



Impact of diesel exhaust particles on infections with *Mycobacterium bovis* BCG in *in vitro* human macrophages and an *in vivo* *Galleria mellonella* model[☆]

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ABSTRACT

There are strong suggestions for a link between pulmonary tuberculosis (TB) and air quality. Diesel exhaust is one of the main contributors to pollution and it is reported to be able to modify susceptibility to lung infections. In this study we exposed THP-1 human macrophages and *Mycobacterium bovis* BCG to diesel exhaust particles (DEPs). High cytotoxicity and activation of apoptosis was found in THP-1 cells at 3 and 6 days, but no effect was found on the growth of *M. bovis* BCG. Infection of THP-1 cells exposed to a non-cytotoxic DEP concentration showed a limited capacity to engulf latex beads. However, *M. bovis* BCG infection of macrophages did not result in an increase in the bacterial burden, but it did result in an increase in the bacteria recovered from the extracellular media, suggesting a poor contentment of *M. bovis* BCG. We also observed that DEP exposure limited the production of cytokines. Using the *Galleria mellonella* model of infection, we observed that larvae exposed to low levels of DEPs were less able to survive after infection with *M. bovis* BCG and had a higher internal bacterial load after 4 days of infection. Unraveling the links between air pollution and impairment of human anti-mycobacterial immunity is vital, because pollution is rapidly increasing in areas where TB incidence is extremely high.

1. Introduction

Tuberculosis (TB) is a worldwide occurring infectious disease that continues to be one of the leading causes of death, causing more than 1.5 million deaths per year (World Health Organization, 2022). Many studies suggest a link between pulmonary TB and air quality (Hiramatsu et al., 2005; Sarkar et al., 2012; Smith et al., 2014; Tremblay, 2007). In fact, air pollution is increasingly becoming a cause of concern when considering infections; not only because it increases susceptibility to

them, but also because it could reduce the efficacy of drugs against the infections (Müller et al., 2021). Indeed, ambient particulate matter (PM), household air pollution and ozone are responsible for the deaths of more than six million people every year (Health Effects Institute, 2018). In Europe, 80% of people live in places where PM levels exceed the recommendations of WHO air quality guidelines (Brugha and Grigg, 2014). Diesel exhaust fumes are a main contributor to urban contamination and operators of excavators, cranes, forklift trucks, workers at bus depots, car mechanics, railway workers or miners, are especially

Abbreviations: (ADC), albumin-dextrose-catalase; (COPD), chronic obstructive pulmonary disease; (DEPs), diesel exhaust particles; (DMSO), dimethyl sulphoxide; (GM-CSF), granulocyte-macrophage colony-stimulating factor; (IFN- γ), interferon gamma; (IL), interleukin; (NIST), National Institute of Standards Technology; (OADC), oleic acid-albumin-dextrose-catalase; (PAH), polycyclic aromatic hydrocarbons; (PMA), Phorbol 12-Myristate 13-Acetate; (PM), particulate matter; (SRM), Standard Reference Material; (TNF), tumor necrosis factor.

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exposed (Hart et al., 2012). Occupational exposure is an important risk factor for chronic obstructive pulmonary disease (COPD); however, not only people at work are at risk, since diesel exhaust is dispersed by transport vehicles such as buses, cars, trains, and especially trucks, it results in air contamination in urban environments (Bujak-Pietrek et al., 2016). In the past rapid industrialization increased the health risk to humans in urban areas (Kim et al., 2019). Today, despite many efforts focused on planning to reduce traffic congestion, there has been no corresponding decrease in the use of fossil fuels used in transport over time, and even though the COVID-19 lockdowns showed temporary reductions, severe air pollution remains a problem all over the world (Magazzino et al., 2020). A study conducted in California concluded that living in proximity to roads with high densities and volumes of traffic, taken as a measure of exposure to traffic related air pollution, was a risk factor for a poor TB treatment outcome (Blount et al., 2017). Other studies have shown that air pollution increased the risk of developing active TB (Lin et al., 2019).

Road traffic PM comes mainly from the incomplete combustion of oil products in fuels such as diesel which are expelled in the exhaust (Magazzino et al., 2020). Apart from the fumes (gas, vapors, and semi-volatile organic and inorganic compounds), diesel engines emit small solid particles (Harrod et al., 2005). Additionally, exhaust gas can condense and form ultrafine particles that can be grouped and aggregate to 15–40 nm particles. The small size of these particles means they can very easily get into the respiratory tract, where they can reach the alveoli (Bujak-Pietrek et al., 2016; Rodríguez-Fernández et al., 2019).

The PM of diesel fumes, called diesel exhaust particles (DEPs), include a variety of organic compounds such as: quinones, polycyclic aromatic hydrocarbons (PAH) and nitro-PAH carcinogenic compounds (Yin et al., 2007). DEPs have a core of 10–30 nm, but they can agglomerate and form aggregates with diameters of 60–200 nm that have a black appearance (Steiner et al., 2016). There is a connection between levels of air pollution and increased adverse health effect, but DEPs have a higher oxidative potential than biomass burning (Longhin et al., 2016). DEPs can modify susceptibility to lung infections, suppress the production of cytokines and inhibit macrophage function (Gilmour, 2012; Hiramatsu et al., 2005). Because DEPs are ultrafine particles and are important constituents of urban air pollution, they can accumulate in the lungs. Accumulation of DEPs is associated with the exacerbation of allergic reactions and various respiratory diseases, including SARS-CoV-2 (Bonay et al., 2006; Estrella et al., 2019; Poyraz et al., 2021). Since *M. tuberculosis* can survive in macrophages of the lungs, and the contention and elimination of the disease depends on the immune status of the host, it is of great importance to know whether DEPs can influence the outcome of *M. tuberculosis* infections (Saxena et al., 2003). Levels of SO₂ are associated with a 7% increment risk in TB incidence (Hwang et al., 2014). TB and air pollution are related in the case of both short- and long-term exposures (Smith et al., 2016; Tremblay, 2007). Perhaps not surprisingly, countries with high diesel consumption like India or China have a high incidence of TB (CEIC, 2022).

In this study, we aimed to understand how DEPs may affect the contention of mycobacterial infections. For that, we have evaluated the impact of DEP exposure in THP-1 macrophages infected with *M. bovis* BCG showing that exposure to DEPs decrease the ability of macrophages in cell culture to phagocytize and contain the infection.

Although *in vitro* models provide high standardization, acceptable reproducibility, low cost and an absence of ethical concerns, they only provide a picture of a small part of what happens in the whole organism, since systemic reactions, buffering capacity, ability to study all toxic compounds or detoxification pathways can only be partly simulated (Rui et al., 2022; Steiner et al., 2016). Recently, a *G. mellonella* model has been proposed as a fast and reproducible way to monitor mycobacterial infections (Asai et al., 2019; Piatek et al., 2021). Here, we show that DEPs lead to a poor survival outcome in *Galleria* sp. larvae, showing the significance of using appropriate models.

