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Milan winter fine particulate matter (wPM2.5) induces IL-6 and IL-8 synthesis in human bronchial BEAS-2B cells, but specifically impairs IL-8 release

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ABSTRACT

Inflammatory responses have an important role in the onset of many lung diseases associated with urban airborne particulate matter (PM). Here we investigate effects and mechanisms linked to PM-induced expression and release of two main interleukins, IL-6 and IL-8, in human bronchial epithelial BEAS-2B cells. The cells were exposed to well characterized Milan city PM, winter PM2.5 (wPM2.5) and summer PM10 (sPM10), representing combustion and non-combustion sources, respectively. Both wPM2.5 and sPM10 increased mRNA-synthesis and intracellular protein levels of IL-6 and IL-8. Exposure to sPM10 also resulted in continuous and time-dependent increases in release of IL-6 and IL-8 for up to 48 h. By comparison, in wPM2.5-exposed cells IL-8 release was not significantly augmented, while extracellular IL-6 levels were increased but remained constant beyond 24 h exposure. Moreover, wPM2.5 also reduced the lipopolysaccharide (LPS)-increased release of IL-8. No cytotoxicity or significant adsorption of cytokines to wPM2.5 were observed. Immunofluorescence microscopy revealed an accumulation of IL-8 in intracellular vesicles and alterations in actin filament organization in wPM2.5 exposed cells, suggesting that the trafficking of vesicles carrying interleukins to the plasma membrane might be inhibited. Thus, wPM2.5 appeared to impair cytokine release in BEAS-2B cells, in particular of IL-8, possibly by damaging cytoskeletal function involved in protein secretion.

1. Introduction

Airborne particulate matter (PM) is among the most important environmental factors linked to adverse health effects world-wide (WHO, 2013). An increased incidence of respiratory and cardiopulmonary diseases has been related to PM exposure (Pope III et al., 2002). These particle-induced effects are considered the result of combined action of various PM properties, giving rise to different cellular damage and type of responses (Kelly and Fussell, 2012). The ability of PM to directly or indirectly increase the cellular level of reactive oxygen species (ROS) and to give rise to inflammatory responses is considered to be central for the onset of many diseases (Nemmar et al., 2013).

The pro-inflammatory effects of PM have been highlighted by several experimental and epidemiological studies, revealing a positive association between PM exposure and increased levels of inflammatory markers in the airways (Upadhyay et al., 2014). PM has been shown to promote the expression and release of a number of inflammatory mediators (including IL-1β, TNF-α, IL-6, IL-8) both in *in vivo* (Farina et al., 2013; Martin et al., 2007; Wegesser and Last, 2008) and *in vitro* models (Camatini et al., 2012; Hetland et al., 2005; Veranth et al., 2006). Coarse PM/PM10 are considered to be more potent than fine (PM2.5) and ultrafine PM. In fact a higher pro-inflammatory potential of coarse PM has been shown *in vitro* in lung cell models (Becker et al., 2005; Gualtieri et al., 2010), *in vivo* in animal models (Cho et al., 2009; Gilmour et al., 2007; Schins et al., 2004) and in humans (Iskandar et al., 2012; Samet et al., 2007). Experimental studies have revealed that the release of coarse PM-induced cytokines is determined by both the composition and size (Hetland et al., 2005; Veranth et al., 2006). However, the organic fraction of diesel PM, a central component of PM2.5, seems to have a higher pro-inflammatory potential than the washed PM (Totlandsdal et al., 2012).

Interestingly, Alfaro-Moreno and collaborators (Alfaro-Moreno et al., 2009) reported an increased release of interleukin-6 (IL-6) combined with a decreased release of IL-8 in Calu-3 cells following

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exposure to city PM2.5. Similar findings have been seen in another human lung epithelial cell line, BEAS-2B cells, following exposure to diesel PM and diesel PM extracts (Totlandsdal et al., 2015; Totlandsdal et al., 2012) and PM2.5 extracts (Fuentes-Mattei et al., 2010), suggesting that some components in fine PMs may differentially affect IL-6 and IL-8.

The interest in PM-induced cytokines and chemokine release resides in the fundamental role these molecules play in orchestrating innate and adaptive immunity. Interleukin-6 is a cytokine not only involved in inflammation and infection responses, but also in the regulation of metabolic, regenerative, and neural processes (Scheller et al., 2011). IL-8 or CXCL8 is the most potent neutrophil-recruiting chemokine (Fox et al., 2005), and is associated with several patho-physiological states in the airways, including broncho-constriction, edema, and neutrophilia (Gangur et al., 2002). The correct balance between pro- and anti-inflammatory responses is necessary for the maintenance of tissues and organs homeostasis, while the suppression of inflammatory responses could impair protective responses against external threats, including bacterial infections.

Using well characterized Milan city PM (Mantecca et al., 2012), we here further explore the effects and mechanisms related to IL-8 and IL-6 release in BEAS-2B cells. Accordingly to other studies, we have previously reported significant differences in pro-inflammatory potency and responses of the various PM size fractions sampled in Milan during different seasons (Gualtieri et al., 2010). In particular, summer PM10 (sPM10) was most effective in inducing interleukins release in all the cell lines investigated (Longhin et al., 2013b). On the contrary, winter PM2.5 (wPM2.5) had minimal effects on this endpoint in various cell lines. In a more recent study on human bronchial BEAS-2B cells, we found that while wPM2.5 slightly but significantly increased the release of IL-6, the level of IL-8 was unaffected. Most interestingly, genome wide analyses suggested that both IL-6 and IL-8 mRNAs were increased (Longhin et al., 2016).

