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Abstract

Exposure to fine atmospheric Particulate Matter (PM) is one of the major environmental causes involved in the development of inflammatory lung diseases, such as chronic obstructive pulmonary disease (COPD) or asthma. When PM is penetrating in the pulmonary system, alveolar macrophages represent the first line of defense, in particular by triggering a pro-inflammatory response, and also by their ability to recruit infiltrating macrophages from the bone marrow. The aim of this in vitro study was to evaluate the gene expression and cytokine production involved in the toxicological and inflammatory responses of infiltrating macrophages, as well as the Extracellular Vesicles (EVs) production, after their exposure to PM. The ability of these EVs to convey information related to PM exposure from exposed macrophages to pulmonary epithelial cells was also evaluated.

Infiltrating macrophages respond to fine particles exposure in a conventional manner, as their exposure to PM induced the expression of Xenobiotic Metabolizing Enzymes (XMEs) such as CYP1A1 and CYP1B1, the enzymes involved in oxidative stress SOD2, NQO1 and HMOX as well as pro-inflammatory cytokines in a dose-dependent manner. Exposure to PM also induced a greater release of EVs in a dose-dependent manner. In addition, the produced EVs were able to induce a pro-inflammatory phenotype on pulmonary epithelial cells, with the induction of the release of IL6 and TNFα proinflammatory cytokines. These results suggest that infiltrating macrophages participate in the pro-inflammatory response induced by PM exposure and that EVs could be involved in this mechanism.

Keywords: infiltrating macrophages, extracellular vesicles, $PM_{2.5}$, lung epithelial cells.

1. Introduction

World Health Organization (WHO) estimates that exposure to air pollution caused around 7 million deaths worldwide in 2012 (WHO, 2017). Air pollutants consist of gaseous compounds such as volatile organic compounds, nitrogen oxides and particulate matter (PM). PM with an equivalent aerodynamic diameter below 2.5 µm ($PM_{2.5}$) is able to penetrate lung until bronchial tubes and alveoli where it can cause the development of respiratory diseases onset such as asthma, Chronic Obstructive Pulmonary Disease (COPD) till lung cancer (Li et al., 2018). In 2013, $PM_{2.5}$ has been classified as a Group 1 carcinogen by the International Agency for Research on Cancer (Cohen et al., 2013). $PM_{2.5}$ is a complex mixture of inorganic (metals, ions…) and organic compounds (PAHs, dioxins…), living organisms (bacteria, pollen…) whose composition depends on the main

contributing sources (urban, industrial, maritime, rural) and of their transport and evolution in the atmosphere (Kfoury et al., 2016; C. Lepers et al., 2014).

Macrophages are heterogeneous cells from different origins showing high plasticity as they can change their phenotype depending on their microenvironment. In the lung, they are established during the embryonic development period and maintained in adulthood through longevity and local self-renewal (Epelman et al., 2014; Sheng et al., 2015). These prenatal-derived macrophages are predominant in lung tissues and can differentiate into Alveolar Macrophages (AMs) localized in the airspaces of the lung (Fathi et al., 2001). AMs are the essential immune effector cells in the lung as they are exposed to the harmful outside environment especially through the inhalation of $PM_{2.5}$. However Goto et al. have shown that the deposition of PM into the lung stimulates the bone marrow and promotes the release of leukocytes into the circulation among which monocytes that are important to control and terminate the inflammatory response (Goto et al., 2004b). These monocytes infiltrate the lung and differentiate into macrophages, which will be called infiltrating macrophages, that are mainly distributed throughout the airways, lung tissues, and alveolar tissues during lung insult (Yamasaki and Eeden, 2018). After PM exposure, the onset of the inflammatory response usually starts with the release of the early responding cytokines, IL1β and TNFα, and continue with the expression of other cytokines and chemokines (IL6, IL8) (Gualtieri et al., 2017). This inflammatory response may be induced by the ability of PM to alter the cells oxidative balance. According to the oxidative stress paradigm, cells respond to low levels of reactive oxygen species (ROS) by increasing the levels antioxidant molecules (such superoxide dismutase (SOD), NAD(P)H quinone dehydrogenase 1 (NQO1), heme oxygenase-1 (HMOX)) thus restoring the intracellular redox homeostasis (Gualtieri et al., 2017). In addition, PAHs present on PM, which require metabolic activation to biologically reactive intermediates to elicit their adverse health effects, are metabolized by the CYP superfamily members, and activate their gene expression through the activation of the Aryl hydrocarbon Receptor (AhR) (Billet et al., 2007). To our knowledge, no study has focused on these effects of $PM_{2.5}$ on infiltrating macrophages. It is thereby interesting to assess the cellular response of infiltrating human macrophages exposed to $PM_{2.5}$ in terms of cytotoxicity, oxidative stress, xenobiotic metabolization and inflammation.

Moreover, recent studies highlight that stimulated or stressed cells possess the ability to release vesicles in the extracellular space. These Extracellular Vesicles (EVs) may have the characteristics of a cell that has just been attacked and are involved in the regulation of several cellular events, with variable phenotypic consequences for recipient cells (Neven et al., 2017). They include a variety of nanoscale membranous vesicles

(exosomes, microvesicles, microparticles, apoptotic bodies) containing key signaling mediators (miRNAs, proteins, metabolites, lipids) transferred from cells of origin to target cells (Jabalee et al., 2018). EVs are known to be critically involved in lung homeostasis as well as inflammation, reflecting crosstalk between AMs and epithelial cells (Haggadone and Peters-Golden, 2018). They are increasingly recognized as a novel mechanism by which the innate immune response is initiated (Lee et al., 2018). Lung exposure to PM may induce the release of EVs originated from various cellular sources, like endothelial cells, epithelial cells and AMs in the alveolar or bronchial space (Benedikter et al., 2018). Up to our knowledge, the effect of fine atmospheric particulate matter on infiltrating human macrophages, the ability of exposed human macrophages to release EVs and the study of the interaction between these released EVs and lung epithelial cells has not been reported yet.

To answer these questions, cellular response of infiltrating human macrophages exposed to $PM_{2.5-0.3}$ collected in an industrial coastal city was assessed. For this, undifferentiated THP-1 monocyte cells were first used to determine the dose and time of exposure at which these cells respond in terms of metabolic activity, oxidative stress and inflammation but also produce EVs. Then the same experiments were carried out on primary macrophages. Finally, EVs produced in response were characterized and their effects on BEAS-2B lung epithelial cells were evaluated.

2. Material and Methods

2.1 PM sampling and handling

Atmospheric PM was collected continuously over a one year period (March 2014-March 2015), in Dunkerque, Northern France (51°1'36" N; 2°22'18" E), using a high volume sampler (68 m^3/h) equipped with a 5 stages cascade impactor (model 235, Staplex®, USA). Dunkerque is a coastal city counting roughly 210,000 inhabitants with its suburbs. The study area is characterized by numerous industries mainly in the fields of metallurgy (integrated and electric steelworks, Al and Mn steelmaking) and organic synthesis (refinery, chemicals manufacturing). Briefly, particles were collected directly on plates (without filter) as published elsewhere (Borgie et al., 2015; Cazier et al., 2016; Landkocz et al., 2017). Two sampling systems were used in parallel and replaced every 14 days over the one year period. With respect to the cut-off diameter of the impaction plates, atmospheric particles deposited on impaction stages 2 to 5 have an aerodynamic diameter in the 0.3 - 2.5 µm size range ($PM_{2.5-0.3}$). After each of the 14 days sampling period, impaction plates were placed in a laminar flow hood for 48 hours. Then particles were recovered from impaction plates (stages 2-5) using a brush and stored at -18°C in a PTFE vial till further use. At the end of the one year campaign, the 14 days $PM_{2.5-0.3}$ samples were pooled together and carefully homogenized using two PTFE balls moved by a magnetic stirrer.

The obtained yearly average $PM_{2.5-0.3}$ sample was then use for chemical characterization and cellular response study.

2.2 PM physico-chemical characterization

Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES, iCAP 6300 DUO, ThermoElectron, UK) and ICP-Mass Spectrometry (ICP-MS, Varian® 820-MS, Varian, USA) were used for quantification of the PM inorganic major and trace components (Al, As, Ba, Cd, Co, Cr, Cu, Fe, Mn, Mo, Ni, P, Pb, Sn, Sr, Ti, V, Zn). Water-soluble ions and cations (F^-, Cl^-, NO_3^-, SO_4^{2-}, Na^+, Ca^{2+}, Mg^{2+}, K^+, NH_4^+) were quantified with ion chromatography (model ICS 5000, ThermoElectron, UK). The PM total carbon (TC) content was measured using the CHNS/O analyzer (FLASH, 2000; ThermoElectron, UK). Finally, concentrations of the 16 priority Polycyclic Aromatic Hydrocarbons (PAHs) listed by the United States Environmental Protection Agency (US-EPA) were quantified using gas chromatography-mass spectrometry (GC-MS,1200 TQ, Varian, USA) after PM Soxhlet extraction with dichloromethane. The whole analytical procedure is detailed in .

2.3 Human Macrophages culture and $PM_{2.5-0.3}$ exposure

THP-1 monocytic cell line was purchased from European Collection of Authenticated Cell Cultures (ECACC, Wiltshire, UK; reference: 88081201) and handled following manufacturer's instructions. Briefly THP-1 were seeded at 4 x 10^5 cells/mL in RPMI 1640 medium supplemented with 1% Penicillin-Streptomycin (PS), 0.05mM 2-mercaptoethanol and 10% Heat-Inactivated Fetal Bovine Serum (HI-FBS).

Peripheral Blood Mononuclear Cells (PBMCs) were purified from fresh whole blood (provided by Etablissement Français du Sang, EFS, Lille, France) of 6 healthy donors through Ficoll gradient centrifugation using Lymphoprep (Axis-Shield). Monocytes were then prepared by a 2h adhesion step, and next cultured in RPMI 1640 medium supplemented with 2mM glutamine, 1% PS, and 10% HI-FBS in the presence of 50 ng/mL GM-CSF during 36h to get infiltrating macrophages.

The concentrations of 12.5 and 50 µg/mL of $PM_{2.5-0.3}$ for THP-1 were determined based on their response to increasing concentrations of $PM_{2.5-0.3}$ measured by quantifying their release of IL8 (supp. Fig. 1). The minimum dose at which THP-1 releases IL8 at the three times of exposure of 6h, 24h and 48h is 12.5 µg/mL (Lowest Observed Effect Concentration (LOEC). A second $PM_{2.5-0.3}$ concentration of exposure for THP-1 has been chosen at a value less than 50% of IL8 release (50 µg/mL) in order to identify a potential dose-response effect. Then the concentration of 12.5 µg/mL for primary macrophages has been chosen according to the obtained results with THP-1.

Each cell type was exposed to $PM_{2.5\text{-}0.3}$ as follows. The supernatants were removed and replaced by RPMI medium supplemented with 10% exosome-depleted FBS (Gibco by ThermoFisher Scientific, Illkirch, France), 2.5% Hepes and 1% antibiotics. $PM_{2.5\text{-}0.3}$ was suspended in cell culture medium and dispersed by 5 min sonication just prior to the exposure. Then, $PM_{2.5\text{-}0.3}$ was or not (non-exposed, NE) added at the indicated concentrations to THP-1 cultured for 6h, 24h or 48h and primary macrophages for 48h.

2.4 Extracellular vesicles (EVs) isolation and quantification

EVs were isolated by differential centrifugation according to Théry et al. with some modifications (Théry et al., 2006). Briefly, THP-1 or macrophages supernatants were centrifuged at 500g for 10 min and at 16,500g for 30 min at 4°C to remove cell debris. In addition, the supernatants were filtered with a 0.2µm syringe filter. For the collection of EVs, the supernatants were ultracentrifuged twice at 110 000g for 70 min at 4°C. The EVs were then washed and suspended in Phosphate Buffer Saline (PBS), aliquoted and stored at −80°C until further exposure or extraction.

To determine the size and the concentration changes of EVs obtained in the different conditions, the NTA measurement was performed using the NS300 Nanosight instrument (Malvern Panalitycal, United Kingdom). All NTA analysis was conducted after thawing of the samples. The samples were diluted in PBS at proper ratios to achieve the optimal detectable concentration (about 10^7 particles per mL). For each sample, 1 mL of the diluted sample was injected into the instrument and then the concentration of EVs as well as the mean and median EVs size was given after 4 measurements by the Nanosight dedicated software.

2.5 BEAS-2B co-culture with EVs

BEAS-2B is a non-cancerous cell line of human bronchial epithelial cells which was obtained from ECACC (Wiltshire, UK; reference: 95102433). They were cultured in LHC-9 Medium (ThermoFisher Scientific, Illkirch, France) as described previously (Héliot et al., 2017). 250,000 cells were exposed to 50µL of EVs suspension (Supp. Fig.1) derived from non-exposed or exposed THP-1 or macrophages. After 48h of exposure, cell-free culture supernatants were collected and quickly frozen at −80°C until further study. Adherent cells were washed twice with 500µL of cold PBS (Gibco by ThermoFisher Scientific, Illkirch, France), and quickly frozen at −80 °C. All experiments were performed in triplicate.

2.6 Cytotoxicity assay

The cytotoxicity of THP-1 and primary macrophages after $PM_{2.5\text{-}0.3}$ exposure, and of BEAS-2B cells after EVs co-culture, was evaluated as described previously (Landkocz et al., 2017), by measuring the extracellular

lactate dehydrogenase (LDH) concentration in the cell culture supernatants (Cytotoxicity Detection Kit LDH, Roche Diagnostics, France). Percentage of cytotoxicity was quantified relatively to 100 % mortality induced by Triton X-100. As a control, increasing concentrations of $PM_{2.5-0.3}$ were suspended in LDH solution (fixed concentration). Results showed that, at the maximum concentration of $PM_{2.5-0.3}$ used (50µg/mL), attenuation of absorbance is below 1%. This allows us to neglect the direct effect of $PM_{2.5-0.3}$ on absorbance measurement values and the artefact related to the adsorption of LDH on PM (Supp. Fig.2).

2.7 Flow cytometry

Phenotypic analysis of primary macrophages was performed using flow cytometry direct immunofluorescence. Cells were first incubated for 30 min in PBS with 5% human autologous serum with FITC-conjugated Ab directed against CD14. Isotypic control labeling was performed in parallel. Cells were then analyzed with an Attune flow cytometer (Applied Biosystems, ThermoFisher Scientific, Illkirch, France) using Kaluza software (Beckman Coulter).

2.8 RNA isolation and RT-qPCR

THP-1, primary macrophages and BEAS-2B RNA and small RNA were extracted using small and large RNA kit (NucleoSpin® miRNA, Macherey Nagel, Hoerdt, France) respectively, according to manufacturer's instructions. The obtained samples were quickly frozen at −80°C until further study.

Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, ThermoFisher Scientific, Illkirch, France).

mRNAs (i.e IL1β, IL6, IL8, CAT, IL12, TNFα, CYP1A1, CYP1B1, CYP2E1, SOD2, CAT, NRF2, NQO1, HMOX) quantification was performed using TaqMan® Gene Expression Assays (ThermoFisher Scientific, Illkirch, France) (Table 1). Real-time quantitative polymerase chain reaction (qPCR) was carried out using a 7500 Fast Real-Time PCR System (Applied Biosystems, ThermoFisher Scientific, Illkirch, France). The relative changes in gene expression were calculated by the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008) and normalized against 18S using the Sequence Detection 7500 Software v2.0.3 (Applied Biosystems, ThermoFisher Scientific, Illkirch, France) (see Table 1 for primer references).

