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Amélie Héliot, Gauthier Trémolet, Yann Landkocz, Dorothée Dewaele, Fabrice Cazier, Frédéric Ledoux, Dominique Courcot, Perrine Martin

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1           **Cellular response and extracellular vesicles characterization of human**  
2           **macrophages exposed to fine atmospheric particulate matter.**

3           **Perrine J. MARTIN<sup>\*,a</sup>, Amélie HÉLIOT<sup>a</sup>, Gauthier TREMOLET<sup>a</sup>, Yann**  
4           **LANDKOCZ<sup>a</sup>, Dorothee DEWAELE<sup>b</sup>, Fabrice CAZIER<sup>b</sup>, Frédéric LEDOUX<sup>a</sup>,**  
5           **Dominique COURCOT<sup>a</sup>.**

6           <sup>a</sup>University of Littoral Côte d'Opale, Unit of Environmental Chemistry and Interactions with Life, UCEIV  
7           EA4492, SFR Condorcet FR CNRS 3417, Dunkerque, France; <sup>b</sup>University of Littoral Côte d'Opale, Common  
8           Center of Measurements, CCM, Dunkerque, France.

9           \*Correspondance to: Dr. Perrine J. Martin, Université du Littoral Côte d'Opale, EA4492 - Unité de Chimie  
10           Environnementale et Interactions sur le Vivant (UCEIV), Maison de la Recherche en Environnement Industriel  
11           2, ULCO, 189A, Avenue Maurice Schumann, 59140 Dunkerque, France.  
12           Phone: +33-3 28 23 76 11, E-mail: Perrine.Martin@univ-littoral.fr

13           Author e-mails:

14           Amelie.Heliot@univ-littoral.fr

15           Gauthier.Tremolet@univ-littoral.fr

16           Yann.Landkocz@univ-littoral.fr

17           Dorothee.Dewaele@univ-littoral.fr

18           [Fabrice.Cazier@univ-littoral.fr](mailto:Fabrice.Cazier@univ-littoral.fr)

19           [Frederic.Ledoux@univ-littoral.fr](mailto:Frederic.Ledoux@univ-littoral.fr)

20           [Dominique.Courcot@univ-littoral.fr](mailto:Dominique.Courcot@univ-littoral.fr)

21

22                    ***Abstract***

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

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

40                    **Keywords:** infiltrating macrophages, extracellular vesicles, PM<sub>2.5</sub>, lung epithelial cells.

41                    ***1. Introduction***

42                    World Health Organization (WHO) estimates that exposure to air pollution caused around 7 million deaths  
43 worldwide in 2012 (WHO, 2017). Air pollutants consist of gaseous compounds such as volatile organic  
44 compounds, nitrogen oxides and particulate matter (PM). PM with an equivalent aerodynamic diameter below  
45 2.5  $\mu$ m (PM<sub>2.5</sub>) is able to penetrate lung until bronchial tubes and alveoli where it can cause the development of  
46 respiratory diseases onset such as asthma, Chronic Obstructive Pulmonary Disease (COPD) till lung cancer (Li  
47 et al., 2018). In 2013, PM<sub>2.5</sub> has been classified as a Group 1 carcinogen by the International Agency for  
48 Research on Cancer (Cohen et al., 2013). PM<sub>2.5</sub> is a complex mixture of inorganic (metals, ions... ) and organic  
49 compounds (PAHs, dioxins...), living organisms (bacteria, pollen...) whose composition depends on the main

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

52 Macrophages are heterogeneous cells from different origins showing high plasticity as they can change their  
53 phenotype depending on their microenvironment. In the lung, they are established during the embryonic  
54 development period and maintained in adulthood through longevity and local self-renewal (Epelman et al., 2014;  
55 Sheng et al., 2015). These prenatal-derived macrophages are predominant in lung tissues and can differentiate  
56 into Alveolar Macrophages (AMs) localized in the airspaces of the lung (Fathi et al., 2001). AMs are the  
57 essential immune effector cells in the lung as they are exposed to the harmful outside environment especially  
58 through the inhalation of PM<sub>2.5</sub>. However Goto et al. have shown that the deposition of PM into the lung  
59 stimulates the bone marrow and promotes the release of leukocytes into the circulation among which monocytes  
60 that are important to control and terminate the inflammatory response (Goto et al., 2004b). These monocytes  
61 infiltrate the lung and differentiate into macrophages, which will be called infiltrating macrophages, that are  
62 mainly distributed throughout the airways, lung tissues, and alveolar tissues during lung insult (Yamasaki and  
63 Eeden, 2018). After PM exposure, the onset of the inflammatory response usually starts with the release of the  
64 early responding cytokines, IL1 $\beta$  and TNF $\alpha$ , and continue with the expression of other cytokines and  
65 chemokines (IL6, IL8) (Gualtieri et al., 2017). This inflammatory response may be induced by the ability of PM  
66 to alter the cells oxidative balance. According to the oxidative stress paradigm, cells respond to low levels of  
67 reactive oxygen species (ROS) by increasing the levels antioxidant molecules (such superoxide dismutase  
68 (SOD), NAD(P)H quinone dehydrogenase 1 (NQO1), heme oxygenase-1 (HMOX)) thus restoring the  
69 intracellular redox homeostasis (Gualtieri et al., 2017). In addition, PAHs present on PM, which require  
70 metabolic activation to biologically reactive intermediates to elicit their adverse health effects, are metabolized  
71 by the CYP superfamily members, and activate their gene expression through the activation of the Aryl  
72 hydrocarbon Receptor (AhR) (Billet et al., 2007). To our knowledge, no study has focused on these effects of  
73 PM<sub>2.5</sub> on infiltrating macrophages. It is thereby interesting to assess the cellular response of infiltrating human  
74 macrophages exposed to PM<sub>2.5</sub> in terms of cytotoxicity, oxidative stress, xenobiotic metabolism and  
75 inflammation.

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

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

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

## 95 **2. Material and Methods**

### 96 *2.1 PM sampling and handling*

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

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

## 112 *2.2 PM physico-chemical characterization*

113 Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES, iCAP 6300 DUO, ThermoElectron,  
114 UK) and ICP-Mass Spectrometry (ICP-MS, Varian® 820-MS, Varian, USA) were used for quantification of the  
115 PM inorganic major and trace components (Al, As, Ba, Cd, Co, Cr, Cu, Fe, Mn, Mo, Ni, P, Pb, Sn, Sr, Ti, V,  
116 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  
117 chromatography (model ICS 5000, ThermoElectron, UK). The PM total carbon (TC) content was measured  
118 using the CHNS/O analyzer (FLASH, 2000; ThermoElectron, UK). Finally, concentrations of the 16 priority  
119 Polycyclic Aromatic Hydrocarbons (PAHs) listed by the United States Environmental Protection Agency (US-  
120 EPA) were quantified using gas chromatography-mass spectrometry (GC-MS, 1200 TQ, Varian, USA) after PM  
121 Soxhlet extraction with dichloromethane. The whole analytical procedure is detailed in .

## 122 *2.3 Human Macrophages culture and $PM_{2.5-0.3}$ exposure*

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

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

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

139 Each cell type was exposed to PM<sub>2.5-0.3</sub> as follows. The supernatants were removed and replaced by RPMI  
140 medium supplemented with 10% exosome-depleted FBS (Gibco by ThermoFisher Scientific, Illkirch, France),  
141 2.5% Hepes and 1% antibiotics. PM<sub>2.5-0.3</sub> was suspended in cell culture medium and dispersed by 5 min  
142 sonication just prior to the exposure. Then, PM<sub>2.5-0.3</sub> was or not (non-exposed, NE) added at the indicated  
143 concentrations to THP-1 cultured for 6h, 24h or 48h and primary macrophages for 48h.

#### 144 *2.4 Extracellular vesicles (EVs) isolation and quantification*

145 EVs were isolated by differential centrifugation according to Théry et al. with some modifications (Théry et  
146 al., 2006). Briefly, THP-1 or macrophages supernatants were centrifuged at 500g for 10 min and at 16,500g for  
147 30 min at 4°C to remove cell debris. In addition, the supernatants were filtered with a 0.2µm syringe filter. For  
148 the collection of EVs, the supernatants were ultracentrifuged twice at 110 000g for 70 min at 4°C. The EVs were  
149 then washed and suspended in Phosphate Buffer Saline (PBS), aliquoted and stored at -80°C until further  
150 exposure or extraction.