Thus, the aim of the present study was to elucidate the differential effects of Milan city PM on IL-6 and IL-8 release. To explore further the role of source and particles chemical composition, both wPM2.5 and sPM10 were used. The study verifies that wPM2.5 and sPM10 induced IL-6 and IL-8 mRNA expression and protein synthesis. However, following wPM2.5 a corresponding enhanced IL-8 release was not observed. More specifically, wPM2.5 reduced the increased release of IL-8 triggered by the potent interleukins inducer lipopolysaccharides (LPS), living IL-6 secretion unaffected. This reduction was not an artefact due to a specific adsorption of IL-8 to PM carbon core, but seemed to be due to impaired IL-8 release. Fluorescence microscopy revealed an accumulation of IL-8 protein in intracellular vesicles and disruption of actin filaments occurring at non-cytotoxic concentrations, suggesting a primary role for cytoskeleton alterations.

2. Materials and methods

2.1. Chemicals and kits

LHC-9 medium was purchased from Gibco, Life Technologies (NY, USA) and PureCol™ collagen from Inamed Biomaterials (Fremont, CA, USA). Cell culture flasks were obtained from Nunc A/S, Roskilde, Denmark and 6-well plates and 10 cm culture discs from Corning, Lowell, MA, USA. Rhodamine phalloidin for actin was purchased from Cytoskeleton Inc. β-tubulin for microtubules staining was purchased from Cell Signaling. Alexafluor secondary antibodies and ELISA CytoSets for IL-6 and CXCL8/IL-8 were purchased from Invitrogen Life Technologies Ltd., UK. IL-8 and IL-6 primary antibodies for microscopy were purchased from Sigma–Aldrich and Life Technologies, respectively.

The Absolutely RNA™ reverse transcription (RT) polymerase chain reaction (PCR) Miniprep kit was from Stratagene California (La Jolla, CA, USA). The predesigned TaqMan® Gene Expression Assays, TaqMan Universal PCR Master Mix, and the High-Capacity cDNA Archive Kit were purchased from Thermo Fisher Scientific. All other chemicals were purchased from commercial sources at the highest purity available.

2.2. PM sampling and preparation for biological experiments

PM samples were collected during winter and summer 2012 at Torre Sarca, a site of urban background for atmospheric pollution in Milan. Samplings were performed by a low volume gravimetric sampler (FAI Instruments, Rome, Italy) on Teflon filters, which were replaced every 24 h and then stored at −20 °C until extraction. Samples from one season were pooled and particles were extracted as previously described (Longhin et al., 2013a). Briefly, filters were put in a glass vial with 2 mL of sterile water and underwent 20 min sonication in an ultrasound bath (SONICA, Soltec). Four sonication cycles were performed and the extraction water was collected and replaced every time. The volumes obtained from the four cycles were put together to obtain a homogeneous sample. Particle suspensions were dried in a desiccator, weighed and stored at −20 °C, and the resulting pellets were re-suspended in sterile water (2 μg/μL) just prior to use.

Data on the PMs chemical composition and physical properties have been previously published (Mantecca et al., 2012). Briefly, chemical characterization was performed on Teflon filter sampled PM, according to standard chemical procedure for airborne PM characterization (Perrone et al., 2010). The results showed that sPM10 had higher metals and endotoxins content, while wPM2.5 was enriched in organic compounds including PAHs.

2.3. Cells culture and exposure

BEAS-2B cells, a SV40-transformed human bronchial epithelial cell line, were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). Cells were maintained in LHC-9 medium at 37 °C with 5% of CO_2, with refreshment of medium every other day. Cells for experiments were seeded at a concentration of 300,000 cells/well into collagen-coated 6-well plates and treated the day after with 10 μg/cm^2 (100 μg/mL) of PM, which is within an *in vitro* dosing range estimated to be relevant for high-dose exposure levels in urban environments (Li et al., 2003). Winter PM2.5 (wPM2.5) and summer PM10 (sPM10) were used to assess the role of PM chemical composition in the IL-8 decreased release. The exposure dose and PMs fractions were selected according to previous results, in order to further investigate the highlighted aspects with regard to interleukins gene expression and release (Longhin et al., 2016). Previous data on PM-cytotoxicity on BEAS-2B cells show no effects on viability at the selected dose (Gualtieri et al., 2010). Lipopolysaccharides standard (LPS; 5.5 EU/well) was added to the medium as a positive control to induce a non-PM triggered inflammatory response in cells. Besides, we also used LPS in combination with particles to investigate the effect of PM on the mechanisms related to interleukins release. After 6 or 24 h (plus 30 and 48 h for some experiments) of exposure to PM and/or LPS the cellular responses were examined.

2.4. Cell viability

Cell viability was assessed by Alamar-Blue assay. Alamar-Blue was directly added to fresh cells culture medium (1:10) and cells were incubated for 2 h at 37 °C. The absorbance of each sample was measured at 570 and 630 nm, and compared to control values (Supplementary data 1). The measured absorbance is proportional to the number of living cells and corresponds to the cells metabolic activity.