Table 1. mRNA Taqman primer references

mRNA	Taqman Reference
18S	Hs99999901_s1
IL1β	Hs01555410_m1
IL6	Hs00985639_m1
IL8	Hs00174109_m1

TNFα	Hs01113624_g1
CYP1A1	Hs01054796_g1
CYP1B1	Hs02382916_s1
CYP2E1	Hs00222327_m1
SOD2	Hs00167309_m1
NRF2	Hs00975961_g1
NQO1	Hs01045993_g1
HMOX	Hs01110250_m1

194

195 *2.9 Cytokines and Extracellular Proteins Concentration Measurement*

196 IL-6, IL-1β, IL-8, TNF-α and sFas concentrations in cell-free culture supernatants were determined using
197 MAGPIX® system and the MILLIPLEX® MAP Human Cytokine Panel 1 and Human CD8+ T Cell
198 (Luminex®, EDM Millipore, Billerica, USA), following the manufacturer's instructions.

199 *2.10Protein Extraction and Western blotting*

200 Cells were centrifuged during 10 min at 2,500g and 1mL of M-PER Reagent was added per 100mg of cells.
201 After stirring for 10 min, the samples were centrifuged again for 15 min at 14,000 g. The supernatant containing
202 the proteins was recovered and Laemmli buffer was added. EVs were directly placed in Laemmli buffer before
203 electrophoresis.

204 Equal volumes of Novex® Tris-Glycine SDS Sample Buffer (2X) (ThermoFisher Scientific, Illkirch,
205 France) were added to the protein samples, boiled for 2 min, applied to SDS-PAGE, and transferred to Novex™
206 4-20% Tris-Glycin Mini Protein Gels (ThermoFisher Scientific, Illkirch, France). After blocking, membranes
207 were incubated with anti-CD63 or anti-CD81 primary antibodies (Novus Biologicals, Littleton, CO, USA) at
208 4°C overnight, washed with PBS-Tween (0.05% Tween 20), and incubated with a Goat anti-Mouse IgG (H+L)
209 secondary antibody, Horseradish Peroxidase (HRP) conjugated (Life Technologies, ThermoFisher Scientific,
210 Illkirch, France) for 60 min. After washing with PBS-Tween, antigen–antibody reactions were detected with a
211 Chemidoc (Biorad, Marnes la Coquette, France) using the ECL Plus Detection kit (Pierce, ThermoFisher
212 Scientific, Illkirch, France).

213 For relative quantification of MSR1 protein on total proteins, macrophages proteins were transferred to
214 Mini-PROTEAN TGX Stain Free Gel (Biorad, Marnes la Coquette, France) and detected with a Chemidoc
215 before membrane transfer. The ImageLab Software was used for relative quantification.

2.11Electron microscopy

EVs in PBS were placed on formvar-coated 300 mesh copper grids during 10 min, rinsed 3 times with H_2O and contrasted with 2% phosphotungstic acid (PTA) during 10 seconds. Images were obtained by transmission electron microscopy with a JEOL 7100F electron microscope (JEOL Ltd, Japan).

2.12 Statistical analysis and controls

Gene and protein expression as well as protein levels were depicted as median and inter-quartile range and graphically represented by bar-graphs. For relative quantification of gene expression, significance RQ> 2 or RQ <0.5 is reported to the value of unexposed control (NE), normalized to 1 with 18S. For each exposure time and concentration of $PM_{2.5\text{-}0.3}$, cytokines measurement data were compared to those obtained for non exposed (NE) cells. The significance of the differences was calculated by the non-parametric Mann-Whitney U test and is indicated by * when $p < 0.05$. For NTA, the significance of the differences was calculated by the T Student's test and is indicated by * when $p < 0.01$.

3. Results

3.1 $PM_{2.5\text{-}0.3}$ major and trace inorganic species

Major and trace inorganic species as well as Total Carbon (TC) contents are reported in Fig. 1A. TC (14.3%), water-soluble NO_3^- (10.0%), SO_4^{2-} (7.5%), Cl^- (6.1%), Ca^{2+} (4.5%), Na^+ (4.3%) and NH_4^+ (1.6%) as well as Fe (3.8%) and Al (2.8%) were the major PM constituents (55% in total). Then five species showed concentrations values between 0.1% and 1%: Mg^{2+} (0.65%), K^+ (0.45%), Mn (0.18%), P (0.14%) and Zn (0.13%). Other species concentrations ranged below 0.1% (Cu, Ti, Ba, Pb, F^-, Cr, Sr, Ni and V) and below 0.01% (Sn, Mo, As Co and Cd).

NO_3^- and SO_4^{2-} mainly derive from the gas-to-particle conversion of NO_X and SO_2. NO_X are emitted by combustion processes related to the use of fossil fuels in cars (diesel) or industrial processes. SO_2 is mainly originated from industrial emissions or related to heavy fuel oil combustion with high sulfur content mainly used in industry or in shipping (Ledoux et al., 2018). NH_4^+ results as well from the conversion of the NH_3 gas known to be mainly from agricultural origin. However, an industrial contribution cannot be excluded with regard to the presence of an integrated steelworks able to emit particles containing NH_4^+ in the study area (Hleis et al., 2013; Kfoury et al., 2016). Na^+, Mg^{2+} and Cl^- are known to be significantly associated with sea-salts (Manders et al., 2010).

Among the considered metals, Al and Fe had the highest levels. These elements can generally be introduced in the atmosphere as a consequence of soil erosion induced by wind as well as industrial emissions (Kfoury et al., 2016; Oravisjärvi et al., 2003; Santacatalina et al., 2010). In particular, steelmaking, metallurgy, cement plants are located in the study area and are known to emit such PM components. Ca is as well found in soil dust (aluminosilicates) as in the raw materials used in the latter industrial activities. Cr, Cu, Mn, Ni, Pb, V and Zn are predominantly associated with anthropogenic origins: Ni, V and Co can be considered as tracers of heavy fuel oil combustion, especially used for shipping (Mazzei et al., 2008; Roche, 2016); Zn, Pb, Cr and Cu elements are more associated with the wear of mobile vehicle parts such as brakes and tires (Gietl et al., 2010; Thorpe and Harrison, 2008). It should also be noted that, in the Dunkirk study area, Fe, Mn, Zn, Pb, Cr and Cd elements could also be related to the iron and steelmaking activities (Kfoury et al., 2016).

3.2 $PM_{2.5-0.3}$ Polycyclic Aromatic Hydrocarbon composition

The average concentrations of the 16 US-EPA priority PAHs compounds are given in Fig. 1B. The predominantly detected compounds were dibenz[a,h]anthracene (8.9 µg/g), followed by indeno[1,2,3-cd]pyrene, chrysene and benzo[b]fluoranthene, with very close concentrations values ranging between 5.3 and 6.0 µg/g. Concentrations of other compounds were below 5 µg/g and below 1µg/g for the 6 lightest PAHs (compounds with 2 and 3 cycles). The main source of dibenz[a,h]anthracene and benzo[b]fluoranthene is road traffic, and more precisely exhaust fumes from diesel engines (Duval and Friedlander, 1981; INERIS, 2005, 2004a). Dibenz[a,h]anthracene is also found in cigarette smoke or in coke oven emissions (INERIS, 2004a). Indeno[1,2,3-cd] pyrene results from fossil fuels as well especially after incomplete combustion of wood, coal, gasoline or diesel fuel (INERIS, 2004b). Chrysene can be emitted into the atmosphere during coal and oil distillation and pyrolysis. It is also one of the predominant PAHs in particulate emissions from household waste incinerators, natural gas appliances and domestic heating devices, particularly those using wood (INERIS, 2004c). The predominantly detected compounds can be explained by at least partly the locally based industries. Indeed, the study area hosts an integrated steel industry (including a coking plant), oil refining (producing marine fuels comparable to heavy fuel oils), a cement factory and a waste incinerator.

3.3 Cellular response of THP-1 exposed to $PM_{2.5}$

The first experiments were done on THP-1 cells. This monocytic cell line was used in a first time to optimize the exposure conditions that the cells will respond in terms of metabolic activity, oxidative stress and inflammation, before switching to primary macrophages. For that, THP-1 were not pre-treated with PMA before exposure to keep a monocytic phenotype and to avoid non-specific activation of THP-1 by PMA as a pro-

inflammatory response (Lund et al., 2016). THP-1 were exposed to $PM_{2.5}$ at two doses (12.5 and 50 µg/mL) and for three times (6h, 24h and 48h) (see section 2.3 for dose justification). Regarding the cytotoxicity, the measurement of the extracellular LDH did not show any significant effect regardless times and doses of exposure (Fig. 2A). For metabolic activation and oxidative stress induction assessment, relative quantification of expression of genes coding for the Xenobiotic Metabolizing Enzymes (XMEs) CYP1B1, CYP1A1, CYP2E1, and for the Superoxide dismutase 2 (SOD2) which transforms toxic superoxide into hydrogen peroxide and diatomic oxygen, the NAD(P)H quinone dehydrogenase 1 (NQO1), an enzyme involved in the reduction of formation of radical species induced by oxidative stress and heme oxygenase (HMOX), was performed (Fig. 2B). The results showed a significant dose-dependent increase of CYP1A1 and CYP1B1 expression after 6h of exposure, with a tendency to decrease with time for CYP1A1 or to reach a plateau for CYP1B1 at 24h and 48h. CYP2E1 expression was not significantly modified after $PM_{2.5}$ exposure. SOD2, NQO1 and HMOX expression was significantly induced proportionally to the doses but not to the time of exposure.

Expression and production of the pro-inflammatory cytokines IL8, IL1β, IL6 and TNFα by THP-1 exposed to $PM_{2.5\text{-}0.3}$ were also quantified (Fig. 2C and D). An early induction of the expression of IL8, IL1β and TNFα was observed decreasing with time and increasing with the doses. For the same cytokines, the release increased with the times and doses of exposure. Concerning IL6, its expression was induced later and its release came only after 48h of exposure to the highest dose of 50 µg/mL. These results demonstrate that undifferentiated THP-1 are able to carry out a metabolic, anti-oxidant, and pro-inflammatory response following $PM_{2.5\text{-}0.3}$ exposure.

3.4 Characterization of extracellular vesicles derived from THP-1 exposed to $PM_{2.5\text{-}0.3}$.

The ability of THP1 to produce EVs after exposure to PM was then evaluated. For that, the EVs produced by the undifferentiated THP-1 after 6h, 24h and 48h of exposure to 12.5 and 50 µg/mL of $PM_{2.5\text{-}0.3}$ were isolated, quantified and characterized. On the one hand, the quantification with the Nanoparticle Tracking Analysis (NTA) of the obtained EVs showed that $PM_{2.5}$ exposure induced an significant increase of the concentration of EVs produced by THP-1 in particular after 48h of exposure, which corresponds also to an increase of the number of EVs released by cell (Fig.3A), suggesting that THP-1 intrinsically produce more EVs if exposed for longer and at higher concentrations of PM. On the other hand, NTA measurement indicated that their average size doesn't significantly vary with the time and doses of exposure and ranges between 130 and 200 nm (Fig. 3A). Electron microscopy confirms the presence of EVs in THP-1 supernatant (Fig. 3B). Finally, it should be noted the presence of exosomes within the EVs produced as evidenced by the western-blot on CD63 and CD81

exosomal proteins (Figure 3C). These results show for the first time that $PM_{2.5}$ exposure enhances EVs release by undifferentiated THP-1.

3.5 Cellular response of infiltrating human macrophages exposed to $PM_{2.5-0.3}$

Goto et al. have shown that one of the cytokine implicated in infiltrating macrophages recruitment into the lung is GM-CSF (Goto et al., 2004b). Just after isolation from blood and before exposure to $PM_{2.5-0.3}$, primary macrophages obtained from 6 donors were incubated for 36h with 50 ng/mL GM-CSF to obtain infiltrating cells. The obtained infiltrating macrophages were then exposed to 12.5µg/mL for 48h as define during the optimization step (section 4), since this was the minimum PM dose at which the THP-1 responded and produced an almost maximal number of EVs. Then as well, metabolic activation, oxidative stress and inflammatory response were studied. Extracellular LDH measurement showed that $PM_{2.5-0.3}$ exposure did not induce significant cytotoxicity (Fig. 4A) in comparison with non exposed (NE) infiltrating human macrophages. Concerning the gene expression, $PM_{2.5-0.3}$ induced a significant increase for CYP1A1, CYP1B1, SOD2, HMOX and NQO1 (Fig. 4B), indicating the setting of a metabolic and antioxidant response. As for THP-1, CYP2E1 expression was not modified by $PM_{2.5-0.3}$ exposure. $PM_{2.5-0.3}$ was also responsible for a significant enhancement of the pro-inflammatory cytokines IL8 and IL1β expression, and IL8, IL1β, IL6 and TNFα release (Fig. 4C and D), as well as an increase of the granularity (SSC) of the exposed macrophages in comparison with the non-exposed one (Fig. 5A). Internalization of the particles by the macrophages was also observed (Fig. 5B) and it is correlated with an increase of the Macrophage Scavenger Receptor 1 (MSR1) protein (Fig. 5C). In conclusion of this section, the results show that infiltrating macrophages, such as undifferentiated THP-1, typically respond to PM exposure. This finding makes it possible to consider the EVs produced in response.

3.6 Characterization of extracellular vesicles derived from infiltrating macrophages exposed to $PM_{2.5-0.3}$.

EVs produced by the macrophages from 6 donors exposed or not to $PM_{2.5-0.3}$ were isolated as described in Methods (sections 2.3 and 2.4) and pooled to form two average EVs samples deriving from exposed and non-exposed macrophages respectively. NTA characterization of the two obtained EVs samples indicated that $PM_{2.5-0.3}$ exposure induced an significant increase of the number of EVs released by infiltrating macrophages as observed for THP-1 (Fig.6A). On the contrary, the quantification revealed that $PM_{2.5-0.3}$ exposure did not affect size distribution (Fig. 6B). Electron microcopy confirmed the presence of EVs (Fig. 6D). Finally, CD63 and CD81 exosomal proteins were detected by western-blot in EVs derived either from NE macrophages or from macrophages exposed to $PM_{2.5-0.3}$ (Fig.6C). These results are in line with those obtained from THP-1 exposed to

$PM_{2.5\text{-}0.3}$. To our knowledge and for the first time, this study shows that $PM_{2.5\text{-}0.3}$ is able to induce EVs release from infiltrating human macrophages as well.

3.7 Effects of EVs derived from human macrophages exposed to $PM_{2.5\text{-}0.3}$ on lung epithelial cells

One of the objectives of this work was to highlight whether EVs produced by exposed macrophages are able to transfer information from this exposure to unexposed lung cells and to activate typical PM exposure response. To do this, BEAS-2B cells co-cultured with EVs from exposed and non-exposed macrophages ($EV_{THP\text{-}1/NE}$ and $EV_{THP\text{-}1/PM}$ for EVs derived from non-exposed and exposed THP-1 and $EV_{HM/NE}$ and $EV_{HM/PM}$ for EVs derived from non-exposed and exposed human macrophages) in proportional number as found in supernatants (Supp. Fig. 3). Surprisingly, an important mortality was observed in BEAS-2B co-cultured with EVs derived from THP-1 regardless their conditions of exposure (data not shown). This cytotoxicity, measured via extracellular LDH activity, was significantly higher ($p<0.1$) for BEAS-2B co-cultured with $EV_{THP\text{-}1}$ in comparison with EV_{HM} (Fig. 7A). In an attempt to identify the involved mechanisms, we measured the concentration of soluble Fas (sFas), an inducer of apoptosis generated by alternative mRNA splicing, in the culture supernatants (Fig. 7B). In addition to membrane-anchored Fas, sFas can antagonize cell-surface Fas function, leading to block the Fas-mediated apoptosis (Nagata, 1994). Here, the results show that, compared to BEAS-2B cells non exposed to EVs, sFas concentration in culture supernatants of BEAS-2B exposed to $EV_{THP\text{-}1}$ (obtained in all conditions) was three times lower, whereas it did not significantly vary in supernatants of BEAS-2B exposed to EV_{HM}.