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

#### 157 *2.5 BEAS-2B co-culture with EVs*

158 BEAS-2B is a non-cancerous cell line of human bronchial epithelial cells which was obtained from ECACC  
159 (Wiltshire, UK; reference: 95102433). They were cultured in LHC-9 Medium (ThermoFisher Scientific, Illkirch,  
160 France) as described previously (Héliot et al., 2017). 250,000 cells were exposed to 50µL of EVs suspension  
161 (Supp. Fig.1) derived from non-exposed or exposed THP-1 or macrophages. After 48h of exposure, cell-free  
162 culture supernatants were collected and quickly frozen at -80°C until further study. Adherent cells were washed  
163 twice with 500µL of cold PBS (Gibco by ThermoFisher Scientific, Illkirch, France), and quickly frozen at -80  
164 °C. All experiments were performed in triplicate.

#### 165 *2.6 Cytotoxicity assay*

166 The cytotoxicity of THP-1 and primary macrophages after PM<sub>2.5-0.3</sub> exposure, and of BEAS-2B cells after  
167 EVs co-culture, was evaluated as described previously (Landkocz et al., 2017), by measuring the extracellular

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

#### 174 *2.7 Flow cytometry*

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

#### 180 *2.8 RNA isolation and RT-qPCR*

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

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

186 mRNAs (i.e IL1β, IL6, IL8, CAT, IL12, TNFα, CYP1A1, CYP1B1, CYP2E1, SOD2, CAT, NRF2, NQO1,  
187 HMOX) quantification was performed using TaqMan® Gene Expression Assays (ThermoFisher Scientific,  
188 Illkirch, France) (Table 1). Real-time quantitative polymerase chain reaction (qPCR) was carried out using a  
189 7500 Fast Real-Time PCR System (Applied Biosystems, ThermoFisher Scientific, Illkirch, France). The relative  
190 changes in gene expression were calculated by the 2<sup>-ΔΔCt</sup> method (Schmittgen and Livak, 2008) and normalized  
191 against 18S using the Sequence Detection 7500 Software v2.0.3 (Applied Biosystems, ThermoFisher Scientific,  
192 Illkirch, France) (see Table 1 for primer references).

#### 193 **Table 1. mRNA Taqman primer references**

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

TNF $\alpha$	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-1 $\beta$ , IL-8, TNF- $\alpha$  and sFas concentrations in cell-free culture supernatants were determined using

197

MAGPIX<sup>®</sup> system and the MILLIPLEX<sup>®</sup> MAP Human Cytokine Panel 1 and Human CD8+ T Cell

198

(Luminex<sup>®</sup>, EDM Millipore, Billerica, USA), following the manufacturer's instructions.

199

### *2.10 Protein 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<sup>®</sup> 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<sup>™</sup>

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.

216 *2.11 Electron microscopy*

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

220 *2.12 Statistical analysis and controls*

221 Gene and protein expression as well as protein levels were depicted as median and inter-quartile range and  
222 graphically represented by bar-graphs. For relative quantification of gene expression, significance  $RQ > 2$  or  $RQ$   
223  $< 0.5$  is reported to the value of unexposed control (NE), normalized to 1 with 18S. For each exposure time and  
224 concentration of PM<sub>2.5-0.3</sub>, cytokines measurement data were compared to those obtained for non exposed (NE)  
225 cells. The significance of the differences was calculated by the non-parametric Mann-Whitney U test and is  
226 indicated by \* when  $p < 0.05$ . For NTA, the significance of the differences was calculated by the T Student's test  
227 and is indicated by \* when  $p < 0.01$ .

228 **3. Results**

229 *3.1 PM<sub>2.5-0.3</sub> major and trace inorganic species*

230 Major and trace inorganic species as well as Total Carbon (TC) contents are reported in Fig. 1A. TC  
231 (14.3%), water-soluble NO<sub>3</sub><sup>-</sup> (10.0%), SO<sub>4</sub><sup>2-</sup> (7.5%), Cl<sup>-</sup> (6.1%), Ca<sup>2+</sup> (4.5%), Na<sup>+</sup> (4.3%) and NH<sub>4</sub><sup>+</sup> (1.6%) as  
232 well as Fe (3.8%) and Al (2.8%) were the major PM constituents (55% in total). Then five species showed  
233 concentrations values between 0.1% and 1%: Mg<sup>2+</sup> (0.65%), K<sup>+</sup> (0.45%), Mn (0.18%), P (0.14%) and Zn  
234 (0.13%). Other species concentrations ranged below 0.1% (Cu, Ti, Ba, Pb, F<sup>-</sup>, Cr, Sr, Ni and V) and below  
235 0.01% (Sn, Mo, As Co and Cd).

236 NO<sub>3</sub><sup>-</sup> and SO<sub>4</sub><sup>2-</sup> mainly derive from the gas-to-particle conversion of NO<sub>x</sub> and SO<sub>2</sub>. NO<sub>x</sub> are emitted by  
237 combustion processes related to the use of fossil fuels in cars (diesel) or industrial processes. SO<sub>2</sub> is mainly  
238 originated from industrial emissions or related to heavy fuel oil combustion with high sulfur content mainly used  
239 in industry or in shipping (Ledoux et al., 2018). NH<sub>4</sub><sup>+</sup> results as well from the conversion of the NH<sub>3</sub> gas known  
240 to be mainly from agricultural origin. However, an industrial contribution cannot be excluded with regard to the  
241 presence of an integrated steelworks able to emit particles containing NH<sub>4</sub><sup>+</sup> in the study area (Hleis et al., 2013;  
242 Kfoury et al., 2016). Na<sup>+</sup>, Mg<sup>2+</sup> and Cl<sup>-</sup> are known to be significantly associated with sea-salts (Manders et al.,  
243 2010).

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

### 254 3.2 *PM<sub>2.5-0.3</sub> Polycyclic Aromatic Hydrocarbon composition*

255 The average concentrations of the 16 US-EPA priority PAHs compounds are given in Fig. 1B. The  
256 predominantly detected compounds were dibenz[a,h]anthracene (8.9 µg/g), followed by indeno[1,2,3-cd]pyrene,  
257 chrysene and benzo[b]fluoranthene, with very close concentrations values ranging between 5.3 and 6.0 µg/g.  
258 Concentrations of other compounds were below 5 µg/g and below 1µg/g for the 6 lightest PAHs (compounds  
259 with 2 and 3 cycles). The main source of dibenz[a,h]anthracene and benzo[b]fluoranthene is road traffic, and  
260 more precisely exhaust fumes from diesel engines (Duval and Friedlander, 1981; INERIS, 2005, 2004a).  
261 Dibenz[a,h]anthracene is also found in cigarette smoke or in coke oven emissions (INERIS, 2004a).  
262 Indeno[1,2,3-cd] pyrene results from fossil fuels as well especially after incomplete combustion of wood, coal,  
263 gasoline or diesel fuel (INERIS, 2004b). Chrysene can be emitted into the atmosphere during coal and oil  
264 distillation and pyrolysis. It is also one of the predominant PAHs in particulate emissions from household waste  
265 incinerators, natural gas appliances and domestic heating devices, particularly those using wood (INERIS,  
266 2004c). The predominantly detected compounds can be explained by at least partly the locally based industries.  
267 Indeed, the study area hosts an integrated steel industry (including a coking plant), oil refining (producing  
268 marine fuels comparable to heavy fuel oils), a cement factory and a waste incinerator.

### 269 3.3 *Cellular response of THP-1 exposed to PM<sub>2.5</sub>*

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

274 inflammatory response (Lund et al., 2016). THP-1 were exposed to PM<sub>2.5</sub> at two doses (12.5 and 50 µg/mL) and  
275 for three times (6h, 24h and 48h) (see section 2.3 for dose justification). Regarding the cytotoxicity, the  
276 measurement of the extracellular LDH did not show any significant effect regardless times and doses of  
277 exposure (Fig. 2A). For metabolic activation and oxidative stress induction assessment, relative quantification of  
278 expression of genes coding for the Xenobiotic Metabolizing Enzymes (XMEs) CYP1B1, CYP1A1, CYP2E1,  
279 and for the Superoxide dismutase 2 (SOD2) which transforms toxic superoxide into hydrogen peroxide and  
280 diatomic oxygen, the NAD(P)H quinone dehydrogenase 1 (NQO1), an enzyme involved in the reduction of  
281 formation of radical species induced by oxidative stress and heme oxygenase (HMOX), was performed (Fig.  
282 2B). The results showed a significant dose-dependent increase of CYP1A1 and CYP1B1 expression after 6h of  
283 exposure, with a tendency to decrease with time for CYP1A1 or to reach a plateau for CYP1B1 at 24h and 48h.  
284 CYP2E1 expression was not significantly modified after PM<sub>2.5</sub> exposure. SOD2, NQO1 and HMOX expression  
285 was significantly induced proportionally to the doses but not to the time of exposure.