2.5. Gene expression

IL-6 and IL-8/CXCL8 mRNA levels were determined by real-time

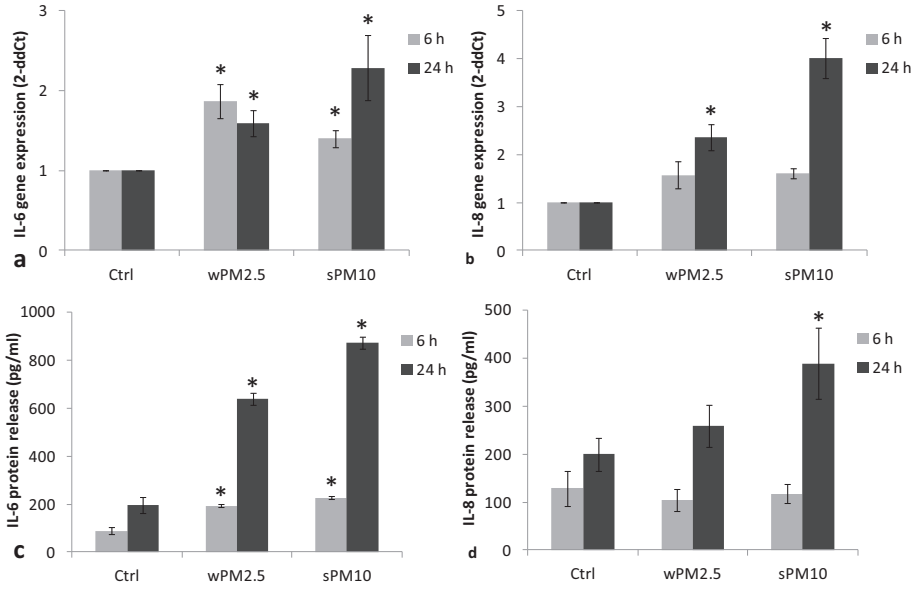

Fig. 1. IL-6 and IL-8 gene expression (a, b) and protein secretion (c, d) in BEAS-2B cells exposed to wPM2.5 and sPM10 for 6 and 24 h. * Statistically significant difference over control (untreated cells). ANOVA, Bonferroni, $p < 0.05$, n = 6 independent biological replicates.

PCR. Total mRNA was isolated from cells according to the supplier's recommendations using the Absolutely RNA™RT-PCR Miniprep kit, and reverse transcribed to cDNA on a PCR System 2400 (PerkinElmer) using a High-Capacity cDNA Archive Kit (Applied Biosystems; Thermo Fisher Scientific). Real-time PCR was performed using the Applied Biosystems 7500 Real-Time PCR System, with predesigned TaqMan Gene Expression Assays (18S rRNA, Hs99999901_s1, IL-6 Hs00174131_m1, and CXCL8, Hs00174103_m1) and TaqMan Universal PCR Master Mix. For these analyses, 1 µg of total RNA was reverse transcribed to complementary (c)DNA using a High-Capacity cDNA Archive Kit. The cDNAs were diluted 1:20 in a solution of nuclease-free water, TaqMan Universal Master Mix, primers, and probe before performing the real-time PCR. The expression of each gene of interest (GOI) in each sample was normalized against housekeeping genes (18S rRNA), and expressed as the fold change compared to the control (untreated cells), as calculated by the ΔΔCt-method: (ΔCt = Ct[Gene of Interest] − Ct[18S]; ΔΔCt = ΔCt[Treated] − ΔCt[Control]; Fold change = 2[−ΔΔCt]).

2.6. ELISA

IL-6 and IL-8/CXCL8 intracellular and released protein levels were measured by ELISA. After exposure, the cells supernatants were collected. The well plates with cells were put overnight at −20 °C. The day after the cells were lysed on ice in RIPA buffer (150 mM NaCl, 1% TritonX-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0 and 0.1% of protease inhibitors, added just before use). Both supernatants and lysates were centrifuged (12,000 rpm, 15 min, 4 °C) and stored at −20 °C. The test was performed according to the manufacturer's guidelines. The absorbance of each sample was measured by Multiplate Reader Ascent (Thermo) at 450 nm and 630 nm, and the amount of proteins in pg/mL calculated on the basis of a standard curve.

Since previous works reported ILs binding on carbonaceous particles (Kocbach et al., 2008), we also carried out experiments to measure this phenomenon in our samples. IL-6 and IL-8 standards at know concentrations have been added to wells containing cell culture media only, and incubated with or without wPM2.5 at the exposure dose (100 µg/mL). wPM2.5 was chosen since it likely has higher adsorption potential, presenting higher content of elemental carbon and smaller particles, *i.e.* higher adsorption surface, with respect to sPM10. After 24 h ELISA was carried out (Supplementary data 2).

2.7. Microscopy

For the detection of IL-8, IL-6, actin and β-tubulin, samples were prepared following common fluorescence microscopy techniques. Briefly, cells grown on cover slips and exposed to wPM2.5 were washed in PBS and fixed with 4% paraformaldehyde for 15 min on ice. Permeabilization and blocking were performed in PBS with 0.5% BSA and 0.2% Triton X-100 for 15 min at room temperature. Cells were then labelled with primary antibodies overnight at 4 °C (IL-8 and IL-6, 10 µg/mL) or with rhodamine phalloidin for actin (100 nM, Cytoskeleton Inc.), β-tubulin (1:100 dilution; Cell Signaling) in PBS at room temperature for 30 min. For detection of ILs, appropriate Alexafluor secondary antibodies (1:500 dilution; Invitrogen) were incubated for 1 h at room temperature. Cells' DNA was counterstained with DAPI. Slides were observed under a fluorescence microscope (AxioObserver, Zeiss Germany) and digital images were taken.