Specifically for the BEAS-2B cultured during 48h in the presence of EV_{HM}, the nucleic acids and culture supernatants were isolated. The expression of 12 genes was measured (Table 2) but none of them showed any difference in expression, whether the cells were cultured in the presence of $EV_{HM/NE}$ or $EV_{HM/PM}$. Interestingly, cytokines quantification showed a significant increase of the secretion for the pro-inflammatory cytokines IL6 and TNFα but not for IL1β (Fig. 7C) while their gene expression do not differ from controls after 48h of exposure. Surprisingly, $EV_{HM/NE}$ significantly induced IL8 secretion by BEAS-2B in comparison with BEAS-2B cultured in the absence of EVs or in the presence of $EV_{HM/PM}$.

Table 2. mRNA expression in BEAS-2B cultured in the presence of EVs from exposed macrophages.
Results of $2^{-\Delta\Delta Ct}$ relative quantification are the medians of duplicates of three independent experiments +/- inter-quartile range. Significance RQ>2 or RQ<0.5 reported to the value of BEAS-2B cultured in the EVs from non exposed macrophages, normalized to 1 with 18S. ND, not detected.

	Relative quantification $\frac{\text{BEAS-2B + EV}_{HM/PM}}{\text{BEAS-2B + EV}_{HM/NE}}$ (Median [Q1-Q3])
IL-1β	0,72 [0,69 – 0,73]
IL-6	0,84 [0,79 – 1,19]
IL-8	0,93 [0,85 – 0,99]
TNFα	ND
TGFβ	0,97 [0,96 – 0,98]
CYP1A1	0,81 [0,57 – 0,91]
CYP1B1	0,80 [0,78 – 0,83]
CYP2E1	ND
NRF2	0,97 [0,90 – 1,02]
HMOX	ND
NQO1	0,91 [0,84 – 0,96]
SOD2	1,03 [0,99 – 1,21]

4. Discussion

Alveolar macrophages are the predominant cells that clean inhaled particulate matter off the lung (Miyata and van Eeden, 2011). In parallel, they produce pro-inflammatory mediators implicated in local as well as systemic inflammatory responses, leading to the recruitment of bone marrow derived macrophages (Hiraiwa and van Eeden, 2013; van Eeden and Hogg, 2002). Whereas many studies have focused on resident alveolar macrophages response when exposed to $PM_{2.5}$, none were dealing with the response of infiltrating macrophages. Here, the PM cytotoxicity and the activation of gene expression of XMEs and enzymes involved in oxidative stress of infiltrating macrophages in response to $PM_{2.5}$ exposure was firstly assessed. To do that, undifferentiated THP-1 cells (non-treated with PMA) and primary macrophages (obtained by treating primary monocytes with GM-CSF during 36h, to mimic their recruitment by alveolar macrophages towards the lung (Goto et al., 2004b, 2004a)) were exposed to $PM_{2.5-0.3}$ (at 0, 12.5 and 50 µ/mL during 6h, 24h and 48h for THP-1 and at 0 and 12.5 µg/mL during 48h for human macrophages). In these conditions, PM exposure was not significantly cytotoxic for both THP-1 and human infiltrating macrophages, compared to non exposed control (Fig.2A and 4A).

Concerning XME expression, previous results have shown that $PM_{2.5-0.3}$ induces high and dose-dependent increase of CYP1A1 expression in alveolar macrophages, which could be related to the presence of PAHs in PM (Abbas et al., 2009; Saint-Georges et al., 2008). In this study, in undifferentiated THP-1 cells, CYP1A1 expression was significantly induced at 6h and decreased with time. Surprisingly, CYP1B1 expression importantly increased as well, whereas it has been shown that, in $PM_{2.5-0.3}$ exposed lung epithelial cells, CYP1B1 was much less induced than CYP1A1 (Capucine Lepers et al., 2014). Concerning CYP2E1, while its expression

has been shown to be significantly induced in alveolar macrophages by $PM_{2.5-0.3}$ exposure (Abbas et al., 2009; Saint-Georges et al., 2008), in THP-1 monocytic cells, no induction of its expression has been observed. By exposing infiltrating primary macrophages, similar results have been obtained to a lesser extent, with a significant induction of CYP1A1 and CYP1B1 expression (RQ>2) and no significant increase of CYP2E1 expression (RQ<2) (Fig. 4B), suggesting that PAHs associated to the collected $PM_{2.5}$ (Fig.1B) are mainly metabolized by the CYP superfamily members CYP1A1 and CYP1B1. In parallel, a role of metals in $PM_{2.5-0.3}$ on the expression of CYP1A1 cannot be excluded. Indeed, it was demonstrated that metal ions as Hg^{2+}, Pb^{2+}, and Cu^{2+} were able to induce its gene expression at transcriptional and posttranscriptional levels (Korashy and El-Kadi, 2005). On the one hand, an AhR-dependent mechanism contributing to the CYP1A1 induction was involved in the presence of metal ions. The latter did not significantly affect mRNA; however, they decreased the degradation rate of its protein, suggesting a posttranslational regulation of the CYP1A1 transcript by heavy metals. In addition, the authors observed a significant reduction in AhR ligand-mediated induction of CYP1A1 activity along with an increase in HMOX and a decrease in cellular heme content. The same research group also found that an increased of ROS production by the AhR ligands potentially generated in the presence of metals, were accompanied by a decrease in the CYP1A1 catalytic activity (Elbekai et al., 2004).

Oxidative stress is a primary effect involved in the $PM_{2.5-0.3}$ toxicity. It is generally correlated to ROS generated by PAHs and/or metals in PM (Cachon et al., 2014; Dergham et al., 2012) (they were detected in the considered PM (Fig.1A and B)) and associated with the induction of enzymes implicated in quinones detoxification like NQO1 or associated with ROS production like HMOX (Jaguin et al., 2015). This oxidative stress also represents an imbalance between the production and the removal of ROS by the antioxidant enzymes such as SOD family enzymes (Michael et al., 2013). In this study, SOD2, NQO1 and HMOX expression was significantly induced in primary macrophages (Fig. 4B), dose-dependent in undifferentiated THP-1 (Fig. 2B). Taken together, these results reveal that human infiltrating macrophages are capable to initiate an antioxidant response after exposure to $PM_{2.5-0.3}$ and have effective metabolic capacities.

Redox-sensitive signaling cascades induced by PM exposure through oxidative stress can further activate an inflammatory response (Gerlofs-Nijland et al., 2009), characterized by pro-inflammatory cytokines production like IL1β, IL6, and TNFα as well as IL8 (Dergham et al., 2012; Miyata and van Eeden, 2011; Yan et al., 2016). In undifferentiated THP-1, in concordance with these previous results, IL8, IL1β and TNFα expression was significantly induced in a dose-dependent manner at the earlier time of 6h (Fig. 2C) and decreased with time. Their secretion was also classically induced in a dose- and time-dependent manner (Fig. 2D). Similar

observations have been done in exposed primary human macrophages (Fig. 4C and D), except for TNFα and IL6 for which 48h could be too late to measure their expression. In a surprising way, IL6 secretion was found to be significantly induced in undifferentiated THP-1 (Fig.2D) but only at the highest dose of 50 µg/mL of PM and at 48h. All of these results suggest a pro-inflammatory reaction of the infiltrating macrophages in response to the $PM_{2.5-0.3}$ exposure.

The time of 48h and the dose of 12.5 µg/mL of PM for the exposure of infiltrating primary macrophages have been chosen by taking into account the results obtained for THP-1. At these time and dose, infiltrating macrophages set up (i) a response allowing the metabolization of PAHs, (ii) the antioxidant system and (iii) a pro-inflammatory response. Moreover, primary macrophages presented an increase of their granularity after exposure (Fig.5A) and even if they were not differentiated, they were able to phagocyte the $PM_{2.5-0.3}$ (Fig.5B) probably through MSR1 receptor found to be increased (Fig.5C). This is in accordance with previous studies, showing a role for scavenger-type receptors in the uptake of unopsonized environmental particles (Hiraiwa and van Eeden, 2013).

Acute and chronic exposure to PM has been associated with increased morbidity and mortality from cardiorespiratory diseases as well as an increase of lung cancer risk (Bentayeb et al., 2015; Raaschou-Nielsen et al., 2013). Different mechanisms have been proposed implicating that the exposed cells act as well as on the lung microenvironment and afar through blood circulation. Among the known mechanisms of action through which PM could impact health, EVs are a well suited candidate as they are produced in the pulmonary environment in response to environmental stimuli (Neven et al., 2017). Among the studies dealing with EVs, some have shown that the production of EVs is increased in response to PM exposure (Bollati et al., 2015, 2010; Cantone et al., 2014; Neri et al., 2016). One objective was to determine if infiltrating macrophages are capable to release EVs in response to $PM_{2.5}$ exposure. To test this hypothesis, EVs were isolated from the supernatants of culture of THP-1 non exposed to $PM_{2.5-0.3}$ or exposed to 12.5 µg/mL, 50 µg/mL for 6h, 24h and 48h and of infiltrating primary human macrophages non exposed or exposed to 12.5 µg/mL for 48h. It was shown for the first time that THP-1 and infiltrating primary human macrophages exposed to $PM_{2.5-0.3}$ produce EVs, in a dose- and time-dependent manner as shown by NTA (Fig. 3A and 6A). Recently, Soni et al. have highlighted the implication of alveolar macrophages-derived EVs in acute lung injury (Soni et al., 2016). Here it can be hypothesized that EVs produced by infiltrating macrophages following $PM_{2.5}$ exposure have a role in $PM_{2.5}$-induced injury. Nevertheless in further studies, it would be essential to ensure that the effects that has been observed in pulmonary epithelial cells is really due to the presence of EVs, or rather due to the presence of free proteins,

RNA molecules, or cell membrane fragments into the medium. Other further studies are necessary to characterized these EVs and identify potential exposure biomarkers.

Lung homeostasis is maintained in large part due to the crosstalk between the alveolar macrophages, predominant lung-resident immune cells, and the lung epithelial cells, the major constituents of the respiratory surface (Haggadone and Peters-Golden, 2018). When exposed to $PM_{2.5}$, this balance is modified: several studies have shown that macrophages exposed to PM induce the expression of several enzymes like CYP1A1 and NQO1 as well as pro-inflammatory cytokines in co-cultured lung cells (Abbas et al., 2009; Wang et al., 2019), indicating that PM-exposed macrophages cause pulmonary cells to develop a phenotype of oxidative stress, metabolic activation, and pro-inflammatory response. Based on this statement, the proposed hypothesis is that macrophages released factors could be extracellular vesicles. The expression of genes involved in xenobiotic metabolization, oxidative stress or inflammation in BEAS-2B lung epithelial cells cultured in the presence of $EV_{HM/NE}$ or $EV_{HM/PM}$ was not significantly different (Table 1). However, in the presence of $EV_{HM/PM}$, the secretion of the two pro-inflammatory cytokines TNFα and IL6 was induced, IL8 secretion was inhibited and no change was observed for IL1β (Fig. 7C). These results suggest that EVs do not act on gene expression but downstream on protein synthesis. It is indeed known that $PM_{2.5}$ exposure directly introduce the pro-inflammatory cytokines IL6 and IL8 release, and to a lesser extend TNFα, by BEAS-2B, whatever the origin of the particles (Cachon et al., 2014; Dergham et al., 2012). These results were confirmed with our $PM_{2.5\text{-}0.3}$, which induce a high increase of IL6, IL8 and TNFα release and not of IL1β (Supp. Fig. 4). In the case of IL6 and TNFα, EVs could act as enhancers of inflammation during the acute phase of the inflammatory response, whereas, as IL8 mediates the recruitment and activation of immune cells, rather act to inhibit this part of the inflammatory response. To answer these questions, further experiments could be performed, by exposing BEAS-2B with EVs derived from exposed macrophages along with $PM_{2.5\text{-}0.3}$.

To go further in the discussion, it is now well established that one of the main components of EVs is miRNAs. A change in the protein expression could be related to the change in the miRNA profile of EVs obtained after exposure to PM. Assuming that, the quantification of miRNAs in EVs of macrophages exposed or not to PM was performed and no significant change was observed (data not shown). Nevertheless, it was shown that $EV_{HM/PM}$ in culture with BEAS-2B cells induced the release of pro-inflammatory cytokines by pulmonary epithelial cells. It suggests that EVs may be involved in the mechanism of the inflammatory response often associated with PM exposure. Moreover, the type of cells from which the EVs originate is preponderant, in particular the fact that they are cancerous or not. Indeed, very interestingly, EVs from THP-1, whether or not

exposed to PM, induce a large cytotoxicity in BEAS-2B compared to EVs from primary macrophages (Fig. 7A). The fact that the level of sFas in the culture supernatant of BEAS-2B grown in the presence of EV_{THP-1} decreases by a factor of 3 compared to BEAS-2B grown in the absence of EVs or in the presence of EV_{HM} suggests that the decrease in sFas could allow Fas to bind to its ligand, which leads to supposed BEAS-2B apoptosis. Coupling these results with post-exposure cytotoxicity measurement, it can be hypothesized that EVs from THP-1, exposed or not to $PM_{2.5-0.3}$, cause BEAS-2B cell death by inhibiting the secretion of sFas. This difference in effect observed between the EV_{THP-1} and the EV_{HM} may be due to the cancerous nature of the THP-1 cell line.

5. Conclusion

This study shows that, as alveolar macrophages, infiltrating macrophages respond to PM exposure by an increase in the expression of the enzymes involved in the metabolization of xenobiotics, in oxidative stress and inflammatory response, as well an increase of the production of extracellular vesicles. These latter were able to induce on pulmonary epithelial cells an increase in the production of proinflammatory cytokines such as IL6 and TNFα suggesting they could play a role in the maintenance of inflammation induced by PM exposure. Although extensive studies would be needed to confirm these findings, it appears that infiltrating macrophages could exacerbate the PM-induced proinflammatory response on alveolar macrophages and maintain it via the action of extracellular vesicles on pulmonary epithelial cells. Further investigations will be carried out on the effects of PM on BEAS-2B, namely their ability to potentially release more extracellular vesicles in response to $PM_{2.5}$ exposure and the effects of these EVs on the cellular environment.

List of abbreviations

AM , Alveolar Macrophages; CAT, catalase; COPD, Chronic Obstructive Pulmonary Disease; EVs, Extracellular vesicles; FBS, Fetal Bovine Serum; GM-CSF, Granulocyte-macrophage colony-stimulating factor; HMOX, heme oxygenase; ICP-OES, inductively coupled plasma- optical emission spectroscopy; MSR1, Macrophage Scavenger Receptor 1; Nrf2, Nuclear factor (erythroid-derived 2)-like 2; NQO1, NAD(P)H quinone dehydrogenase 1; NTA, Nanoparticle Tracking Analysis; PAHs, Polycyclic Aromatic Hydrocarbons; PBMC, Peripheral Blood Mononuclear Cell; PM, Particulate Matter; PMA, Phorbol myristate acetate; ROS, Reactive Oxygen Species; SOD2, Superoxide Dismutase 2; USEPA, United States Environmental Protection Agency; WHO, World Health Organization; XME, Xenobiotic Metabolizing Enzymes.

Declarations

Ethics approval and consent to participate

Not applicable.

Conflict of Interest

The authors declare that they have no conflict of Interest.