2. Materials & methods

2.1. THP-1 cell line and mycobacteria growth conditions

The human monocytic cell line THP-1 (ATCC TIB-202™) was maintained in RPMI 1640 GlutaMax (Gibco, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum, penicillin 10,000 units/ml, streptomycin 10 mg/ml and Fungizone® 25 µg/ml. The cells were passaged every 3 days. For the experiments, RPMI without antibiotics was used. THP-1 monocytes were stimulated to become macrophages using 0.1 µM of Phorbol 12-Myristate 13-Acetate (PMA, Sigma, St Louis, USA) for 72 h (37 °C in 5% CO₂). RPMI was replaced by fresh medium 24 h before the start of the experiments.

Mycobacterium bovis BCG strain was grown in Middlebrook 7H9 supplemented with 0.05% Tween 80, 0.08% of glycerol and 10% albumin-dextrose-catalase (ADC), or on 7H10 agar supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC). Cultures were incubated at 37 °C without agitation. *M. bovis* BCG-GFP was kindly provided by Carlos Martin from the University of Zaragoza, Spain.

2.2. Preparation of diesel exhaust particles

DEPs (Standard Reference Material (SRM) 2975) from the National Institute of Standards Technology (NIST) (Gaithersburg, MD, USA) were kindly provided by MJ Cruz from the Pulmonology Service, Hospital Universitari Vall d'Hebron, Barcelona. The reported mean diameter of these particles was 11.2 ± 0.1 µm by area distribution, and the surface area, as determined by nitrogen gas adsorption, was 0.538 ± 0.006 m²/cm³. DEPs are black in appearance, odorless and insoluble in organic solvents.

DEPs (5 mg/ml) were dissolved in dimethyl sulphoxide (DMSO, Sigma-Aldrich, St Louis, MO, USA) and exposed to ultrasonic irradiation for 20 min and vigorous agitation. Once prepared, they were stored frozen at –20 °C. No changes were observed after resuspension compared to the descriptive properties listed in the physical and chemical properties of the supplier's safety data sheet. For a complete revision of the physical and chemical properties of DEPs we recommend the revision of Wang et al. (2019) (Wang et al., 2019).

2.3. Cytotoxicity assay

To determinate the cytotoxic effect of the DEPs on THP-1 macrophages, viable cells were counted with trypan blue using an optical microscope. THP-1 monocytes were seeded at a concentration of 3 × 10⁵ cells per well in 24-well tissue culture plates with clear bottoms (Falcon®, Tewksbury, USA) in RPMI containing PMA. The plates were incubated for 72 h, and then the medium was changed (37 °C in 5% CO₂ atmosphere). Different concentrations of DEPs (0–50 µg/ml) were added to the wells, and cells were incubated for 24 h, 72 h and 6 days, respectively, before counting. Fresh medium was added at 72 h (with DEPs) for the wells used at day 6. The results are expressed as viability percentage using cells untreated as a control.

2.4. *M. bovis* BCG growth curve

M. bovis BCG was adjusted to OD_{600nm} 0.01 in 7H9 medium supplemented with 0.05% Tween 80, 0.08% of glycerol and 10% albumin-dextrose-catalase (ADC), with DEPs (6 or 25 µg/ml) or without. The absorbance was measured every 24 h for two weeks.

2.5. Macrophage infection with *M. bovis* BCG

THP-1 macrophages were infected as previously reported (Gómez et al., 2020). Briefly, 3 × 10⁵ cells per well were PMA stimulated and seeded in 24-well tissue culture plates with clear bottoms (Falcon®). DEPs warmed to 37 °C (6.25 µg/ml) were added to the cells and

incubated for 3 h. For the infection, mid-log phase *M. bovis* BCG or latex beads were used at a multiplicity of infection (MOI) of 10:1 in DEPs 6.25 µg/ml in a total volume of 200 µl/well. After 3 h' incubation, macrophages were treated with 200 µg/ml of amikacin for 1 h and washed three times with DPBS to eliminate any extracellular bacteria. Lastly, 0.5 ml of RPMI with DEP 6.25 µg/ml was added to each well and incubated at 37 °C in 5% CO₂. Fresh medium with DEPs was added at day 3. Extracellular growth was assessed with serial dilution in PBS-tween plating onto Middlebrook 7H10 solid medium on days 0, 1, 2, 3 and 6. Intracellular growth was assessed by lysis of the monolayers by the addition of 500 µl of water followed by a 30 min incubation at room temperature and then plating on 7H10 medium. Colonies were counted after 3–4 weeks incubation at 37 °C and the average CFU/ml determined was determined.

For fluorescent microscopy, infection was performed and analyzed as previously described (Gómez et al., 2020), and for flow cytometry as reported previously by our group (Rodríguez-Fernández et al., 2021).

2.6. Cytokine detection

THP-1 cells were seeded as indicated above and exposed to DEPs (6.25 µg/ml) for 3 or 18 h before *M. bovis* BCG infection. Supernatants were then collected 0, 3 and 6 days post-infection, and cytokine production was assessed using the human magnetic Luminex assay kit (LXSAHM, R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. Analysis included detection of tumor necrosis factor (TNF)-α, interleukin (IL)-8, IL-1β, granulocyte-macrophage colony-stimulating factor (GM-CSF) and interferon (IFN)-γ. Supernatants were recovered from infection assays with *M. bovis* BCG. For the supernatants of day 6, 500 µl extra of fresh RPMI (with or without DEPs) were added on day 3 (this extra volume was taken in account in the calculation of cytokine production). Cytokine levels were calculated using the Luminex® 200™ system and the xPONENT® 3.1 software (Luminex Technologies, Inc., Austin, USA).

2.7. *Galleria mellonella* infections

G. mellonella larvae were purchased from Livefoods Direct Ltd (Sheffield, UK). Larvae of 2–3 cm in length were infected with a final volume of 10 µl, containing DEPs (0.25–37.5 mg/kg) and 6.4×10^6 CFU *M. bovis* BCG, into the hemocoel via the last proleg with a 30G needle. Infected larvae were incubated at 37 °C. Survival of infected larvae (N = 15 per group) following treatment was recorded every 24 h for 96 h. Larvae was considered dead when they failed to respond to touch. Uninfected groups were injected with 10 µl of PBS-0.05% Tween. Kaplan-Meier survival curves were plotted using data pooled from a minimum of five independent experiments.

To calculate the internal burden of bacteria, live worms at 96 h post-infection were homogenized in a FastPrep-24 machine for 1 min at maximum potency in 2 ml tubes containing 800 µl PBS-0.05% Tween, 0.05 ml of 1 mm glass beads. Then, 300 µl of the sample was decontaminated with 150 µl of 1M NaOH and amphotericin B to a final concentration of 50 µg/ml for 15 min. Samples were then centrifuged at maximum speed for 3 min, and the pellet was resuspended in 90 µl PBS-0.05% Tween. All experimental points were plated onto 7H10 agar plates as 10-fold dilutions in triplicate with worms pooled from at least three independent experiments.

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism software. Statistics are log-rank (Mantel-Cox) for survival curves of *Galleria* or 2way ANOVA with Sidak's multiple comparisons tests for the rest of the plots unless stated otherwise in the figure caption. The statistical significance of data is denoted on graphs by asterisks (*) where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ or ns = not significant.