2.8. Statistical analyses

Mean and standard error of mean (SEM) of at least three independent experiments (independent biological replicates) are reported. Statistical analyses were performed using Sigma Stat 3.1 software, using one-way ANOVA with Bonferroni or Dunn's as a *post hoc* test. Values of $p < 0.05$ were considered statistically significant.

3. Results

In order to investigate the inflammatory mechanisms activated by urban PM, the gene expression and protein secretion of two key mediators, IL-6 and IL-8, were measured in bronchial BEAS-2B cells exposed to particles for 6 and 24 h. At these time points there are no sign of cytotoxicity as determined by the Alamar-Blue assay (Supplementary data 1).

The expression (mRNA) and release of IL-6 were increased at the investigated time-points, from cells exposed to both wPM2.5 and sPM10 (Fig. 1a and c). Both PM also increased the expression of *IL-8* after 24 h of exposure, with sPM10 apparently somewhat more potent than wPM2.5 (Fig. 1b). Interestingly, statistically significant increases in levels of IL-8 protein were only seen in the supernatants of cells treated with sPM10, and not after wPM2.5 (Fig. 1d).

Protein secretion was further measured at 30 and 48 h of exposure,

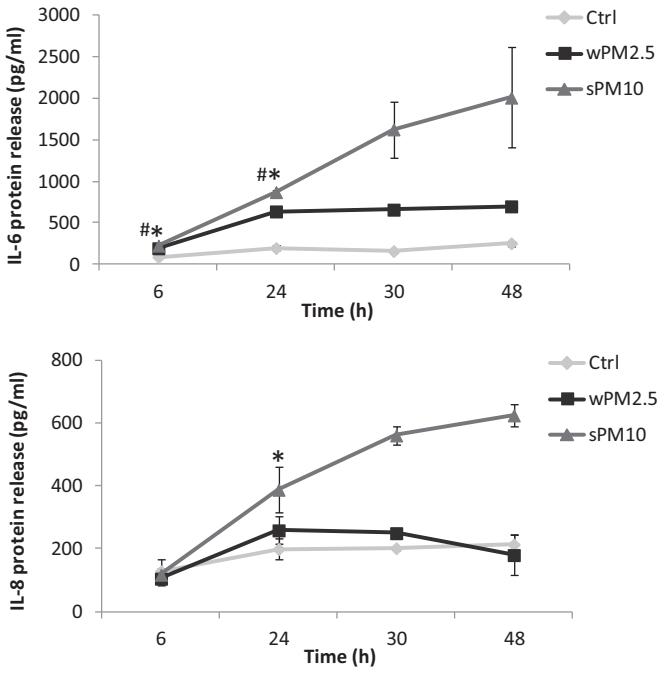

Fig. 2. IL-6 and IL-8 secretion in BEAS-2B cells exposed to wPM2.5 and sPM10 for 6, 24, 30 and 48 h. The time points 6 and 24 h are mean +/− SE of 6 independent biological replicates (repeated from Fig. 1 to aid the comparison); 30 and 48 are means of 2 independent biological replicates, with 2 technical replicates each. (#) wPM2.5 and (*) sPM10 are statistically significant different over control (untreated cells), respectively. ANOVA, Bonferroni, $p < 0.05$.

to explore the possibility of a delayed IL-8 release following wPM2.5 exposure (Fig. 2). wPM2.5-induced IL-6 release was constant at the later time points, however, an increase in IL-8 secretion was still not observed. In contrast, sPM10 further increased the secretion of both IL-6 and IL-8 also at the later time points.

To further explore the mismatch between *IL-8* gene expression and IL-8 protein release, intracellular protein levels were next analysed (Fig. 3a). The results show that wPM2.5 increased the synthesis of both IL-6 (3.3 fold) and IL-8 proteins (2.5 fold), as judged by their increased intracellular levels compared to untreated control cells. However, only IL-6 was secreted, confirming the previous data. The intracellular accumulation of IL-8 was also validated by microscopy investigations (Fig. 3b), showing an augmented signal of immunostained protein, organized in spots that suggest compartmentalization in vesicles. In particular, exposure to wPM2.5 seemed to increase the number and size of the spots. In contrast, no clear compartmentalization of IL-6 following wPM2.5 could be seen.

IL-6 and IL-8 adsorption on particles was measured to check its possible contribution to the apparent lack of IL-8 secretion. A slight IL-8 adsorption was observed. Reduction of measured standard in presence of PM was 20 ± 8% with 400 pg/mL of IL-8 standard and 13 ± 0.1% with 100 pg/mL of IL-8 standard (Supplementary data 2). Considering the intracellular amount of IL-8 (151 pg/mL, FI 2.5 over control), adsorption on particles cannot explain the complete lack of extracellular protein. No IL-6 adsorption was observed.