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References

Abbas, I., Saint-Georges, F., Billet, S., Verdin, A., Mulliez, P., Shirali, P., Garçon, G., 2009. Air pollution particulate matter (PM2.5)-induced gene expression of volatile organic compound and/or polycyclic aromatic hydrocarbon-metabolizing enzymes in an in vitro coculture lung model. Toxicol. In Vitro 23, 37–46. https://doi.org/10.1016/j.tiv.2008.09.020

Benedikter, B.J., Wouters, E.F.M., Savelkoul, P.H.M., Rohde, G.G.U., Stassen, F.R.M., 2018. Extracellular vesicles released in response to respiratory exposures: implications for chronic disease. J. Toxicol. Environ. Health B Crit. Rev. 1–19. https://doi.org/10.1080/10937404.2018.1466380

Bentayeb, M., Wagner, V., Stempfelet, M., Zins, M., Goldberg, M., Pascal, M., Larrieu, S., Beaudeau, P., Cassadou, S., Eilstein, D., Filleul, L., Le Tertre, A., Medina, S., Pascal, L., Prouvost, H., Quénel, P., Zeghnoun, A., Lefranc, A., 2015. Association between long-term exposure to air pollution and mortality in France: A 25-year follow-up study. Environ. Int. 85, 5–14. https://doi.org/10.1016/j.envint.2015.08.006

Billet, S., Garçon, G., Dagher, Z., Verdin, A., Ledoux, F., Cazier, F., Courcot, D., Aboukais, A., Shirali, P., 2007. Ambient particulate matter (PM2.5): Physicochemical characterization and metabolic activation of the organic fraction in human lung epithelial cells (A549). Environ. Res. 105, 212–223. https://doi.org/10.1016/j.envres.2007.03.001

Bollati, V., Angelici, L., Rizzo, G., Pergoli, L., Rota, F., Hoxha, M., Nordio, F., Bonzini, M., Tarantini, L., Cantone, L., Pesatori, A.C., Apostoli, P., Baccarelli, A.A., Bertazzi, P.A., 2015. Microvesicle-associated microRNA expression is altered upon particulate matter exposure in healthy workers and in A549 cells. J. Appl. Toxicol. JAT 35, 59–67. https://doi.org/10.1002/jat.2987

Bollati, V., Marinelli, B., Apostoli, P., Bonzini, M., Nordio, F., Hoxha, M., Pegoraro, V., Motta, V., Tarantini, L., Cantone, L., Schwartz, J., Bertazzi, P.A., Baccarelli, A., 2010. Exposure to Metal-Rich Particulate Matter Modifies the Expression of Candidate MicroRNAs in Peripheral Blood Leukocytes. Environ. Health Perspect. 118, 763–768. https://doi.org/10.1289/ehp.0901300

Borgie, M., Dagher, Z., Ledoux, F., Verdin, A., Cazier, F., Martin, P., Hachimi, A., Shirali, P., Greige-Gerges, H., Courcot, D., 2015. Comparison between ultrafine and fine particulate matter collected in Lebanon: Chemical characterization, in vitro cytotoxic effects and metabolizing enzymes gene expression in human bronchial epithelial cells. Environ. Pollut. Barking Essex 1987 205, 250–260. https://doi.org/10.1016/j.envpol.2015.05.027

Cachon, B.F., Firmin, S., Verdin, A., Ayi-Fanou, L., Billet, S., Cazier, F., Martin, P.J., Aissi, F., Courcot, D., Sanni, A., Shirali, P., 2014. Proinflammatory effects and oxidative stress within human bronchial epithelial cells exposed to atmospheric particulate matter (PM(2.5) and PM(>2.5)) collected from Cotonou, Benin. Environ. Pollut. Barking Essex 1987 185, 340–351. https://doi.org/10.1016/j.envpol.2013.10.026

Cantone, L., Angelici, L., Bollati, V., Bonzini, M., Apostoli, P., Tripodi, A., Bertazzi, P.A., Baccarelli, A.A., 2014. Extracellular histones mediate the effects of metal-rich air particles on blood coagulation. Environ. Res. 132, 76–82. https://doi.org/10.1016/j.envres.2014.03.029

Cazier, F., Genevray, P., Dewaele, D., Nouali, H., Verdin, A., Ledoux, F., Hachimi, A., Courcot, L., Billet, S., Bouhsina, S., Shirali, P., Garçon, G., Courcot, D., 2016. Characterisation and seasonal variations of particles in the atmosphere of rural, urban and industrial areas: Organic compounds. J. Environ. Sci. 44, 45–56. https://doi.org/10.1016/j.jes.2016.01.014

Cohen, A.J., Samet, J.M., Straif, K., International Agency for Research on Cancer, 2013. Air pollution and cancer. International Agency for Research on Cancer, Lyon.

Dergham, M., Lepers, C., Verdin, A., Billet, S., Cazier, F., Courcot, D., Shirali, P., Garçon, G., 2012. Prooxidant and Proinflammatory Potency of Air Pollution Particulate Matter (PM2.5–0.3) Produced in Rural, Urban, or Industrial Surroundings in Human Bronchial Epithelial Cells (BEAS-2B). Chem. Res. Toxicol. 25, 904–919. https://doi.org/10.1021/tx200529v

Duval, M.M., Friedlander, S.K., 1981. Source resolution of polycyclic aromatic hydrocarbons in the Los Angeles atmosphere: application of a chemical species balance method with first order chemical decay. Final report Jan-Dec 80 (No. PB-82-121336). California Univ., Los Angeles (USA). Dept. of Chemical, Nuclear, and Thermal Engineering.

Elbekai, R.H., Korashy, H.M., Wills, K., Gharavi, N., El-Kadi, A.O.S., 2004. Benzo[a]pyrene, 3-methylcholanthrene and beta-naphthoflavone induce oxidative stress in hepatoma hepa 1c1c7 Cells by an AHR-dependent pathway. Free Radic. Res. 38, 1191–1200. https://doi.org/10.1080/10715760400017319

Epelman, S., Lavine, K.J., Randolph, G.J., 2014. Origin and functions of tissue macrophages. Immunity 41, 21–35. https://doi.org/10.1016/j.immuni.2014.06.013

571 Fathi, M., Johansson, A., Lundborg, M., Orre, L., Sköld, C.M., Camner, P., 2001. Functional and morphological
572 differences between human alveolar and interstitial macrophages. Exp. Mol. Pathol. 70, 77–82.
573 https://doi.org/10.1006/exmp.2000.2344
574 Gerlofs-Nijland, M.E., Rummelhard, M., Boere, A.J.F., Leseman, D.L.A.C., Duffin, R., Schins, R.P.F., Borm,
575 P.J.A., Sillanpää, M., Salonen, R.O., R. Cassee, F., 2009. Particle Induced Toxicity in Relation to
576 Transition Metal and Polycyclic Aromatic Hydrocarbon Contents. Environ. Sci. Technol. 43, 4729–
577 4736. https://doi.org/10.1021/es803176k
578 Gietl, J.K., Lawrence, R., Thorpe, A.J., Harrison, R.M., 2010. Identification of brake wear particles and
579 derivation of a quantitative tracer for brake dust at a major road. Atmos. Environ. 44, 141–146.
580 https://doi.org/10.1016/j.atmosenv.2009.10.016
581 Goto, Y., Hogg, J.C., Shih, C.-H., Ishii, H., Vincent, R., van Eeden, S.F., 2004a. Exposure to ambient particles
582 accelerates monocyte release from bone marrow in atherosclerotic rabbits. Am. J. Physiol.-Lung Cell.
583 Mol. Physiol. 287, L79–L85. https://doi.org/10.1152/ajplung.00425.2003
584 Goto, Y., Ishii, H., Hogg, J.C., Shih, C.-H., Yatera, K., Vincent, R., van Eeden, S.F., 2004b. Particulate Matter
585 Air Pollution Stimulates Monocyte Release from the Bone Marrow. Am. J. Respir. Crit. Care Med. 170,
586 891–897. https://doi.org/10.1164/rccm.200402-235OC
587 Gualtieri, M., Ledoux, F., Verdin, A., Billet, S., Martin, P.J., Courcot, D., 2017. Particulate Matter Physico-
588 Chemical Characterization and in vitro Toxicological Effects. Airborne Part. Orig. Emiss. Health
589 Impacts.
590 Haggadone, M.D., Peters-Golden, M., 2018. Microenvironmental Influences on Extracellular Vesicle-Mediated
591 Communication in the Lung. Trends Mol. Med. 24, 963–975.
592 https://doi.org/10.1016/j.molmed.2018.08.006
593 Héliot, A., Landkocz, Y., Roy Saint-Georges, F., Gosset, P., Billet, S., Shirali, P., Courcot, D., Martin, P.J.,
594 2017. Smoker extracellular vesicles influence status of human bronchial epithelial cells. Int. J. Hyg.
595 Environ. Health 220, 445–454. https://doi.org/10.1016/j.ijheh.2016.12.010
596 Hiraiwa, K., van Eeden, S.F., 2013. Contribution of Lung Macrophages to the Inflammatory Responses Induced
597 by Exposure to Air Pollutants. Mediators Inflamm. 2013, e619523.
598 https://doi.org/10.1155/2013/619523
599 Hleis, D., Fernández-Olmo, I., Ledoux, F., Kfoury, A., Courcot, L., Desmonts, T., Courcot, D., 2013. Chemical
600 profile identification of fugitive and confined particle emissions from an integrated iron and
601 steelmaking plant. J. Hazard. Mater. 250–251, 246–255. https://doi.org/10.1016/j.jhazmat.2013.01.080
602 INERIS, 2005. Institut National de l'Environnement Industriel et des Risques. Fiche de données toxicologiques
603 et environnementales des substances chimiques, Benzo[b]fluoranthène. INERIS –DRC-02-25590-
604 02DF47, 36p.
605 INERIS, 2004a. Institut National de l'Environnement Industriel et des Risques. Fiche de données toxicologiques
606 et environnementales des substances chimiques, dibenzo[a,h]anthracène. INERIS –DRC-01-25590-
607 01DR125, 37p.
608 INERIS, 2004b. Institut National de l'Environnement Industriel et des Risques. Fiche de données toxicologiques
609 et environnementales des substances chimiques, Indeno[1,2,3-cd]pyrène. INERIS–DRC-01-25590-
610 01DR122, 35p.
611 INERIS, 2004c. Institut National de l'Environnement Industriel et des Risques. Fiche de données toxicologiques
612 et environnementales des substances chimiques, Chrysène. DRC-11-117259-10328A, 46p.
613 Jabalee, J., Towle, R., Garnis, C., 2018. The Role of Extracellular Vesicles in Cancer: Cargo, Function, and
614 Therapeutic Implications. Cells 7. https://doi.org/10.3390/cells7080093
615 Jaguin, M., Fardel, O., Lecureur, V., 2015. Exposure to Diesel Exhaust Particle Extracts (DEPe) Impairs Some
616 Polarization Markers and Functions of Human Macrophages through Activation of AhR and Nrf2.
617 PLOS ONE 10, e0116560. https://doi.org/10.1371/journal.pone.0116560
618 Kfoury, A., Ledoux, F., Roche, C., Delmaire, G., Roussel, G., Courcot, D., 2016. PM2.5 source apportionment
619 in a French urban coastal site under steelworks emission influences using constrained non-negative
620 matrix factorization receptor model. J. Environ. Sci. China 40, 114–128.
621 https://doi.org/10.1016/j.jes.2015.10.025
622 Korashy, H.M., El-Kadi, A.O.S., 2005. Regulatory mechanisms modulating the expression of cytochrome P450
623 1A1 gene by heavy metals. Toxicol. Sci. Off. J. Soc. Toxicol. 88, 39–51.
624 https://doi.org/10.1093/toxsci/kfi282
625 Landkocz, Y., Ledoux, F., André, V., Cazier, F., Genevray, P., Dewaele, D., Martin, P.J., Lepers, C., Verdin, A.,
626 Courcot, L., Boushina, S., Sichel, F., Gualtieri, M., Shirali, P., Courcot, D., Billet, S., 2017. Fine and
627 ultrafine atmospheric particulate matter at a multi-influenced urban site: Physicochemical
628 characterization, mutagenicity and cytotoxicity. Environ. Pollut. 221, 130–140.
629 https://doi.org/10.1016/j.envpol.2016.11.054

Ledoux, F., Roche, C., Cazier, F., Beaugard, C., Courcot, D., 2018. Influence of ship emissions on NOx, SO2, O3 and PM concentrations in a North-Sea harbor in France. J. Environ. Sci. China 71, 56–66. https://doi.org/10.1016/j.jes.2018.03.030

Lee, H., Abston, E., Zhang, D., Rai, A., Jin, Y., 2018. Extracellular Vesicle: An Emerging Mediator of Intercellular Crosstalk in Lung Inflammation and Injury. Front. Immunol. 9, 924. https://doi.org/10.3389/fimmu.2018.00924

Lepers, Capucine, André, V., Dergham, M., Billet, S., Verdin, A., Garçon, G., Dewaele, D., Cazier, F., Sichel, F., Shirali, P., 2014. Xenobiotic metabolism induction and bulky DNA adducts generated by particulate matter pollution in BEAS-2B cell line: geographical and seasonal influence. J. Appl. Toxicol. 34, 703–713. https://doi.org/10.1002/jat.2931

Lepers, C., Dergham, M., Armand, L., Billet, S., Verdin, A., Andre, V., Pottier, D., Courcot, D., Shirali, P., Sichel, F., 2014. Mutagenicity and clastogenicity of native airborne particulate matter samples collected under industrial, urban or rural influence. Toxicol. Vitro Int. J. Publ. Assoc. BIBRA 28, 866–874. https://doi.org/10.1016/j.tiv.2014.03.011

Li, R., Zhou, R., Zhang, J., 2018. Function of PM2.5 in the pathogenesis of lung cancer and chronic airway inflammatory diseases. Oncol. Lett. 15, 7506–7514. https://doi.org/10.3892/ol.2018.8355

Lund, M.E., To, J., O'Brien, B.A., Donnelly, S., 2016. The choice of phorbol 12-myristate 13-acetate differentiation protocol influences the response of THP-1 macrophages to a pro-inflammatory stimulus. J. Immunol. Methods 430, 64–70. https://doi.org/10.1016/j.jim.2016.01.012

Manders, A.M.M., Schaap, M., Querol, X., Albert, M.F.M.A., Vercauteren, J., Kuhlbusch, T.A.J., Hoogerbrugge, R., 2010. Sea salt concentrations across the European continent. Atmos. Environ. 44, 2434–2442. https://doi.org/10.1016/j.atmosenv.2010.03.028

Mazzei, F., D'Alessandro, A., Lucarelli, F., Nava, S., Prati, P., Valli, G., Vecchi, R., 2008. Characterization of particulate matter sources in an urban environment. Sci. Total Environ. 401, 81–89. https://doi.org/10.1016/j.scitotenv.2008.03.008

Michael, S., Montag, M., Dott, W., 2013. Pro-inflammatory effects and oxidative stress in lung macrophages and epithelial cells induced by ambient particulate matter. Environ. Pollut., Selected Papers from Urban Environmental Pollution 2012 183, 19–29. https://doi.org/10.1016/j.envpol.2013.01.026

Miyata, R., van Eeden, S.F., 2011. The innate and adaptive immune response induced by alveolar macrophages exposed to ambient particulate matter. Toxicol. Appl. Pharmacol. 257, 209–226. https://doi.org/10.1016/j.taap.2011.09.007

Nagata, S., 1994. Fas and Fas ligand: a death factor and its receptor. Adv. Immunol. 57, 129–144.