3. Results

3.1. DEPs are highly toxic and increase the apoptosis in THP-1 cells

We evaluated a range of DEP concentrations (6.25–50 µg/ml) to determine the maximum non-cytotoxic dose in THP-1 macrophages. THP-1 cells were exposed to DEPs dissolved in RPMI, and cell viability was assessed by trypan blue exclusion test at days 1, 3 and 6. After 24 h of exposure, viability of cells exposed to 25 µg/ml and 50 µg/ml DEPs decreased ($p < 0.008$), but the viability of cells exposed to concentrations of 12.5 µg/ml or lower did not (Fig. 1A). However, at days 3 and 6, viability of THP-1 was inversely correlated with the dose of DEPs (Fig. 1A). We selected 6.25 µg/ml DEPs as the concentration to be used in the experiments since THP-1 viability over 80% at all time points (Fig. 1A). After exposing the macrophages for 3 and 6 days to DEPs, we also measured the apoptosis and death of THP-1 macrophages by flow cytometry staining the cells with annexin-V and 7AAD (Fig. 1B). When cells had been exposed to DEPs for 3 days, the percentage of apoptotic cells increased, and when exposed to DEPs for 6 days, the percentage of attached dead cells decreased ($p < 0.0234$) (Fig. 1B).

Additionally, we observed that DEPs tended to form aggregates, were efficiently phagocytized, and accumulated inside the macrophages in a dose-dependent way (Fig. 1C, Figure S-1).

3.2. DEPs do not impair the growth of *M. bovis* BCG but impair the phagocytosis of beads

We also evaluated the impact of DEPs on *M. bovis* BCG growth. Bacterial cultures were exposed to DEP 6.25 µg/ml for two weeks and OD_{600nm} was measured every 24 h. Growth of *M. bovis* BCG was not affected by the presence of DEPs in the RPMI media (Fig. 2A). *M. bovis* BCG was also exposed to a higher concentration of DEPs in the media (25 µg/ml), and no significant impairment in the growth was observed (Fig. 2A).

We then looked at if exposure to DEPs had a direct effect on the ability of macrophages to phagocytize. For this, macrophages were exposed to DEPs for 3 h, as an average of the time found in previous studies (Jaspers et al., 2005; Rivas-Santiago et al., 2015; Zarcone et al., 2017) and then infected with EGFP latex beads (MOI 10:1). We assessed the ratio of infection and the particle load at 0 h post-infection by confocal microscopy. A decrease in the infection percentage was observed in the macrophages exposed to DEPs compared to the untreated ones ($p = 0.0002$) (Fig. 2B). Interestingly, we also observed that the particle load was lower in infected cells exposed to DEPs ($p = 0.0274$) (Fig. 2C and D), highlighting the fact that the total number of engulfed beads was lower in the population exposed to DEPs. We also studied the effect of 1% DMSO without DEPs, and observed that was not responsible of the results attributed to the DEPs (Figure S-2).

3.3. DEPs do not impair the control of mycobacteria infection in macrophages

Next, we studied if this reduced ability to engulf particles after exposing macrophages to DEPs was correlated with a poor contention of the burden of *M. bovis* BCG in THP-1 cells. For this, we infected THP-1 macrophages at a MOI 10:1 for 3 h, and then removed the extracellular bacteria with intensive DPBS washings. Extracellular bacteria and bacterial burden were evaluated by determining the mycobacterial CFU of extracellular media or lysates of infected macrophages for up to six days. No significant changes were observed in the intracellular burden of macrophages exposed or not exposed to DEPs (Fig. 3A). However, extracellular bacteria recovery was higher at day 6 in cells exposed to DEPs ($p = 0.032$) (Fig. 3B). Importantly, intracellular recovered bacteria were always higher than extracellular recovered bacteria by at least one order of magnitude (Fig. 3A and B). When we observed the internalized bacteria by fluorescence microscopy, the percentage of infection