Thus, our data suggest that wPM2.5 specifically impaired the secretion of the synthetized IL-8, while IL-6 secretion seems to be unaffected. To test this hypothesis, BEAS-2B cells were exposed to LPS with or without wPM2.5 (Fig. 4). Statistically significant induction in IL-8 release induced by LPS was lost after co-treatment with wPM2.5. In contrast, no effects of wPM were observed on the intracellular and secreted levels of IL-6 in LPS-exposed cells.

Next, we investigated if particle exposure could affect the cytoskeleton organization, as this is an essential element for vesicular transport within the cells. Fig. 5a shows alterations in actin organization in cells exposed to particles after 24 h (Fig. 5a-iv), but not after 6 h (Fig. 5a-iii). Untreated cells (Fig. 5a-i,ii) and cells exposed for 6 h (Fig. 5a-iii) present actin filaments in the cytoplasm and near the cell periphery, while samples exposed to wPM2.5 for 24 h (Fig. 5a-iv) almost completely lack these structures. Fine and short actin projections could also be observed in these cells, along with a slightly alteration of morphology, appearing as cellular shrinkage. Interestingly, these alterations were not obviously present after 6 h of exposure, nor in samples exposed to sPM10 up to 24 h (Fig. 5a-v, vi) or in LPS exposed samples (Supplementary data 3). Staining of microtubules' β-tubulin did not highlight major alterations of these structures, as long filaments can be observed in both control and exposed cells, and the total intensity of fluorescence signal, as measured by AxioVision Microscope Software, was similar in control cells and exposed samples (data not shown). However, a slight difference can be observed in the morphological organization of the microtubules that in control cells present a perinuclear grouping, likely departing from the area of the microtubule organizing center (MTOC, Fig. 5b, arrow heads). This area is more difficult to detect in wPM2.5 exposed cells (Fig. 5b).

4. Discussion

The correct functioning of the inflammatory responses is important for the maintenance of tissues and organs homeostasis. Here we investigated the inflammatory processes induced by well- characterized ambient Milan wPM2.5 and sPM10 (Mantecca et al., 2012) in bronchial epithelial BEAS-2B cells, by analyzing two main interleukins, IL-6 and IL-8. Interestingly, both urban PMs increased mRNA expression and intracellular protein levels of IL-6 and IL-8 at non-cytotoxic concentrations. While wPM2.5 induced only the release of IL-6, sPM10 increased the release of both proteins. Similarly, exposure to wPM2.5 specifically reduced the release of LPS-induced IL-8 synthesis. ELISA and immunofluorescence microscopy analysis revealed that wPM2.5 resulted in an accumulation of IL-8 in intracellular vesicles. This effect was accompanied by alterations in the actin filament organization, suggesting that wPM2.5 reduced the transport of IL-8 vesicles to the plasma membrane.

sPM10 presents mostly mineral particles with high content of metals and endotoxins, while wPM2.5 originating from combustion of organic material including diesel are agglomerates of smaller carbon particles with organic chemicals including PAHs attached (Mantecca et al., 2012). It is well-known that various mineral PM as such induces cytokines (Øvrevik et al., 2009). Accordingly, we here find increased expression and excretion of IL-6 and IL-8 following exposure to Milan sPM10. We have previously reported that these inflammatory reactions partly are depending on the endotoxin content (Camatini et al., 2012). In contrast, we have found that diesel PM-induced IL-6 and IL-8/CXCL8 responses in BEAS-2B cells are caused by methanol extractable constituents, in particular found in the non-polar fraction (Bach et al., 2015; Totlandsdal et al., 2012; Totlandsdal et al., 2010). In contrast, the residual diesel PM gave almost no inflammatory responses (Totlandsdal et al., 2012). Furthermore, the increased expression of cytokines were found to be at least partly depending on CYP-activity and formation of reactive molecules/ROS, suggesting a role for compounds such as PAHs (Bach et al., 2015). It is, however, important to note that the inflammatory potency of various combustion PMs are highly variable and obviously much depending on the specific composition of chemicals adsorbed (Becker et al., 2005; Schins et al., 2004; Totlandsdal et al., 2015; Totlandsdal et al., 2014). This also seems to be the case for IL-8/CXCL8 responses in BEAS-2B cells, as both increased (Rodríguez-Cotto et al., 2013; Yan et al., 2016) and decreased IL-8 release after exposure to PM2.5 have been reported (Gioda et al., 2011; Rodríguez-Cotto et al., 2014). Thus, the different chemical composition of the various urban air PM2.5 used is likely to explain most of the diverse results reported. In fact, particles composition is not only depending on the source, but