Neri, T., Pergoli, L., Petrini, S., Gravendonk, L., Balia, C., Scalise, V., Amoruso, A., Pedrinelli, R., Paggiaro, P., Bollati, V., Celi, A., 2016. Particulate matter induces prothrombotic microparticle shedding by human mononuclear and endothelial cells. Toxicol. Vitro Int. J. Publ. Assoc. BIBRA 32, 333–338. https://doi.org/10.1016/j.tiv.2016.02.001

Neven, K.Y., Nawrot, T.S., Bollati, V., 2017. Extracellular Vesicles: How the External and Internal Environment Can Shape Cell-To-Cell Communication. Curr. Environ. Health Rep. 4, 30–37. https://doi.org/10.1007/s40572-017-0130-7

Oravisjärvi, K., Timonen, K.L., Wiikinkoski, T., Ruuskanen, A.R., Heinänen, K., Ruuskanen, J., 2003. Source contributions to PM2.5 particles in the urban air of a town situated close to a steel works. Atmos. Environ. 37, 1013–1022. https://doi.org/10.1016/S1352-2310(02)01048-8

Raaschou-Nielsen, O., Andersen, Z.J., Beelen, R., Samoli, E., Stafoggia, M., Weinmayr, G., Hoffmann, B., Fischer, P., Nieuwenhuijsen, M.J., Brunekreef, B., Xun, W.W., Katsouyanni, K., Dimakopoulou, K., Sommar, J., Forsberg, B., Modig, L., Oudin, A., Oftedal, B., Schwarze, P.E., Nafstad, P., De Faire, U., Pedersen, N.L., Ostenson, C.-G., Fratiglioni, L., Penell, J., Korek, M., Pershagen, G., Eriksen, K.T., Sørensen, M., Tjønneland, A., Ellermann, T., Eeftens, M., Peeters, P.H., Meliefste, K., Wang, M., Bueno-de-Mesquita, B., Key, T.J., de Hoogh, K., Concin, H., Nagel, G., Vilier, A., Grioni, S., Krogh, V., Tsai, M.-Y., Ricceri, F., Sacerdote, C., Galassi, C., Migliore, E., Ranzi, A., Cesaroni, G., Badaloni, C., Forastiere, F., Tamayo, I., Amiano, P., Dorronsoro, M., Trichopoulou, A., Bamia, C., Vineis, P., Hoek, G., 2013. Air pollution and lung cancer incidence in 17 European cohorts: prospective analyses from the European Study of Cohorts for Air Pollution Effects (ESCAPE). Lancet Oncol. 14, 813–822. https://doi.org/10.1016/S1470-2045(13)70279-1

Roche, C., 2016. Etude des concentrations et de la composition des PM10 sur le littoral du Nord de la France - Evaluation des contributions maritimes de l'espace Manche-Mer du Nord.

Saint-Georges, F., Abbas, I., Billet, S., Verdin, A., Gosset, P., Mulliez, P., Shirali, P., Garçon, G., 2008. Gene expression induction of volatile organic compound and/or polycyclic aromatic hydrocarbon-metabolizing enzymes in isolated human alveolar macrophages in response to airborne particulate matter (PM2.5). Toxicology 244, 220–230. https://doi.org/10.1016/j.tox.2007.11.016

689 Santacatalina, M., Reche, C., Minguillón, M.C., Escrig, A., Sanfelix, V., Carratalá, A., Nicolás, J.F., Yubero, E.,
690 Crespo, J., Alastuey, A., Monfort, E., Miró, J.V., Querol, X., 2010. Impact of fugitive emissions in
691 ambient PM levels and composition: a case study in Southeast Spain. Sci. Total Environ. 408, 4999–
692 5009. https://doi.org/10.1016/j.scitotenv.2010.07.040
693 Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative C(T) method. Nat.
694 Protoc. 3, 1101–1108.
695 Sheng, J., Ruedl, C., Karjalainen, K., 2015. Most Tissue-Resident Macrophages Except Microglia Are Derived
696 from Fetal Hematopoietic Stem Cells. Immunity 43, 382–393.
697 https://doi.org/10.1016/j.immuni.2015.07.016
698 Soni, S., Wilson, M.R., O'Dea, K.P., Yoshida, M., Katbeh, U., Woods, S.J., Takata, M., 2016. Alveolar
699 macrophage-derived microvesicles mediate acute lung injury. Thorax 71, 1020–1029.
700 https://doi.org/10.1136/thoraxjnl-2015-208032
701 Théry, C., Amigorena, S., Raposo, G., Clayton, A., 2006. Isolation and characterization of exosomes from cell
702 culture supernatants and biological fluids. Curr. Protoc. Cell Biol. Editor. Board Juan Bonifacino Al
703 Chapter 3, Unit 3.22. https://doi.org/10.1002/0471143030.cb0322s30
704 Thorpe, A., Harrison, R.M., 2008. Sources and properties of non-exhaust particulate matter from road traffic: a
705 review. Sci. Total Environ. 400, 270–282. https://doi.org/10.1016/j.scitotenv.2008.06.007
706 van Eeden, S.F., Hogg, J.C., 2002. Systemic inflammatory response induced by particulate matter air pollution:
707 the importance of bone-marrow stimulation. J. Toxicol. Environ. Health A 65, 1597–1613.
708 https://doi.org/10.1080/00984100290071685
709 Wang, G., Zhang, X., Liu, X., Zheng, J., Chen, R., Kan, H., 2019. Ambient fine particulate matter induce
710 toxicity in lung epithelial-endothelial co-culture models. Toxicol. Lett. 301, 133–145.
711 https://doi.org/10.1016/j.toxlet.2018.11.010
712 WHO, 2017. Burden of disease from Ambient Air Pollution for 2012 (Report). World Health Organization
713 (WHO).
714 Yamasaki, K., Eeden, S.F. van, 2018. Lung Macrophage Phenotypes and Functional Responses: Role in the
715 Pathogenesis of COPD. Int. J. Mol. Sci. 19, 582. https://doi.org/10.3390/ijms19020582
716 Yan, Z., Wang, J., Li, J., Jiang, N., Zhang, R., Yang, W., Yao, W., Wu, W., 2016. Oxidative stress and
717 endocytosis are involved in upregulation of interleukin-8 expression in airway cells exposed to PM2.5.
718 Environ. Toxicol. 31, 1869–1878. https://doi.org/10.1002/tox.22188

719

720

Figure captions

Fig. 1: $PM_{2.5-0.3}$ composition. A) Major and trace inorganic species concentrations of the $PM_{2.5-0.3}$ collected in Dunkerque (March 2014 - March 2015). B) Polycyclic Aromatic Hydrocarbon concentrations in the $PM_{2.5-0.3}$ collected in Dunkerque (March 2014 - March 2015).

Fig. 2: Cellular response of THP-1 exposed to $PM_{2.5-0.3}$. In each experiment, THP-1 were exposed 6h, 24h or 48h to 0 (NE), 12.5 or 50 µg/mL of $PM_{2.5-0.3}$. A) LDH cytotoxicity quantification in percentage of 100% mortality. mRNA expression of B) CYP1A1, CYP1B1, CYP2E1, SOD2, NQO1, HMOX and C) IL8, IL1β, IL6 and TNFα was measured by RT-qPCR. Results of $2^{-\Delta\Delta Ct}$ relative quantification are the medians of duplicates of three independent experiments +/- inter-quartile range. Significance RQ> 2 or RQ <0.5 reported to the value of unexposed control (NE), normalized to 1 with 18S. D) Measurement of IL8, IL1β, IL6 and TNFα secretion of THP-1 exposed 6h, 24h or 48h to 0 (NE), 12.5 or 50 µg/mL to $PM_{2.5-0.3}$. Results are the medians in pg/mL of duplicates of three independent experiments. Significant differences (*$p<0.05$) were evaluated by Mann Whitney U test.

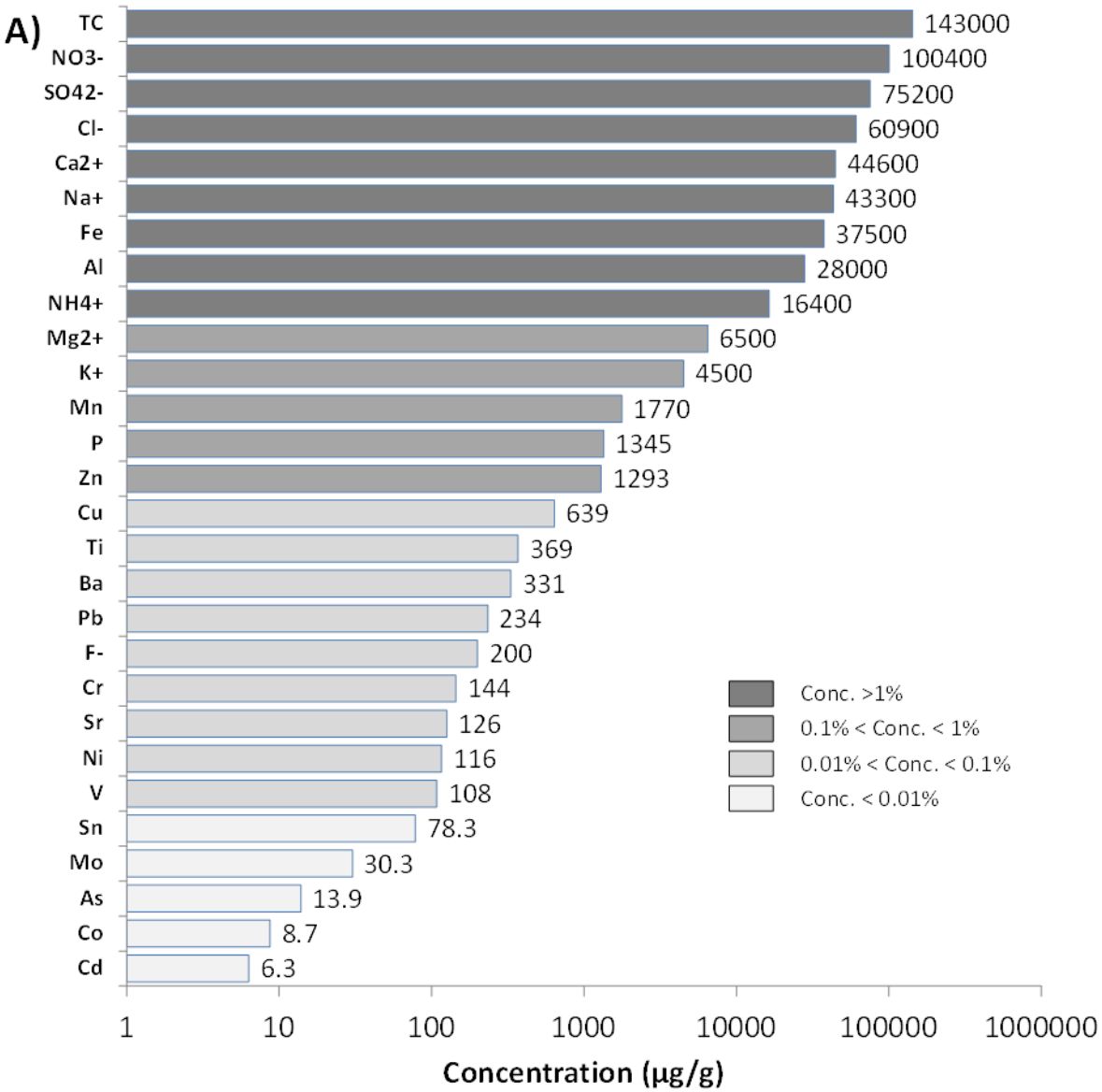
Fig. 3: Characterization of extracellular vesicles derived from THP-1 exposed to $PM_{2.5-0.3}$. EVs were obtained from THP-1 exposed 6h, 24h or 48h to 0 (NE), 12.5 or 50 µg/mL of $PM_{2.5-0.3}$. A) Graphs representing the amounts and the mean of size of EVs measured by nanoparticle tracking analysis. Significant differences were evaluated by Student's T test (*$p<0.01$); B) Transmission electronic microscopy images of EVs (bar = 100nm); C) Western-blot using CD63 and CD81 antibodies on EVs obtained from each condition.

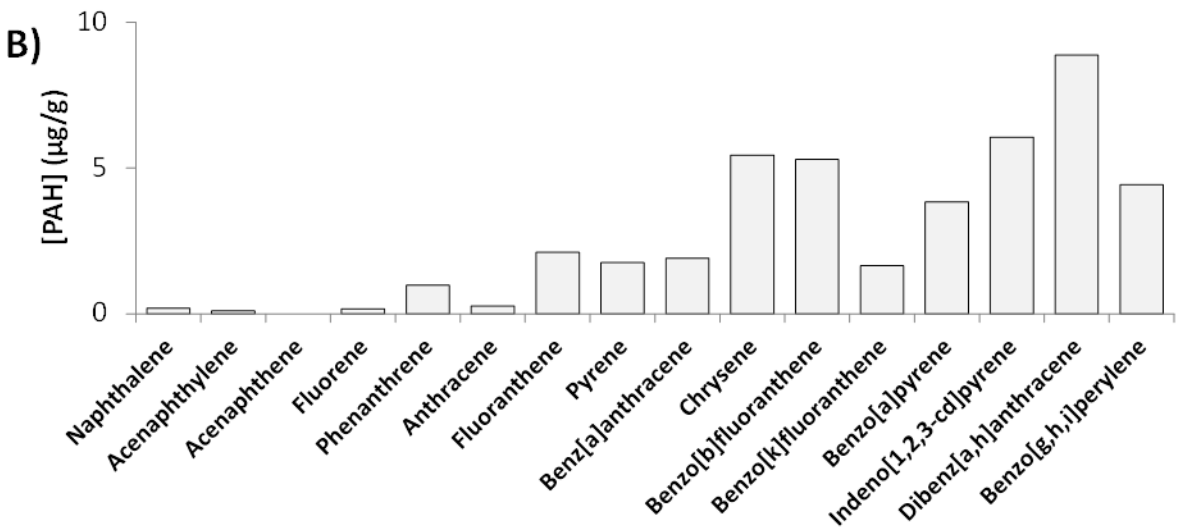
Fig. 4: Cellular response of infiltrating macrophages exposed to $PM_{2.5-0.3}$. In each experiment, primary macrophages from 6 healthy donors were exposed 48h to 0 (NE) or 12.5 µg/mL of $PM_{2.5-0.3}$.A) LDH cytotoxicity quantification in percentage of 100% mortality. mRNA expression of B) CYP1A1, CYP1B1, CYP2E1, SOD2, NQO1, HMOX and C) IL8, IL1β, IL6 and TNFα was measured by RT-qPCR. Results of $2^{-\Delta\Delta Ct}$ relative quantification are the medians of duplicates of three independent experiments +/- inter-quartile range. Significance RQ> 2 or RQ <0.5 reported to the value of unexposed control (NE), normalized to 1 with 18S. D) IL8, IL1β, IL6 and TNFα secretion of primary macrophages exposed 6h, 24h or 48h to 0 (NE), 12.5 or 50 µg/mL to $PM_{2.5-0.3}$. Results are the medians in pg/mL of duplicates of three independent experiments. Significant differences (*$p<0.05$) were evaluated by Mann Whitney U test.

Fig. 5: Morphology of infiltrating macrophages exposed to $PM_{2.5-0.3}$. A) Dot plots of macrophages (black dots in the circle) before PM exposure, non-exposed (NE), or exposed to 12.5 µg/mL of $PM_{2.5}$. Upon PM exposure, the granularity of macrophages increase as evidenced by the shift of SSC-H in comparison to control (NE) macrophages. B) Optical microscopy images of macrophages before PM exposure, non-exposed (NE), or exposed to 12.5 µg/mL of $PM_{2.5}$ showing particles inside the cells. C) Western-blot using MSR1 antibody on proteins from primary macrophages from 5 donors non-exposed (NE), or exposed to 12.5 µg/mL of $PM_{2.5-0.3}$. Relative quantification has been performed using stain-free gel of total proteins.

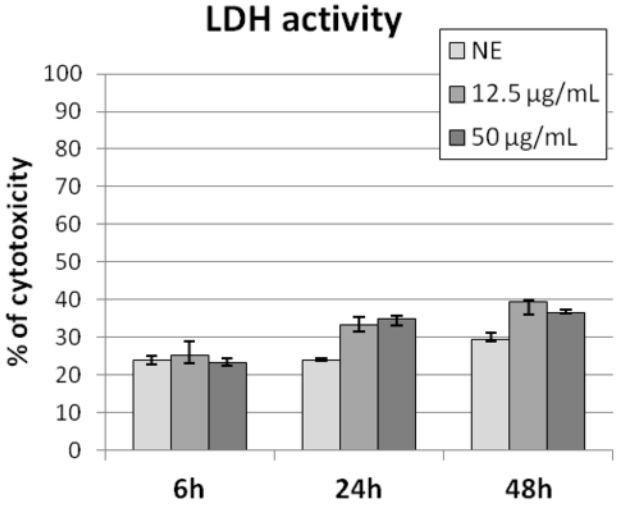
Fig. 6: Characterization of extracellular vesicles derived from primary macrophages exposed to $PM_{2.5-0.3}$. EVs were obtained from pooled supernatants of primary macrophages isolated from 6 donors and exposed 48h to 0 (NE) or 12.5 µg/mL of $PM_{2.5-0.3}$. A) Graph representing the amounts of EVs measured by nanoparticle tracking analysis. Significant differences were evaluated by Student's T test (*$p<0.01$); B) Graphical representation of size distribution of EVs produced by non exposed (NE) and exposed (12.5 µg/mL) primary macrophages; C) Western-blot using CD63 and CD81 antibodies on EVs obtained from each condition; D) Transmission electronic microscopy images of EVs (bar = 100nm except when indicated).