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

#### 292 *3.4 Characterization of extracellular vesicles derived from THP-1 exposed to PM<sub>2.5-0.3</sub>.*

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

303 exosomal proteins (Figure 3C). These results show for the first time that PM<sub>2.5</sub> exposure enhances EVs release  
304 by undifferentiated THP-1.

### 305 *3.5 Cellular response of infiltrating human macrophages exposed to PM<sub>2.5-0.3</sub>*

306 Goto et al. have shown that one of the cytokine implicated in infiltrating macrophages recruitment into the  
307 lung is GM-CSF (Goto et al., 2004b). Just after isolation from blood and before exposure to PM<sub>2.5-0.3</sub>, primary  
308 macrophages obtained from 6 donors were incubated for 36h with 50 ng/mL GM-CSF to obtain infiltrating cells.  
309 The obtained infiltrating macrophages were then exposed to 12.5µg/mL for 48h as define during the  
310 optimization step (section 4), since this was the minimum PM dose at which the THP-1 responded and produced  
311 an almost maximal number of EVs. Then as well, metabolic activation, oxidative stress and inflammatory  
312 response were studied. Extracellular LDH measurement showed that PM<sub>2.5-0.3</sub> exposure did not induce significant  
313 cytotoxicity (Fig. 4A) in comparison with non exposed (NE) infiltrating human macrophages. Concerning the  
314 gene expression, PM<sub>2.5-0.3</sub> induced a significant increase for CYP1A1, CYP1B1, SOD2, HMOX and NQO1 (Fig.  
315 4B), indicating the setting of a metabolic and antioxidant response. As for THP-1, CYP2E1 expression was not  
316 modified by PM<sub>2.5-0.3</sub> exposure. PM<sub>2.5-0.3</sub> was also responsible for a significant enhancement of the pro-  
317 inflammatory cytokines IL8 and IL1β expression, and IL8, IL1β, IL6 and TNFα release (Fig. 4C and D), as well  
318 as an increase of the granularity (SSC) of the exposed macrophages in comparison with the non-exposed one  
319 (Fig. 5A). Internalization of the particles by the macrophages was also observed (Fig. 5B) and it is correlated  
320 with an increase of the Macrophage Scavenger Receptor 1 (MSR1) protein (Fig. 5C). In conclusion of this  
321 section, the results show that infiltrating macrophages, such as undifferentiated THP-1, typically respond to PM  
322 exposure. This finding makes it possible to consider the EVs produced in response.

### 323 *3.6 Characterization of extracellular vesicles derived from infiltrating macrophages exposed to* 324 *PM<sub>2.5-0.3</sub>.*

325 EVs produced by the macrophages from 6 donors exposed or not to PM<sub>2.5-0.3</sub> were isolated as described in  
326 Methods (sections 2.3 and 2.4) and pooled to form two average EVs samples deriving from exposed and non-  
327 exposed macrophages respectively. NTA characterization of the two obtained EVs samples indicated that PM<sub>2.5-  
328 0.3</sub> exposure induced an significant increase of the number of EVs released by infiltrating macrophages as  
329 observed for THP-1 (Fig.6A). On the contrary, the quantification revealed that PM<sub>2.5-0.3</sub> exposure did not affect  
330 size distribution (Fig. 6B). Electron microcopy confirmed the presence of EVs (Fig. 6D). Finally, CD63 and  
331 CD81 exosomal proteins were detected by western-blot in EVs derived either from NE macrophages or from  
332 macrophages exposed to PM<sub>2.5-0.3</sub> (Fig.6C). These results are in line with those obtained from THP-1 exposed to

333 PM<sub>2.5-0.3</sub>. To our knowledge and for the first time, this study shows that PM<sub>2.5-0.3</sub> is able to induce EVs release  
334 from infiltrating human macrophages as well.

### 335 *3.7 Effects of EVs derived from human macrophages exposed to PM<sub>2.5-0.3</sub> on lung epithelial cells*

336 One of the objectives of this work was to highlight whether EVs produced by exposed macrophages are able  
337 to transfer information from this exposure to unexposed lung cells and to activate typical PM exposure response.  
338 To do this, BEAS-2B cells co-cultured with EVs from exposed and non-exposed macrophages (EV<sub>THP-1/NE</sub> and  
339 EV<sub>THP-1/PM</sub> for EVs derived from non-exposed and exposed THP-1 and EV<sub>HM/NE</sub> and EV<sub>HM/PM</sub> for EVs derived  
340 from non-exposed and exposed human macrophages) in proportional number as found in supernatants (Supp.  
341 Fig. 3). Surprisingly, an important mortality was observed in BEAS-2B co-cultured with EVs derived from THP-  
342 1 regardless their conditions of exposure (data not shown). This cytotoxicity, measured via extracellular LDH  
343 activity, was significantly higher (p<0.1) for BEAS-2B co-cultured with EV<sub>THP-1</sub> in comparison with EV<sub>HM</sub> (Fig.  
344 7A). In an attempt to identify the involved mechanisms, we measured the concentration of soluble Fas (sFas), an  
345 inducer of apoptosis generated by alternative mRNA splicing, in the culture supernatants (Fig. 7B). In addition to  
346 membrane-anchored Fas, sFas can antagonize cell-surface Fas function, leading to block the Fas-mediated  
347 apoptosis (Nagata, 1994). Here, the results show that, compared to BEAS-2B cells non exposed to EVs, sFas  
348 concentration in culture supernatants of BEAS-2B exposed to EV<sub>THP-1</sub> (obtained in all conditions) was three  
349 times lower, whereas it did not significantly vary in supernatants of BEAS-2B exposed to EV<sub>HM</sub>.

350 Specifically for the BEAS-2B cultured during 48h in the presence of EV<sub>HM</sub>, the nucleic acids and culture  
351 supernatants were isolated. The expression of 12 genes was measured (Table 2) but none of them showed any  
352 difference in expression, whether the cells were cultured in the presence of EV<sub>HM/NE</sub> or EV<sub>HM/PM</sub>. Interestingly,  
353 cytokines quantification showed a significant increase of the secretion for the pro-inflammatory cytokines IL6  
354 and TNF $\alpha$  but not for IL1 $\beta$  (Fig. 7C) while their gene expression do not differ from controls after 48h of  
355 exposure. Surprisingly, EV<sub>HM/NE</sub> significantly induced IL8 secretion by BEAS-2B in comparison with BEAS-2B  
356 cultured in the absence of EVs or in the presence of EV<sub>HM/PM</sub>.

#### 357 **Table 2. mRNA expression in BEAS-2B cultured in the presence of EVs from exposed macrophages.**

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

361

362

	<b>Relative quantification</b> <b>BEAS-2B + EV<sub>HM/PM</sub></b> <b>BEAS-2B + EV<sub>HM/NE</sub></b> <b>(Median [Q1-Q3])</b>
<b>IL-1<math>\beta</math></b>	0,72 [0,69 – 0,73]
<b>IL-6</b>	0,84 [0,79 – 1,19]
<b>IL-8</b>	0,93 [0,85 – 0,99]
<b>TNF<math>\alpha</math></b>	ND
<b>TGF<math>\beta</math></b>	0,97 [0,96 – 0,98]
<b>CYP1A1</b>	0,81 [0,57 – 0,91]
<b>CYP1B1</b>	0,80 [0,78 – 0,83]
<b>CYP2E1</b>	ND
<b>NRF2</b>	0,97 [0,90 – 1,02]
<b>HMOX</b>	ND
<b>NQO1</b>	0,91 [0,84 – 0,96]
<b>SOD2</b>	1,03 [0,99 – 1,21]

#### 363 **4. Discussion**

364 Alveolar macrophages are the predominant cells that clean inhaled particulate matter off the lung (Miyata  
365 and van Eeden, 2011). In parallel, they produce pro-inflammatory mediators implicated in local as well as  
366 systemic inflammatory responses, leading to the recruitment of bone marrow derived macrophages (Hiraiwa and  
367 van Eeden, 2013; van Eeden and Hogg, 2002). Whereas many studies have focused on resident alveolar  
368 macrophages response when exposed to PM<sub>2.5</sub>, none were dealing with the response of infiltrating macrophages.  
369 Here, the PM cytotoxicity and the activation of gene expression of XMEs and enzymes involved in oxidative  
370 stress of infiltrating macrophages in response to PM<sub>2.5</sub> exposure was firstly assessed. To do that, undifferentiated  
371 THP-1 cells (non-treated with PMA) and primary macrophages (obtained by treating primary monocytes with  
372 GM-CSF during 36h, to mimic their recruitment by alveolar macrophages towards the lung (Goto et al., 2004b,  
373 2004a)) were exposed to PM<sub>2.5-0.3</sub> (at 0, 12.5 and 50  $\mu$ /mL during 6h, 24h and 48h for THP-1 and at 0 and 12.5  
374  $\mu$ g/mL during 48h for human macrophages). In these conditions, PM exposure was not significantly cytotoxic  
375 for both THP-1 and human infiltrating macrophages, compared to non exposed control (Fig.2A and 4A).