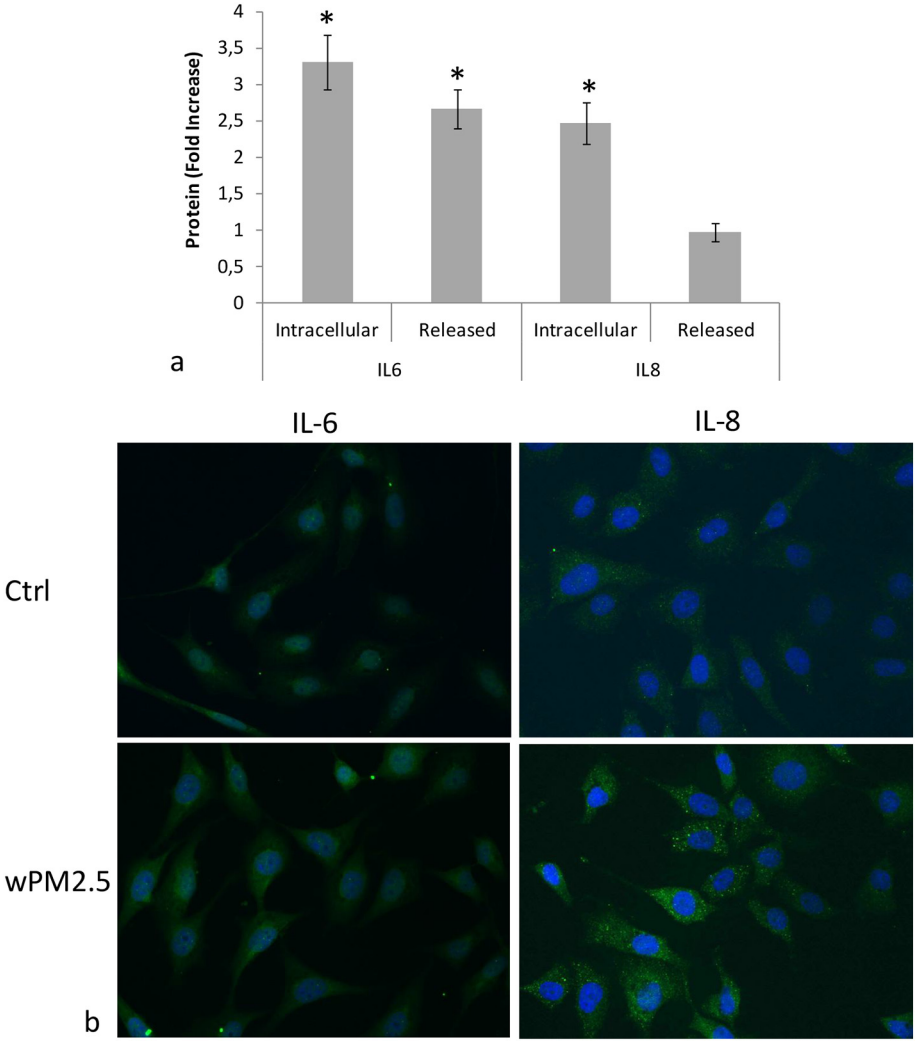

Fig. 3. (a) Intracellular and secreted IL-6 and IL-8 proteins in BEAS-2B cells exposed to wPM2.5 for 24 h. * Statistically significant difference over control (untreated cells). ANOVA, Dunn's, $p < 0.05$, $n = 10$ independent biological replicates. (b) IL-6 and IL-8 immunostaining (green) in BEAS-2B cells exposed to wPM2.5 for 24 h. DNA was counter-stained with DAPI (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

also on the sampling and extraction procedures.

Also a mismatch between IL-8 induced gene expression and lack of increased release has previously been reported following exposure to urban PM2.5 (Alfaro-Moreno et al., 2009; Kocbach et al., 2008). Similar effects have more specifically also been seen after exposure to diesel PM (Totlandsdal et al., 2012). A suggested explanation for this observation has been a specific adsorption of the IL-8 protein to carbonaceous particles (Kocbach et al., 2008) or to other dust particles (Bauer et al., 2012). However, incubating purified recombinant IL-8 with wPM2.5 only resulted in a slight adsorption of IL-8 to wPM2.5. This reduction was certainly not sufficient to explain the near complete lack of enhanced IL-8 release following exposure to Milan wPM2.5, and supports previous findings by Alfaro-Moreno et al. (2009).

In the present study, we have further elucidated possible mechanisms linked to the lacking of increased IL-8-release following exposure to PM2.5 by specifically analyzing the effects on gene expression and intracellular content of IL-8. Here we find that wPM2.5 not only up-regulated *IL-8* gene expression comparable to its effect on *IL-6*, but also stimulated IL-8 protein synthesis, as evident from the increased intracellular levels identified by both ELISA and immunofluorescence microscopy. Furthermore, wPM2.5 specifically reduced the LPS-increased release of IL-8, leaving IL-8 synthesis and IL-6 synthesis and release unaffected. This supports the hypothesis of an inhibitory effect of wPM2.5 specifically affecting IL-8 release. Of the 2 PM samples used in this study, sPM10 has the highest content of crustal elements while wPM2.5 has a higher content of organic matter (Mantecca et al., 2012). It is therefore tempting to speculate that the inhibition of IL-8 release could be due to PAHs or other organic chemicals of the wPM2.5 fraction. In support of this, previous studies have shown that soluble extracts of PM (Fuentes-Mattei et al., 2010) and diesel exhaust PM (Totlandsdal et al., 2014) also suppress IL-8 release. Also in accordance with this, Rodríguez-Cotto and colleagues report a decrease of both IL-6 and IL-8 release in BEAS-2B cells exposed to industrial PM2.5, and demonstrate that heavy metals are not responsible for this effect since it is not reverted by treatment with the metal chelator deferoxamine mesylate (Rodríguez-Cotto et al., 2014).