Fig. 7: Effects of EVs derived from human macrophages exposed to $PM_{2.5-0.3}$ on lung epithelial cells. In each experiment, BEAS-2B were co-cultured or not (w/o EV) during 48h with EVs isolated from exposed and non-exposed (NE) THP-1 or primary macrophages to $PM_{2.5}$ at the indicated conditions. A) LDH cytotoxicity quantification in percentage of 100% mortality; B) Quantification sFas secretion by BEAS-2B co-cultured with EVs with ELISA multiplex. Results are the medians in pg/mL of duplicates of three independent experiments. C) Quantification of IL8, IL1β, IL6 and TNFα secretion by BEAS-2B co-cultured with EVs with ELISA multiplex. Results are the medians in pg/mL of duplicates of three independent experiments. Significant differences (*$p<0.05$) were evaluated by Mann Whitney U test.

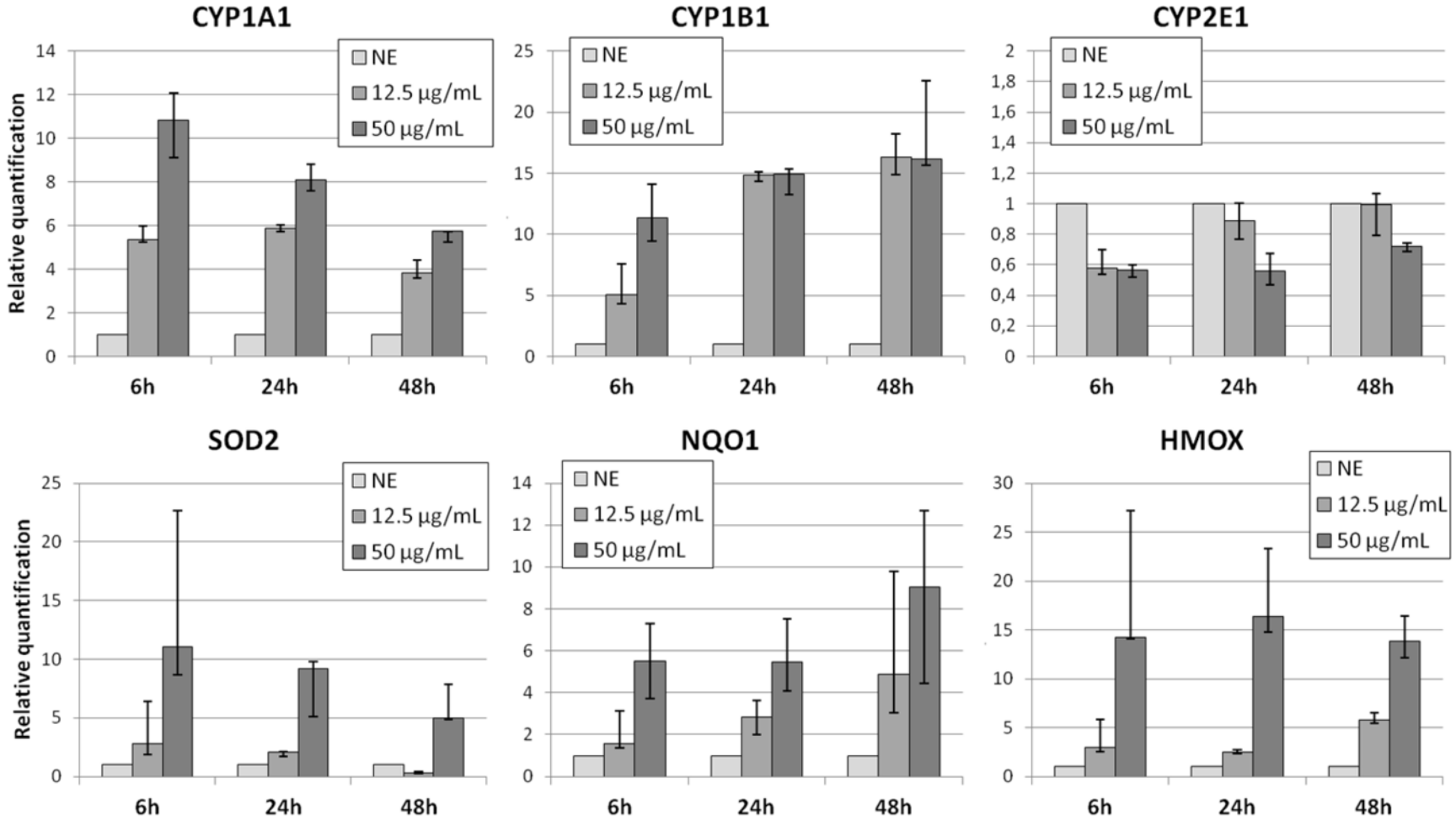

A)
TC
NO3-
SO42-
Cl-
Ca2+
Na+
Fe
Al
NH4+
Mg2+
K+
Mn
P
Zn
Cu
Ti
Ba
Pb
F-
Cr
Sr
Ni
V
Sn
Mo
As
Co
Cd
143000
100400
75200
60900
44600
43300
37500
28000
16400
6500
4500
1770
1345
1293
639
369
331
234
200
144
126
116
108
78.3
30.3
13.9
8.7
6.3
Conc. >1%
0.1% < Conc. < 1%
0.01% < Conc. < 0.1%
Conc. < 0.01%
1
10
100
1000
10000
100000
1000000
Concentration (µg/g)


B)
[PAH] (µg/g)
10
5
0
Naphthalene
Acenaphthylene
Acenaphthene
Fluorene
Phenanthrene
Anthracene
Fluoranthene
Pyrene
Benz[a]anthracene
Chrysene
Benzo[b]fluoranthene
Benzo[k]fluoranthene
Benzo[a]pyrene
Indeno[1,2,3-cd]pyrene
Dibenz[a,h]anthracene
Benzo[g,h,i]perylene

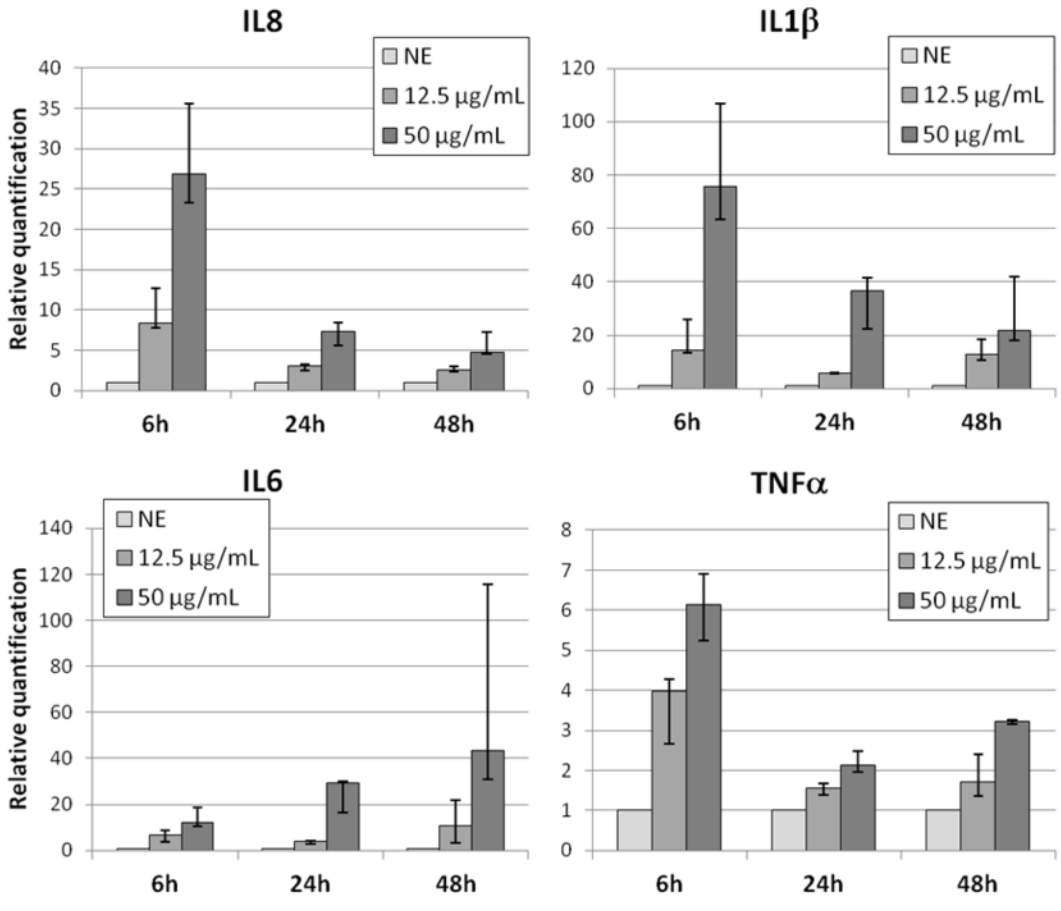
A)


LDH activity
NE
12.5 µg/mL
50 µg/mL
% of cytotoxicity
100
90
80
70
60
50
40
30
20
10
0
6h
24h
48h

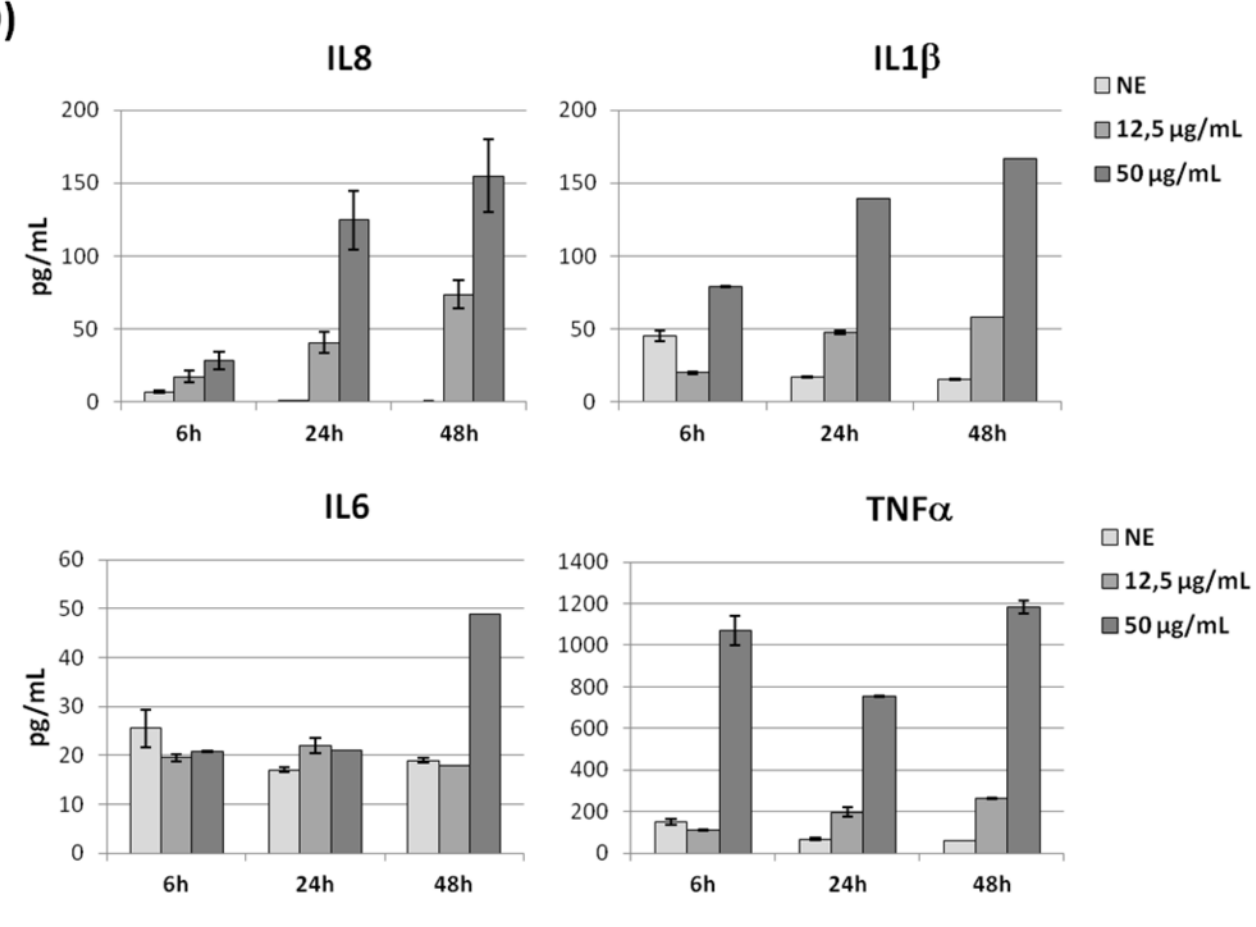
B)

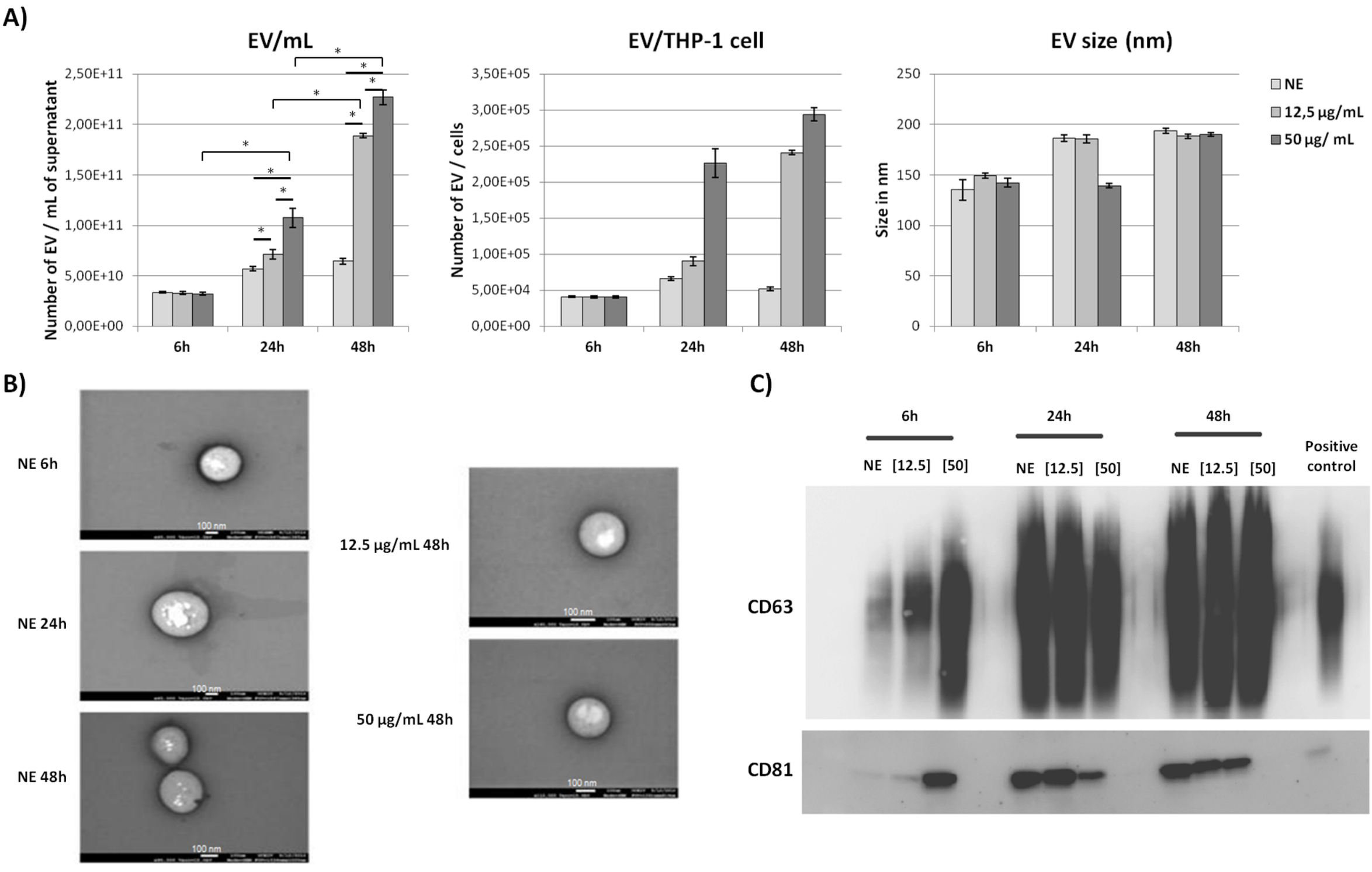

CYP1A1
CYP1B1
CYP2E1
SOD2
NQO1
HMOX
NE
12.5 µg/mL
50 µg/mL
Relative quantification
6h
24h
48h