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

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

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

406 Redox-sensitive signaling cascades induced by PM exposure through oxidative stress can further activate an  
407 inflammatory response (Gerlofs-Nijland et al., 2009), characterized by pro-inflammatory cytokines production  
408 like IL1 $\beta$ , IL6, and TNF $\alpha$  as well as IL8 (Dergham et al., 2012; Miyata and van Eeden, 2011; Yan et al., 2016).  
409 In undifferentiated THP-1, in concordance with these previous results, IL8, IL1 $\beta$  and TNF $\alpha$  expression was  
410 significantly induced in a dose-dependent manner at the earlier time of 6h (Fig. 2C) and decreased with time.  
411 Their secretion was also classically induced in a dose- and time-dependent manner (Fig. 2D). Similar

412 observations have been done in exposed primary human macrophages (Fig. 4C and D), except for TNF $\alpha$  and IL6  
413 for which 48h could be too late to measure their expression. In a surprising way, IL6 secretion was found to be  
414 significantly induced in undifferentiated THP-1 (Fig.2D) but only at the highest dose of 50  $\mu$ g/mL of PM and at  
415 48h. All of these results suggest a pro-inflammatory reaction of the infiltrating macrophages in response to the  
416 PM<sub>2.5-0.3</sub> exposure.

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

425 Acute and chronic exposure to PM has been associated with increased morbidity and mortality from  
426 cardiorespiratory diseases as well as an increase of lung cancer risk (Bentayeb et al., 2015; Raaschou-Nielsen et  
427 al., 2013). Different mechanisms have been proposed implicating that the exposed cells act as well as on the lung  
428 microenvironment and afar through blood circulation. Among the known mechanisms of action through which  
429 PM could impact health, EVs are a well suited candidate as they are produced in the pulmonary environment in  
430 response to environmental stimuli (Neven et al., 2017). Among the studies dealing with EVs, some have shown  
431 that the production of EVs is increased in response to PM exposure (Bollati et al., 2015, 2010; Cantone et al.,  
432 2014; Neri et al., 2016). One objective was to determine if infiltrating macrophages are capable to release EVs in  
433 response to PM<sub>2.5</sub> exposure. To test this hypothesis, EVs were isolated from the supernatants of culture of THP-1  
434 non exposed to PM<sub>2.5-0.3</sub> or exposed to 12.5  $\mu$ g/mL, 50  $\mu$ g/mL for 6h, 24h and 48h and of infiltrating primary  
435 human macrophages non exposed or exposed to 12.5  $\mu$ g/mL for 48h. It was shown for the first time that THP-1  
436 and infiltrating primary human macrophages exposed to PM<sub>2.5-0.3</sub> produce EVs, in a dose- and time-dependent  
437 manner as shown by NTA (Fig. 3A and 6A). Recently, Soni et al. have highlighted the implication of alveolar  
438 macrophages-derived EVs in acute lung injury (Soni et al., 2016). Here it can be hypothesized that EVs  
439 produced by infiltrating macrophages following PM<sub>2.5</sub> exposure have a role in PM<sub>2.5</sub>-induced injury.  
440 Nevertheless in further studies, it would be essential to ensure that the effects that has been observed in  
441 pulmonary epithelial cells is really due to the presence of EVs, or rather due to the presence of free proteins,

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

444 Lung homeostasis is maintained in large part due to the crosstalk between the alveolar macrophages,  
445 predominant lung-resident immune cells, and the lung epithelial cells, the major constituents of the respiratory  
446 surface (Haggadone and Peters-Golden, 2018). When exposed to PM<sub>2.5</sub>, this balance is modified: several studies  
447 have shown that macrophages exposed to PM induce the expression of several enzymes like CYP1A1 and  
448 NQO1 as well as pro-inflammatory cytokines in co-cultured lung cells (Abbas et al., 2009; Wang et al., 2019),  
449 indicating that PM-exposed macrophages cause pulmonary cells to develop a phenotype of oxidative stress,  
450 metabolic activation, and pro-inflammatory response. Based on this statement, the proposed hypothesis is that  
451 macrophages released factors could be extracellular vesicles. The expression of genes involved in xenobiotic  
452 metabolism, oxidative stress or inflammation in BEAS-2B lung epithelial cells cultured in the presence of  
453 EV<sub>HM/NE</sub> or EV<sub>HM/PM</sub> was not significantly different (Table 1). However, in the presence of EV<sub>HM/PM</sub>, the  
454 secretion of the two pro-inflammatory cytokines TNF $\alpha$  and IL6 was induced, IL8 secretion was inhibited and no  
455 change was observed for IL1 $\beta$  (Fig. 7C). These results suggest that EVs do not act on gene expression but  
456 downstream on protein synthesis. It is indeed known that PM<sub>2.5</sub> exposure directly introduce the pro-inflammatory  
457 cytokines IL6 and IL8 release, and to a lesser extend TNF $\alpha$ , by BEAS-2B, whatever the origin of the particles  
458 (Cachon et al., 2014; Dergham et al., 2012). These results were confirmed with our PM<sub>2.5-0.3</sub>, which induce a  
459 high increase of IL6, IL8 and TNF $\alpha$  release and not of IL1 $\beta$  (Supp. Fig. 4). In the case of IL6 and TNF $\alpha$ , EVs  
460 could act as enhancers of inflammation during the acute phase of the inflammatory response, whereas, as IL8  
461 mediates the recruitment and activation of immune cells, rather act to inhibit this part of the inflammatory  
462 response. To answer these questions, further experiments could be performed, by exposing BEAS-2B with EVs  
463 derived from exposed macrophages along with PM<sub>2.5-0.3</sub>.

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

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

## 479 **5. Conclusion**

480 This study shows that, as alveolar macrophages, infiltrating macrophages respond to PM exposure by an  
481 increase in the expression of the enzymes involved in the metabolism of xenobiotics, in oxidative stress and  
482 inflammatory response, as well an increase of the production of extracellular vesicles. These latter were able to  
483 induce on pulmonary epithelial cells an increase in the production of proinflammatory cytokines such as IL6 and  
484 TNF $\alpha$  suggesting they could play a role in the maintenance of inflammation induced by PM exposure. Although  
485 extensive studies would be needed to confirm these findings, it appears that infiltrating macrophages could  
486 exacerbate the PM-induced proinflammatory response on alveolar macrophages and maintain it via the action of  
487 extracellular vesicles on pulmonary epithelial cells. Further investigations will be carried out on the effects of  
488 PM on BEAS-2B, namely their ability to potentially release more extracellular vesicles in response to PM<sub>2.5</sub>  
489 exposure and the effects of these EVs on the cellular environment.

## 490 **List of abbreviations**

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

## 499 **Declarations**

500 *Ethics approval and consent to participate*

501 Not applicable.

502 *Conflict of Interest*

503 The authors declare that they have no conflict of Interest.

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720

721 **Figure captions**

722 **Fig. 1: PM<sub>2.5-0.3</sub> composition.** A) Major and trace inorganic species concentrations of the PM<sub>2.5-0.3</sub> collected in  
723 Dunkerque (March 2014 - March 2015). B) Polycyclic Aromatic Hydrocarbon concentrations in the PM<sub>2.5-0.3</sub>  
724 collected in Dunkerque (March 2014 - March 2015).