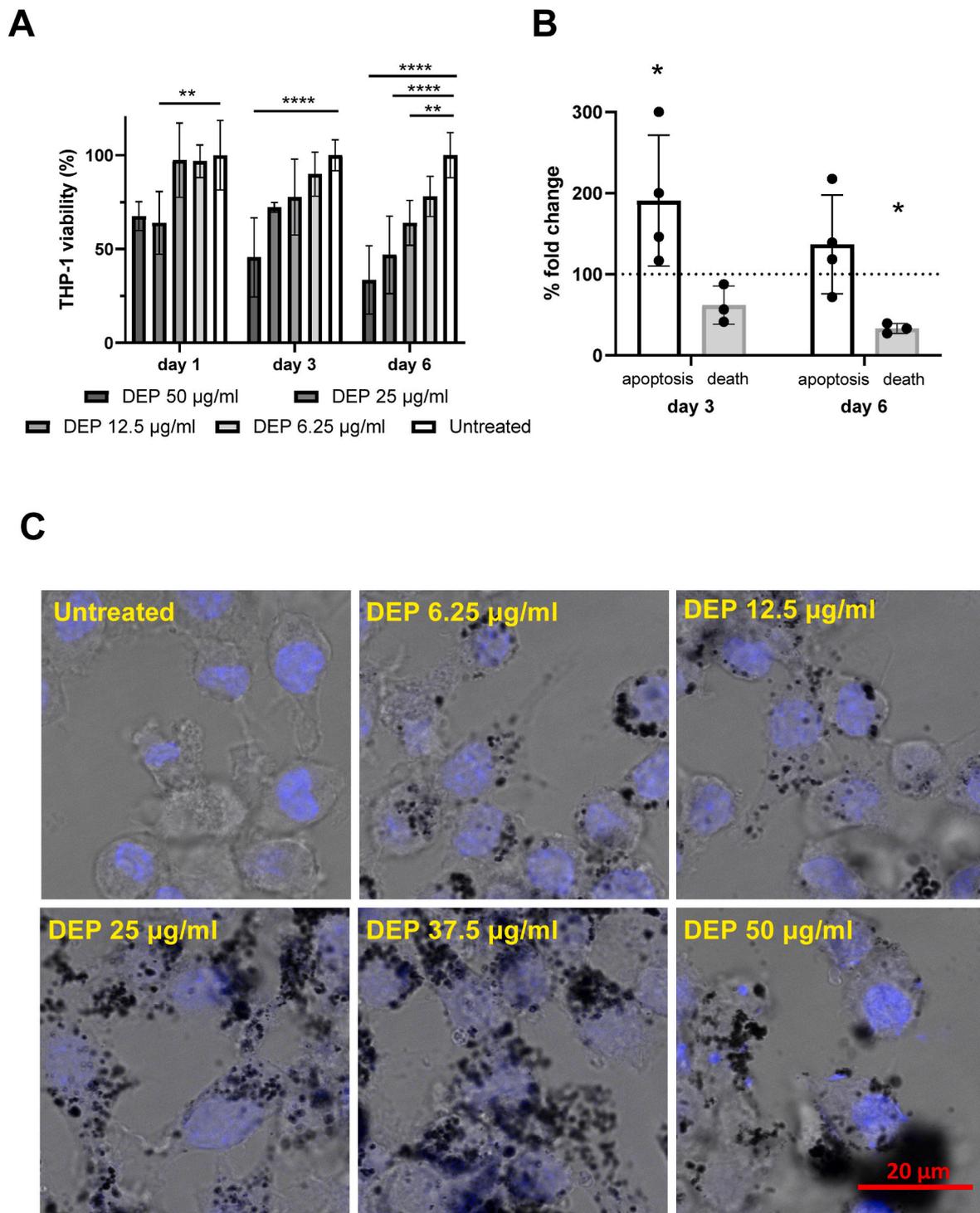


Fig. 1. Cytotoxicity of THP-1 macrophages exposed to DEPs. (A) Viability of THP-1 cells exposed to different concentrations of DEPs (0–50 µg/ml) measured by trypan blue exclusion test after 1, 3, or 6 days. Data shows mean with SD of three technical replicates of at least two independent experiments. **(B)** Fold change of apoptosis and death of uninfected THP-1 cells exposed to DEP 6.25 µg/ml measured by flow cytometry compared to untreated. Values of unexposed cells were then 100% and are not represented. Data shows average with SD of at least three independent experiments. Stars over the DEPs conditions indicate the significance compared to unexposed. **(C)** Representative images of THP-1 cells one day after exposure to different concentrations of DEPs (0–50 µg/ml). After 24h of exposing cells to DEPs, cells were fixed with 4% PFA, nuclei were stained with DAPI and coverslips were transferred to a slide for imaging in a SPS-8 Confocal Microscopy.

increased from day zero (13.5%) to day six post-infection (18.5%) ($p < 0.0047$) in untreated macrophages, but cells exposed to DEPs did not show significant differences over time. However, no differences in the percentages of infection were observed in macrophages exposed or not exposed to DEPs at a specific time point, although the percentage tended to decrease in cells exposed to DEPs after 6 days post infection (Fig. 3C

and D).

We also infected cells exposed to DEPs and measured infection, apoptosis, and death ratios by flow cytometry. For this, we detached the cells from the plate and then stained the cells with Annexin/7AAD. No changes were observed in infection, apoptosis, or death due to exposure of cells to DEPs either in the global population, or when considering only

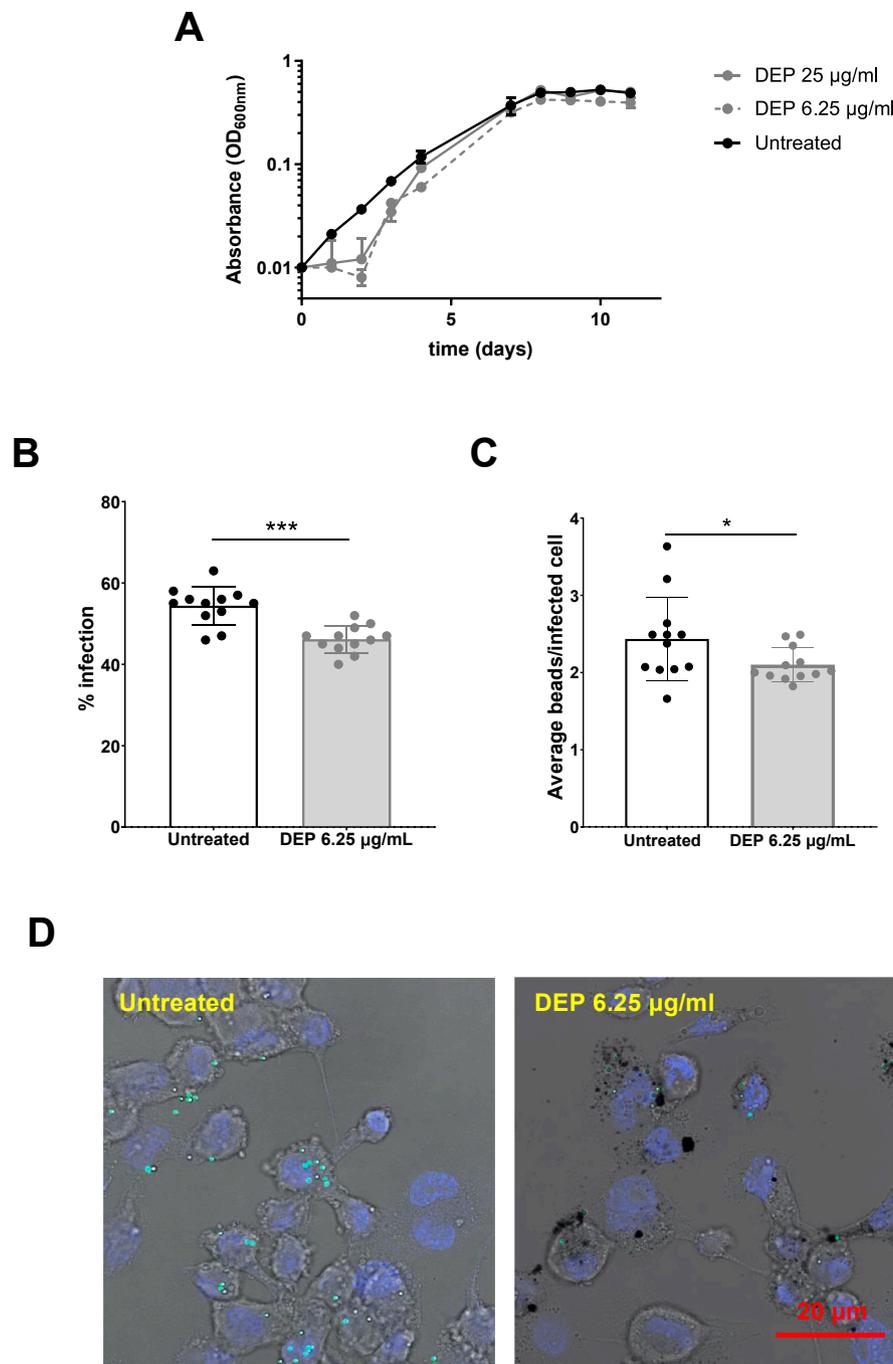


Fig. 2. Effect of DEPs in the growth of bacteria and in the engulfment of particles by macrophages. **(A)** Acellular growth of *M. bovis* BCG in Middlebrook 7H9 supplemented with 10% ADC with and without DEPs at different concentrations (6.25–25 µg/ml) monitored over 11 days by optical density at 600 nm. Data shows mean with SD of at least two independent experiments. **(B)** Percentage of THP-1 macrophages containing GFP latex beads calculated from confocal images at 0 h post-infection. Cells were exposed to DEPs for 3h and then incubated with the latex beads for 4 h. Then, cells were fixed with 4% PFA and nuclei were stained with DAPI for 15 min before mounting the slides. Data shows mean with SD of at least three independent experiments with three technical replicates. **(C)** Internal particle-load of infected macrophages analyzed from confocal microscopy. The percentage was calculated acquiring images under the conditions of 2B and dividing the number of beads of 100 infected macrophages (per replicate) per 100. Data shows mean with SD of at least three independent experiments with three technical replicates. **(D)** Representative confocal images of THP-1 cells infected with latex beads exposed to DEP 6.25 µg/ml. After 3h of exposing cells to DEPs, and incubating cells for 4 h extra with GFP latex beads (MOI 10:1) cells were fixed with 4% PFA, nuclei were stained with DAPI and coverslips were transferred to a slide for imaging in a SPS-8 Confocal Microscopy.

the infected subpopulation (Fig. 3E).

Overall, macrophages exposed to DEPs increase the extracellular mycobacterial population when infected and we observed by microscopy that the infection percentage tended to be lower.