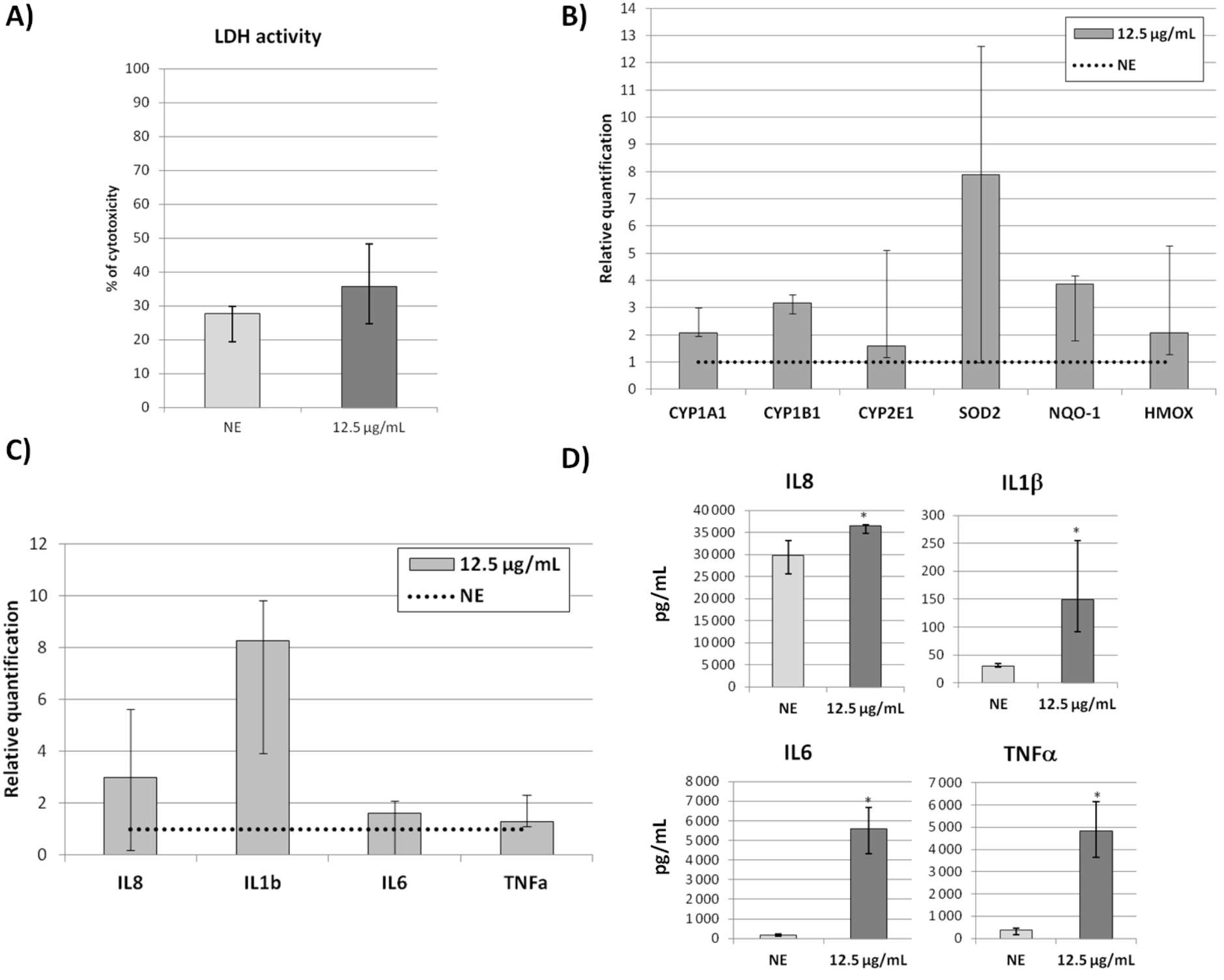
C)


IL8
IL1β
IL6
TNFα
NE
12.5 µg/mL
50 µg/mL
Relative quantification
6h
24h
48h

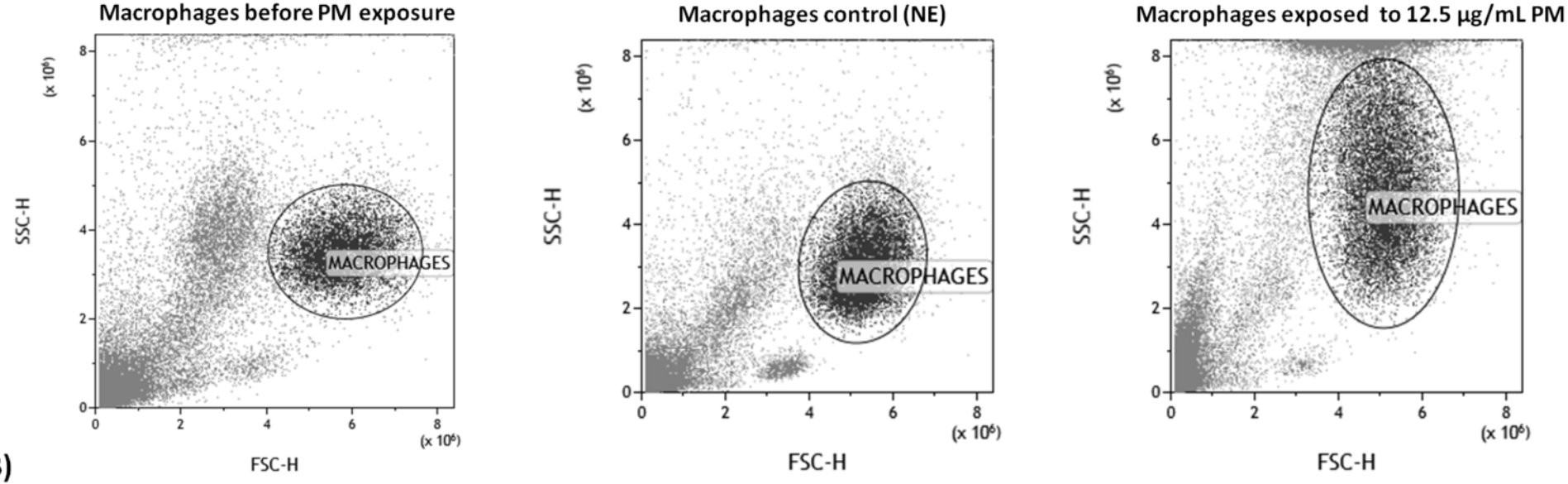
D)


IL8
IL1β
IL6
TNFα
NE
12,5 µg/mL
50 µg/mL
pg/mL
6h
24h
48h

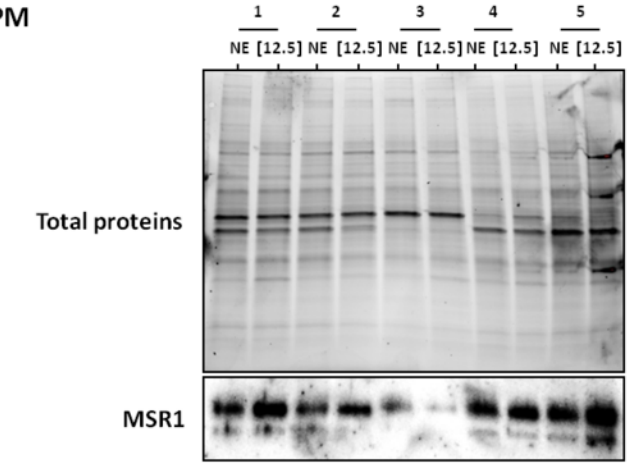

A)
EV/mL
Number of EV / mL of supernatant
2,50E+11
2,00E+11
1,50E+11
1,00E+11
5,00E+10
0,00E+00
6h
24h
48h
EV/THP-1 cell
Number of EV / cells
3,50E+05
3,00E+05
2,50E+05
2,00E+05
1,50E+05
1,00E+05
5,00E+04
0,00E+00
EV size (nm)
Size in nm
250
200
150
100
50
0
NE
12,5 µg/mL
50 µg/ mL
B)
NE 6h
NE 24h
NE 48h
12.5 µg/mL 48h
50 µg/mL 48h
100 nm
C)
6h
24h
48h
NE [12.5] [50]
Positive control
CD63
CD81


A)
LDH activity
% of cytotoxicity
100
90
80
70
60
50
40
30
20
10
0
NE
12.5 µg/mL
B)
Relative quantification
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
12.5 µg/mL
NE
CYP1A1
CYP1B1
CYP2E1
SOD2
NQO-1
HMOX
C)
Relative quantification
12
10
8
6
4
2
0
12.5 µg/mL
NE
IL8
IL1b
IL6
TNFa
D)
IL8
pg/mL
40 000
35 000
30 000
25 000
20 000
15 000
10 000
5 000
0
*
NE
12.5 µg/mL
IL1β
300
250
200
150
100
50
0
*
NE
12.5 µg/mL
IL6
pg/mL
8 000
7 000
6 000
5 000
4 000
3 000
2 000
1 000
0
*
NE
12.5 µg/mL
TNFα
7 000
6 000
5 000
4 000
3 000
2 000
1 000
0
*
NE
12.5 µg/mL

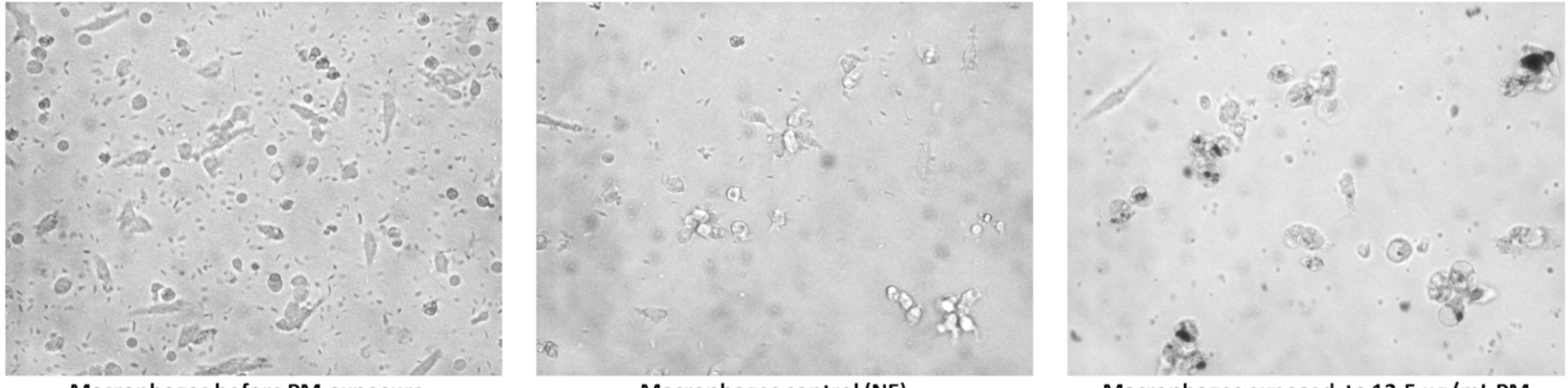
A)

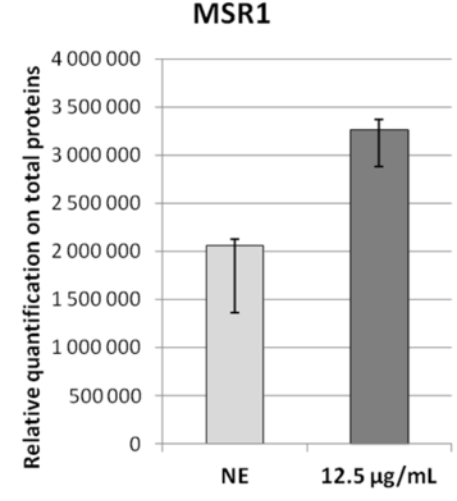

Macrophages before PM exposure
Macrophages control (NE)
Macrophages exposed to 12.5 µg/mL PM
MACROPHAGES
SSC-H
FSC-H
(x 10⁶)

C)

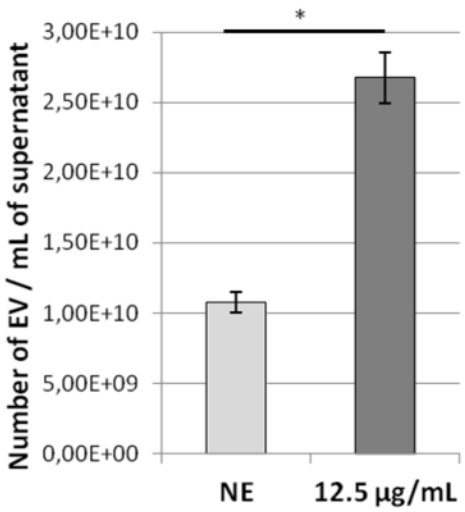

1
2
3
4
5
NE [12.5] NE [12.5] NE [12.5] NE [12.5] NE [12.5]
Total proteins
MSR1

B)