725 **Fig. 2: Cellular response of THP-1 exposed to PM<sub>2.5-0.3</sub>.** In each experiment, THP-1 were exposed 6h, 24h or  
726 48h to 0 (NE), 12.5 or 50 µg/mL of PM<sub>2.5-0.3</sub>. A) LDH cytotoxicity quantification in percentage of 100%  
727 mortality. mRNA expression of B) CYP1A1, CYP1B1, CYP2E1, SOD2, NQO1, HMOX and C) IL8, IL1β, IL6  
728 and TNFα was measured by RT-qPCR. Results of 2<sup>-ΔΔCt</sup> relative quantification are the medians of duplicates of  
729 three independent experiments +/- inter-quartile range. Significance RQ > 2 or RQ < 0.5 reported to the value of  
730 unexposed control (NE), normalized to 1 with 18S. D) Measurement of IL8, IL1β, IL6 and TNFα secretion of  
731 THP-1 exposed 6h, 24h or 48h to 0 (NE), 12.5 or 50 µg/mL to PM<sub>2.5-0.3</sub>. Results are the medians in pg/mL of  
732 duplicates of three independent experiments. Significant differences (\*p<0.05) were evaluated by Mann Whitney  
733 U test.

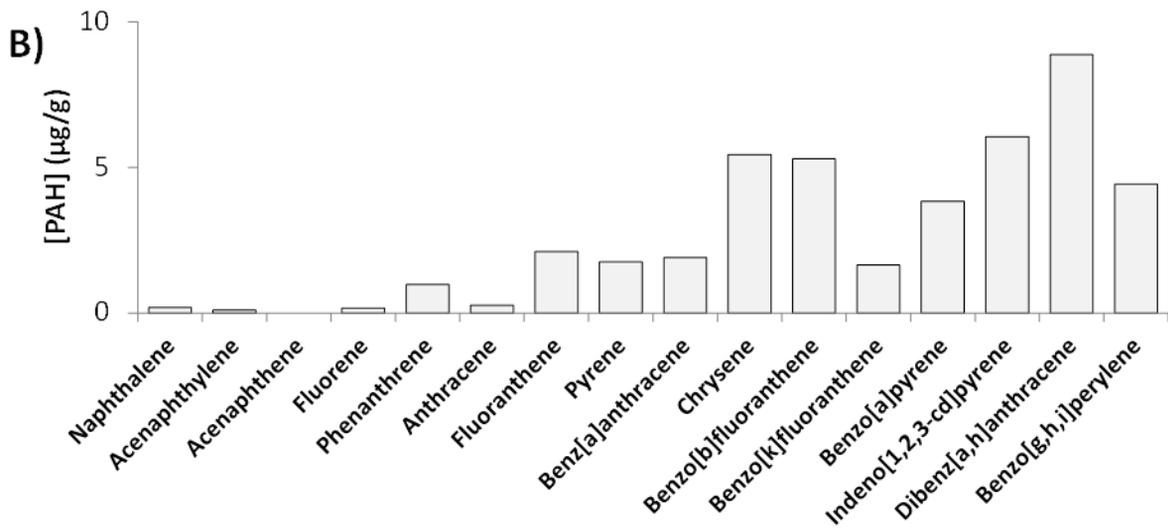
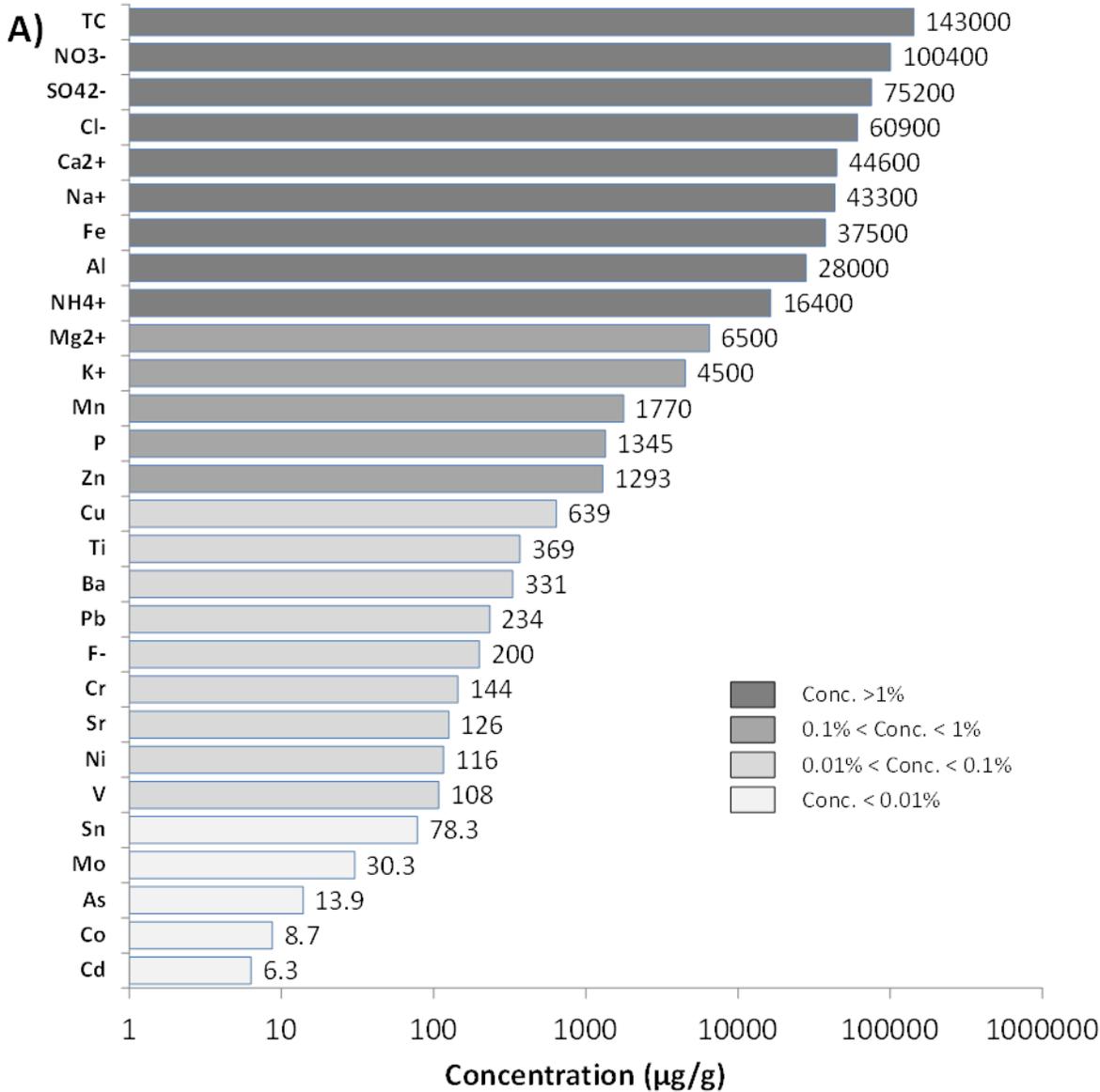
734 **Fig. 3: Characterization of extracellular vesicles derived from THP-1 exposed to PM<sub>2.5-0.3</sub>.** EVs were  
735 obtained from THP-1 exposed 6h, 24h or 48h to 0 (NE), 12.5 or 50 µg/mL of PM<sub>2.5-0.3</sub>. A) Graphs representing  
736 the amounts and the mean of size of EVs measured by nanoparticle tracking analysis. Significant differences  
737 were evaluated by Student's T test (\*p<0.01); B) Transmission electronic microscopy images of EVs (bar =  
738 100nm); C) Western-blot using CD63 and CD81 antibodies on EVs obtained from each condition.