3.4. Exposure to DEPs reduced the inflammatory response in the macrophage model

Previously, production of TNF- α , IL-1 β , IL-8, IFN- γ and GM-CSF has been described as altered by the exposure to DEPs (Rodríguez-Fernández et al., 2019). We wanted to investigate if exposure to DEPs could modify

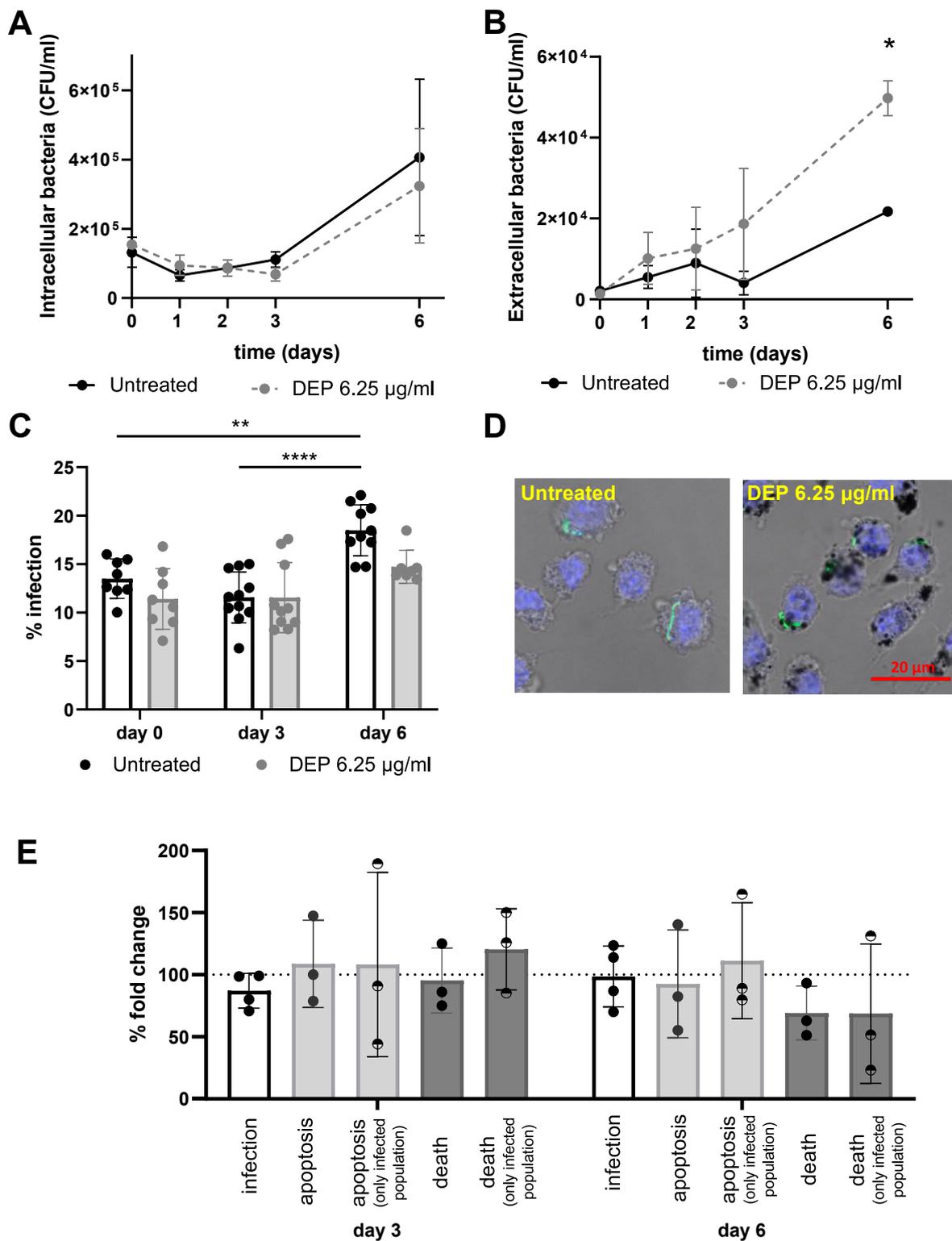


Fig. 3. Infections of THP-1 cells with *M. bovis* BCG. (A) Intracellular recovery of *M. bovis* BCG at days 0, 1, 2, 3, and 6 after infection. THP-1 macrophages were exposed to DEPs, infected with *M. bovis* BCG MOI 10:1 for 4 h, and the macrophages were lysed at the indicated post-infection times (B) Extracellular *M. bovis* BCG recovery from supernatants recovered from the wells before lysing the macrophages. Data shows mean with SD of at least three independent experiments with three technical replicates (C) Percentage of infection calculated from fluorescent images. Cells were fixed at 0, 3 and 6 days post-infection with 4%PFA, and nuclei were stained with DAPI before mounting and acquire the images. Each dot in the plot represents the number of infected cells in 100 cells. Data shows mean with SD of at least three independent experiments with three technical replicates. (D) Representative confocal images of THP-1 cells infected with *M. bovis* BCG exposed to DEP 6.25 µg/ml at three days post-infection. Cells were fixed and stained with DAPI before image acquisition in a SPS-8 Confocal microscope (E) Fold change in infection, apoptosis or death (THP-1 exposed to DEPs comparing nonexposed) calculated by flow cytometry. Values of cells nonexposed to DEPs were then 100% and are not represented. Pooled data from at least three independent experiments.

the inflammatory response by changing the cytokine response, and exposure to different concentrations of DEPs (6.5 or 12.5 $\mu\text{g}/\text{ml}$) or longer exposures to the same concentration of DEPs prior to the infection (3 or 18 h) may have a major impact in the production of cytokines. To investigate this, we exposed THP-1 macrophages to DEPs, infected them with *M. bovis* BCG MOI 10:1, and measured the cytokine concentrations in the supernatants at day 0, 1, 2, 3 and 6 post-infection. The experiment finished at day 3 when cells were exposed to DEPs at 12.5 $\mu\text{g}/\text{ml}$, as longer exposures resulted in very high cytotoxicity (Fig. 1A). In general, TNF- α and IL-1 β production increased as the result of the infection (Figure S-3A & B), THP-1 expressed high levels of IL-8 (Figure S-3C) and the levels of IFN- γ and GM-CSF were very low during the whole experiment (Figure S-3D & E). Although we did not observe a strikingly significant difference in the production of a cytokine when exposing to DEPs (Figure S-1), we observed a general trend in which cells exposed to DEPs produced lower amounts of cytokines at day 3 post infection, and this reduction was more visible in uninfected cells (Fig. 4). Longer incubations or doubling the concentration of DEPs did not exacerbate the reduction of any cytokine, indeed exposing cells to DEPs at 12.5 $\mu\text{g}/\text{ml}$ increased the production of TNF- α and IL-1 β (Fig. 4).

Exposure to DEPs decreases the survival of *M. bovis* BCG infected *Galleria mellonella* and increases the internal bacterial burden.

