflammatory stimuli and mimic a pathogen infection, with a strong leukocyte recruitment (Benard et al., 2012; Cafora et al., 2022; Nguyen-Chi et al., 2014). The analysis of neutrophil and macrophage behavior in this double-exposed model showed a reduced leukocyte migratory capacity after exposure to the first trigger (i.e., $PM_{2.5}$ exposure). In line with our results, the study conducted in the mouse model by Chen and colleagues illustrated that long-term exposure to $PM_{2.5}$ disrupted macrophage polarization, resulting in macrophage dysfunction. This, in turn, impaired the production of pro-inflammatory cytokines in response to *Pneumococcus*, promoting infection and ultimately worsening pulmonary pathogenesis (Chen et al., 2020). To our knowledge, no other study has investigated the functional effects of short-term PM exposure on subsequent infectious stimuli in the zebrafish model.

In addition, we showed decreased ROS levels following local inflammatory stimulus in $PM_{2.5}$ pre-exposed larvae. $PM_{2.5}$ exposure is known to trigger an impairment in ROS production (Neophytou et al., 2013; Wellenius et al., 2013) and this condition may foster the exacerbation of pathogen infection (Phan et al., 2018; Segal, 2005; Sheshachalam et al., 2014). The evidence obtained helps to explain why short-term $PM_{2.5}$ exposure is associated not only with an increased risk of pathogen infections but also with greater severity of the disease, as observed by us and others in previous in population studies (Liang et al., 2014; Liu et al., 2019; Milani et al., 2022; Sheppard et al., 2023). Although the number of recruited neutrophils at the injection site was reduced in pre-exposed larvae, they were able to produce the same amount of ROS compared to pre-unexposed controls. We speculate that the activation of neutrophils, triggered by prior exposure to $PM_{2.5}$, might result in attenuated responsiveness upon subsequent proinflammatory stimuli. However, this initial activation might contribute to sustained ROS production within the neutrophil population. This prolonged ROS generation might be higher than in controls previously not exposed to $PM_{2.5}$, thus leading to cellular damage and an ensuing impairment in their ability to effectively counteract a subsequent pathogenic trigger.

We acknowledge some limitations of the study. First, zebrafish larvae were exposed to the whole mixture of $PM_{2.5}$, composed of both organic and inorganic elements, thus preventing us from elucidating the specific effect of the different components. Indeed, our study highlights the effects determined by exposure to $PM_{2.5}$ as a whole but we may not exclude that the different PM components might exert specific effects in the biological mechanisms we observed. Second, the exposure of zebrafish larvae through immersion raises questions about whether the observed outcomes were a result of the interaction between $PM_{2.5}$ components with the skin or their internalization (Smoot et al., 2022). Notably, zebrafish larvae breathe through passive diffusion of oxygen through their skin, allowing for immersion-based $PM_{2.5}$ exposure. Previous research has explored the developmental toxicity of PM, including $PM_{10}$ and $PM_{2.5}$, through immersion methods, aligning with the approach taken in our study (Chen et al., 2020; Manjunatha et al., 2021; Smoot et al., 2022). Third, in the present study, we tested a narrow range of PM exposure concentrations (i.e., 15–100 μg/ml), excluding lower doses that might elicit different effects than those described in the present study. Additionally, both the LPS assay and macrophage measurements were conducted at only a single exposure concentration, limiting the breadth of insights into potential dose-dependent responses. Finally, in this study, we focused on a bacterial-origin stimulus, while the effects of viral stimuli were not explored. We chose the LPS molecule because it is widely accepted to replicate the activation of immune and inflammatory processes triggered by pathogenic bacteria (Bertani and Ruiz, 2018). Although zebrafish larval models of human virus infection have been developed (Ding et al., 2011; Sullivan et al., 2017; Van Dycke et al., 2019), their use is not widespread, and the difference between fish and human pathogenic viruses could hamper definite conclusion.

## 5. Conclusion

The present study helps to understand, from a functional perspective, the cumulative effect of two different pro-inflammatory environmental exposures. We generated a zebrafish model of short-term exposure to a specific $PM_{2.5}$, collected in the northern metropolitan area of Milan, Italy. Taken together, our results indicate that $PM_{2.5}$ exposure alters the immune microenvironment and affects the inflammatory processes by inducing the expression of cytokines and provoking hyper-activation of both neutrophil and macrophage cell populations. These $PM_{2.5}$-induced alterations can ultimately hamper the resistance to subsequent pathogen infections. Indeed, when we examined the impact of a secondary infectious pro-inflammatory stimulus (*Pa*-LPS), we showed that innate immunity was less responsive. These effects are particularly detrimental in hyper-susceptible populations, such as immunocompromised individuals and infants who often have not yet developed sufficient immune memory to fight pathogen infections. Therefore, it is crucial to support monitoring campaigns and initiatives aimed at improving air quality to safeguard these vulnerable populations.

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## CRediT authorship contribution statement

**Marco Cafora:** Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Sabrina Rovelli:** Methodology. **Andrea Cattaneo:** Supervision. **Anna Pistocchi:** Writing – review & editing, Supervision, Investigation, Conceptualization. **Luca Ferrari:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Formal analysis, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2024.123841.

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