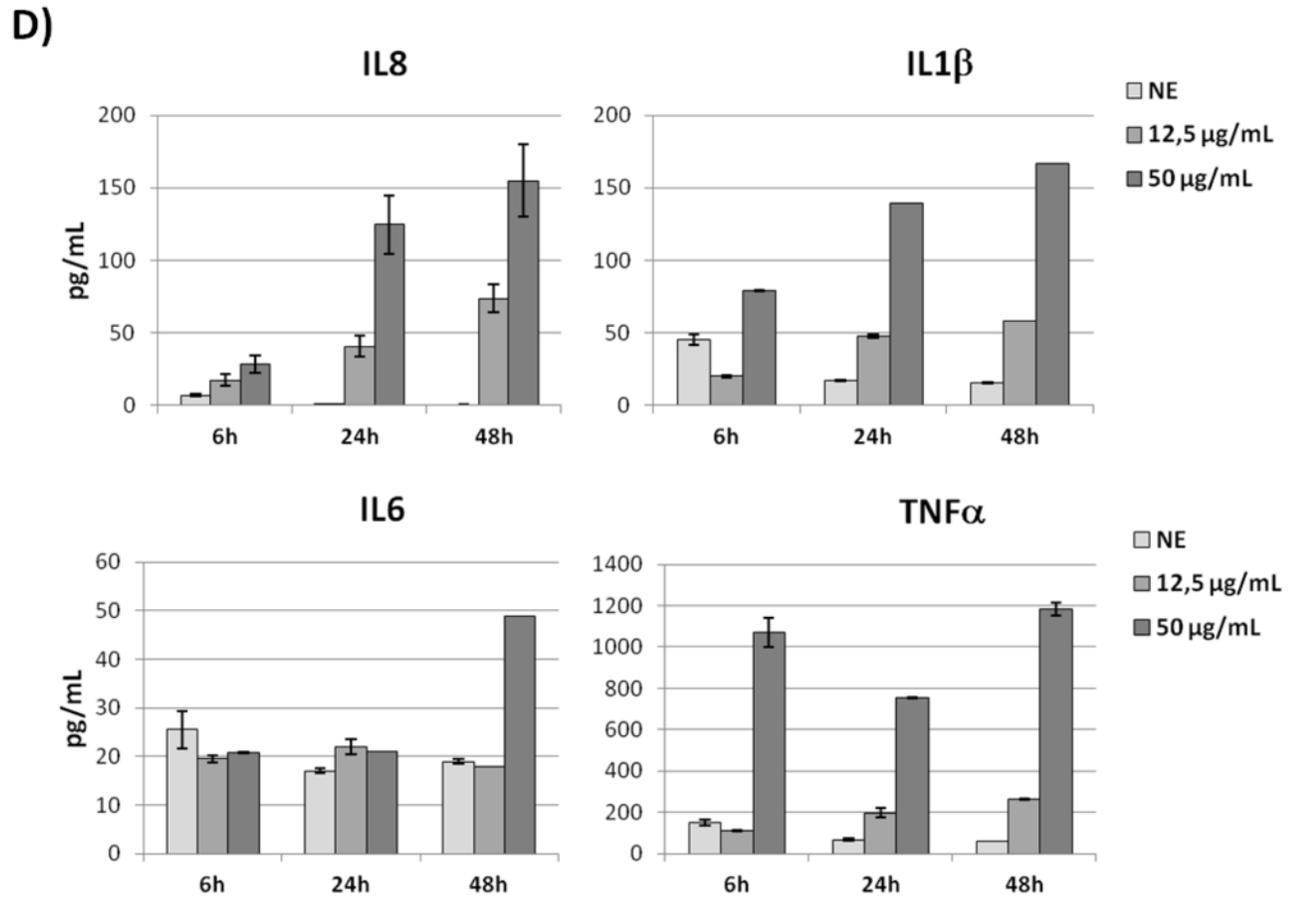
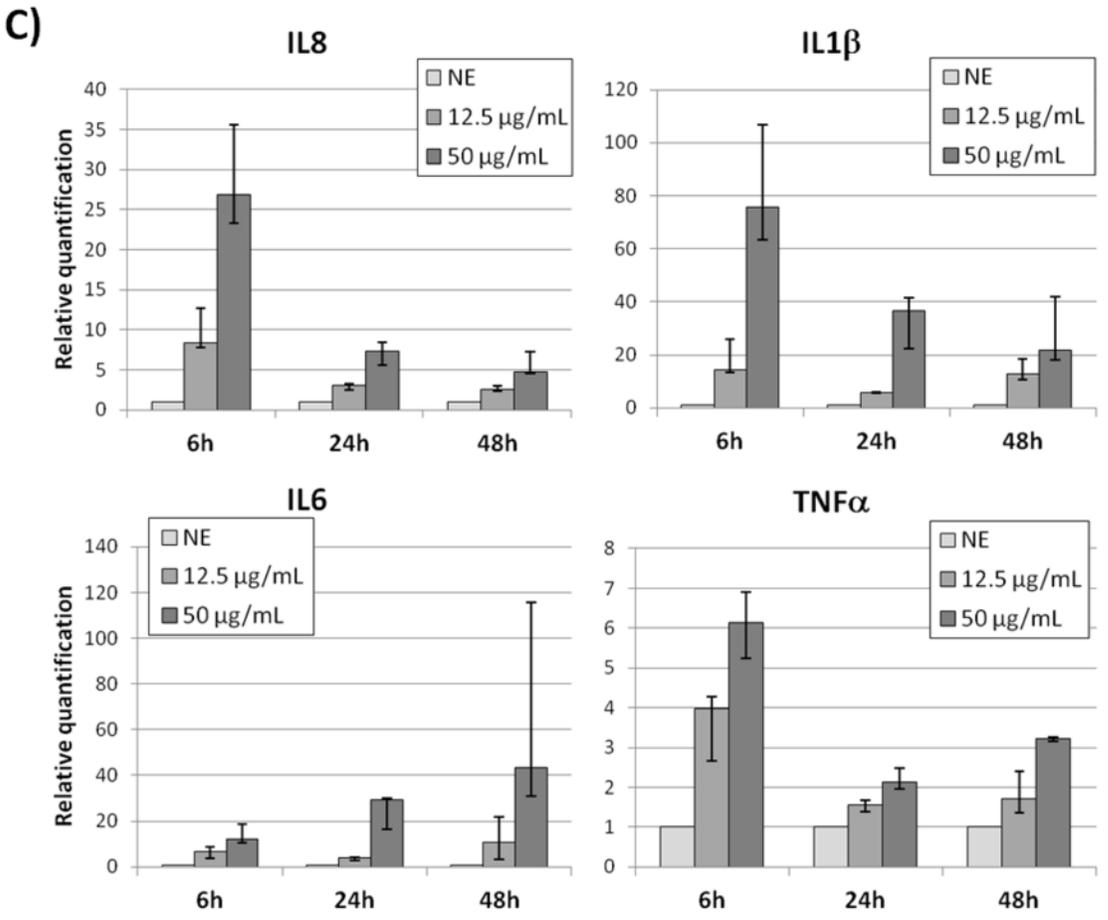
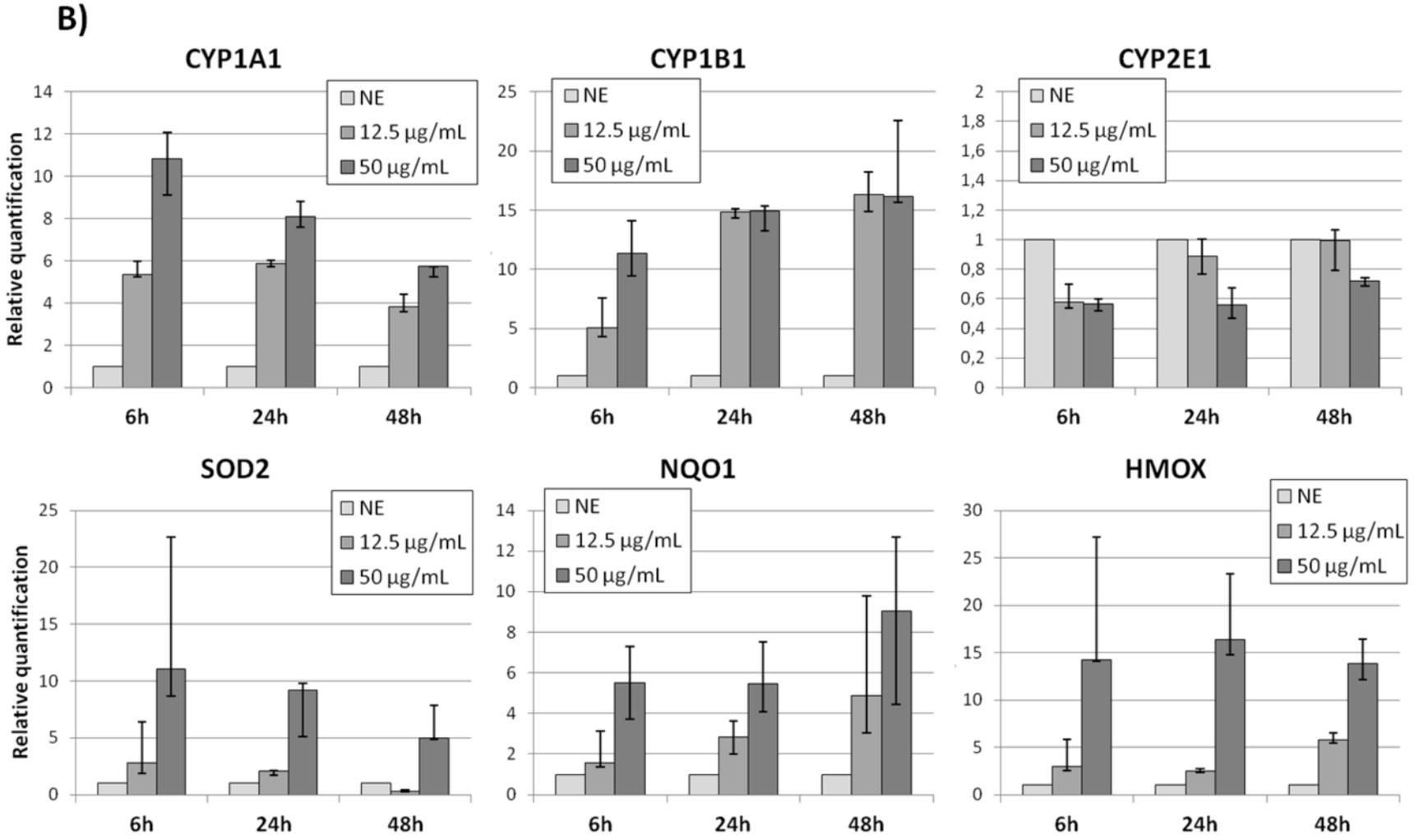
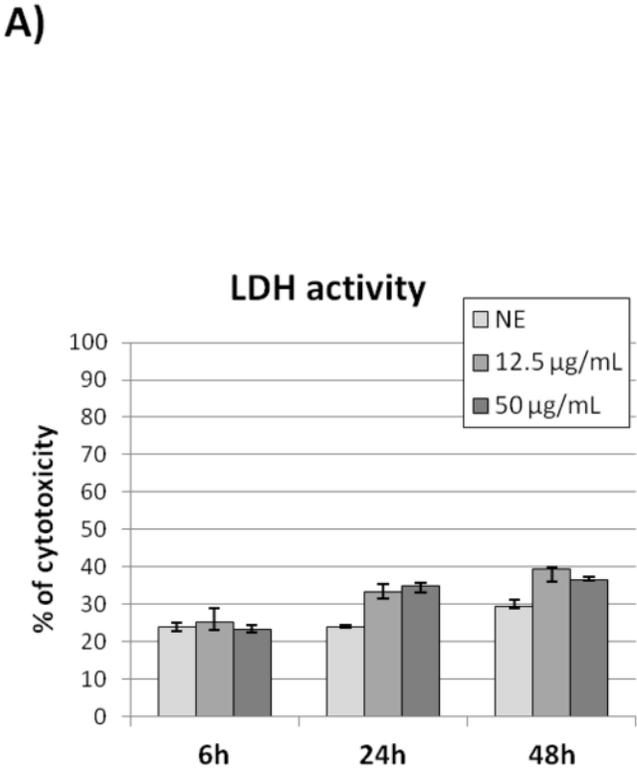
739 **Fig. 4: Cellular response of infiltrating macrophages exposed to PM<sub>2.5-0.3</sub>.** In each experiment, primary  
740 macrophages from 6 healthy donors were exposed 48h to 0 (NE) or 12.5 µg/mL of PM<sub>2.5-0.3</sub>. A) LDH cytotoxicity  
741 quantification in percentage of 100% mortality.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