The differential effects of wPM2.5 on IL-6 and IL-8 release, suggest that different mechanisms may regulate the secretion of these two cytokines in BEAS-2B cells. This hypothesis is supported by recent literature reporting that cellular secretion of various cytokines occurs through different pathways (Stanley and Lacy, 2010). Indeed, different pathways have previously been described for IL-6 and IL-8 secretion in different cell types. IL-6 has been related to constitutive exocytosis in macrophages (Manderson et al., 2007; Murray and Stow, 2014) and mast cells (Lacy and Stow, 2011), where it has been shown to be directly secreted after synthesis by a mechanism that is independent from

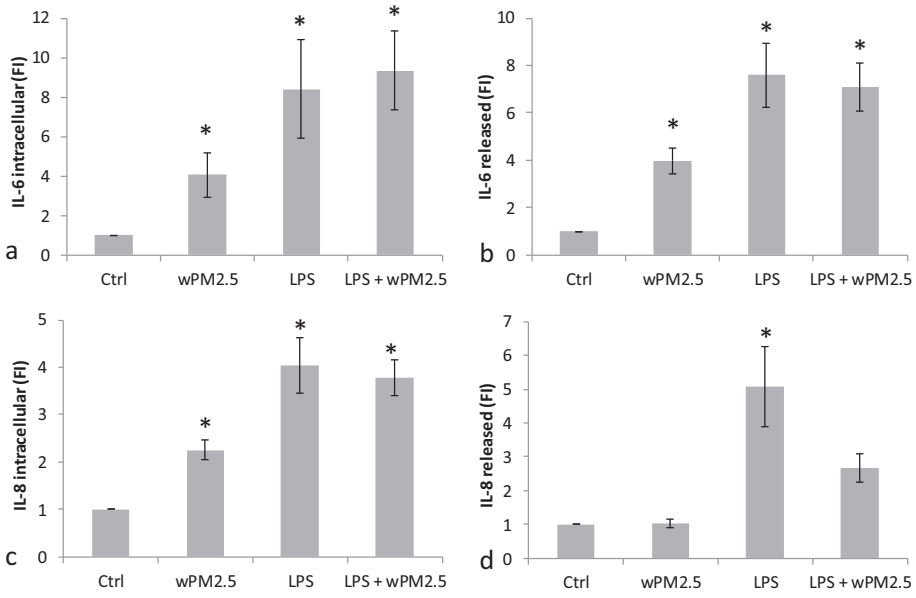

Fig. 4. IL-6 (a - intracellular, and b - released) and IL-8 (c - intracellular, and d - released) protein levels in BEAS-2B cells exposed to LPS and/or wPM2.5 for 24 h. * Statistically significant difference over control (untreated cells). ANOVA, Dunn's, $p < 0.05$, n = 4 independent biological replicates.

Ca^{2+} influx and degranulation (Kandere-Grzybowska et al., 2003). In endothelial cells IL-8 was found to be pre-stored in specific storage granules, Weibel-Palade bodies, and released after stimulation as cAMP and Ca^{2+} increase (Rondaij et al., 2006). The cytoskeleton remodeling has an important role in proteins exocytosis. Actin fibers were reported to participate in the motility of secretory granules, while it has been suggested that microtubules, but not actin filaments, are responsible for transport and fusion dynamics of constitutive post-Golgi secretory vesicles (Schmoranzer and Simon, 2003). Significantly, IL-8 secretion from epithelial cells has been associated to Rho GTPases Rac1, Rac2, and Cdc42, which modulate the actin cytoskeleton (Hobert et al., 2002). Furthermore, it has recently been reported that the disruption of centrosome, which organizes microtubules, leads to defects in IL-6 secretion, while it does not have a significant effect on IL-6 synthesis (Vertii et al., 2016).

We have previously reported a modulation of genes involved in cytoskeleton organization and actin/tubulin polymerization (*CDC42EP2, RAB7L1*, and *ARHGAP26*) in Milan wPM2.5 exposed BEAS-2B cells, accompanied by increased levels of the active phosphorylated form of the actin-binding protein HSP27 (regulating cytoskeleton organization, Longhin et al., 2016), and centrosome amplification (Longhin et al., 2013a). Here we observe alterations of actin cytoskeleton, consisting of fibre loss and formation of membrane projections after 24 h of wPM2.5 exposure. Cellular morphology is also affected with apparent shrinkage, suggesting that cell adhesion to the substrate might be partly compromised, likely in relation to actin alteration (Iwamoto and Calderwood, 2015). As the correct functioning of the cytoskeleton is fundamental for numerous cell functions, here actin alteration might be involved in lack of IL-8 secretion due to impaired granules trafficking. Long microtubules filaments can be observed in both control and exposed cells, supporting the idea that microtubules-dependent IL-6 release is unaffected. However, the images seem to present a weaker staining for β-tubulin in the area of the microtubule organizing center (MTOC) in wPM2.5 exposed cells, suggesting a possible effect on centrosomes similar to the one reported by Vertii et al. (2016). Notably, wPM2.5-induced IL-6 secretion seemed to be triggered prior to IL-8 release, and did not increase beyond 24 h (in contrast to sPM10-induced IL-6 release) when the cytoskeletal effects were observed. Thus, the contrasting impact on IL-6 and IL-8 could also be related to the difference in timing of synthesis and release of these two cytokines, upon wPM2.5 exposure. The possibility that cytoskeletal modulation could have a more broad impact on secretory mechanism in general, should therefore not be completely omitted. Additionally, up-regulation of some genes and pathways, as well as mucin proteins in the mucosal airway surfaces, has been suggested to reduce PM-induced IL-8 secretion following *in vivo* exposure (Fuentes-Mattei et al., 2010).