Effects of DEPs were also studied *in vivo* by infecting *Galleria mellonella* with *M. bovis* BCG in a solution with DEPs. Firstly, we injected a range of concentrations of DEPs (0.25–37.5 mg/kg) to the *Galleria* larvae and observed that all DEP concentrations tested were well tolerated by the larvae during the four days of the experiment (Fig. 5A). Then, we infected the larvae with *M. bovis* BCG and DEPs and observed that survival of *G. mellonella* decreased, despite having been infected with the same amount of bacteria when DEPs at a concentration of 1.25 mg/kg or more were injected into the larvae ($p < 0.0478$) (Fig. 5B). To better understand the survival plots, we then recovered the *M. bovis* BCG from the surviving worms at day 4. Larvae were homogenized, samples were decontaminated with sodium hydroxide and amphotericin β , and CFU was determined by plating onto 7H11 medium. We observed that the number of bacteria recovered was higher in larvae exposed to DEPs (at 1.25 mg/kg or higher), in a non-dose dependent relation (Fig. 5C). Representative images of the waxworms are shown in Figure S-4. In Fig. 6 we summarize the key effects of DEPs on macrophages and

G. mellonella larvae.

4. Discussion

Several studies have demonstrated a correlation between PM exposure and lung inflammation, asthma, decreased pulmonary function, and COPD (Velasquez et al., 2020). Additionally, DEP exposure is related to susceptibility to bacterial infections in the lung (Castranova et al., 2001; Saito et al., 2002; Yin et al., 2007). Indeed, exposure to DEPs has been predicted to mildly increase the incidence and prevalence of tuberculosis and non-tuberculous mycobacteria infections, but the authors caution that the increases would only be detectable in large population studies (Ghio, 2014). We investigated the effects of exposing macrophages to DEPs in mycobacterial infections. We observed that DEPs increased apoptosis of cells, decreased the ability of macrophages to engulf particles, decreased the inflammatory response, and reduced the ability of *Galleria* larvae to cope with the mycobacterial infection.

We first examined cytotoxicity of DEPs in THP-1 cells up to 50 $\mu\text{g}/\text{ml}$, since this is the most frequent value found on the literature (Castranova et al., 2001; Rivas-Santiago et al., 2015). We found high cytotoxicity values similar to those already reported (Dumax-Vorzet et al., 2015). Toxicity effects can be explained because macrophages engulf DEPs very efficiently (internalization is described as over 99%), and sedimentation of DEPs occurs rapidly (Bonay et al., 2006). DEPs deposition on lung phagocytes has also been observed in mice (Harrod et al., 2005). Studies have shown that DEPs are able to disturb alveolar epithelial cell differentiation and induce oxidative stress damage, through activating the expression of nicotinamide adenine phosphate oxidases (Kim et al., 2019; Poyraz et al., 2021). Using flow cytometry, we also observed that exposure to DEPs increased the number of apoptotic cells, decreasing the percentage of dead cells. We suggest that the decrease in necrotic cells in macrophages exposed to DEPs is explained by the fact that dead cells tend to detach and are excluded from the analysis. This acute toxicity and accumulation of the DEPs suggests that cessation of exposure will decrease but not reverse the associated risk for infections (Ghio, 2014). In a study with Influenza virus in A549 cells, the oxidative stress generated by DEPs was related to an increased susceptibility to viral infection due to better attachment and more particles entering the respiratory cells (Jaspers et al., 2005). DEPs are also associated with

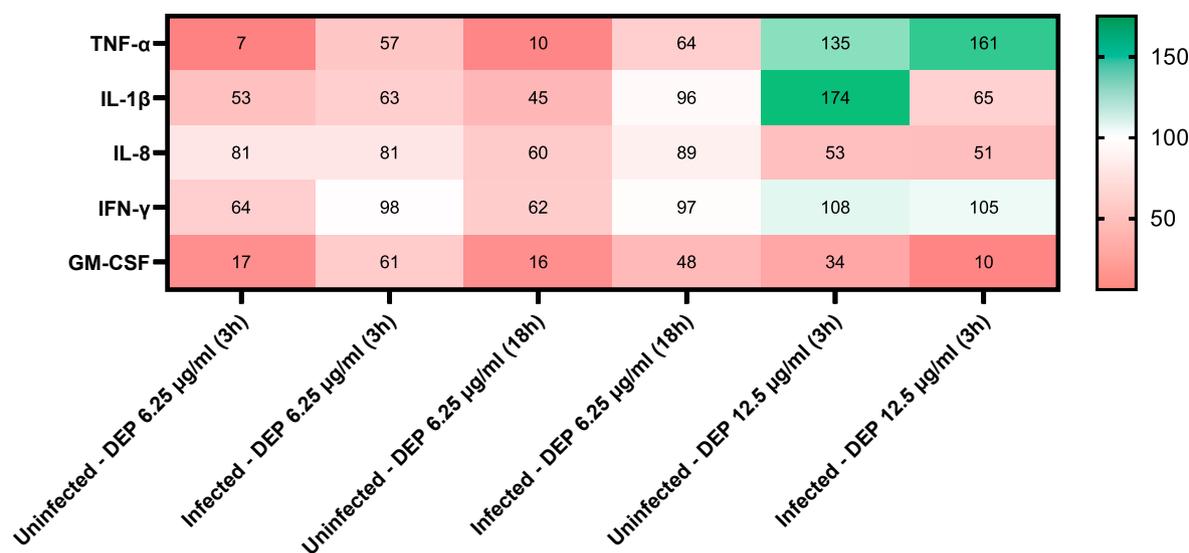


Fig. 4. Heat map showing the relative production (%) of cytokines by THP-1 exposed to DEPs at day 3 compared to the untreated. THP-1 macrophages were exposed to DEP 6.25 or 12.5 $\mu\text{g}/\text{ml}$, 18h or 3h prior to infection, and supernatants were taken at 3 days post-infection, to detect the amounts of TNF- α , IL-1 β , IL-8, IFN- γ and GM-CSF by Luminex. Each value resulted of dividing the cytokine level of a supernatant exposed to DEPs multiplied by 100 between the average of the cytokine production of untreated macrophages. Red values show a reduction in the cytokine production compared to the untreated, and green values show an increase. By definition, untreated values are 100. Number shows the average of three independent replicates.