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

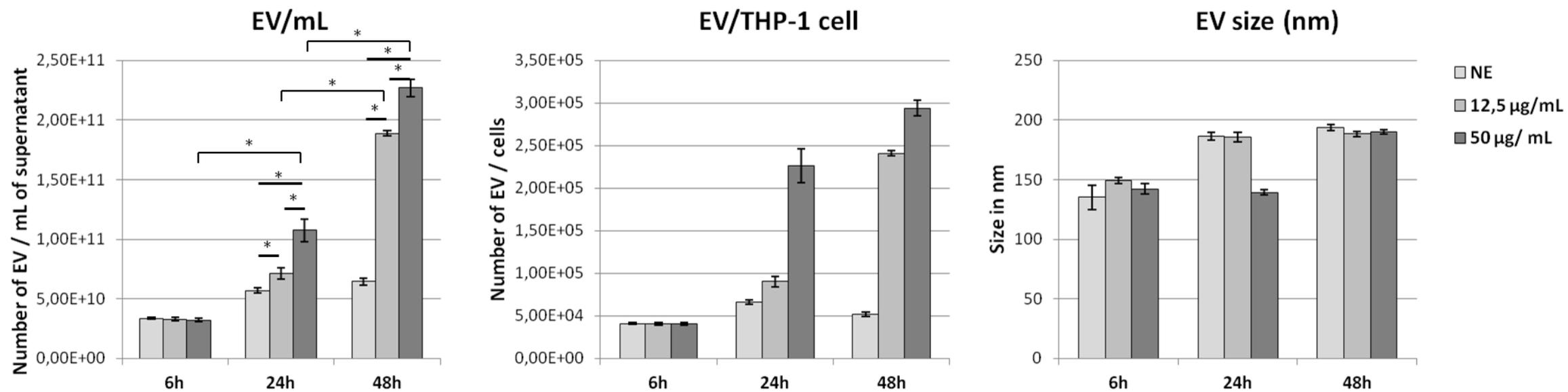
755 **Fig. 6: Characterization of extracellular vesicles derived from primary macrophages exposed to PM<sub>2.5-0.3</sub>.**  
756 EVs were obtained from pooled supernatants of primary macrophages isolated from 6 donors and exposed 48h to  
757 0 (NE) or 12.5 µg/mL of PM<sub>2.5-0.3</sub>. A) Graph representing the amounts of EVs measured by nanoparticle tracking  
758 analysis. Significant differences were evaluated by Student's T test (\*p<0.01); B) Graphical representation of  
759 size distribution of EVs produced by non exposed (NE) and exposed (12.5 µg/mL) primary macrophages; C)  
760 Western-blot using CD63 and CD81 antibodies on EVs obtained from each condition; D) Transmission  
761 electronic microscopy images of EVs (bar = 100nm except when indicated).