A possible important implication of these PM2.5-effects could be reduced and/or unbalanced immune responses following pulmonary infections, as underscored by the suppression of LPS-increased IL-8 release. This may have further implications for the recruitment of immune cells and thus potentially increase susceptibility to lung diseases. Despite the fact that IL-6 could be measured in the cells supernatants, further investigations are required to verify possible effects on the release of this and other inflammatory mediators. It would also be important to clarify whether sustained exposure to lower PM concentrations, more realistic of real-life exposure conditions, could suppress the release of inflammatory mediators and impair secretory functions in a similar manner.

In conclusion, here we demonstrate that Milan wPM2.5 exposure, despite being able to initiate pro-inflammatory responses in BEAS-2B cells, impairs the expected enhanced release of IL-8. This effect seems to be due to alterations of the cells cytoskeleton leading to impaired trafficking of secretory vesicles.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tiv.2018.07.016.

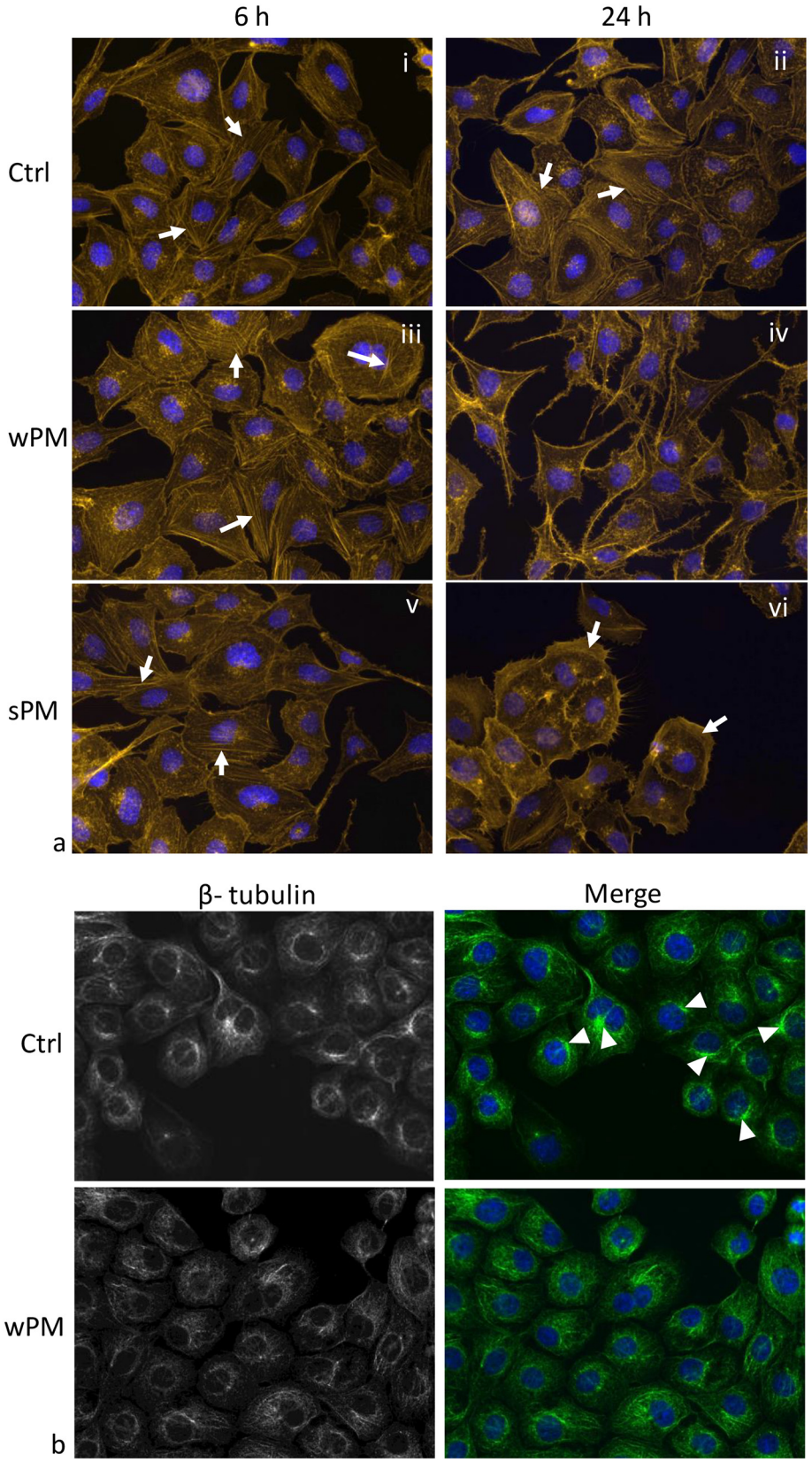

Fig. 5. a) Actin organization (red) in BEAS-2B cells exposed to wPM2.5 (iii and iv) and sPM10 (v and vi) for 6 (i, iii, v) and 24 h (ii, iv, vi). Arrow: actin filaments. b) β-tubulin (green) in BEAS-2B cells exposed to wPM2.5 for 24 h. DNA was counter-stained with DAPI (blue). Arrow heads: perinuclear grouping of microtubules, likely departing from the microtubules organizing center (MTOC). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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