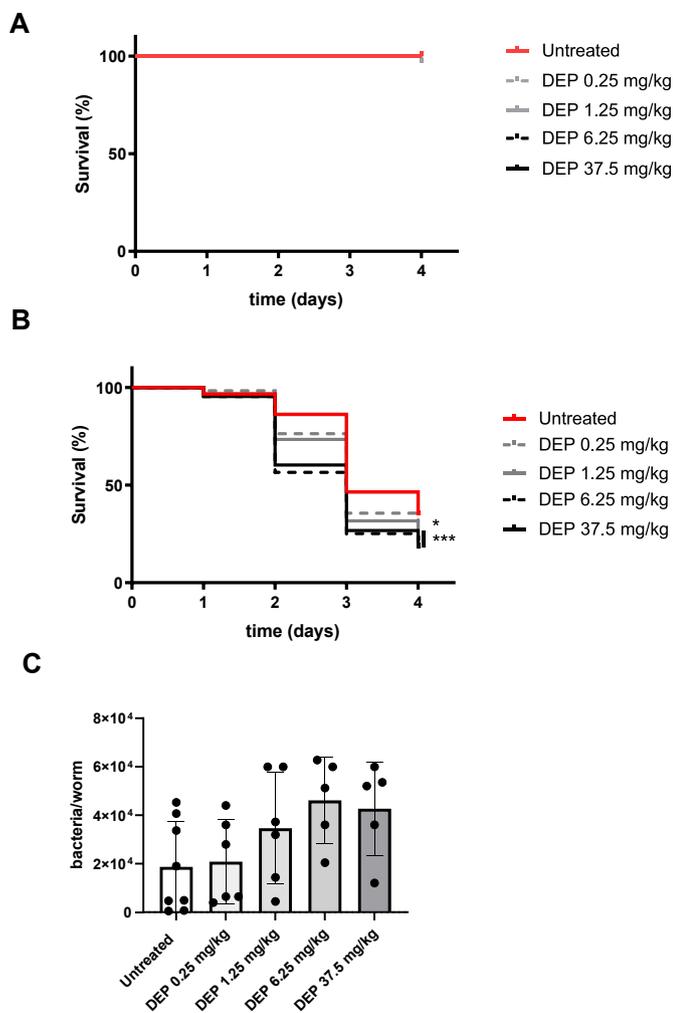


Fig. 5. Infection of *G. mellonella* with *M. bovis* BCG at different concentrations of DEPs (0.25–37.5 mg/kg). (A) Kaplan-meier survival curves of uninfected larvae and exposed to DEPs. Data are polled from two experiments. (B) Kaplan-meier survival curves of larvae infected with *M. bovis* BCG and exposed to DEPs. Data are polled from a minimum of five experiments. (C) Effect of exposure to different concentrations of DEPs on the burden of *M. bovis* BCG within *G. mellonella*. From each group, at least five larvae from two independent experiments were homogenized 96 h post-infection and plated to calculate the live bacteria.

decreasing host defense against non-typeable *Haemophilus influenzae* and by modulating stress responses (Zarcone et al., 2017).

Ability to phagocytize and prevent intracellular replication is critical for containment and removal of intracellular pathogens like *M. bovis* BCG. Our results showed no differences in the infection rate in macrophages exposed to DEPs over the six days, while macrophages not exposed to DEPs increased their infection rate at day 6. However, we observed no differences in the intracellular burden, but we did see an increase in the extracellular bacteria. Taken together, these results suggest that the ability of macrophages to control the intracellular replication is impaired, as the quantity of intracellular bacteria is the same in both groups, although the percentage of infected cells tends not to be. Additionally, more internalized bacteria should be able to escape from the cells or are prevented from being re-phagocytosed as we observed an increase in the extracellular population over time in cells exposed to DEPs. Another explanation is that overburden of bacteria in macrophages caused by a decreased killing ratio could lead to some death in macrophages, which increases the numbers of extracellular bacilli but not that of the intracellular ones. Although this hypothesis

seems contradictory to our flow cytometry data, which suggests no change in cell death caused by DEP exposure, a small increase in macrophage deaths does not need to correlate with a higher death ratio, as dead cells tend to detach from the plate and are excluded from the analysis.

Additionally, studies with alveolar macrophages exposed to DEPs showed impaired phagocytosis of heat-killed fluorescent yeast particles or inert latex particles (Chen et al., 1980; Rudell et al., 1999). Other studies with *Streptococcus pneumoniae* showed that the killing rates for intracellular bacteria in alveolar macrophages and J774 cells are similar in the presence of concentrated ambient particles, although they detected an inhibition in internalization of bacteria. Consequently, they detected more surviving bacteria (extracellular and intracellular) in DEP exposed samples (Zhou and Kobzik, 2007). Yin et al. infected alveolar macrophages from rats and observed a decrease in the phagocytosis and killing of *Listeria monocytogenes* (Yin et al., 2007). Similarly, Zhi et al. demonstrated a reduced phagocytic capacity of bone marrow derived macrophages upon DEPs exposure, which led to a decreased *Streptococcus pyogenes* internalization and bacterial clearance impairment (Zhi et al., 2022). We did not observe any significant effect of DEP exposure on the uptake of bacteria at day 0, but we observed that uptake of inert beads was reduced when macrophages were exposed to DEPs.

Similar to our results, *in vitro* studies with mycobacteria, exposing macrophages to DEPs for three days before infection with *M. bovis* BCG, showed no changes in survival of mycobacteria before day 10. Bonay et al. exposed human monocytes to DEPs at the same time as they were infected, and they obtained similar results, suggesting that no functional changes, such as activation of cells or impairing phagocytosis, take place (Bonay et al., 2006). They concluded that although DEPs are a major pollutant with adverse health effects, they do not seem to influence the ability of macrophages to eliminate mycobacteria (Bonay et al., 2006). Rivas-Santiago et al. exposed A549 cells to different PM sizes and concentrations for 18 h followed by 18 h of infection with *M. tuberculosis* at different MOI. CFU recovered was higher when exposed to 10 and 50 $\mu\text{g/ml}$ PM (but not in lower concentrations), and the impact was more visible when the MOI was higher and the PM smaller (Rivas-Santiago et al., 2015). Therefore, another question to consider, which may explain the discrepancies between the results of different experiments, is whether a longer exposure time is needed to observe the effects of DEPs. Long-term DEP exposure in animal studies show a relation between it and pulmonary inflammation, airway remodeling in the lungs and oxidative stress (Zhang et al., 2017). However, with SARS-CoV-2, short or long-term exposure to PM_{2.5} and NO₂ increased infection and associated mortality equally (Poyraz et al., 2021).

Bacterial killing by alveolar macrophages is associated with the production of reactive oxygen and nitrogen species, cytokines, chemokines and interferon (Castranova et al., 2001; Yin et al., 2007). Previous studies have shown that reactive oxygen species decreased in macrophages exposed to DEPs (Castranova et al., 2001), and we have observed a reduction in the production of cytokines. DEPs have been observed to inhibit macrophage function and reduce cytokine production *in vitro* and *in vivo* (Hiramatsu et al., 2005). Production of cytokines such as TNF- α , IFN- γ and IL-12, which play important roles in combating mycobacterial infections, are altered in mice exposed to DEPs (Hiramatsu et al., 2005). DEPs are described as slightly increasing IL-1 β , IL-8, IL-17A, TNF- α and IL-6 levels and decreasing IFN- γ and IL-10, although not in all cases (Estrella et al., 2019; Müller et al., 2021). In fact, IL-17A increased expression in the lung is associated with chronic inflammation with neutrophil infiltration and disintegration of the bronchiolar wall (Singh et al., 2023). We have observed a trend in that DEPs reduced the IL-1 β , but maybe this discrepancy can be explained by the low concentration of DEPs used, as the higher concentration of DEPs that we used (12.5 $\mu\text{g/ml}$) increased the production of IL-1 β and TNF- α .