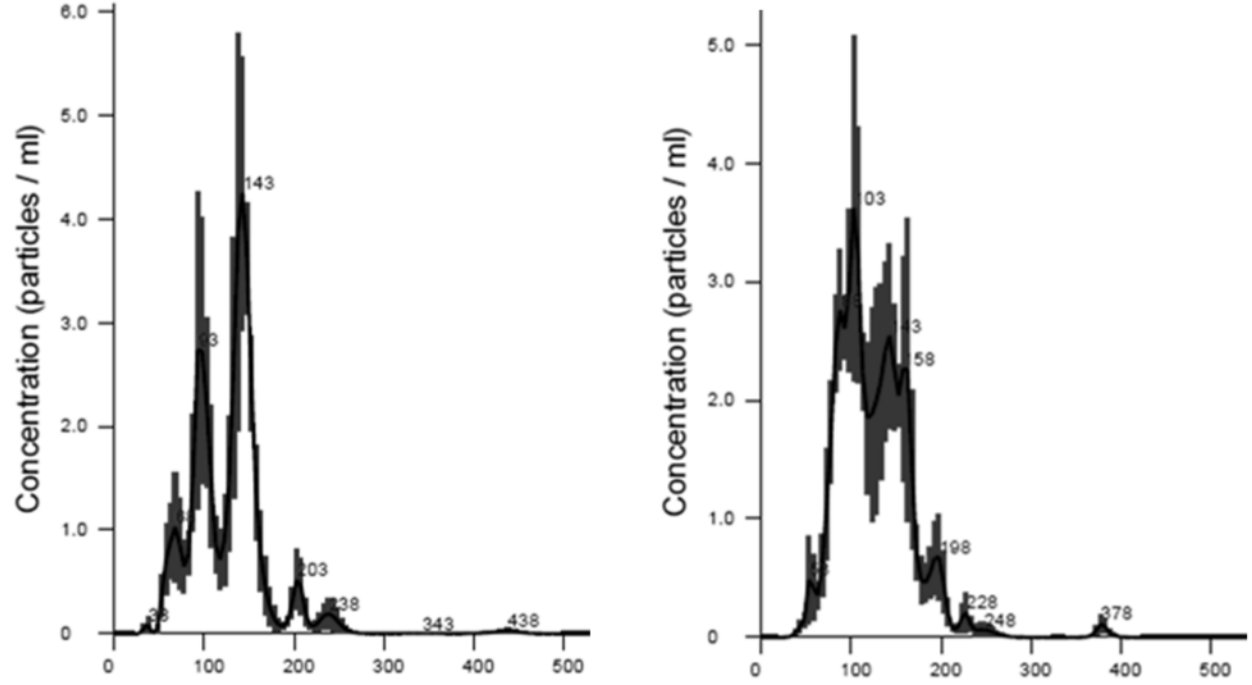

Macrophages before PM exposure
Macrophages control (NE)
Macrophages exposed to 12.5 µg/mL PM


MSR1
Relative quantification on total proteins
4 000 000
3 500 000
3 000 000
2 500 000
2 000 000
1 500 000
1 000 000
500 000
0
NE
12.5 µg/mL

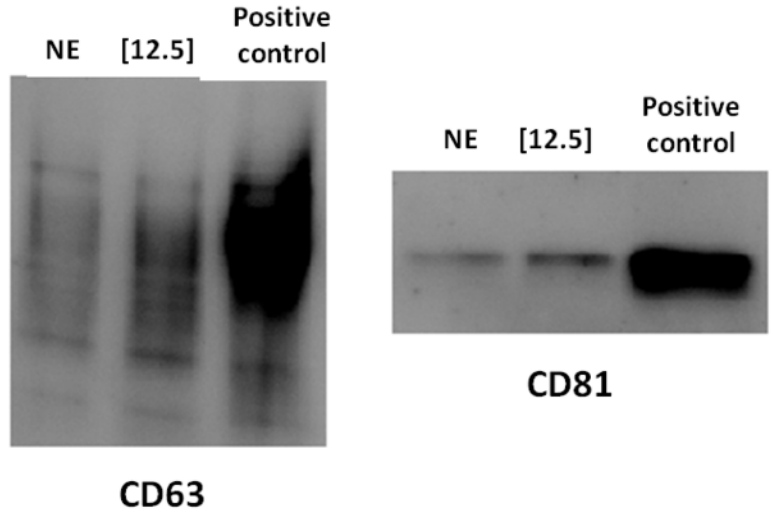
A)


EV/mL
*
Number of EV / mL of supernatant
3,00E+10
2,50E+10
2,00E+10
1,50E+10
1,00E+10
5,00E+09
0,00E+00
NE
12.5 µg/mL

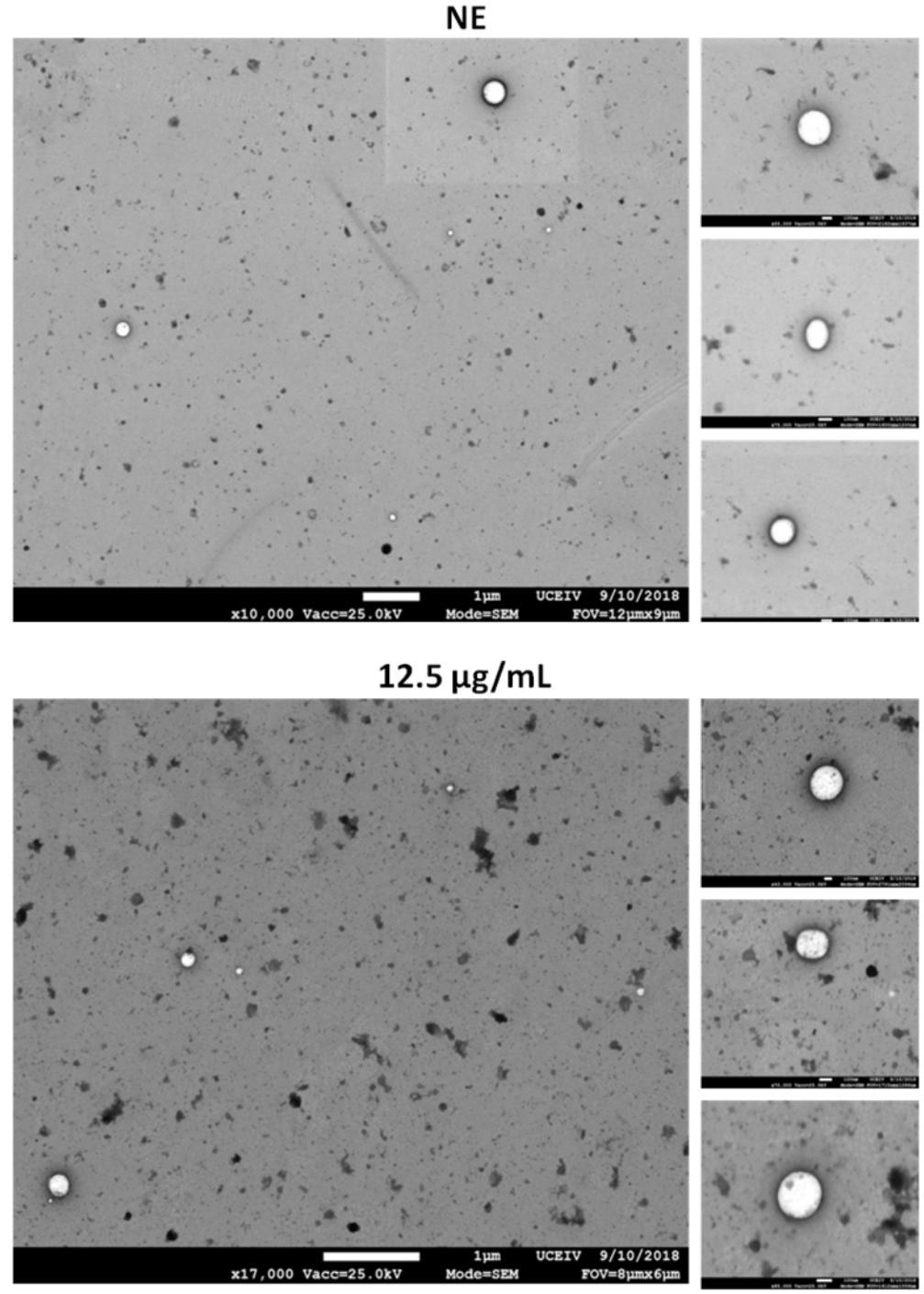
B)

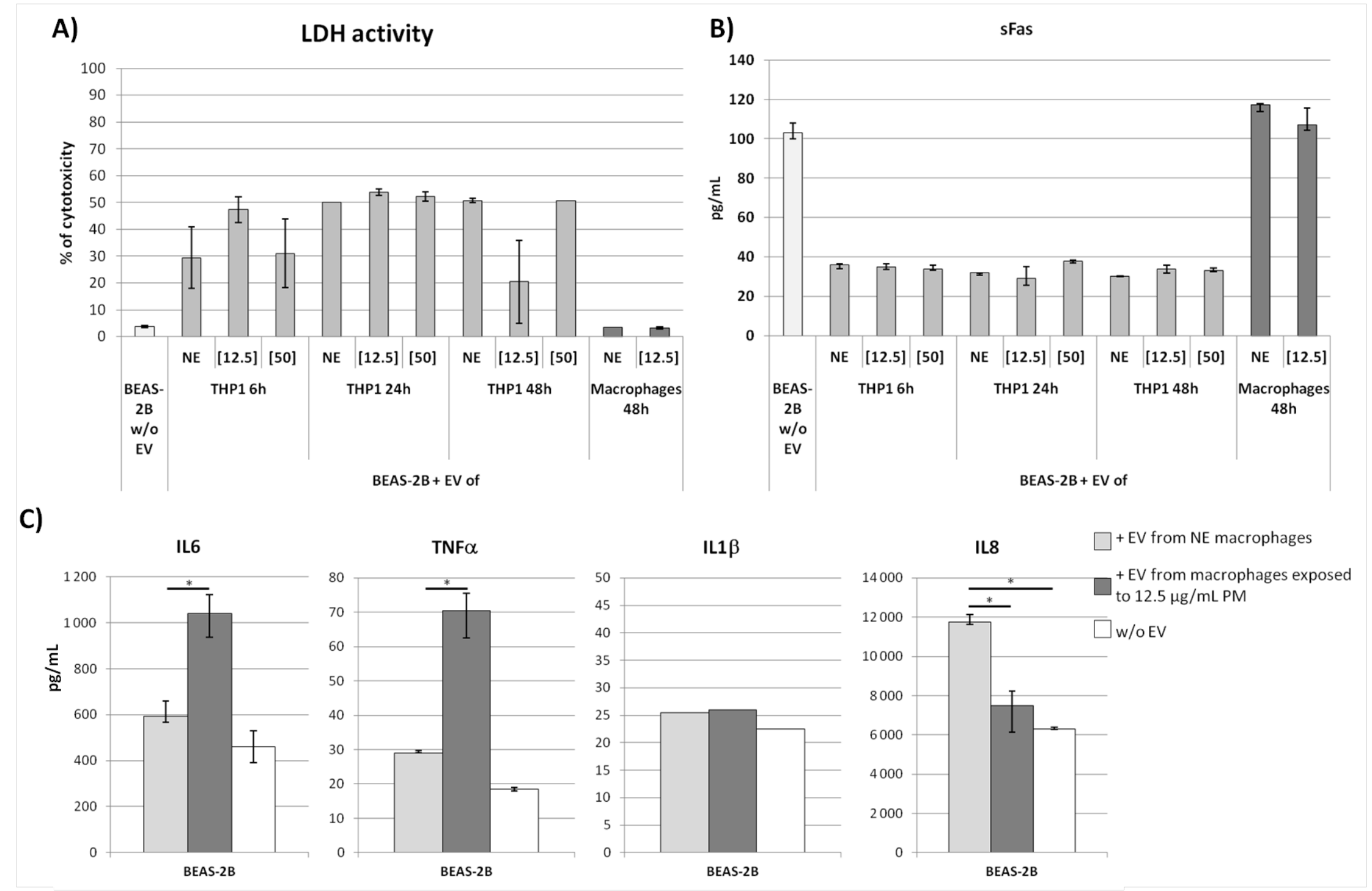

NE
12.5 µg/mL
Concentration (particles / ml)
143
203
343
438
198
228
248
278

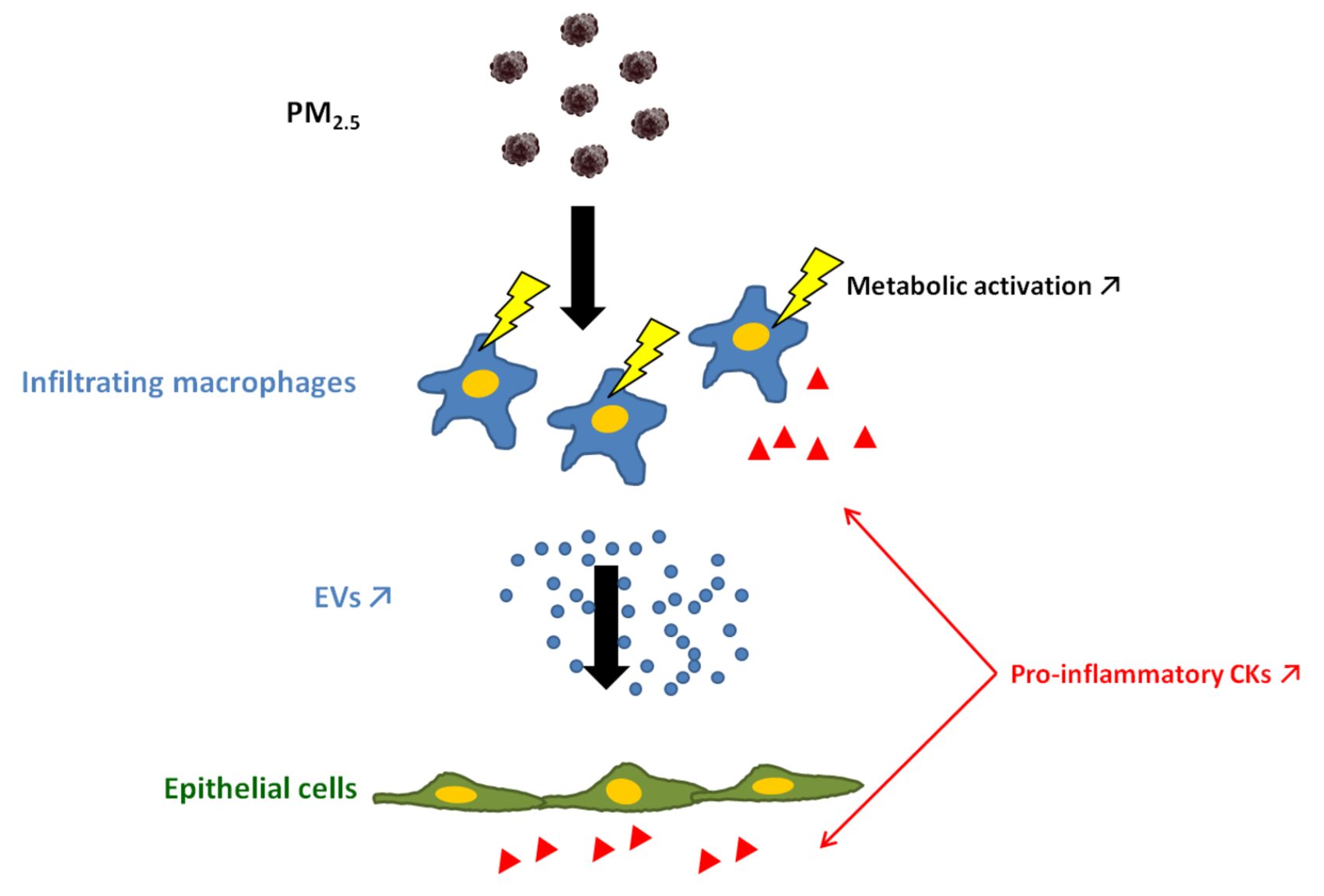
C)


NE
[12.5]
Positive control
CD63
NE
[12.5]
Positive control
CD81

D)


NE
1µm UCEIV 9/10/2018
x10,000 Vacc=25.0kV Mode=SEM FOV=12µmx9µm
12.5 µg/mL
1µm UCEIV 9/10/2018
x17,000 Vacc=25.0kV Mode=SEM FOV=8µmx6µm


A)
LDH activity
% of cytotoxicity
100
90
80
70
60
50
40
30
20
10
0
NE
[12.5]
[50]
BEAS-2B w/o EV
THP1 6h
THP1 24h
THP1 48h
Macrophages 48h
BEAS-2B + EV of
B)
sFas
pg/mL
140
120
100
80
60
40
20
0
C)
IL6
TNFα
IL1β
IL8
pg/mL
BEAS-2B
+ EV from NE macrophages
+ EV from macrophages exposed to 12.5 µg/mL PM
w/o EV


PM2.5
Metabolic activation ↗
Infiltrating macrophages
EVs ↗
Pro-inflammatory CKs ↗
Epithelial cells