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

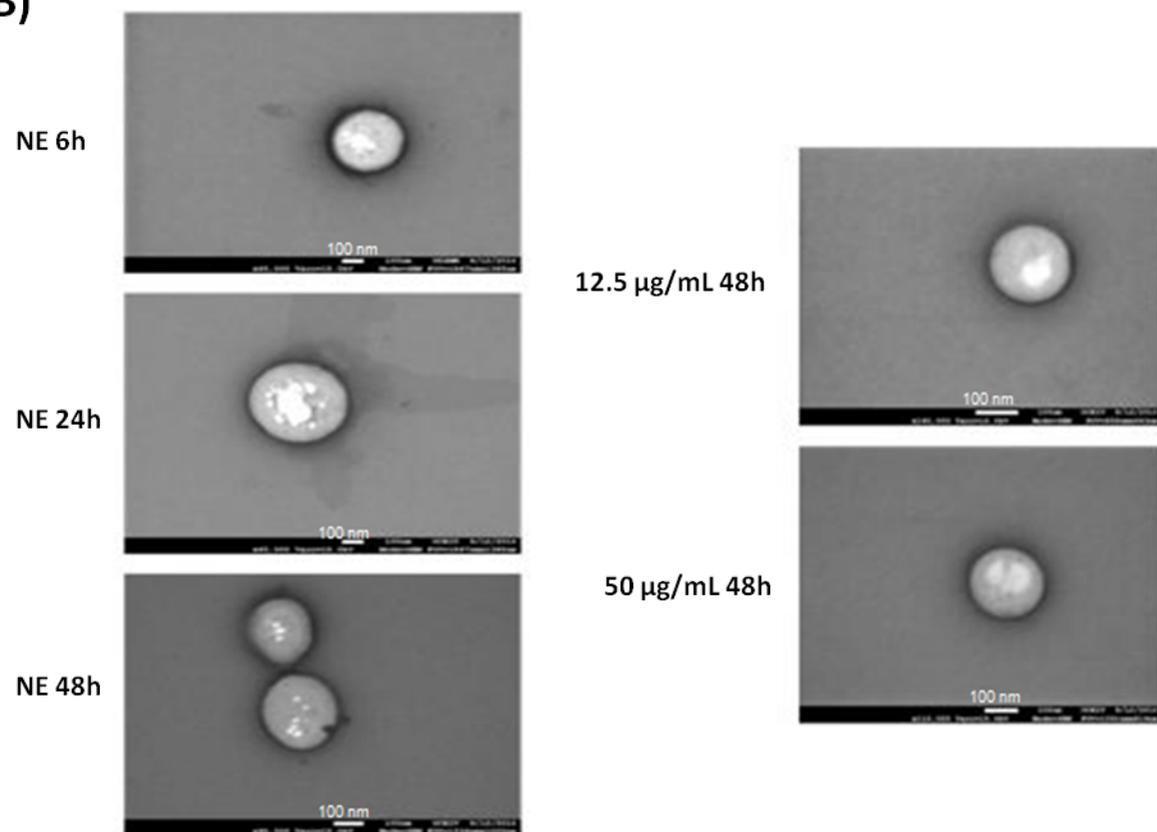




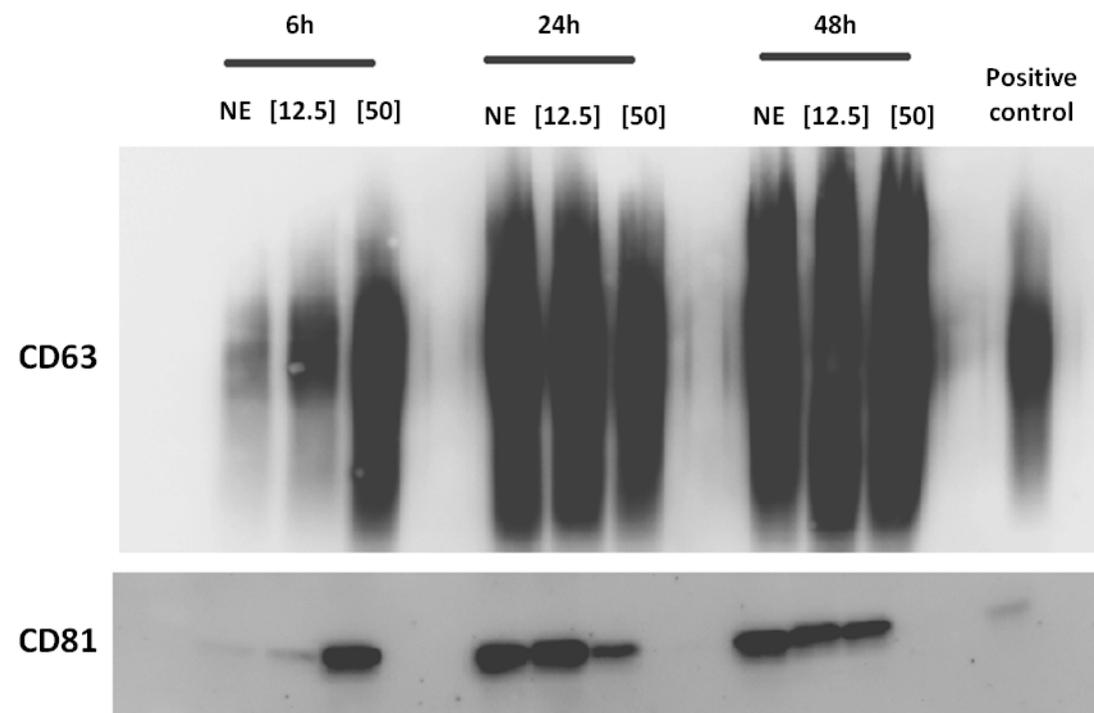
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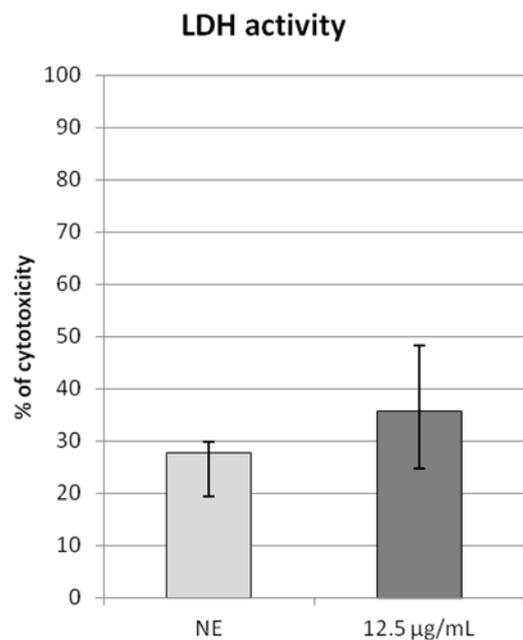
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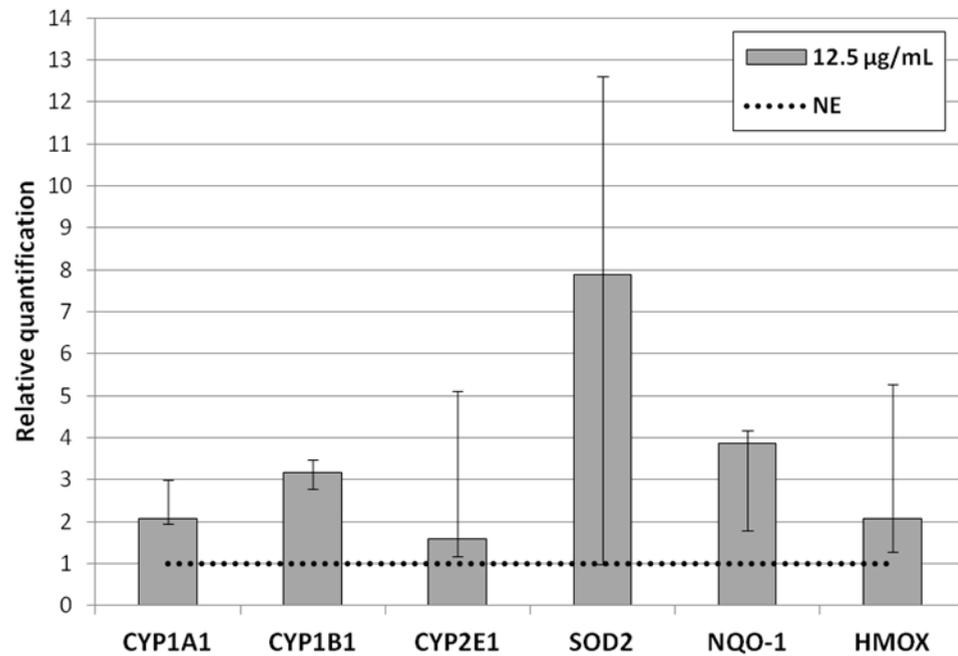
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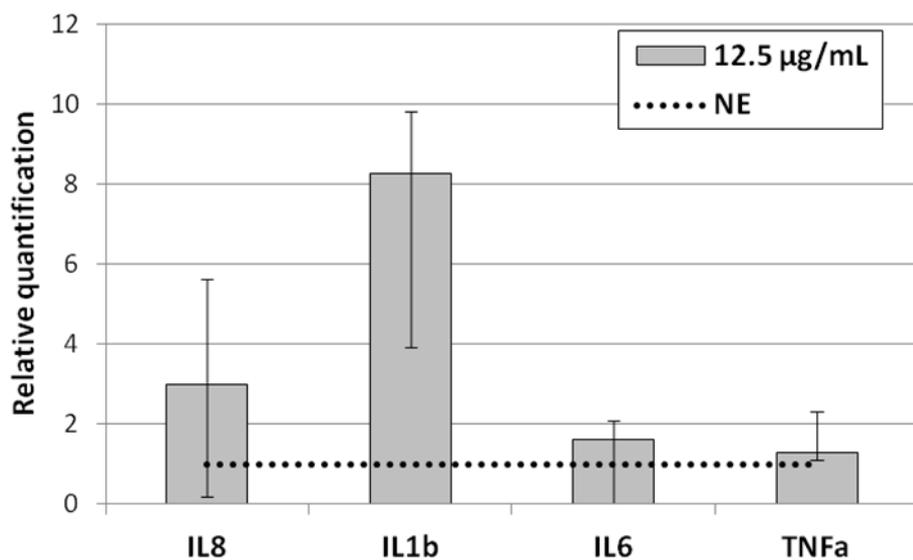
A)



B)



C)



D)