Altered levels of IL-1 β are described as being related with lung diseases caused by DEP exposure (Kim et al., 2019). Levels of TNF- α and IL-12 were reduced *in vitro* in murine alveolar macrophages exposed to

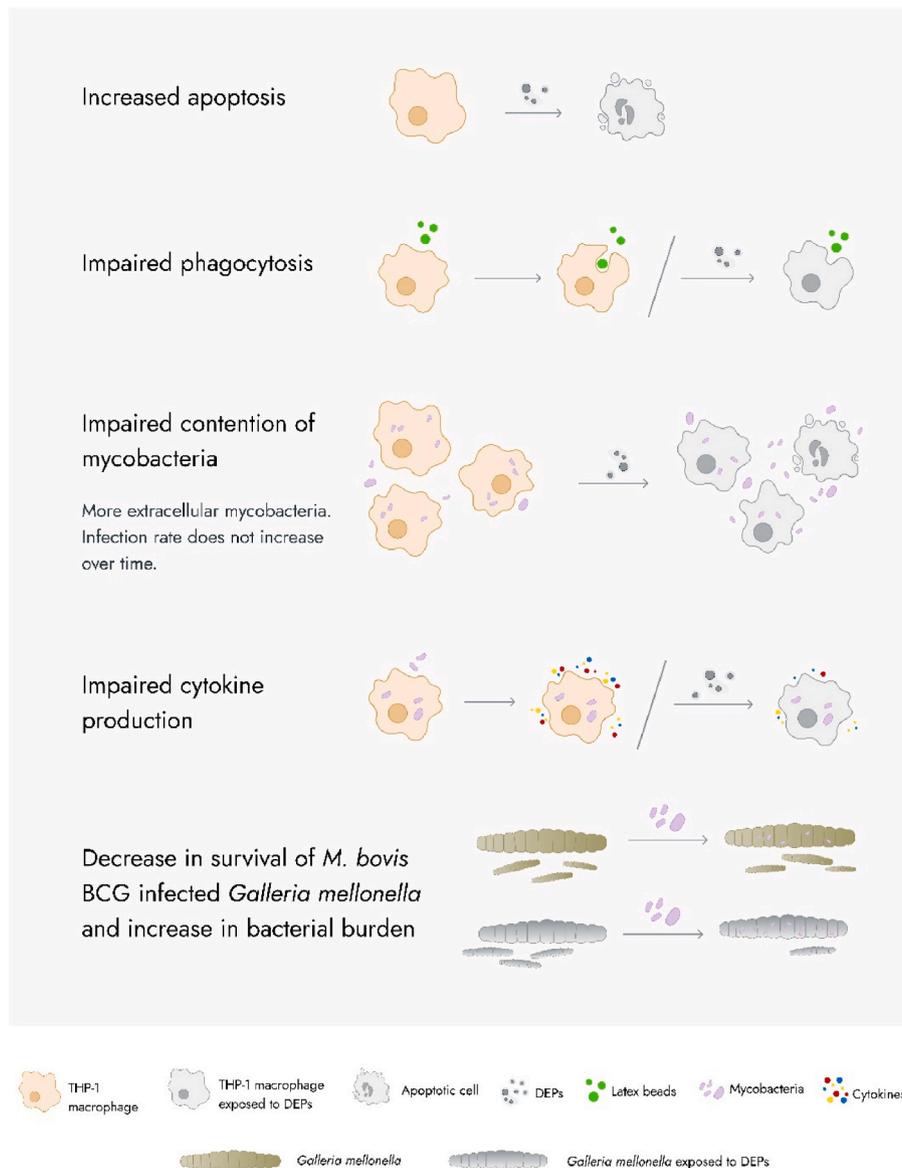


Fig. 6. Summary of key discoveries of the impact of DEPs on macrophages uninfected, infected with latex beads or *M. bovis* BCG, and of the impact of DEPs on *Galleria mellonella* larvae.

DEPs and subsequently infected with *M. bovis* BCG compared with macrophages only infected with *M. bovis* BCG alone (Saito et al., 2002). Additionally, the same study showed a reduction in the levels of mRNA of TNF- α in RAW264.7 macrophages (Saito et al., 2002). IL-8 was reduced in alveolar macrophages exposed to high DEP concentrations and in A549 cells infected with *M. tuberculosis* when treated with PM (Rivas-Santiago et al., 2015). All of these changes in cytokines resulted in a reduced macrophage bactericidal activity and prolonged bacterial survival (Yin et al., 2007). In addition, levels of nitric oxide (NO) in response to IFN- γ are reduced in alveolar macrophages exposed to DEPs. This response is a fundamental mediator of antibacterial action of activated macrophages (Hiramatsu et al., 2005; Saxena et al., 2003). We and others have already observed that other pollutants, such as cigarette smoke, also modify the profile of proinflammatory cytokines (Gómez et al., 2020; Rui et al., 2022). However, DEPs may trigger local pulmonary inflammation rather than a systemic response as one study has shown no changes in IL-6, IL-1 β or TNF- α mRNA expression in the liver (Jeong et al., 2021).

Then, we further explore the effects of DEPs in the *in vivo* *Galleria mellonella* model. We observed that a single dose of DEPs as low as 1.25

mg/kg reduced the probability of survival of the larvae and increased the quantity of mycobacteria recovered from the worm. Other experiments *in vivo* showed that when *M. bovis* BCG and DEPs are inoculated in mice, *M. bovis* BCG is present at higher quantities in lungs, liver and lymph nodes after five weeks when animals are treated with DEPs (Saxena et al., 2003). Another study showed that exposure to DEPs by inhalation for one month does not increase the mycobacterial CFUs in the lungs of mice, two months increases them slightly and 6 months increases them significantly (Hiramatsu et al., 2005). In mice infected with *Pseudomonas aeruginosa*, a study describes that DEPs increase susceptibility to lung infection, mice have higher levels of inflammation and the clearance of the bacteria from the lungs is impaired in a DEPs dependent manner (Harrod et al., 2005). Jeong et al. (2021) also observed an anxious behavior in mice exposed to DEPs due to accumulation of DEPs in the lungs; a decrease in body weight, whereas lung weight increases; and circulatory pathophysiological effects (Jeong et al., 2021). They also observed from bronchoalveolar lavages (BAL), that the percentage of macrophages in recovered cells were lower in comparison with mice not exposed to DEPs (Jeong et al., 2021), again highlighting the toxicity of DEPs.

In summary, we have shown that DEP exposure is one contributor to a poorer outcome in mycobacterial infections. Unsurprisingly, due to the lung damage associated with DEPs, an increase in emergency visits associated with DEP exposure for other more frequently studied pathogens like influenza and pneumonia have been observed (Croft et al., 2020). However, validation of the present study with a primary immune cell line, the use of a more robust animal model naturally susceptible to mycobacterial infections, and an airborne DEP exposure would better mimic the physiological conditions. Nevertheless, our results provide evidence that DEPs exposure play a critical role in the immunity against infections. Deciphering the links between air pollution and impairment of human antimycobacterial immunity is vital, since pollution is rapidly increasing in areas where TB incidence is extremely high (Rivas-Santiago et al., 2015) and this study contributes to the evidence that DEPs make the removal of mycobacterial infections more difficult.

CRedit authorship contribution statement

Pablo Rodríguez-Fernández: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Iris Romero-Andrada:** Formal analysis, Investigation. **Barbara Molina-Moya:** Formal analysis, Investigation. **Irene Latorre:** Formal analysis, Investigation. **Alícia Lacoma:** Resources, Supervision. **Cristina Prat-Aymerich:** Conceptualization, Writing – review & editing, Supervision. **Lydia Tabernerro:** Resources, Writing – review & editing, Funding acquisition. **José Domínguez:** Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2023.122597>.

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